Mechanism of Milk Peptide Growth Factor

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Abstract

Milk peptide is a fragment of αS_2-casein of bovine milk, which has previously been shown to stimulate DNA synthesis in rat mammary fibroblasts (Rama 27 cells). The mechanism of milk peptide was not known. This thesis focuses on confirming that milk peptide is a novel growth factor in fibroblast cells and characterizing the major intracellular signaling pathway(s) and early phosphorylation events which are activated by milk peptide in fibroblast cells.

To confirm that milk peptide is a novel growth factor for Rama 27 cells, milk peptide has been highly purified from bovine milk, and it gave good stimulation of DNA synthesis in fibroblast cells (Rama 27 cells and Swiss 3T3 cells). In addition, recombinant milk peptide in *E coli* was made to confirm its identity.

In order to find the mechanism of milk peptide, several small peptides with growth promoting activity were used to induce DNA synthesis in some cell lines. These included milk peptide; 'KVIPY', a synthetic milk peptide; 'VREKS', a synthetic peptide from basic Fibroblast Growth Factor; Bombesin, which is a tetradecapeptide originally isolated from the skin of the European frog Bombina and Epidermal Growth Factor (EGF). The maximal stimulation of DNA synthesis obtainable with 10 ng/ml of milk peptide was 37 fold higher than control, and higher than 2% FCS in Rama 27 cells.

To find the intracellular signaling pathway of milk peptide, early phosphorylation events on tyrosine have been investigated in fibroblast cells (Rama 27 cells and Swiss 3T3 cells). The experiments suggested that milk peptide activated a tyrosine kinase receptor similar to that of EGF rather than a G protein coupled receptor and bFGF receptor. Besides, milk peptide was shown to activate phosphorylation of EGF receptor in Rama 27 cells although it might activate other receptor(s) in CHO cells which lack endogenous EGF receptor.

The major mitogenic pathway of milk peptide was investigated. Inhibition of milk peptide stimulated DNA synthesis was observed when incubated with PD-098095, a MAP kinase pathway inhibitor. The Western blotting experiments showed that milk peptide activated the phosphorylation of P42/p44^{MAPK}, and this phosphorylation was shown to be abolished by PD-098095, but not by Wortmannin, an inhibitor of PI3 kinase.

Acknowledgements

To my husband and my son, with love and thanks.

Abbreviations

AR	Amphiregulin
ATP	Adenosine triphosphate
bFGF	Basic FGF or FGF-2
BMB	Bombesin
BTC	Betacellulin
BSA	Bovine serum albumin
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-related kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GFs	Growth factors
GRP	Gastrin releasing peptide
G-protein	GTP-binding protein
HBGF	Heparin-binding growth factor
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor-1
INF	Interferon
IR	Insulin receptor
IRS	Insulin receptor substrate
kDa	KiloDaltons
KVIPY	KVIPYVRYL

LF	Lactoferrin
MAPK	mitogen activated protein kinase
MAPKAP-K1	MAPK-activated protein kinase 1, also called p90^{RSK}
MAPKAP-K2	MAPK-activated protein kinase 2
MEK	MAPK kinase, also called MAPKK
MBWE	Mitogenic bovine whey extract
NDF	Neu differentiation factor
NMB	Neuromedin B
NRG	Neuregulin
p42/p44^{MAPK}	The p42 and p44 ERK MAPK proteins
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
pET	Plasimid for expression by T7 RNA polymerase
Phe	Phenylalanine
pI	Isoelectric point
PI3-kinase	phosphatidylinositol 3'-kinase
PIP$_2$	phosphoinositol 4,5,-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PTB	Phosphotyrosine binding domain
PVDF	Polyvinylidene filter
Rama	Rat mammary
RM	Routine medium
SD	Standard deviation
SDM	Step-down medium
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SH2	Src homology 2
TCA	Trichloroacetic acid
TEMED	N,N,N',N',Tetramethylethylene diamine
TGF	Transforming growth factor

Thr	Threonine
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl) methylamine
Tween-20	Polyethlenesorbitan monolaurate
Tyr	Tyrosine
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
VREKS	VREKSDPHIKGGC
w/v	Weight per volume

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Chapter 1

General Introduction

1.1 Introduction

Polypeptide growth factors represent a diverse group of hormone-like agents that affect a variety of cellular processes including metabolic regulation, cell division, and extension of processes. The peptide growth factors often promote cell growth, but they also can inhibit it, moreover, they regulate many other processes that have little to do with growth itself. Modern study of peptide growth factors began when it was shown that extracts of tumors and salivary glands promoted neurite outgrowth from cultured nerve ganglia (Levi-Montalcini and HamBurger, 1953, Cohen 1960). Subsequently studies provided the insight into mechanisms by which growth factors themselves produce a variety of effects and also provided the characterized model systems (such as EGF) for studying the interaction of peptide growth factors with their membrane associated receptors. It is apparent that peptide growth factors provide an essential means for a cell to communicate with its immediate environment and to ensure that there is proper local homeostatic balance between the numerous cells that comprise a tissue. Since a cell must adjust its behavior to changes in its environment, it needs mechanisms to provide this adaptation. Thus, cells use sets of peptide growth factors as signaling molecules to communicate with each other and alter their behavior to respond appropriately to their biological context. Since peptide growth factors act by binding to functional receptors which transduce their signal, the peptides themselves may be viewed as bifunctional molecules: they have both an afferent function (conveying information to the receptor, providing it with information from the outside) as well as an efferent function (inception of the latent biochemical activity of the receptor) (Sporn et al., 1990).

The isolation and characterization of polypeptide growth factors parallels the major developments in protein chemistry itself. The earliest factors to be identified were only scantily characterized with functional information far outstripping molecular characterization. When structural information became available, it was first obtained with substances that could be isolated in relatively large amounts. Only as the methods of protein chemistry advanced and more sensitive techniques became available (beginning of the early 1970s) were smaller and smaller quantities required to produce detailed structural information. With the advent of cloning techniques it became possible to obtain this information even with vanishingly small samples of the hormone. In turn these methodologies, through the creation of expression systems, have allowed the production of large amounts of any identified growth factor. Thus the isolation and structural characterization of a polypeptide growth factor now only depends upon the ingenuity of the investigator to first craft an appropriate assay to identify the activity to be ultimately assured of obtaining complete characterization, usually within a relatively short time span.

1.2 Growth Promoting Activities in Milk

Milk proteins have long been considered only as food proteins for young mammals. However, in addition to a nutritional role, milk proteins have physiological importance and are a source of biologically active peptides. This aspect has been studied since 1979 and numerous peptides of known sequence that are derived from milk proteins have been shown to exhibit various activities, such as opiate activity, antihypertension, immunomodulation, bioconversion of ions (Ca^{2+}), antithrombotic activities, antibacterial properties and growth promoting activities (Fiat et al., 1993). A wide range of regulatory peptides (e.g. gastrin) and hormones (e.g., growth hormone), and growth factors have been identified in the milk of various animal species (Koldovsky 1994a). The biological significance of these polypeptides is uncertain (Murphy et al., 1998).

Milk is a biological fluid that stimulates the growth of cells in culture. When serum is replaced by milk, a whole variety of cell types will proliferate in milk supplemented medium, including epithelial cells, normal and transformed fibroblasts, smooth muscle cells, chondrocytes, and fetal small intestinal cell lines (Steimer and Klagsbrun, 1981; Steimer et al., 1981; Sereni and Baserg, 1981). The growth promoting properties of milk are due in part to the presence of growth factors, mitogens capable of stimulating DNA synthesis and cell division in cultured cells. These growth factors are found in both human (Klagsbrun, 1978) and bovine (Klagsbrun and Neumann, 1979) milk.

1.2.1 EGF - a major growth factor in human milk

The mitogenic activity of milk has been studied for long time. Klagsbrun (1978) recognised the mitogenic activity of milk when activity that stimulated DNA synthesis in fibroblasts was demonstrated. Subsequent studies revealed the existence of many growth factors in milk. As first reported by Shing and Klagsbrun (1984) human and bovine milk contain very different sets of growth factor. One of these, designated human milk-derived growth factor (HMGF) III, constitutes over 75% of the total growth factor activity of human milk. HMGF III has a molecular weight of 6 kDa and pI between 4.4 and 4.7. Comparative biochemical studies strongly suggest that is human epidermal growth factor (EGF). Human EGF isolated from urine (urogastrone) has a molecular weight of about 6000 Da and a pI of 4.5 (Hollenberg, 1979). Carpenter (1980) demonstrated that antibodies directed against human urine-derived EGF reduced the mitogenic activity of milk by 67%, corresponding to the amount contributed by the presence of HMGF III. This evidence suggested that the major mitogen in human milk was EGF (Shing and Klagsbrun 1984, Carpenter 1980). Two other higher molecular weight growth factors, HMGF I (mol wt 100,000-120,000) and HMGF II (mol wt 30,000-35,000), accounted for the other 20-25% of human milk-derived growth factor activity. Therefore, EGF is a major growth factor in the human milk (Carpenter 1980). EGF in human milk is considered to be physiologically important for the development of the digestive tract of the infant (Berseth, 1987, Falconer, 1987).

1.2.2 EGF is absent in bovine milk

In contrast to the numerous studies on human milk EGF, little work has been published on bovine milk EGF. Only two reports have estimated the EGF concentration in bovine milk and milk -based infant formulas (Read et al., 1985; Yagi et al., 1986). In the absence of an anti-bovine EGF antibody and the apparent lack of cross-reactivity of bovine EGF with anti-human or anti- mouse EGF antibodies, both reports used RRA instead of RIA. They only estimated biochemical EGF-like activity in an unknown sample based on the ability to displace the binding of $[^{125}I]$- EGF from its receptor. Iacopetta et al.(1992) reported that EGF had not been unequivocally detected in bovine colostrum or milk. Bastian et al., (2001) reported the presence of betacellulin by homologous RIA in bovine colostrums and milk, and suggested that bovine milk does not contain EGF but instead low levels of betacellulin, and that this growth factor may be responsible in part for the bovine milk EGF receptor binding activity detected in the studies by Read et al. (1984) and Iacopetta et al. (1992).

1.2.3 Growth factors in bovine milk

Several mitogenic factors have been identified in bovine milk, including: Insulin-like Growth Factor (IGF) I and II (Francis et al. 1986); Transforming Growth Factor-ß (Cox and Burk 1991); Bovine Colostrum Growth Factor (BCGF) structurally related to Platelet- Derived Growth Factor (PDGF) (Shing and Klagsbrun, 1984, Petrides et al., 1985); Heparin-Binding Growth Factor (HBGF) that may be an example of the Fibroblast Growth Factor (Sandoswki et al, 1993).

1.2.4 Growth factors in the bovine whey

In 1995, Francies et al., reported that growth factor activity in bovine milk is retained in the whey fraction from where it can be enriched some 100- to 200-fold using a single-step cation-exchange procedure. The major protein species isolated by this process are lactoperoxidase and immuno-globulin (IgG), although no cell growth activity could be attributed to these proteins. This fraction (termed mitogenic bovine whey extract; MBWE) was shown to possess potent growth- promoting activity for

cells of mesodermal origin, although it was inhibitory to the growth of epithelial cells (Belford et al., 1995).

To characterise further the growth factor component of milk, which relates the activity of factors known to be present in bovine milk (IGF-I, and IGF-II, PDGF, FGF-1 and FGF-2 and TGF- α) to cell growth activity of concentrated whey-derived fraction, Belford et al., (1997) concentrated the cells growth activity from the whey fraction using cation-exchange chromatography. The resultant fraction has been shown to be remarkably potent in stimulating the growth *in vitro* of myoblast and fibroblast cells including rat L6 myoblasts and Balb/c3T3 fibroblasts (Belford et al., 1995). Several growth factors have been identified in this extract, including fibroblast growth factors (FGF1 and FGF-2) (Roger et al., 1995), IGF-I and IGF-II, PDGF and TGF- ß (Rogers et al., 1996). However, a cocktail of recombinant growth factors containing those growth factors stimulated the growth of Balb/c3T3 cells to a level equivalent to only 50% of that of WGFE (whey growth factor extract) (Belford et al., 1997). Results here suggested that much of the Balb/c3T3 cell-stimulating activity present in WGFE remains uncharacterised.

1.2.5   Identification of betacellulin (BTC) as a major peptide growth factor in the bovine whey

To further characterize growth-promoting factors in bovine whey extract, Dunbar et al., (1999) discovered the presence of a Balb/c 3T3 cell growth-promoting factor capable of displacing ^{125}I-rhEGF from EGF receptors present on AG2804 cells. Then this growth factor has been identified from bovine whey, and by implication from bovine milk, as BTC.

BTC was originally identified as a growth factor in the conditioned medium of mouse pancreatic b-cell carcinoma (insulinoma) cell line (Shing et al., 1993) and has since been identified in humans (Sasada et al., 1993). The mature form of mouse BTC exists as a 32kDa glycoprotein composed of 80 amino acid residues derived from a 178-residue membrane-bound precursor. The amino acid sequence of BTC

exhibits significant overall similarity with other members of the EGF family. BTC is a recently described member of the EGF family (Pathak et al., 1995).

Purified bovine BTC (bBTC) from WGFE migrated on SDS –PAGE at approx. 21-22kDa (Dunbar et al., 1999). This is in contrast with 32 kDa of mature BTC. This difference is probably due to variable states of glycosylation between two molecules. bBTC contains the six–cysteine consensus motif ($CX_7CX_4CX_{10}CX_1CX_8C$) characteristic of other members of the EGF family. The key residue between the fourth and fifth cysteine residues, which defines the EGF receptor contact point (Campion et al., 1993), was identified as a valine residue in bBTC.

1.2.6 Other growth promoting activity in milk

Hagiwara et al. (1995) demonstrated that both bovine and human Lactoferrin (LF) had the ability to promote proliferation of intestinal epithelial cells and LF was an activator of DNA synthesis in some cell lines (Hagiwara et al., 1995). In recent studies of peptides from casein that stimulate DNA synthesis, several active peptides were isolated from a tryptic hydrolysate of ß-casein, including Arg-Glu-Thr-Ile-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Pro-Glu-Tyr-Lys (human ß-casein, positions 1-18) (Azuma et al., 1989), Gln-Pro-Thr-Ile-Pro-Phe-Phe-Asp-Pro-Gln-Ile-Pro-Lys (human ß-casein, positions 105-117) (Azuma et al., 1989), and Ala-Val-Pro-Tyr-Pro-Gln-Arg (bovine ß-casein, positions 177-183)(Nagaune et al., 1989). Hagiwara et al. (1995) isolated a peptide with cell growth promoting activity from bovine LFH whose sequence was Ala-Glu-Ile-Tyr-Gly-Thr-Lys-Glu-Ser-Pro-Gln-Thr-His- Tyr - Tyr, corresponding to residues 79-93 of bovine LF.

1.2.7 Discovery of milk peptide fragment from αs_2- casein (milk peptide) as a mitogenic factor for rat mammary fibroblast cell line (Rama 27)

In 1996, Liu, Wilkinson and Smith (1996) purified growth promoting activity to homogeneity from bovine milk. Milk peptide was purified by a combination of ion-exchange chromatography, hydrophobic interaction chromatography and reverse phase HPLC. These techniques, in conjunction with more conventional separation

techniques such as acidic extraction, salting out, gel filtration, and preparative sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), formed the basis for the original milk peptide growth factor purification protocols.

It has been shown that bovine milk contained growth promoting activity for a rat fibroblast cell line (Rama 27), that is not significantly stimulated by IGF or PDGF (M[c]Andrew, 1993; Liu et al., 1996). Amino acid sequencing showed that this growth factor activity of bovine milk is due to C-terminal fragments of αs_2- casein (see below). That the peptides isolated from bovine milk were active was confirmed by chemical synthesis of the peptide (Liu et al., 1996). Since milk peptide is a novel growth factor in bovine milk, whether this milk peptide would stimulate DNA synthesis in other cell lines and their mechanism of action are unknown.

1.2.8 The function of growth promoting activity in milk

In addition to nutrients, vitamins and minerals, milk contains a variety of growth factors that are thought to be important in the regulation of growth and secretory functions of maternal mammary tissue and, in the newborn, regulation of growth, development, and maturation of the gut and immune system (Grosvenor et al., 1992).

In human milk, EGF, TGF-α, TGF-β, insulin-like growth factor-1, and others have been detected (Koldovsky, 1994b). Establishing the biological significance of these polypeptides has proved very difficult (Murphy, 1998). In agricultural animals the stimulatory effects of colostrum and milk on gastrointestinal growth and development have long been recognized (Odle et al., 1996). Calf mortality rates of almost 10 % are common, and in about half of these, persistent diarrhoea has been a contributory factor. The potential importance of growth factors in milk is therefore a major focus of research.

There are large variations in growth factor concentrations in the milk of different species. EGF, for example, is present at 10 times greater concentration in mouse milk than in rat and human milk (Koldovsky, 1994b). EGF is prone to proteolytic digestion in the gut lumen, and so there are difficult questions with regard to

bioavailability. It may be that in young infants in whom the digestive processes are relatively immature, the survival of EGF in the gut lumen may be greater. EGF breakdown may also be reduced if it is ingested with other dietary proteins because these may competitively inhibit the digestive enzymes. Finally, it has been suggested that the immature neonatal mucosal barrier may allow EGF to cross the suface epithelium, thus enabling it to bind to the basolateral EGF receptor (Murphy, 1998).

Studies have shown that oral administration of EGF can stimulate gastric and duodenal mucosal growth in adult rats, and in neonatal animals increased growth of stomach, small intestine, and colon have been reported (Rao et al., 1991). Interpretation of such studies and of their significance in the human infant is difficult. The true significance of EGF and other growth factors in human milk requires further study (Murphy, 1998).

In addition, a number of studies indicate that milk-derived EGF is required as regulator of postnatal gut development in the suckling young of rats, mice and pigs (Berseth 1987, Popliker et al. 1987, Shen et al. 1996, 1998), and influences the growth and development of the post-natal liver (McCuskey et al, 1997), and spontaneous intestinal bacterial translocation in the newborn (Okamoto et al. 1985).

Recently many studies have focused on the function of growth factor in bovine whey/milk protein. Whey protein contains active components that can activate osteoblast cell proliferation and differentiation. These active components can probably permeate or be absorbed by intestines. Takada et al., (1996) proposed the possibility that the active component in whey protein plays an important role in bone formation by activating osteoblasts. Rayner et al. (2000) demonstrated that mitogenic extract derived from bovine whey had considerable wound healing activity, and, given the mixture of growth factors present, they speculate that it may have potential as a clinical treatment to stimulate healing of chronic ulcers and other problem wounds. Besides, a polymeric diet containing active TGF-β2 was effective in inducing clinical and biochemical remission in adolescent Crohn's disease patients (Donnet-Hughes et al., 2000).

Although the significance of betacellulin occurring in bovine milk for the growth and development of suckling neonate is presently unknown, accumulating evidence supports a role for betacellulin in gut development (Bastian et al., 2001). The erb B receptors are known to bind betacellulin in gut and in gastrointestinal cancers (Quirke et al., 1989, Jankowski et al. 1992, Prigent et al. 1992, Kataoka et al. 1998, Noguchi et al. 1999), while betacellulin itself is expressed in adult gastrointestinal tract (Seno et al. 1996). Betacellulin can stimulate proliferation of rat IEC-6 cells *in vitro* and gastrointestinal epithelial cells in vivo following systemic administration to rats (Bastian et al., 2001).

1.3   Growth Factor Receptors

It has long been known that the biological actions of peptide growth factors are mediated by cell surface receptors, but only within the last few years has intimate knowledge of these membrane glycoprotein structures become available. The induction of DNA synthesis and biological effects of growth factors depends on the interaction of the growth factor with specific, high-affinity receptors located in the plasma membrane of the target cells. The main ' families' of growth factor receptors are the tyrosine kinases, the G-protein associated seven-helical domain class, the multichain 'cytokine' family, and the serine/threonine kinases. Each family exhibits fundamental similarities in their mechanism of action which explains how different types of growth factors can elicit apparently similar cellular responses.

1.3.1   G-protein -linked receptors

Many intercellular signals such as bombesin are received at the surface by specific receptors, which upon activation transduce the signal to the appropriate cellular effector system via GTP-binding proteins (G-proteins). The sequenced receptors that are known to function via G-protein mediation all display a common seven transmembrane helical topology and comprise a family of several hundred receptors (Rens-Domiano and Hamm, 1995). This family of receptors resembles the light-activated receptor rhodopsin and α-adrenergic membrane-spanning domains linked

by polypeptide loops. The primary sequence identity in the transmembrane domain of these receptors ranges from 85-95% for species homologs of a given receptor, to 60-80% for related subtypes of same receptor, to 35-45% for other members of the same family, down to 20-25% for unrelated G-protein-coupled receptors (Strader et al., 1994). In the case of the bombesin and related mitogenic receptors the coupled 'downstream event' is the hydrolysis of membrane phosphatidylinositol lipids by phospholipase C, liberating inositolpolyphosphates and diacylglycerol (DAG). Thus although the design of the G-protein linked receptor is quite distinct from the tyrosine kinase receptor, both families induce DAG and inositol polyphosphates. The major biochemical function of DAG is the activation of a key signalling enzyme, protein kinase C.

1.3.2   Receptor tyrosine kinases (RTK)

Stimulation of quiescent cells by specific polypeptide growth factors, such as EGF/TGFα, PDGF, or FGF leads to the rapid phosphorylation of tyrosine residues of a specific set of proteins. Molecular characterisation of the cell surface receptors for growth factors reveals that within the portion of the receptor located in the cytoplasm of the cell, they all contain a polypeptide domain with tyrosine kinase activity. Binding of the growth factor to this kind of receptor leads to activation of intrinsic receptor-associated tyrosine kinase activity and concomitant phosphorylation of tyrosine residues of cellular proteins (Heath, 1993). It is clear that tyrosine phosphorylation plays some important function in the cellular signalling mechanism of growth factor receptors. Protein tyrosine phosphorylation, which represents only about 0.1% of total cellular protein phosphorylation, plays a crucial role in the regulation of growth, differentiation, and cell metabolism.

1.3.2.1 Overall structure and domain organization

RTKs comprise an extracellular portion which binds polypeptide ligands, a transmembrane helix and a cytoplasmic portion which possesses catalytic activity and sites for protein-protein interactions. RTKs catalyze the transfer of the γ-phosphate of adenosine triphosphate (ATP) to the side-chain hydroxyl group of tyrosine residues in protein substrates, which include the receptors themselves (autophosphorylation).

The major RTKs consist of a single polypeptide chain and are monomeric in the absence of ligand (Figure. 1.1).

The members of the RTK family can be classified into at least 14 different subgroups based on the details of their structural organization (Figure 1.1). The members of RTK subclass I include EGF receptor family (which are discussed in detail in Section 1.3), are characterized by presence of two cysteine-rich clusters in the extracellular region and an uninterrupted tyrosine kinase domain. In contrast, subclass II RTKs, which include the insulin receptor (IR), insulin-like growth factor I receptor (IGF-II-R) and the insulin-related receptor (IRR), function as heterotetrameric structures composed of two α and β subunits that are linked by disulphide bounds (Figure 1.1) (Yarden and Ullrich, 1988; Fantl et al., 1993; Kavanaugh and Williams, 1996).

The extracellular portion of RTKs exhibits considerable diversity across the family, and typically contains a linear array of discrete folding modules such as immunoglobulin (Ig)-like domains, cysteine-rich domains, fibronectin type III-like domains and EGF-like domains. In contrast, the domain organization in the cytoplasmic portion of RTKs is more uniform. After the transmembrane helix is the so-called juxtamembrane region, followed by the tyrosine kinase catalytic domain and finally a C-terminal region. The tyrosine kinase insertion as well as juxtamembrane and C-terminal regions typically contain tyrosine residues that are autophosphorylated upon ligand binding to the receptor (Hubbard, 1999).

The hallmark feature which distinguishes the RTK family from other receptor classes is the presence of a tyrosine kinase domain in the intracellular portion. Although the overall amino acid sequences of the tyrosine kinase domain is conserved among member of the RTK family, in three subfamilies the kinase domain is interrupted by an inserted sequence (Figure 1.1). The kinase insert regions participate in the recruitment of cytoplasmic molecules into a receptor-based signalling complex (Kavanaugh and Williams, 1996).

Figure 1.1 The vertebrate receptor tyrosine kinase family
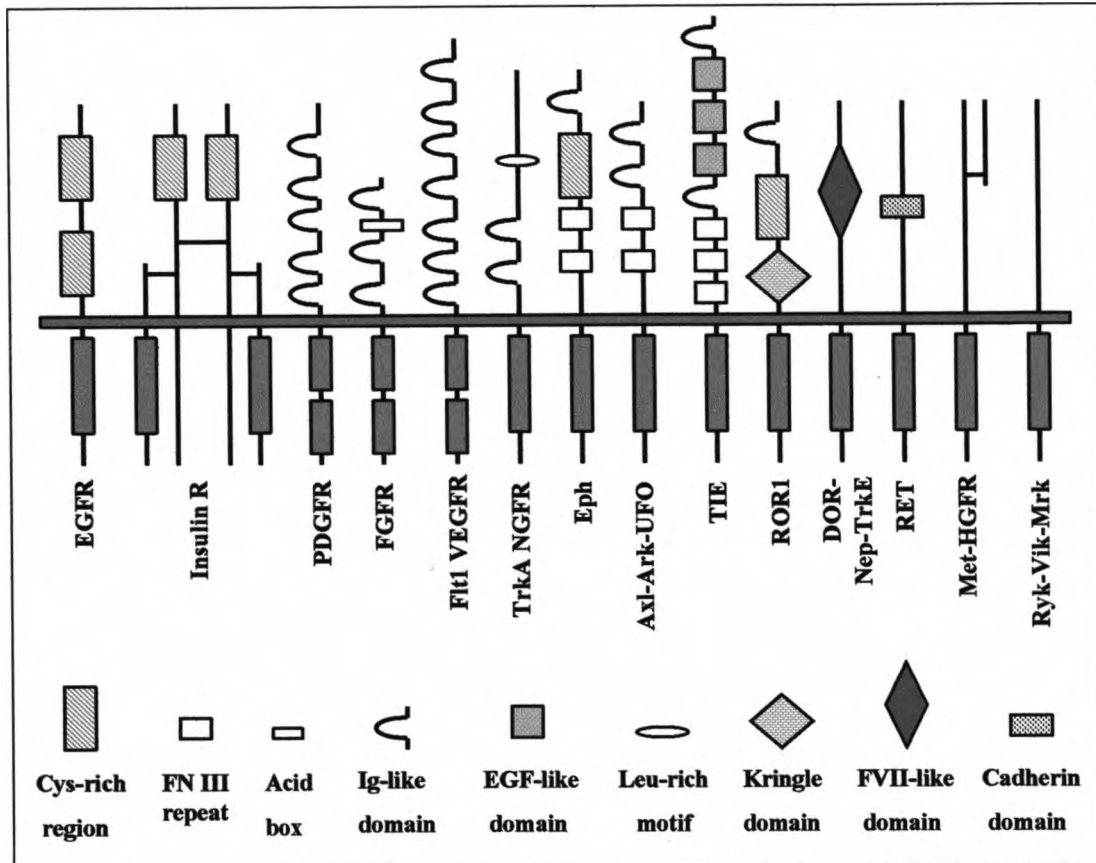
Figure 1.1 is a proposed classification scheme based on structural features. Motifs in the extracellular domains are indicated in the legend; grey boxes represent the tyrosine kinase domains. Receptors representative of each class are indicated; multiple names that have been given to apparently identical proteins are included. Abbreviations: Cys-rich, cysteine-rich; FN III, fibronectine type III repeat; Ig-like, immounoglobulin-like domain; leu-rich, leucine-rich domain; FVII-like, factor VII-like domain. (Adapted from Kavanaugh and Williams, 1996).

1.3.2.2 Initiation of signaling: dimerization and phosphorylation

In general, activation of RTKs requires two processes: stimulation of receptor catalytic activity and creation of docking sites for downstream signalling proteins. In most cases, autophosphorylation of tyrosines in the so-called activation loop (A-loop) in the tyrosine kinase domain leads to stimulation of catalytic activity (Figure 1.2), while autophosphorylation of other tyrosines generates binding sites for proteins containing phosphotyrosine-recognition domains, such as Src homology 2 (SH 2) or the phosphotyrosine-binding (PTB) domain (Pawson, 1995). All RTKs identified to date contain between one and three tyrosines in the A-loop of the tyrosine kinase domain, comprising subdomains VII and VIII of the protein kinase catalytic core (Hanks et al., 1991). Phosphorylation of these tyrosines has been shown to be critical for stimulation of catalytic activity and biological function for RTKs such as the insulin receptor (Ellis et al., 1986), the IGF I receptor (Kato et al., 1994), the FGF receptor (Mohammadi et al., 1996) and so on. The major exception to catalytic enhancement via A-loop autophosphorylation appears to be the EGF receptor subfamily. Although a tyrosine in the A-loop is conserved in this subfamily, substitution with phenylalanine has no demonstrable effect on the signalling properties of the EGF receptor (Gotoh et al, 1992).

Dimerization, by bringing two receptor molecules into close proximity, promotes autophosphorylation, in which each member of the pair trans-phosphorylates its partner (Figure 1.2) (Heldin, 1995).

1.3.2.2.1 Dimerization in extracellular domain

Ligand binding to the extracellular portion of RTKs leads to noncovalent dimerization of monomeric receptors or a change in the quaternary structure of heterotetrameric receptors, which facilitates tyrosine autophosphorylation between cytoplasmic domains (Ullrich et al., 1990, Heldin, 1995). Dimeric ligands such as PDGF or VEGF, which are disulfide-linked, bind to their respective receptors to form a symmetric dimer, with one dimeric ligand engaging two receptor molecules (PDGF:PDGFR ratio of 1:2) via two equivalent binding sites (Hubbard, 1999). Growth factors such as EGF are monomeric and appear to possess two nonequivalent

receptor binding sites which result in formation of a complex with an EGF: EGFR ratio of 2:2 (Lemmon et al., 1997). Thus, several different configurations of ligand and receptor serve to stabilize a receptor dimer.

1.3.2.2.2 Dimerization in the cytoplasmic domain

Although ligand binding stabilizes a dimeric configuration (symmetric or asymmetric) of the extracellular domains, the spatial relationship between the cytoplasmic domains in the receptor pair is not clear (Hubbard, 1999). In the case of EGF receptor, where dimerization stimulates kinase activity independent of A-loop phosphorylation, formation of a cytoplasmic dimer could stabilize an A-loop conformation favourable for catalysis. In this case, due to steric constraints, autophosphorylation of at least some tyrosines would presumably have to occur between pairs of dimers rather than within dimers. Biochemical evidence for phosphorylation occurring between EGF receptor dimers has been reported (Sherrill, 1997). A hydrophobic patch on the surface of the C-terminal lobe of EGF receptor tyrosine kinase domain is conserved among EGF receptor subfamily members but not generally in the RTK family.

Many chimeric receptors in which the extracellular portion of one RTK is fused with the cytoplasmic portion of another RTK have been engineered to study various aspects of RTK signaling (Riedel et al., 1986; Seedorf et al., 1991; McCloskey et al., 1994; Isakoff et al., 1996; Nakamura et al., 1996). In nearly all cases, ligand-dependent autophosphorylation of the chimeric receptor readily occurs. These results would suggest that either the cytoplasmic domains need not form a stable dimer (transient association is sufficient), or there is adequate flexibility in the regions separating the extracellular and tyrosine kinase domains to allow coexistence of proper cytoplasmic dimer configuration with several different extracellular dimer configurations (Hubbard, 1999).

1.3.2.2 3 Dimerization in the transmembrane helix

The discovery that an oncogenic form of the EGF subfamily member Neu (also known as Erb B2/Her2) results from a substitution of glutamic acid for valine (Val664Glu) in the transmembrane helix spurred interest in the role of the

transmembrane helix in receptor activation (Bargmann et al., 1986). This substitution causes ligand-independent dimerization and activation of the receptor. Whether the transmembrane helix physically interacts with its partner during RTK dimerization is not well understood.

1.3.2.3 Formation of signaling complexes

A second consequence of receptor autophosphorylation is the creation of binding sites on the receptor for signalling molecules that interact specifically with phosphotyrosine (Figure 1.2).

SH2 domains were the first class of protein domains to be recognized that specifically bind tyrosine-phosphorylated target. In some cases, the SH2 domain-containing protein has a known enzymatic function, such as phospholipase C-γ, the Ras GTPase activating protein (Ras-GAP), or the tyrosine-specific phosphatase SH-PTP2 (Syp). All of these proteins are often tyrosine kinase substrates themselves, and therefore bind not only to autophosphorylated RTKs, but also to each other and to other phosphorylated proteins. These complex interactions are highly specific. The specificity of an individual SH2 domain for its target is determined by the first 1-3 residues carboxy-terminal to the phosphotyrosine (Pawson, 1995).

Another domain identified was phosphotyrosine binding (PTB) domain. PTB domain was first identified in the amino terminus of the adapter protein Shc and a related molecule, Sck (Kavanaugh and Williams, 1994). The PTB domain binds specifically to the tyrosine-phosphorylated form of several signalling proteins and is thought to represent an alternative to SH2 domains for mediating the assembly of signalling complexes during tyrosine kinase signalling. The structure of the PTB domain is different from SH2; the PTB domain of Shc recognizes residues amino-terminal to the phosphotyrosine within the motif NXXpY, in contrast to SH2 domans, which bind to carboxy-terminal residues domains (Kavanaugh et al.,1995; Gustafson et al., 1995). However, the solution structure of the Shc PTB domain is similar to that of pleckstrin homology (PH) domains which is often found together with SH2 and SH3 domains (Musacchio et al., 1993). It has been suggested that Shc and Sck might be

members of a large family of PTB domain-containing proteins (Kavanaugh et al., 1995).

SH3 domain-mediated interactions are important for proper signalling by RTKs (Pawson, 1995). One or more SH3 domains are often found in proteins that also contain SH2 domains. The adapter protein Grb2/Sem-5 contains two SH3 domains which bind to the guanine nucleotide exchange factor Sos. The Grb2-Sos complex can influence Ras activity and therefore activation of the Ras/Raf/Mek/MAP kinase signalling pathway. Whether SH3 domain-mediated interactions are regulated during RTK signalling is unknown.

Figure 1.2 Initiation of signalling by receptor tyrosine kinase
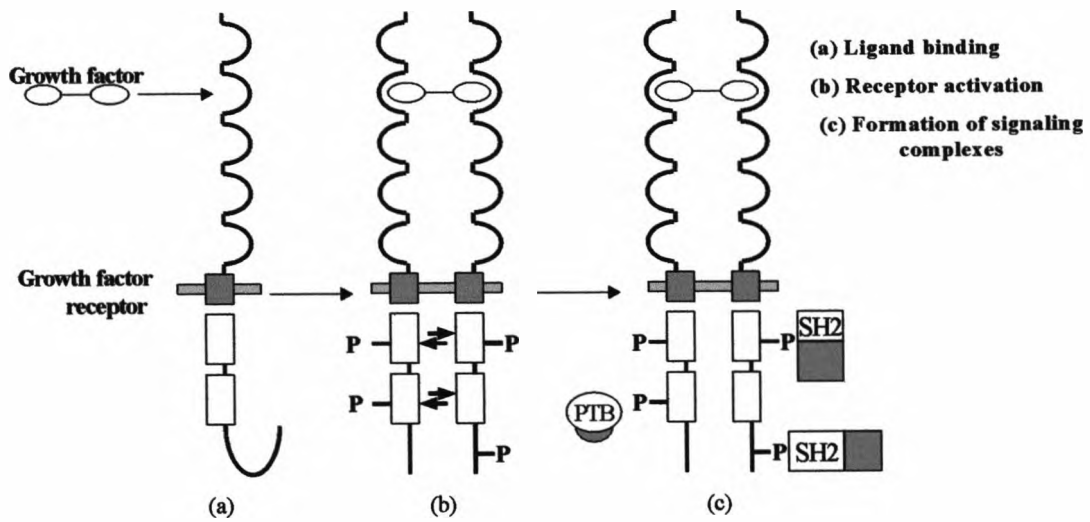
(a) A growth factor binds to the extracellular domain of its receptor, stable dimers are formed.

(b) Dimerization promotes autophosphorylation which induces a conformational change.

(c) Intracellular proteins containing SH2 or PTB domains then bind to phosphotyrosines and adjacent residues, creating signalling complexes.

1.3.2.4 Major pathways in RTK signaling

1.3.2.4.1 The PLC-γ pathway

One of the first signaling pathways to be identified in RTK signalling involved PLC-γ. PLC-γ hydrolyzes PtdIns (4,5) P_2 into diacylglycerol, an activator of protein kinase C, and inositol-1,4,5-triphosphate, which mobilizes calcium from intracellular stores. PLC-γ associates with most RTKs through its SH2 domains at specific sequence motifs. This association is thought to facilitate its phosphorylation and subsequent activation. Activation of PLC-γ is not necessary for mitogenesis in response to FGF in L6 myoblasts (Peters et al., 1992). Thus, the relationship of the PLC-γ pathway to mitogenesis is unclear.

1.3.2.4.2 PI3 kinase pathway

PI3-kinase catalyses the addition of phosphate to the 3'-position of phosphatidylinositol and other phosphoinositols. PI3-kinase was first signalling molecule to be shown to associate physically with RTKs (Coughlin et al., 1989). PI3-kinase usually consists of a heterodimer of 85-kDa proteins and a 110-kDa subunit. There are at least two 85-kDa proteins (p85α and p85β), which contain one Src homology domain 2 (SH2) (Panayotou and Waterfield, 1993). The latter binds with high specificity and affinity to tyrosine phosphorylated sequences (Koch et al., 1991), p85α associates with growth factors and mT-cSrc complex via its SH2 domains (McGlade etal., 1992; Hu et al., 1992; Klippel et al., 1992; Yoakim et al., 1992).

The evidence for a key role of PI3-kinase in the mitogenic response has come from a variety of experimental approaches, including the demonstration that the enzyme is activated following treatment of cells with various growth factors (Kapellar et al., 1994; Stephens et al., 1995) such as the PDGF (Auger et al., 1989). In addition, certain mutations in the PDGF receptor, which prevent its ability to interact with PI3K, also prevent activation of cell division. Restoration of the defect allows PDGF to activate the mitogenic response through its receptor (Valius & Kazloukas, 1993). Roche et al. (1994) has microinjected antibodies specific for the p110α subunit of PI3-kinase into quiescent fibroblasts and tested their effect on the ability of growth

factors to stimulate exit from quiescence and entry into S phase. The results indicated that PDGF and EGF required the PI3-kinase for inducing DNA synthesis, but colony-stimulating factor 1 (whose receptor is closely related to the PDGF receptor) could induce DNA synthesis in the absence of active PI3-kinase, as could two growth factors (bombesin and lysophosphatidic acid) whose receptors are functionally coupled to G-proteins.

1.3.2.4.3 Ras/ MAP kinase

MAP kinase pathway is a protein kinase cascade. Each cascade consists of no fewer than three enzymes that are activated in series: the MAP kinase or ERK (extracellular signal-related protein kinase) is activated by a MAP/ERK kinase or MEK which is itself activated by a MEK kinase or MEKK (Cobb, 1999).

The initial identification of MAP kinase (mitogenic-activated protein kinase) as a protein serine/threonine kinase was based on its capacity to phosphorylate microtubule-associated protein. Ray and Sturgill (1987) showed that insulin treatment of 3T3-L1 adipocytes led to the activation of a novel protein kinase. The activity was detected on the basis of the ability of kinase to phosphorylate a microtubule-associated protein (MAP-2) leading to the kinase being designated MAP-2 kinase. Subsequent studies revealed that this kinase activity, known as MAP kinase or ERK (extracellular signal-regulated kinase), is related to a 42 kDa protein that becomes transiently phosphorylated on tyrosine after stimulation of fibroblasts by a variety of mitogens, including EGF, PDGF, phorbol ester and insulin-like growth factor II (Rossomando et al., 1989). The induction of tyrosine phosphorylation on MAP kinase by such diverse mitogenic agents suggests that it may play an important role in the pathway of signaling events responsible for the G_0-G_1 transition in the cell cycle.

Following the description of the early steps leading from growth factor signalling, particularly EGF, an almost complete picture of Ras/MAP kinase pathway has been built up. This pathway involves key interaction between the Src-homology (SH)2- and SH3-domain-containing proteins (Pawson, 1995), which lead to activation of Raf and thus the rest of the kinase cascade. The final steps of the pathway involve direct

regulation of gene transcription through phosphorylation by MAP kinase of transcription factors (Karin and Hunter, 1995).

The details of the mechanism that links receptor occupancy to Raf activation vary for different receptors. In the PDGF and Insulin receptor pathways, different SH2-domain-containing proteins act in linking to Ras/Raf activation. In the case of insulin they interact with insulin receptor substrate-1, which is tyrosine-phosphorylated by the insulin receptor (Myers et al., 1994). In addition, it is increasingly apparent that the pathway contains many more branch points and interconnections (Pawson, 1995) than suggested by the linear pathway described in Figure1.3.

More recently, it is reported that adapter molecules containing SH2 or other phosphotyrosine binding domains, usually Grb2 and Shc, bind to the receptors (Pawson and Scott, 1997). SH3 domains of the adapters link receptors to a proline-rich region of guanine nucleotide exchange protein 'son of sevenless' (Sos) (Li et al., 1993; Pawson and Scott, 1997). Sos enhances GDP release and GTP binding to Ras. The GTP-bound form of Ras binds to Raf bringing it to the plasma membrane where its protein kinase activity is increased and the kinase cascade is activated (Vojtek et al., 1993; Morrison and Cutler, 1997). Once activated, Raf phosphorylates and activates the dual specificity protein kinase MEK1 and MEK2 (Ahn et al., 1991; Seger et al., 1992; Zheng and Guan, 1993; Wu et al., 1993). MEKs 1 and 2 are the activators of the MAP kinases ERK 1 and ERK 2. Kinase Suppressor of Ras or KSR is the most recently discovered cascade component (Therrien et al., 1995; Michaud et al., 1997). The mechanism of action of KSR remains elusive; however, it interacts with multiple components of the cascade including Raf and and stimulates Raf and downstream ERK activity in a manner dependent on its cysteine-rich domain but independent of its own protein kinase activity (Michaud et al., 1997).

Figure 1.3 The components of the Ras/MAP kinase pathways and their interactions following activation of the EGF receptor
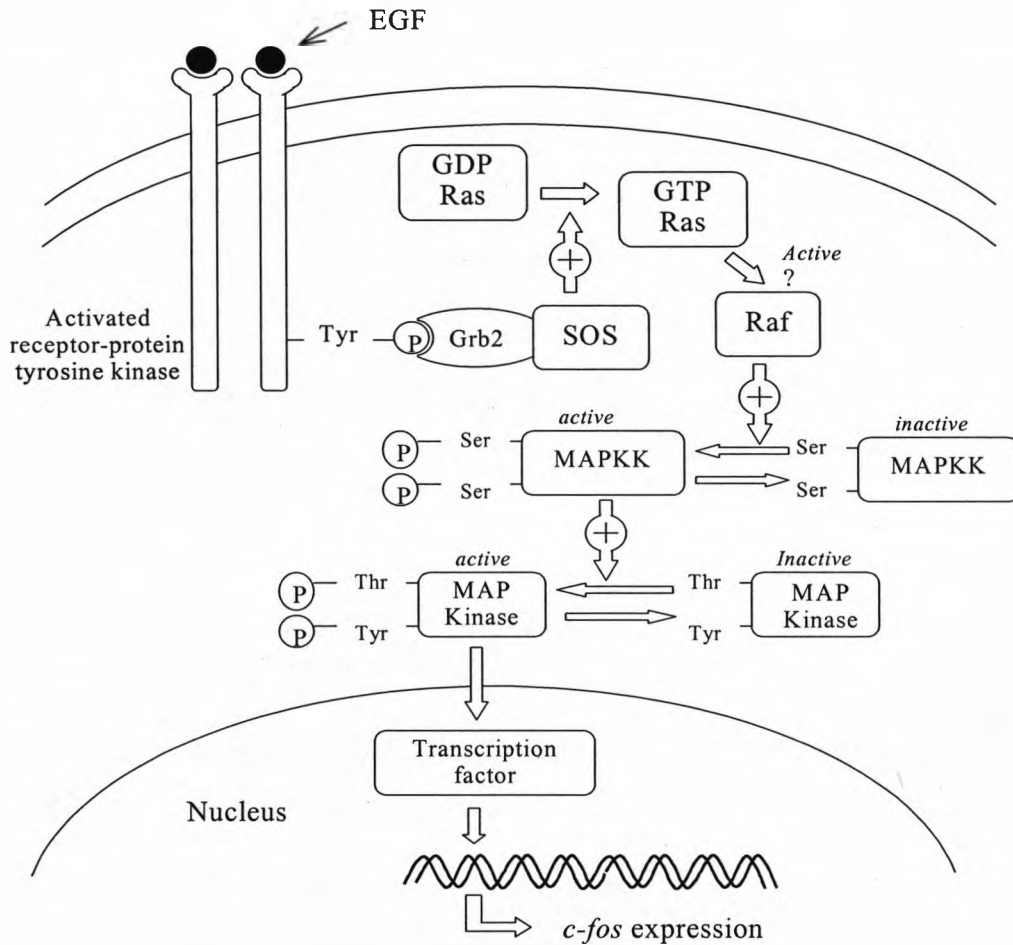
Following EGF binding to the receptor, autophosphorylation of the receptor on tyrosine allows recruitment of the SH2-containing protein, Grb2, and SOS. SOS acts to stimulate guanine nucleotide exchange on Ras. The GTP-bound form of Ras activates Raf, leading to stimulation of the entire kinase cascade and phosphorylation by MAP kinase of transcription factors.

Extensive work has now elucidated the principles of signal transduction pathways from receptor tyrosine kinases. A different way to approach the question of critical signaling pathways comes from genetic studies. Strikingly for the let-23, sevenless, and torso signaling pathways, defects in ligand or receptor can be compensated for by gain-of-function alleles of Ras (Fortini et al., 1992), Raf (Dickson et al., 1992; Han et al., 1994), Mek (Tsuda et al., 1993), or extracellular signal-regulated kinase (ERK) (Brunner et al., 1994), all of which lie in the same signaling pathway. Such results are perhaps not so surprising with Ras or Raf for which it has been known for some time that oncogenic forms can liberate signal transduction from the need for extracellular signals. Injection of oncogenic Ras into quiescent fibroblasts stimulates DNA synthesis in the absence of mitogenic growth factors (Morris et al., 1989). Oncogenic Ras and Raf will also mimic the effect of nerve growth factor (NGF) in stimulating neurite outgrowth in PC12 cells (Bar-Sagi and Ferasmisco, 1985; Noda et al., 1985; Wood et al., 1993). It now appears that Ras may have at least two effects, on Raf and PI3-kinase (Rodriguez-Viciana et al., 1994), and activated Ras would therefore be expected to be able to exert a multiplicity of effects. However, it is more surprising that gain-of-function mutants of MEK and ERK overcome receptor defects since they lie on a single signal transduction pathway downstream of Ras and Raf and would be expected to have more restricted effects. Experiments with PC 12 cells suggested that the duration of ERK activation is critical for cell signal decisions (Marshall, 1995).

1.4   The Epidermal Growth Factor Receptor (EGFR) Subfamily

The receptor for EGF, also called Erb B1 or HER1, is the prototype of the type I subfamily of RTKs, which includes three additional members: ErbB2/neu/HER2, ErbB3/HER3 and ErbB4/HER4. Whereas Erb B1 binds multiple distinct ligands that share the EGF-like motif (Marikovsky et al., 1995), all of known ligands of Erb B3 and Erb B4 are isoforms of the Neu differentiation factor (NDF, or Neuregulin) (Carraway et al., 1994; Peles and Yarden, 1993; Sternberg and Gullick, 1990), and no completely characterized ligand binds to Erb B2 (Dougall et al., 1994).

1.4.1   ErbB ligands and dimerization

ErbB receptors are activated by a number of ligands, referred to as EGF-related peptide growth factors (reviewed in Peles and Yarden, 1993; Riese and Stern, 1998) These are produced as transmembrane precursors, and are processed and released by proteolysis (Massague and Pandiella, 1993). There are numerous ErbB-specific ligands, each with an EGF-like domain that is sufficient to confer binding specificity. These include EGF, amphiregulin (AR) and transforming growth factor-α (TGF-α), which bind specifically to ErbB1, and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which exhibit dual specificity in that they bind both ErbB1 and ErbB4. The neuregulins (NRG) comprise the third ligand family. NRG-1 and NRG-2 (Riese et al., 1995; Busfield et al., 1997 Carraway et al., 1997; Chang et al., 1997) both bind ErbB3 and ErbB4, whereas the more recent additions to the NRG family, NRG-3 (Zhang et al., 1997) and NRG-4 (Harari et al., 1999), bind ErbB4 but not ErbB3 (Olayioye et al., 2000). Despite the large number of ligands so far identified for ErbB1, 3, and 4, as well as intensive efforts, no direct ligand for ErbB2 has yet been discovered. However, increasing evidence suggests that the primary function of ErbB2 is as a coreceptor.

Heterodimerization is governed by a hierarchy with ErbB 2 being the preferred partner of all ErbB proteins. In the absence of ErbB2, NDF (Neu differentiation factor) can trigger heterodimerization between ErbB1 and ErbB4 but neither ErbB1 nor ErbB4 appear to interact with ErbB3 (Graus-Porta et al., 1997; Tzahar et al., 1996). Heterodimers are generally biologically more active than homodimers. Co-expression of ErbB2 with Erb B1 or Erb B3 can increase the affinity of Erb B1 and Erb B3 for their cognate ligands (Tzahar et al., 1996) and increase the duration and intensity of MAP kinase activation evoked by ligand binding (Aroian and Sternberg, 1991; Beerli et al., 1995; Graus-Porta et al., 1995).

The signaling properties of the receptor dimer are modulated by the specific nature of the ligand and the individual components of the dimer. In the presence of Erb B1 and EGF, Erb B2 exhibits a different profile of tyrosyl phosphorylation and association with signaling effectors than in the presence of Erb B4 and NDF (Olayioye et al.,

1998). Even within the context of the same heterodimer, distinct ligands can differentially affect a receptor's signaling properties. In an Erb B1-Erb B4 heterodimer, NDF promotes more threonine phosphorylation and less tyrosyl phosphorylation of Erb B1 than EGF (Olayioye et al., 1998). This difference correlated with a temporally monophasic rather than a biphasic association of Erb B1 with the p85 subunit of PI3 kinase, and a failure of NDF to promote GRB2 association with Erb B1. Similarly, in the context of an Erb B2-Erb B3 heterodimer, NRG1β and NRG2β promote equivalent association of Shc and SHP2 with Erb B3 but NRG1β rather than NRG 2β stimulates stronger association of p85, Shc, and SHP2 with Erb B2 (Sweeney et al., 1998).

Among ligands that stabilize the same receptor dimer, differences in signalling properties can be accounted for at least partly by differences in the affinities of the ligand-receptor interactions. Each neuregulin has a distinct affinity for Erb B3 and Erb B4 that can be further modulated by the presence of Erb B2. Thus, heterodimers between Erb B2 and either Erb B3 or Erb B4 are bound with higher affinity by NRG2β and NRG2α than NRG1α and NRG2β (Pinkas-Kramarski et al., 1998). These differences in affinities are tightly correlated with differences in the duration and intensity of MAPK activation and proliferative responses. Thus, a broad range of strengths of ligand-receptor interactions is generated that ultimately allows a tremendous range in signal output (Moghal and Sternberg, 1999).

1.4.2 Major signaling pathways activated by Erb B receptors

Ligand binding drives receptor dimerization, leading to activation of the intrinsic tyrosine kinase and autophosphorylation of specific, C-terminal tyrosine residues (reviewed in Heldin, 1995; Weiss and Schlessinger, 1998) that provide docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Shoelson, 1997; Sudol, 1998). These include adaptor proteins such as Shc, Crk, Grb2, Grb7 and Grb1; kinases such as Src, Chk and phosphatidylinositol 3-kinase (PI3K; via the p85 regulatory subunit); and the protein tyrosine phosphatases SHP1 and SHP2. Each ErbB receptor displays a distinct pattern of C-terminal autophosphorylation sites. Examination of binding preferences of a variety of

different SH2 and PTB domains, using BioCore or phosphopeptide competition assays, combined with studies utilizing receptor autophosphorylation site mutants and co-immunoprecipitation approaches, has enabled identification of effector proteins that coupled to specific Erb B phosphotyrosine residues (Olayioye et al., 2000). It has become evident that there is a great deal of overlap in the signaling pathways activated by the four ErbB receptors. All Erb B family members, including the Drosophila and C. elegans homologs DER and Let23, couple via Shc and /or Grb2 to the MAP kinase pathway. However, there are also examples of preferential modulation of specific pathways. For example, due to the presence of multiple binding sites for p85, Erb B3 is the most efficient activator of PI 3 kinase (Prigent and Gullick, 1994). It is clear that Erb B receptors couple to specific downsteam, intracellular pathways with different efficiencies, thus affording great signaling possibilities. (Olayioye et al., 2000).

1.4.3 Crosstalk

Despite the absence of a direct ligand, the heterologous ligand, EGF, (King et al., 1998; Stancovski et al., 1994) can elevate tyrosine phosphorylation of Erb B2. This transphosphorylation reaction is preceded by the formation of noncovalent heterodimers betweeen the ligand-occupied Erb B1 and Erb B2 resulting in synergistic transforming effects that were accompanied by the appearance of high affinity binding sites for EGF (Kokai et al., 1989). These interactions are reminiscent of cross talk that occurs between the insulin receptor and the insulin-like growth factor 1 receptor (Solotoff et al., 1994) and the two types of the PDGF receptors (Hammacher et al., 1989). However, inter-receptor interactions in the ErbB family are not limited to the Erb B1-Erb B2 heterodimers because synergistic transforming activity was also induced by coexpression of Erb B3 together with Erb B2 (Alimandi et al., 1995; Wada et al., 1990). As in the case of the ErbB1 –Erb B2 combination, an increase in NDF binding affinity accompanied co-overexpression of Erb B2 and Erb B3 (Slamon et al., 1989). NDF and EGF receptors have been reported to interact with each other. NDF binding to certain tumor cells can inhibit EGF binding in a temperature-independent manner (Karunagaran et al., 1995), and EGF-dependent recruitment of PI3 kinase to Erb B3 has been demonstrated in certain cell lines (Kim

et al., 1994; Sliwkowski et al., 1994). In an effort to elucidate the role of Erb B2 in NDF and EGF receptor signaling, the expression of the protein has been selectively blocked by using intracellular antibodies (Beerli et al., 1994). This resulted in significant reduction of NDF- as well as EGF-induced intracellular signals (Graus-Porta et al., 1995; Karunagaran et al., 1996) due to reduction of dissociation rates of both ligands from their receptors (Karunagaran et al., 1996).

Apart from the network of inter-Erb B cross talk, cells integrate information from distinct signaling pathways; the EGF receptor is one point of such cross talk (Moghal and Sternberg, 1999). Mitogenic agonists of G-protein-coupled receptors stimulate proliferation through ligand- independent activation of the EGFR. These agonists strongly promote EGFR, Shc, and Gab1 tyrosyl phosphorylation, and the activation of MAP kinase (Stancoski et al., 1994; Stern and Kamps, 1988). Cytokine receptors have been implicated both in the positive and negative regulation of the EGFR activity. Growth hormone can promote tyrosyl phosphorylation of the EGFR and MAP kinase activation in tissue culture cells as well as in the livers of whole mice (Yamauchi et al., 1997).

In summary, signalling mediated by the EGFR can no longer be simply thought of as a direct linear pathway from the cell surface to the nucleus. Strikingly, regulatory mechanisms appear to exist at essentially every step. Understanding the complexities of EGFR signalling networks will help to understand the mechanism of action of milk peptide.

1.5 Insulin Receptor Subfamily

The insulin receptors are a subclass II of RTKs, where α and β chains already exist as a dimers in the absence of ligand. The human insulin receptor (IR), like the receptors for EGF and PDGF, contains intrinsic tyrosine kinase activity and undergoes tyrosine autophosphorylation during ligand stimulation (Rosen, 1987). However, unlike EGF and PDGF receptors, the human IR does not associate strongly with signalling proteins containing Src homology 2 (SH2) domains (Myers et al., 1994a; Pawson, 1995). Instead, the IR phosphorylates IR substrate (IRS) proteins (IRS-1 and IRS-2) on multiple tyrosine residues (Myers et al., 1994a; Sun et al., 1991). Many of the signalling pathways activated by the human IR require the presence of an IRS protein (Myers et al., 1994b): IRS-1 binds and activates PI3 kinase, Grb-2/Sos, SH-PTP2, and nck (White, 1994); IRS-1 also mediates insulin-stimulated $p70^{s6k}$, probably through the activation of PI3 kinase (Chung et al., 1994; Myers et al., 1994a). IRS-1 also plays a role in the stimulation of glucose uptake by insulin (Araki et al., 1994). However, not all insulin-stimulated pathways require IRS proteins, as Shc is phosphorylated directly by the human IR and then binds Grb2/Sos, providing one of the pathways to activate $P21^{Ras}$ and the MAP kinase cascade (Myers et al., 1994a,b).

The insulin-like growth factor-I (IGF-I) receptor, homologous with the insulin receptor, is a heterotetrameric protein, consisting of two $\alpha\beta$ heterodimers. The α subunit is located extracellularly and has a molecular mass of 120-130 kDa, whereas the β subunit, which is mainly binds to IGF-I localized intracellularly, is a 93 kDa protein. IGF-I binds to the IGF-I receptor with high affinity. The crossreactions of IGF-II and insulin with the IGF-I receptor are 5-10% and 0.1-1% respectively (Roth and Kiess, 1994).

The IGF-I receptors also belong to the tyrosine kinase growth factor receptor family. Tyrosine kinase activity and tyrosine phosphorylation of the receptor and intracellular substrates are essential for all actions mediated by the IGF-I receptor (Kato et al., 1994, Li et al., 1994). Binding of IGF-I to its receptor induces autophosphorylation of the receptor and phosphorylation of intracellular substrates, catalysed by the intrinsic

tyrosine kinase in the intracellular domain of receptor β subunits (Kato et al., 1994 Jones and Clemmons, 1995). In certain cell types, IGF-I stimulates phosphorylation of two types of β subunits of IGF-I receptors with molecular masses of about 97 kDa and 95 kDa (Moxham et al., 1989). The immunological properties and protein microsequencing of the two proteins have revealed the presence of IGF-I/insulin hybrid receptors (Siddle et al., 1994, Kasuya et al., 1993). The hybrid receptors can be formed in vitro using purified heterodimers from insulin receptors and IGF-I receptors (Treadway et al., 1989). Several intracellular proteins have been found to be phosphorylated after activation of IGF-I receptors (Oh et al. 1993, Yenush and White 1997). The major intracellular substrate for activated IGF-I receptor is IRS-I as well as for insulin receptor. IRS-I cDNA has been cloned and sequenced (Sun et al., 1991). Phosphorylated IRS-1 is capable of interacting with and activating several SH-2 domain-containing proteins, such as the regulatory subunit PI3 kinase, growth factor receptor-binding protein 2 (Grb2) (Cantley et al. 1991, LeRoith et al., 1995), protein tyrosine phosphatase (SHP2) (Rocchi et al. 1996, Yenush and White 1997) and other intracellular proteins (Jones and Clemmons 1995). In mice carrying a disrupted IRS-I gene, insulin and IGF-I stimulate phosphorylation of another docking protein, IRS-2 associated with activation of PI3 kinase (Araki et al. 1994, Tobe et al., 1995). This pathway becomes an alternative, IRS-1 independent pathway in insulin or IGF-I signaling.

In comparison with the insulin receptor, the IGF-I receptor is more effective in mediating cell growth (LeRoith et al. 1995). IGF-I receptors are present in insulin-secreting cells (Van Schravendijk et al. 1987). Exogenous IGF-I stimulates proliferation of rat islets (Swenne et al. 1987, Hogg et al. 1993), in which IGF-I was shown to have a more potent effect than IGF-II on DNA synthesis (Hogg et al., 1993). A proliferative effect of IGF-I was also demonstrated in the rat insulin-secreting cell line, RINm5F (Dereli et al., 1988; Zhang et al. 1998).

Although the insulin and IGF-I receptors have many structural and functional similarities, they play different roles in development and physiology. Insulin receptors are mainly distributed in tissues that are responsible for regulation of glucose homeostasis and metabolism, whereas IGF-I receptors are expressed on many

cell types and are involved in cell growth and differentiation. It has been suggested that the differences in the physiological effects of insulin and IGF-I may be due to the differential expression of their receptors in various tissues (Gronborg et al., 1993; Czech, 1989; Prager and Melmed, 1993). However, both insulin and IGF-I receptors are found in muscle, a major site of glucose disposal. In addition, insulin receptors are found at low levels on many cells that express IGF-I receptors. The substantial differences in the signal transduction pathways stimulated by these two receptors have not yet been identified (Mastick et al., 1994).

1.6 Small Peptide Growth Factors

Most peptide growth factors are really small proteins that work via tyrosine kinase receptors. There are few other small peptides that can also stimulate DNA synthesis. Among these small peptide growth factors only Bombesin and related peptides are well studied so far. A background to these studies is reviewed below.

1.6.1 Bombesin

Bombesin family of peptides are a group of small peptides that can act as mitogens. This has been shown *in vitro* in Swiss 3T3 murine embryonal fibroblasts (Rozengurt and Sinnett-Smith, 1983) and *in vivo* in human small cell lung carcinoma as an autocrine growth factor (Carney et al., 1987; Cuttitta et al., 1985; Maruno et al., 1989). The 14-amino-acid peptide bombesin was isolated in 1971 from the skin of the European frog Bombina bomina, using a smooth muscle contraction assay (Anastasi et al. 1971). A number of bombesin-related peptides were isolated from different amphibian species and grouped into three subfamilies according to their C-terminal tripeptide (Erspamer et al, 1978). In 1978, a structurally related peptide was isolated from porcine gastric tissue, using the rise of plasma immunoreactive gastrin-releasing peptide (GRP) (McDonald et al., 1979) as assay. Bombesin and related peptides that act through a seven transmembrane receptor are the only small peptide growth factors known at present.

1.6.1.1 Mechanism of action of Bombesin

Bombesin is a potent mitogen for Swiss 3T3 cells (Rozengurt, 1988). In serum-free medium it stimulates DNA synthesis and cell division in the absence of other growth-promoting agents. The ability of bombesin, like PDGF, to act as a sole mitogen for these cells contrasts with other growth factors which are active only in synergistic combinations (Rozengurt, 1986). The mitogenic effects of bombesin are markedly potentiated by insulin, which both increases the maximal response and reduces the bombesin concentration required for half-maximal effect. Structurally related peptides found in mammals (GRP, NMB-neuromedin B, NMC) have similar mitogenic effects (Zachary and Rozengurt, 1985).

To determine how bombesin/GRP stimulates mitogenesis, Rozengurt's group (1991) sought specific receptors on Swiss 3T3 cells, using radiolabelled [^{125}I]GRP. Binding measurements and chemical cross-linking experiments using this ligand show that bombesin-like peptides interact with specific, high-affinity receptors located on the cell surface. [^{125}I]GRP binding is inhibited by various bombesin-like peptides in proportion to their ability to stimulate DNA synthesis but not by structurally unrelated mitogens (Zachary and Rozengurt, 1985). Thus, bombesin and related peptides interact with receptors that are distinct from those for other mitogens in Swiss 3T3 cells. The properties of the mitogenic bombesin receptor have been examined in membrane preparations from Swiss 3T3 cells, i.e. in the absence of ligand internalisation and degradation (Sinnett-Smith et al., 1990). [^{125}I]GRP binding to such membranes is specific, saturable and reversible. The results are consistent with the existence of a homogeneous population of bombesin/GRP binding sites in membranes of Swiss 3T3 cells.

The physical properties of the bombesin / GRP receptor have been investigated using an affinity labelling method (Rozengurt, 1991). The bombesin / GRP receptor is a glycoprotein of apparent M_r 75000-85000 with N-linked carbohydrate side-chains and a polypeptide core of M_r 43000. The availability of membrane preparations that retain specific bombesin receptors is useful in the identification of the signal-transduction mechanism(s) that couples the receptor for these neuropeptides with the generation of intracellular events. A decrease in ligand affinity for receptors produced

by added guanine nucleotides was seen that is characteristic of a receptor G protein interaction (Bourne et al., 1988). Sinnett-Smith et al. (1990) demonstrated that the non-hydrolysable GTP analogue GTP-y-S causes a specific and concentration-dependent inhibition of [^{125}I]GRP binding and cross-linking to 3T3 cell membranes. The effect is due primarily to an increase in the equilibrium dissociation constant (K_d) rather than to a decrease in the number of receptors. This modulation of ligand affinity by guanine nucleotides strongly suggests that a G protein couples the mitogenic bombesin receptor with intracellular effector systems.

In this study, we want to use Bombesin as a model to try finding out whether the milk peptide acts in the same way as Bombesin to stimulate the DNA synthesis.

1.6.2 Milk peptide

Milk peptide is fragment of αS-2 casein (192-222), which stimulates DNA synthesis in the rat mammary fibroblast cell line (Rama27) (Liu et al, 1996). The sequences of peptides are:

KVIPYVRYL
TKVIPYVRYL
KTKVIPYVRYL
KPWIQPKTKVIPYVRYL
PQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

They are a nested series of sequences of 5 peptides. They corresponded to the C-terminus of bovine αS-2 casein. In this study, they are termed as milk peptide. Whether milk peptide has activity in other cell lines is not clear. How this peptide can stimulate DNA synthesis in Rama 27 cells is also not known.

1.6.3 Synthetic peptides

To assess structure-function relationships in the EGF-like family of molecules, several groups have successfully synthesized biologically active molecules corresponding to complete sequence of one of the EGF-like growth factors: mouse EGF (Akaji et al. 1985; Heath and Merrifield 1986), human TGF-α (Tam et al. 1986), rat TGF-α (Tam et al. 1984). This demonstrates that proper folding of the polypeptide chain and formation of correct disulfides can occur in vitro.

There is understandable interest in determining whether defined fragments of the EGF structure might be capable of acting as agonists or antagonists of EGF. One study (Komoriya et al. 1984) reported limited success with peptides corresponding to mouse EGF sequences Cys20-Cys31 (no disulfide) and Tyr14-Cys31 (one disulfide). Also, Leu15-Arg53, containing two disulfides corresponding to the second and third disulfide loops, was reported to have EGF-like activity (Heath and Merrifield 1986) as was the rat TGF-α sequence Cys33-Cys 42 (no disulfide) (Nestor et al. 1985). However, the potency displayed by any of these synthetic peptides was very low, about 0.01% of the potency of the intact molecule. Subsequent studies have suggested even lower or nondetectable activities for these and other synthetic fragments corresponding to the N-terminus, C terminus, and each disulfide loop (Komoriya et al. 1984; Nestor et al, 1985; Heath and Merrifield 1986; Defeo-Jones et al. 1988; Darlak et al. 1988). The results of these studies suggest that it will probably be necessary to include all three disulfide loops in any synthetic derivatives of this growth factor that retain significant biological activity.

1.6.3.1 VREKS

'VREKS' is a fragment of bFGF which is a synthesized peptide. The basic fibroblast growth factor (bFGF) was characterized as a 146 amino acid polypeptide with mitogenic properties for fibroblasts as well as for a variety of neuroectoderm- and mesoderm-derived cell types (Gospodarowicz et al., 1986, 1987; Gospodarowicz, 1989; Thomas, 1987). VREKS contains 12 amino acids and has the ability to stimulate DNA synthesis in Rama 27 cells. The sequence is: VREKSDPHIKGGC. It

is clear that bFGF stimulates DNA synthesis through tyrosine kinase receptors. However, the mechanism of VREKS action is not clear.

1.6.3.2 KVIPY

'KVIPY' is a synthetic peptide from the milk natural material. To confirm milk peptide activity was from C-terminal fragments of αs_2- casein, the effect of synthetic milk peptide was tested in Rama 27 cells. It was shown that synthetic milk peptide can stimulate DNA synthesis in Rama 27 cells (Liu, 2002) to a modest degree. The sequence is KVIPYVRYL.

1.7 Aims and Objectives

Although bovine milk containing growth factor activity for a fibroblast cell line derived from normal rat mammary tissue has been reported, and the activity is known to be from fragment of αS_2-casein (179-222), the mechanisms by which it exerts this effect remain unknown. The overall aim of this project is to verify milk peptide as a novel growth factor for rat fibroblasts (Rama 27), to investigate its mechanism of action and the major signalling pathway which is activated in the response to milk peptide in this cell line. In this thesis the specific objectives were: verification that the milk peptide is a novel growth factor by the purification of highly pure milk peptide from bovine milk, and by cloning and expressing milk peptide in *E coli* to overcome the difficulties raised by the poor efficacy of the synthetic peptide; Understanding the action of milk peptide through study of the response of receptor molecules and of additional phosphorylation events and by the use of major pathway inhibitors to identify signalling pathways which seem to be involved in the response to milk peptide.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General materials

2.1.1.1 Growth factors

Mouse EGF purified from submaxillary glands	Pepsyn Ltd (Liverpool)
KVIPY	Pepsyn Ltd
VREKS	Pepsyn Ltd
bFGF	Produced in *E. coli* and purified by heparin-affinity chromatography (Ke et al., 1992)
Bombesin	Sigma
Insulin	Sigma
Insulin – like growth factor II	Sigma

2.1.1.2 Routine culture of Rama 27 cells

Bovine serum albumin (BSA)	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dulbecco's modified Eagle's medium (DMEM)	Life Technologies
Fetal Calf serum (FCS)	Harlan Sera-labs
Hydrocortisone	Sigma-Aldrich
Insulin	Sigma-Aldrich
Phosphate buffered saline (PBS)	Life Technologies
Trypsin –EDTA	Life Technologies
Tissue culture plastic ware	Falcon
Filters	Millipore (Bedford, MA, USA).

Geneticin (G418 sulphate)   Gibco.

L-glutamine (200mM)   Sigma.

Isoton   Coulter Electronics (Luton, UK).

Penicillin (10,000 IU/ml) and streptomycin   Gibco. (10 mg/ml) in normal saline

Sodium bicarbonate (7.5% w/v)   Gibco.

Sterilin tubes   Bibby Serilin Ltd (Stone, UK).

TCA   BDH

Trypsin, 2.5% (w/v) in PBS   Gibco.

Versene (200 mg/ml EDTA in PBS, pH 7.2)   Gibco.

Dithiothreitol   Sigma

Sephadex G25   Pharmacia.

Chloramine T   Sigma

$[^{125}I]$ NaI (100 mCi/ml)   Amersham International.

KI   Sigma

$Na_2S_2O_5$ Sigma

2.1.1.3 $[^3H]$-thymidine incorporation DNA synthesis assays

Methyl $[^3H]$-thymidine (1 mCi/ml)   ICN

Ethanol   Sigma.

NaOH BDH

Trichloroacetic acid (TCA)   BDH

ULTIMA scintillant   Packard Canberra

2.1.1.4 Signalling pathway inhibitors

PD-098059   Calbiochem

Wortmannin   Calbiochem

2.1.1.5 SDS-PAGE

Acrylamide / bis-acrylamide mixture   Severn Biotech Ltd

Ammonium persulphate   BDH

β-mercaptoethanol   BDH

Brilliant blue stain   Sigma-Aldrich

Bromophenol blue   Sigma-Aldrich

Glacial acetic acid	BDH
Glycerol	Sigma-Aldrich
Glycine	BDH
N,N,N',N',Tetramethylethylene diamine (TEMED)	BDH
Polyethylenesorbitan monolaurate (Tween-20)	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	BDH
Tricine	Sigma
Silver nitrate	BDH
EDTA	Sigma
Sodium carbonate	Sigma
Sodium thiosulphate	Sigma
Sodium acetate	Sigma
Formaldehyde	BDH
Glutaraldehyde	BDH

2.1.1.6 Western blotting

ECL Western blotting detection reagent	Amersham International.
Fast green stain	Sigma-Aldrich
Methanol	BDH
NaCl	BDH
Tris(hydroxymethyl)methylamine (Tris)	BDH
Polyvinylidene filter (PVDF) membrane	Millipore
X-ray film	Kodak
Hybond-C	Amersham International.
"Marvel", fat-free, dried skimmed milk	Premier Brands UK Ltd. (Stafford, UK)

2.1.1.6.1 Antibodies

Monoclonal anti-phosphotyrosine (PY 20)	Transduction Laboratories
Anti-phospho-EGF Receptor (y1173) (Mouse)	Upstate biotechnology
Phosphorylated p42/p44 MAP Kinase	
E10 Monoclonal Antibody (Mouse)	Cell signaling Technology
Anti-mouse horseradish peroxidase	
(HRP)-conjugated second antibody	DAKO Ltd

2.1.1.6.2 Molecular weight markers

Prestained SDS-PAGE Standards low range	Bio-Rad
Ultra-low molecular weight range	Sigma
Color molecular weight marker	Sigma

2.1.2 Materials for purification of protein

CM-sepharose	Pharmacia
Butyl sepharose	Pharmacia
C_4 column	Beckman
HCl	Sigma
$(NH_4)_2SO_4$	Sigma
Dialysis tube	Scientific laboratory supplies Ltd (UK)
Acetonitrile (HPLC 'far UV' analytical grade)	Fisons
TFA	Sigma
NaH_2PO_4	Sigma
NaCl	Sigma

All the chemicals were appropriate Analar quality.

2.1.3 Materials for molecular biology procedures

λDNA size markers	RocheDiagnostics (Germany)
DNA molecular weight marker VIII	RocheDiagnostics (Germany)
Agarose	Pharmacia.
Luria broth	Bio gene limited.
Luria agar	Bio gene limited.
Ampicillin	Sigma.
Caesium chloride	BDH
Ethidium bromide	Sigma
Isopropylthio-b-D-galactoside	Novabiochem

DH5α	Dr P Turner, University of Liverpool
BL21	Novagen
pET-16b vector	Novagen
Restriction endonucleases and compatible buffer	Boehringer Mannheim New England Biolabs Inc
Sucrose	BDH
T4 DNA ligase	New England Biolab, Inc.
Taq polymerase	Promega (Southampton, UK)
Isopropanol	BDH

2.1.4 Chemical synthesis of oligonucleotides

Fusion milk peptide gene	MWG
PCR oligonucleotide primer	MWG

2.1.5 Cultured cell lines

Name of cell line	Description	Reference
Rama 27	Fibroblastic in character	Rudland et al., 1984
Rama 29	Elongated myoepithelial-like	Bennett et al., 1978
Rama 37	Cuboidal epithelial	Dunnington et al., 1983
Rama 37 E8	Myoepithelial - like	
Swiss 3T3	Mouse fibroblasts	Rozengurt, 1986
CHO	Chinese hamster ovary	Tzahar et al., 1996
CB1	Chinese hamster ovary transformed with EGFR	Tzahar et al., 1996

2. 2   Composition of Reagents and Buffers

2.2.1   General reagents and buffer

2.2.1.1 Cell culture

Insulin stock solution	1 mg/ml Insulin
	5 mM HCl
	150 mM NaCl
Routine medium (RM)	DMEM
	5% (v/v) FCS
	50 ng/ml Insulin
	50 ng/ml Hydrocortisone
	0.75% (w/v) sodium bicarbonate
	20 mM l-glutamine
	1000 IU/ml penicillin
	1 mg/ml streptomycin
Step Down medium (SDM)	DMEM
	250 µg/ml BSA
Freezing medium	DMEM
	20% (v/v) FCS
	7.5% (v/v) DMSO
Hydrocortisone stock	5 µg/ml in 25% (v/v) ethanol in PBS.
Insulin stock	5 µg/ml in 1 mM HCl/PBS.
PBS	137 mM NaCl, 2.7 mM KCI, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4.
Trypsin/EDTA solution	25 ml versene (0.53 mM EDTA) containing 0.5 ml trypsin, 2.5% (w/v).

2.2.1.2. Signalling pathway inhibitor reagents

PD-098059 stock solution	10 mM in 100% anhydrous DMSO
Wortmannin	1 mM in 100% anhydrous DMSO

2.2.1.3 [^{3}H]-thymidine DNA synthesis assay reagents

[^{3}H]-thymidine stock solution	40 μCi/ml [^{3}H]-labelled thymidine containing 40 μM unlabelled thymidine
TCA	5% TCA
Ethanol	95% ethanol
NaOH	0.2M NaOH

2.2.1.4 SDS-PAGE
2.2.1.4.1 Laemmli method

Acrylamide stock mixture	30% (w/v) acrylamide 0.8% (w/v) bis-acrylamide mixture
SDS-PAGE stacking gel	4 % (v/v) acrylamide stock solution 0.125 M Tris-HCl, pH 6.8 0.1% (w/v) SDS 0.1% (v/v) TEMED 0.05% (w/v) ammonium persulphate
SDS-PAGE resolving gel	7.5% (v/v) acrylamide stock solution 0.375 M Tris-HCl, pH 8.8 0.1% (w/v) SDS 0.033% (w/v) ammonium persulphate 0.033% (v/v) TEMED

Electrophoresis buffer	25 mM Tris-HCl
	250 mM glycine
	0.1% (w/v) SDS
	pH 8.3
Sample buffer	0.0625 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue

2.2.1.4.2 Schagger and Von Jagow method

Resolving gels	16.5% (w/v) acrylamide, 10% acrylamide, 1M Tris-HCl (pH 8.0), 0.1% SDS
Stacking gels	6 % (w/v), 1M Tris-HCL (pH 8.0), 0.1% SDS
Anode buffer	0.2 M Tris-HCl (pH 8.9)
Cathode buffer	0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (w/v) SDS (pH 8.25)
Sample buffer	0.05 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue
Gel Stain	0.025% (w/v) Coomassie brilliant blue
	40% (v/v) methanol
	10% (v/v) glacial acetic acid
Gel destaining solution	40% (v/v) methanol

2.2.1.5 Western blotting

Lysis buffer	0.125 M Tris, pH6.8
	4% (w/v) SDS
	20% (v/v) glycerol
	0.002% (v/v) Bromophenol blue
	5% (v/v) β-mercaptoethanol
	(added just before use)
Transfer buffer	25 mM Tris
	190 mM Glycine
	0.1% (w/v) SDS
	25 mM methanol
Tris-buffered saline (TBS)	20 mM Tris
	0.9% (w/v) NaCl
	pH 6.8-7.2
TBS-Tween (TBST)	20 mM Tris
	0.9% (w/v) NaCl
	0.1% (v/v) Tween-20
Blocking buffer (TBST-milk)	20 mM Tris
	0.9% (w/v) NaCl
	0.1% (v/v) Tween-20
	5% (w/v) non-fat milk powder
Membrane Stain	1 mg/ml Fast Green stain
	40% (v/v) iso-propanol
	10% (v/v) glacial acetic acid
Membrane destaining solution	40% (v/v) iso-propanol
	10% (v/v) glacial acetic acid
Coomassie blue dye stain	0.2% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol
	1% (v/v) glacial acetic acid
Destaining solution	50% methanol

2.2.2 Buffers for purification

CM sepharose column	0.02 M NaH_2PO_4, pH6.0, 0.05-0.7 M NaCl.
Butyl sepharose column	0.02 M NaH_2PO_4, 4M NaCl, pH6.5, 4 M NaCl-0 M NaCl.
Reverse phase HPLC	0.1% TFA, 0.1% TFA, CH_3CN.

2.2.3 Buffers for molecular biological studies

LB-broth	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.
LB-agar	LB-broth containing 1.5% (w/v) bacto-agar.
TBE buffer	89 mM Tris, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3.
Agarose gel loading buffer	0.1% (w/v) bromophenol blue, 20% (v/v) glycerol in sterile water.
SOC medium	2% (w/v) tryptone, 10 mm NaCl, 2.5 mM KCl, 0.5% (w/v) yeast extract, 10 mM MgCl2, 20mM glucose, pH 7.0.
Lysis buffer	50 mM NaH_2PO_4 (pH7.8), 300 mM NaCl and lysozyme (5 μg/ml).

2.3 Methods

2.3.1 General methods

2.3.1.1 Cell culture

2.3.1.1 1 Rat mammary cells culture

The purpose of tissue culture is to provide a source of healthy responsive cells for bioassay. The cell lines used were Rama 27, Rama 29,Rama 37, Rama 37E8. Cells were grown in a monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, Scotland) supplemented with 5% Fetal Calf Serum (FCS; Imperial Labs), FCS is a mixture of nutrients and certain growth factors, 50ng / ml Insulin and 50 ng /ml Hydrocortisone. This medium is referred as 'routine medium' (RM). The cells were maintained at 37°C in a humidified incubator gassed with 10% carbon dioxide in air. Cells were subcultured when 80-90% confluent. The purpose of this was to produce a constant supply of healthy growing cells and provide back up plates in case of accidental biological contaminations.

The process of passaging involves the removal of the routine medium from the culture plate using a vacuum line in sterile conditions. The exposed cellular monolayer is then washed twice with 10 ml Phosphate Buffered Saline (PBS). The PBS was then removed in the same manner as the routine medium, and the cells are allowed to incubate at 37°C with 1 ml per plate trypsin/EDTA for 3-5 minutes, until the majority of the cells had rounded up and detached from the surface of the dish, then 8 ml routine medium was added to each plate. The cells were resuspended in this volume by vigorous pipetting. This suspension was divided into 8 new plates, and a further 10 ml of routine medium added. But, for Rama 37, cells were incubated 5 minutes and washed in versene, before using trypsin/EDTA; the versene performs this task by acting as a calcium ion chelater.

2.3.1.1.2 Swiss 3T3 cell culture

Stock culture Swiss 3T3 cells were maintained in DMEM containing 10% FCS. For experimental purposes cells were subcultured in the above medium at least 6 days after the last change of medium (Rozengurt and Sinnett-Smith, 1983), this is to allow

cells entering Go phase to accumulate. Or medium was changed to one containing 2/3(v/v) DMEM, 1/3(v/v) Waymouth medium, 5% FCS and 1% Newborn Calf Serum and incubated for 3 days.

2.3.1.1.3 CHO culture

Stock culture CHO cells were maintained in DMEM containing 10% FCS.

2.3.1.2 Maintenance of cell stock

2.3.1.2 1 Freezing of cells

The medium was replaced 24 h prior to freezing. Cells were trypsinised and resuspended as described above. The contents of 2-4 dishes were resuspended in 20 ml routine medium. 0.5 ml of this suspension was counted in 10 ml Isoton on a Coulter counter (model ZF, Coulter Electronics), and the remainder centrifuged on an MSE bench centrifuge at 800 rpm for 5 minutes. Then the medium was aspirated, the cell pellet was resuspended in a suitable volume of DMEM containing 20% FCS and 10% DMSO (dimethylsulfoxide, the cryopreservative) to provide a concentration of about 1.2×10^6 cells per ml. 1ml aliquots were dispensed into 1.8 ml Cryotubes and frozen in dry ice for at least 1 hour, then transfered to a -70°C and then into a -140°C freezer.

2.3.1.2 2 Thawing of cells

After extraction from the -140°C freezer, the contents of a Crytotube were thawed as quickly as possible in the 37°C water bath until the last ice crystal had just disappeared. Since the membranes of cells in this condition are easy disrupted, care was taken to avoid agitation of the vial. Then the crytotube contents were immediately transfered to a 20 ml sterile plastic universal bottle containing 20 ml routine medium with 20% FCS. The universal was centrifuged in an MSE bench centrifuge at 800 rpm for 5 minutes, the supernatant discarded and the pellet resuspended in routine medium containing 10% FCS. The cells were plated out into three plates at a cell density of approximately 1×10^6 cells per plate, and subcultured three days later.

2.3.1.3 [^{3}H]-thymidine DNA synthesis assays

Measurement of the level of incorporation of labelled thymidine over one hour at the point of maximal DNA synthesis was employed both as a routine means of ensuring cell viability and growth factor activity; and also to test the effects of signalling pathway inhibitors.

2.3.1.3.1 Preparation of cells for DNA synthesis assays

Cells in routine medium were plated out at 1.5×10^{4} per 1.5 cm-diameter well in 24-well plates in 0.5 ml medium. For Rama 27, Rama 29,Rama 37, Rama 37E8 cells, after 24 hour the cell monolayer was washed twice with 0.5 ml PBS and then 0.5 ml Step Down Medium (SDM, DMEM containing 0.25 mg/ml bovine serum albumin) was added. The cells were incubated for a further 24 h to induce cells into quiescence, then medium was replaced with 0.5 ml fresh SDM at 37°C.

Swiss 3T3 cells in D/W medium or DMEM containing 10 % FCS as described above were plated out at 1.5×10^{4} per 1.5 cm-diameter well in 24-well plates in 0.5 ml of the same medium, after 5-7 days, the cultures were confluent and quiescent.

CHO cells were grown in 10% FCS DMEM/ F12 medium about 60% confluent as described above were plated out at 1.5×10^{4} per 1.5 cm-diameter well in 24-well plates in 0.5 ml of the same medium. After 24 hour the cell monolayer was washed twice with 0.5 ml PBS and then 0.5 ml DMEM was added and the cells were incubated for further 48 hours.

These cells were arrested in the G_1 / G_o phase of the cell cycle. Serum or growth factor was added to stimulate DNA synthesis.

2.3.1.3.2 Addition of signalling pathway inhibitors and growth factors

For Rama 27, Rama 29 Rama37 and Rama 37 E8 cells, after 24 h, SDM was replaced with fresh SDM containing growth factors at appropriate concentrations. In experiments in which inhibitors of signalling pathways were used, SDM was replaced with SDM containing inhibitor stock solutions at appropriate dilutions 15 mins prior to the addition of growth factors in fresh inhibitor-containing SDM. Although most of the inhibitors were first dissolved in anhydrous DMSO, the

subsequent dilution into SDM was sufficient to reduce the concentration of DMSO in cell medium to negligible levels·

For Swiss 3T3 cells, after 5-7 days culture in DMEM containing 10 % FCS, the medium was replaced with fresh DMEM containing growth factors at appropriate concentrations.

For CHO cells, after 48 hours culture in DMEM when cultures into quiescence, the medium was replaced with fresh DMEM containing growth factors at appropriate concentrations.

2.3.1.3.3 Incorporation of [^{3}H]-thymidine

18 hours after the addition of growth factors (the point of maximal DNA synthesis in Rama 27 cells, (Chen 1995)), 20 µl of 40 µCi/ml [^{3}H]-labelled thymidine (containing 40 µM unlabelled thymidine) was added to the medium in each well (0.8 µCi per well).

24 hours after the addition of growth factors in Swiss 3T3 cells or 16 hours after the addition of growth factor in CHO cells, 20 µl of 40 µCi/ml [^{3}H]-labelled thymidine (containing 40 µM unlabelled thymidine) was added to the medium in each well (0.8 µCi per well).

Incorporation of labelled thymidine was allowed to proceed for 1 hour for Rama 27 cells (2 hours for CHO cells and 4 hours for Swiss 3T3 cells) before medium was removed and cell monolayers were washed twice in ice-cold PBS. Ice-cold 5% (w/v) TCA (500 µl) was then added to each well and dishes were incubated at 4°C for 30 min to precipitate macromolecules.

Following TCA precipitation, cells were washed once in fresh cold TCA and dried by washing twice in cold 95% ethanol. The final wash was removed and the residue was allowed to evaporate at room temperature for 0.5-1 hour. Once dry, TCA precipitates were solubilised in 0.2 M NaOH (500 µl per well) at 37°C for 60 min; and 300 µl of each solubilised TCA precipitate was transferred to a scintillation vial.

ULTIMA scintillant (1 ml) was added to each vial and incorporated radioactivity was measured in a Packard TRI 1900TR scintillation counter using a 10 minute counting program.

2.3.1.4 Counting cells

Cells were detached as described previously and counted using a Coulter counter. For the Coulter counter, trypsinised cells were resuspended in RM and 1ml of the cell suspension added to 19ml of isoton for counting.

2.3.1.5 Detection of proteins by inmmunoblotting

2.3.1.5.1 Preparation of total cell lysates for immunoblotting.

Cells to be used in western blotting experiments were seeded into 6 cm culture dishes in RM. After 24 h, RM was removed, cells were washed twice with PBS, and step-down medium (SDM) was added. After 24 h the cells were treated with growth factors. Medium was removed and replaced with fresh SDM containing the growth factor of interest at the appropriate concentration. Fresh SDM was added to untreated cells to control for the effects of changing the medium.

Growth factor incubation periods varied according to the aims of the experiment. At the end of the incubation period each plate was washed twice with ice-cold PBS before the cells were scraped into 100 µl of ice-cold lysis buffer using a rubber policeman. The cell lysate was transferred to a micro-centrifuge tube and placed into a boiling water bath for 3 min. In most cases the lysate was then sonicated to solubilise the cells. Lysates were either kept on ice for immediate western blot analysis or frozen at -20°C for future analysis. Frozen lysates were re-heated to 60°C for 1 min before loading onto a gel to ensure full SDS-coating of the proteins.

2.3.1.5.2 SDS-PAGE and transfer of proteins to PVDF membrane

Cell lysates were resolved by electrophoresis 30 mA through 7.5% or 15% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels (according to different experiments) until the dye front reached the bottom of the gel. The resolved proteins were transferred to PVDF membrane (Millipore) in a Bio-Rad wet transfer apparatus at 30 mA for 1 h at 4°C.

2.3.1.5.3 Chemiluminescent detection of bound antibodies.

Equal amounts of the two chemiluminescent reaction components were mixed according to the manufacturer's instructions and the membrane was gently shaken in the solution for 1 min before exposure to X-ray film. Exposure times were adjusted for each membrane but were typically 1-5 min. Finally, the membrane was incubated in Fast Green protein stain for 5 min, and washed in membrane destaining buffer until the background was white, to reveal the polypeptide bands.

2.3.1.5.4 Detection of tyrosine phosphorylated proteins by immmunoblotting.

Cells were grown in the 6-cm-diameter culture-dishes to near confluency. The RM was removed and the cells washed twice with PBS. The cells were re-fed with DMEM containing 250 μg/ml BSA (SDM) and incubated in this medium for 24 hours to induce quiescence. The cells were cultured in the absence or presence of 100ng/ml EGF for 15 minutes at 37°C prior to preparation.

The cell monolayers were washed twice with ice cold PBS, and then scraped into a fresh 0.2 ml of the lysis buffer. The cells were collected by centrifugation for 1 minute at 12,000 g_{max} at 4°C. SDS-PAGE and subsequent immunoblotting were carried out using 7.5% (w/v) acrylamide resolving gels and 4% (w/v) acrylamide stacking gels according to Method 2.3.1.5.2 and 2.3.1.5.3.

2.3.1.5.5 Addition of signalling pathway inhibitors and growth factors

In experiments in which inhibitors of signalling pathways were used, cells were grown in the 6-cm-diameter culture-dishes to near confluency. The RM was removed and the cells washed twice with PBS. The cells were re-fed with SDM. After 24 h, SDM was replaced with fresh SDM containing inhibitor stock solutions at appropriate dilutions 15 mins prior to the addition of growth factors in fresh inhibitor-containing SDM. For the control experiment, one same culture dish was treated with inhibitor alone without growth factors. Method 2.3.1.5.1 was used for preparation of total cell lysates for immunoblotting. SDS-PAGE and subsequent immunoblotting were carried out using 15 % (w/v) acrylamide resolving gels and 4% (w/v) acrylamide stacking gels according to Method 2.3.1.5.2 and 2.3.1.5.3.

2.3.1.5.6 Quantification of immunoblots and SDS-PAGE

In order to quantify the signal obtained upon immunoblotting or the concentration of proteins from gels, the developed film and gels were scanned by laser scanner (300A Computing Densitometer Fast Scan).

2.3.1.6 Binding assay

2.3.1.6.1 Iodination of EGF

EGF was iodinated by the chloramine T method (Hollenberg and Cuatrecasas, 1975). The following were added to an Eppendorf tube: 1μl of 1 μg/μl EGF dissolved in PBS; 50μl 0.5M phosphate buffer (pH 7.4); 100μCi[^{125} I] NaI. The reaction was initiated by the addition of 5 μl 5 mg/ml chloramine T and allowed to proceed for 1 minute. The addition of 10 μl 100 mM $Na_2S_2O_5$ terminated the reaction, 20μl of 100 mM KI was added to dilute the isotope, and 70 μl (w/v) BSA dissolved in PBS was added as carrier for the EGF. The [^{125} I]-EGF was separated from the unincorporated [^{125} I] NaI by centrifugal desalting (Christopherson, 1983), at $1600g_{max}$ for 3 minutes through a 1 ml Sephadex G25 column which had previously been equilibrated in 1% BSA (w/v) dissolved in PBS. The biological activity of iodinated EGF was determined by using non-radioactive NaI in the iodination reaction. The [^{125} I] EGF was used immediately or stored at –20°C for up to 3 weeks.

2.3.1.6.2 Binding of [^{125} I] EGF to cell-surface receptors

Rama 27 cell line was plated at 5×10^4 cells /well in 24 well culture-dishes into 0.5 ml RM. After 24 hours the monolayers were washed twice with PBS to remove any traces of EGF in the RM. The cells were re-fed with 0.5 ml DMEM containing 250 μg/ml BSA. Then 18hours later the cells were washed twice with PBS (4°C), and once with Binding Medium (DMEM (1x), 1%(w/v) BSA, 25mM HEPES, pH 7.4) (4°C), which was then replaced with 200 μl Binding Medium (4°C). For displacement binding experiments increasing concentrations of [^{125} I] EGF were added to multiwells. Natural materials and peptides were respectively added to multiwells. After 2 hours incubation at 4°C on an orbital shaker, when binding of [^{125} I] EGF was maximal, the cell were washed twice with Binding Medium (4°C) and three times with PBS (4°C). Cell-associated radioactivity was solubilised with 0.2M NaOH and determined in a Wilj gamma counter.

2.3.2 Molecular biological methods

2.3.2.1 Isolation of plasmid DNA

Plasmid DNA (pET-16b) was isolated from bacterial culture using the Concert miniprep or midiprep methods (Gibco).

2.3.2.1.1 Concert miniprep

A single colony containing the required plasmid was used to inoculate 5 ml LB-broth. 1 to 3ml of the overnight culture was centrifuged at 6,000 rpm in a Sorval SS-34 rotor for 10 min at 4°C. The bacterial cell pellet was resuspended in 0.4ml of buffer E1 [50mM Tris-HCl (pH 8.0), 10mM EDTA] and mixed with 0.4ml of buffer E2 [200mM NaOH, 1% (w/v) SDS] and incubated for 5min at room temperature. 0.4ml of buffer E3 [3.1M potassium acetate (pH5.5)] was added, mixed by inverting the tube and the mixture centrifuged at 12,000g for 10mins at room temperature. The resulting supernatant was loaded onto an equilibrated column and the flow through discarded, following which the column was washed twice with 2.5ml of buffer E5 [800mM NaCl, 100mM sodium acetate (pH5.0)] and the flow through discarded. The DNA was eluted with 0.9ml of buffer E6 [1.25M NaCl, 100mM Tris-HCl (pH 8.5)], 0.63 ml of isopropanol was added to the eluate and centrifuged at 12,000xg at 4°C for 30min and the resulting plasmid DNA pellet washed with 1ml of 70% (v/v) ethanol and centrifuged at 12,000xg at 4°C for 5 min. The pellet was air dried and resuspended in 50 µl of sterile distilled H_2O.

2.3.2.1.2 Concert midiprep

200 µl of culture containing the required plasmid was used to inoculate 100ml of LB-Broth. 15 to 25ml of the overnight culture was pelleted at 6,000 rpm in a Sorval SS-34 rotor for 10 min at 4°C. The cell pellet was resuspended in 4ml of buffer E1 [50mM Tris-HCl (pH 8.0), 10mM EDTA] and 4ml of buffer E2 [200mM NaOH, 1% (w/v) SDS] added and mixed by inverting the tube. 4ml of buffer E3 [3.1M potassium acetate (pH5.5)] was added and the tube mixed as before, following which the mixture was spun at 13,000 rpm for 10 min at room temperature in a Sorvall SS-34 rotor. The supernatant was put onto the equilibrated column and the flow through discarded, following which the column was washed twice with 10ml of buffer E5

[800mM NaCl, 100mM sodium acetate (pH5.0)] and then flow through discarded. The DNA was eluted from the column with 5ml of buffer E6 [1.25M NaCl, 100mM Tris-HCl (pH 8.5)], 3.5ml of isopropanol added and the mixture centrifuged at 11,000 rpm for 30 min at 4°C in a Sorvall SS-34 rotor. The supernatant was removed and the plasmid DNA pellet was washed in 2 ml 70% ethanol and recentrifuged for 5 min. The pellet was dried, resuspended in an appropriate volume of sterilised water and quantified.

2.3.2.2 Growth and storage of bacterial stocks

Single colonies of *E. coli* DH5α containing a plasmid or not, were incubated into 3 ml LB-broth, containing 50 µg/ml ampicillin if appropriate, and incubated at 37°C shaking incubator overnight. Sterile glycerol was added to 15% (w/v) and the culture stored at –70°C in cryotubes. When required cultures were thawed on ice, and a small amount streaked to produce single colonies onto an LB-agar plate and then incubated at 37°C overnight.

2.3.2.3 Preparation of competent bacterial cells

One colony from a freshly prepared LB-agar plate of single *E. coli* DH5α colonies, was transferred to 30 ml LB-broth and incubated at 37°C overnight in a shaking incubator. Periodically samples of the culture were removed and the absorbance at 600 nm was measured. When the absorbance reached between 0.5 and 0.8 units, a value that corresponded to the exponential growth phase of this bacterial strain, the culture was transferred to ice-cold tubes and centrifuge at $1600g_{max}$ in a Sorvall HS-4 rotor for 10 minutes at 4°C. The L-broth was poured off and all traces allowed to drain away. The cells were gently resuspended in 20 ml ice-cold 0.1M $CaCl_2$ and then stored on ice for 10 minutes. Centrifugation was repeated as above, and the pellet was resuspended in 1 ml ice-cold 0.1M $CaCl_2$, 10% (w/v) glycerol. The cells were either used immediately or frozen on dry-ice, and then transferred to -70°C in 50 µl samples.

2.3.2.4 Transformation of competent bacterial cells

50 ng of DNA in a volume of 10 µl or less was added to 200 µl of competent cells and incubated on ice for 30min, followed by a 90s incubation at 42°C. Tubes were

put on ice for 1-2min and 800 µl of SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast, 0.05% NaCl, pH 7.0, 20mM glucose] added and culture incubated for 45min at 37^{0}C. Up to 200 µl of transformed competent cells were transferred onto agar SOB [SOB broth containing 1.5% (w/v) bacto-agar] and spread over the surface of the plate. Plates were incubated at 37°C for 12-16h until colonies had formed.

2.3.2.5 Restriction endonuclease digestions

Restriction enzyme digests were performed in conditions recommended by the manufacturer, using the buffers provided with the enzymes. Typically reactions were carried out using 1-20 µg of DNA in a volume of 10-20 µl, 1 unit enzyme/µg DNA and incubated at 37°C. Plasmid DNA digests was incubated for 1-2 hours.

2.3.2.6 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis on an agarose gel containing 0.5µg/ml of ethidium bromide, in 0.5x TBE buffer [0.045M Tris-borate, 0.001M EDTA]. The agarose concentration of the gel was varied from 0.8% to 1.5% (w/v) agarose, according to DNA size. DNA was loaded following addition of one-fifth of the volume of agarose gel loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in water]. 1Kb ladder and λDNA digested with *Hind*III were used as DNA size markers for agarose gel electrophoresis and DNA was visualised on an U.V transilluminator (302 nm).

2.3.2.7 Ligation of plasmid DNA fragments

Ligation was performed using T4 DNA ligase, and ligation buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 20 mM DTT, 2 mM ATP, pH 7.8) in total volume of 10 µl. Reactions contained 400 Weiss units (Weiss et al., 1968) and were incubated at 15 ^{o}C overnight. Control ligations consisted of insert alone and vector alone with and without ligase. Ligation of plasmid DNA fragments were sized by agarose gel.

2.3.2.8 Purification of DNA from agarose gels

DNA fragments were separated by agarose gel electrophoresis as descibed earlier, the required bands excised and purified (Qiagen). The excised gel slice was mixed with 3 volumes Buffer QG (100µl buffer/100µg of gel) and incubated at 50°C for 10

min with occasional vortex mixing. The melted agarose and DNA solution was applied to a QIAquick spin column and centrifuged for 1 min at 13,000 rpm in a microcentrifuge. DNA bound to the membrane was washed, eluted from the column and quantified as described above.

2.3.2.9 Identification of recombinant plasmids by PCR

PCR (Saiki et al., 1988) was used to verify the identity of recombinant plasmids. Single bacterial colonies were transferred to 3 ml L-broth containing 100µg/ml ampicillin and incubated at 37°C overnight in a shaking incubator. The next day 0.5 ml of each culture was moved to a microfuge tube, and the cells collected by centrifugation at 12,000g_{max} in a microfuge for 1 minute. The remaining 2.5 ml was stored at 4°C. The cell pellet was resuspended in 100µl sterile, double-distilled water. The bacteria were lysed by incubating in boiling water bath for 5 minutes, and a 2µl sample was removed for use in the PCR reactions. PCR reactions were carried out in a total volume of 50µl, and each reaction contained four deoxynucleoside triphosphates at a concentration of 3.3 mM, 100pM of each primer, 5µl 10x reaction buffer (supplied with the enzyme) and 1 unit of Taq polymerase. The primers used were synthetic oligonucleotides (Method 2.3.1.6), and reactions were incubated on a Techne programmable dri-block (model PHC-1) for 30 cycles of: (i) 92°C-30 seconds, (ii) 60°C-2 minutes and (iii) 72°C-2 minutes. A negative control reaction of 2µl sterile, double-distilled water was included, but a positive control was not possible as the construct being sought had not been previously produced. One-fifth volume of agarose gel loading buffer was added to each PCR and one-fifth of the total volume was analysed by agarose gel electrophoresis (Method 2.2.2.6).

2.3.2.10 Purification of PCR amplified DNA

PCR products were purified using the QIAquick PCR purification kit (Qiagen). Briefly, the PCR reaction was mixed with 5 volumes of Buffer PB and applied to a QIAquick spin column. DNA was bound to the silica-gel matrix by centrifuging for 1 min at 13,000 rpm in a microcentrifuge. The DNA was washed by applying 750 µl Buffer PE to the column and repeating the centrifugation twice, and eluted by applying 20µl sterile dH$_2$O to the centre of the membrane, incubating for 1 min and repeating the centrifugation. Eluted DNA was quantitated by eye, by electrophoresis

alongside the DNA molecular weight marker VIII (Roche) and subsequent comparison to the intensity of staining of DNA fragments of known concentration.

2.3.2.11 Induction and analysis of expressed recombinant material

2.3.2.11.1 Expression of recombinant material

Purified plasmid pET-16b containing the cloned, full-length fusion milk peptide gene (pET-16b-milk peptide) or pET-16b without the fusion milk peptide gene was used to transform *E. coli* strain BL21 made competent as previously described (2.3.2.4). Colonies of transformed bacteria were grown at 37 °C for overnight on LB-agar plates containing ampicillin (50 μg/ml). For production of recombinant milk peptide, transformed colonies were incubated in LB medium at 37°C until the optical density of the cultures at 600 nm was 0.5-0.7. At this point, expression of cloned gene was induced by adding 1 mM IPTG for 2 hours, after which time the bacterial cells were collected by centrifugation and resuspended in PBS containing 1 mM PMSF. Bacterial suspensions were stored at –70°C for up to 1 month.

2.3.2.11.2 Isolation and analysis of inclusion bodies

After incubation at 37 °C for 2 hours with IPTG induction, samples of the bacterial cultures were centrifuged and the cells resuspended in a lysis buffer containing [50 mM NaH_2PO_4 (pH7.8), 300 mM NaCl] and lysozyme (5 μg/ml) and left on ice. After 1 hour, the samples were subjected to sonication. Insoluble material was collected by centrifugation and resuspended in lysis buffer. SDS-PAGE analysis was performed to determine the presence of the recombinant protein in either the soluble or insoluble fraction.

2.3.2.11.3 Analysis of proteins expressed recombinant material by electrophoresis

SDS-PAGE was carried out on resolving gels 16.5 acrylamide (w/v) with 4% (w/v) stacking gel using Bio-Rad mini protean gel system (10 cm x 7 cm x 0.1 cm). Samples were analysed in the presence of 2-mercaptoethanol. For the analysis of protein with low molecular weight, the method of Schagger and Von Jagow (1987) was used in preference to the method of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie blue to visualize the separated proteins and destained in the presence of 40% (v/v) methanol, 7% (v/v) acetic acid. Protein

standards were treated in the same manner. Silver staining of gels was performed as a more sensitive method (2.3.2.11.4).

2.3.2.11.4 Method of silver staining acrylamide gels

Following electrophoresis, the acrylamide gels were fixed for 30 minutes in 40% Ethanol, 10% acetic acid. The gels were washed twice for 10 minutes with distilled water then sensitized for 30 minutes in 30% ethanol, 0.2% (w/v) sodium thiosulphate, and 6.8% (w/v) sodium acetate containing 2% (v/v) glutaraldehyde. The gels were incubated for 30 minutes at room temperature with a solution containing 0.2% (w/v) silver nitrate and colour was developed by washing gels with a solution of 2.5% (w/v) sodium carbonate containing 0.1% (v/v) formaldehyde. The reaction was stopped using 1.5% (w/v) EDTA.

2.3.2.12 Purification of recombinant milk peptide

The *E.coli* cell suspensions were removed from storage at $-70^{\circ}C$ and thawed rapidly by being placed in a $37^{\circ}C$ water bath. Once thawed, the samples were stored on ice. Cell debris was removed by centrifugation at 12,000g for 30 minutes. The resulting supernatant was purified by the same method described for the purification of natural milk peptide (Section 3.1.2) except smaller sized columns were used.

2.3.2.13 Transfer of proteins to PVDF for amino acid sequencing

Proteins were separated by SDS-PAGE using the Bio-Rad mini protean gel system (10 cm x 7 cm x 0.1 cm) and electrophoretically transferred to an Immobilon polyvinylidene difluoride (PVDF)-based membrane using the method of Towbin et al. (1979). Transfer was performed in a Bio-Rad mini transblot apparatus at 75 V for 1 hour with cooling.

2.3.2.14 Amino acid sequence determination

Automated Edman degradation of the peptide was carried on an Applied Biosystems 471A protein sequencer with on-line detector.

2.3.3 Methods for purification of milk peptide from bovine milk

See Section 3.1.2.

2.3.4 Assay of protein

The concentrations of the purified proteins were determined using the method of Bradford (1976) using the Coomassie reagent from Bio-Rad DC Protein Assay. This was carried out according to the manufacturers instructions.

2.3.5 Synthesis of peptides

Peptides were synthesized on a Milligen/Biosearch 9050 Peptide Synthesizer (Millipore, Watford, UK), using Fmoc chemistry and pentaflurophenyl esters, according to the standard protocol.

Chapter3

Identification of Milk Peptide

As chapter 1 described, milk peptide, like other growth factors, has been found to induce the stimulation of DNA synthesis for a rat mammary fibroblast cell line (Rama 27). Total purification of milk peptide has been done by Liu et al. (1996). It was shown that the activity has been identified as a nested series of sequences of 5 peptides. Those peptides corresponded to the C-terminus of bovine α_{S2}-casein. The amino acid sequences are:

KVIPYVRYL
TKVIPYVRYL
KTKVIPYVRYL
KPWIQPKTKVIPYVRYL
PQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

That these peptides isolated from bovine milk were active was confirmed by chemical synthesis of the peptides (Liu, 2002).

The present study was designed to look at the mechanism of action of the milk peptide. To achieve this, milk peptide was purified to the point at which there was a single active peak eluted, ie not contaminated by other growth factors. At this point, it is good enough for studies of mechanism.

However, the problem with relying on purification from bovine milk alone is that you can never be entirely sure that there is not an undetectably small contaminant that is responsible for the observed activity. Although chemically synthesised peptide had been used earlier by Liu (2002), it had not given entirely satisfactory results. Hence, a methodology of recombinant milk peptide was developed based on milk peptide sequence, in order to obtain sufficient pure material to allow its identification by sequence analyses in this study.

Once that recombinant material so obtained was active, it was confirmed to be identical to the milk peptide.

The overall objective of this chapter was to show that stimulation of DNA synthesis for Rama 27 cells could be obtained 'by synthesis' as well as 'by purification', and to produce enough material for studying its mechanism.

In this chapter, to provide partially and highly purified peptide from bovine milk the purification of milk peptide was studied. In addition, recombinant milk peptide was made and purified.

3. 1   Purification of Milk Peptide from Bovine Milk

3.1.1   Introduction

Bovine milk is a rich source of several classes of growth factors, and a wide variety of methods of purification of milk activity has been previously employed. It was shown that cation-exchange chromatography effectively concentrates the growth factor component of bovine milk (Francis, et al., 1995), because in contrast to major whey proteins, growth factors generally have basic isoelectric points. This whey growth factor extract has been shown to be remarkably potent in stimulating the growth *in vitro* of cells of mesodermal origin, including rat L6 myoblasts and mouse Balb/c 3T3 fibroblasts (Belford et al, 1995). Francis et al., (1995) used cation-exchange chromatography with Sepharose Fast flow-S resin to yield an extract containing only 1 to 2% whey protein but substantial growth-promoting activity for cells. Dunbar et. al., (1999) have identified Betacellulin as a novel growth factor in bovine milk for Balb/c 3T3 cells, and this was purified by cation-exchange chromatography combined with other chromatographic methods. Liu et al. (1996) used cation-exchange to purify the extract from bovine milk which stimulated DNA synthesis in Rama 27 cells. The activity has been identified as a nested series of sequences of 5 peptides. Those peptides corresponded to the C-terminus of bovine α_{S2}-casein. The amino acid sequence of this extract of bovine milk revealed that the

peptides are cationic, their content in milk is very low, and the molecular weight of this peptide growth factor is very low. Thus, the purification of this peptide has proven difficult. However, the partial purification of milk peptide from bovine milk is possible following the established protocol (Liu et al., 1996). The role of the milk peptide is much less clear, although it has been suggested that the peptide identified here would appear to be the replacement for EGF in bovine milk (Liu et al., 1996).

To study the mechanism of action of milk peptide, the preparation should not be contaminated with other proteins that have the ability to stimulate DNA synthesis, so the purification of milk peptide from bovine milk was based on several stages of chromatographic methods in this study.

3.1.2 Experimental and results

The method for the purification of milk peptide is described in Materials and Methods (2.3.3). It consists of several steps. Since milk peptide is cationic, after acid extraction and salt precipitation, it is first bound to Carboxy methyl-sepharose and growth promoting activity was recovered in the fractions eluted by the salt gradient. These fractions were then further purified by chromatography on Butyl sepharose, Reverse Phase High-Performance-Liquid-Chromatography (RP-HPLC) and gel filtration. Figure 3.1 is a summary of the overall purification scheme, leading to the isolation of the pure homogenous milk peptide.

Purification procedures were performed around neutral pH because milk activity is destroyed at extreme pH values (pH 1). To improve the recovery, the purification was designed to minimize dialyses and concentrations between each step.

3.1.2.1 Growth factor activity of bovine milk

As starting material, semi-skimmed bovine milk was used. It has been shown that semi-skimmed milk gave the highest stimulatory activity on Rama 27 cells of the different sorts of crude milk (Liu et al. 1996). Mitogenic activity is monitored by measuring the incorporation of [*methyl-^{3}H*] thymidine into the DNA of subconfluent Rama 27 cells in culture. The crude milk was tested for the ability to stimulate DNA synthesis in Rama 27 cells (Figure 3.2). The growth factor activities found in crude

semi-skimmed milk were dose-dependent. A unit of growth factor activity, defined as the concentration of growth factor required to stimulate half-maximal DNA synthesis (obtained with 1% FCS), was obtained with about 264 μg/ml of bovine milk protein.

3.1.2.2 Acid extraction and salt precipitation of milk peptide

When bovine milk was acidified to pH 3.0, precipitation of protein occurred. It is known that growth factors are stable at pH 3.0, whereas the large bulk of protein in milk including some caseins are unstable at this pH being moved from their normal physiological operating environment. After centrifugation, 68% of the growth factor activity was found in the supernatant fraction which containing 7% of proteins. This step removed 93% of the total proteins (Table 3.1).

Then supernatant was precipitated by ammonium sulphate. Ammonium sulphate is used here because its high solubility permits the achievement of solutions with high ionic strengths. Solid $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 22% (v/v) while it was stirred at 4°C. After all the added $(NH_4)_2SO_4$ was dissolved, it was left for 2 hours without further stirring. Then, after precipitate was removed by centrifugation as described above, the supernatant was withdrawn from the pellet. The above procedure was repeated again in order to increase the concentration of $(NH_4)_2SO_4$ to 35% (v/v) which gave the higher recovery of growth factor activity (Liu et al. 1996). Finally, the supernatant was discarded and the pellet was redissolved in distilled water and dialysed.

The dialysed pellet was found to be mitogenically active and the bioassay results showed that it produced a 135 % stimulation compared with SDM control when 1 % (v/v) sample solution was added to the cells in the serum-free medium (data not shown). This procedure produced a 51 % recovery of activity (Table 3.1).

3.1.2.3 Ion-exchange chromatography

For the separation of milk peptide from the most abundant whey proteins by the ion-exchange process used in this investigation, whey clarity was a requirement for reproducible chromatography and reduced column fouling. Glass wool was therefore employed as pretreatment to remove fat without substantial loss of protein.

A column of Carboxyl Methyl-Sepharose Fast Flow was equilibrated with a buffer of 20 mM NaH_2PO_4 at pH 6.0. In preliminary studies this resin was evaluated for good separation of interested protein from the bulk of other contaminating whey proteins (Liu et al.1996). The experiment shown in Figure 3.4 demonstrated that a huge amount of protein was washed out during the wash step, proteins that bound to the column under these conditions were eluted by the application of a linear gradient of 0.05-0.7 M NaCl in 20 mM NaH_2PO_4 (Figure 3.3a).

DNA assay analysis of the collected fractions showed the presence of one peak of fractions which stimulated DNA synthesis in Rama 27 cells (Figure 3.3b). As Fig 3.4a shows protein eluted from CM-Sepharose Fast Flow with 0.29 M NaCl plus 20 mM NaH_2PO_4 gave a markedly stimulatory growth activity. The protein recoveries from this experiment are shown in Table 3.1. The recovery of the activity was 97.3%. The specific bioactivity was increased 318.5 fold.

SDS-PAGE analysis of fractions of peaks which stimulated DNA synthesis showed that major whey proteins were eliminated, such as caseins (19-25kDa), α-lactalbumin (14 kDa), β-lactogloblin (18.5 kDa), and bovine serum albumin (66kDa) (Figure 3. 4). The bands with high molecular mass seem to be enriched.

3.1.2.4 Hydrophobic interaction chromatography

Those fractions containing the bioactivity were pooled and applied to hydrophobic interaction chromatography on a Butyl-sepharose column. The proteins were applied to this column in 4 M sodium chloride at pH 6.5. Under these conditions the active fractions bound to the hydrophobic interaction column (whereas the majority of contaminating protein did not bind), and were subsequently eluted by a 4-0 M linear gradient of NaCl (Figure 3.5 a). As Figure 3.5 b shows, the active fractions eluted at about 2.1M NaCl. This purification step yielded a 14.8% recovery of mitogenic activity from the previous step and an enrichment of 2851 fold in total (Table 3.1).

When examined by SDS-PAGE, only two bands with high molecular masses were detected in the eluted fractions with bioactivity. These bands were not changed under non-reducing conditions. There were no other detectable bands after silver staining

even when Tricine gel was used to allow visualisation of low molecular weight peptides (Figure 3.4).

3.1.2.5 Reverse phase HPLC

After the butyl-sepharose chromatography step, fractions containing growth activity were subjected to C_4 reverse phase HPLC (an aquapore butyl bonded phase, Brownlee column, 25mm x 4.6mm) with a 0-30% (v/v) acetonitrile gradient in 30 min (Figure 3.6a). The C_4 column gave excellent resolution leading to a high degree of purification, 17752.6 fold in total, and 66 % recovery of activity. As Figure 3.6 shows resolution of those fractions into several peaks. The peak of activity was eluted at 23% acetonitrile (Figure 3.6b); it is separated very well from the late eluting major peaks. This small active peak eluted from the HPLC column is very sharp. This step eliminated most of the contaminating proteins and a single peak of protein coincident with a peak of milk peptide activity was obtained.

Difficulties were encountered in accurately measuring the protein concentration of the milk peptide eluted from the final HPLC purification step because SDS-PAGE failed to detect the band from the eluted fractions. Estimating the concentration of milk peptide was based on the analysis of standard protein (Lysozyme) in the same conditions by Reverse-Phase HPLC.

The purified fraction from RP-HPLC was examined by electrospray mass spectrometry (Figure 3.6c). The mass spectrum is not useful for determining quantities, but does show evidence of the presence of expected peptides (arrows). There is no evidence of a family of derivative lines that would indicated the presence of a peptide of mass around 6 kDa.

Stimulation of DNA synthesis by highly pure milk peptide is dose dependent. Increasing volume of milk peptide added to quiescent Rama 27 cells caused increasing stimulation of DNA synthesis (Figure 3.7). On addition of 5 ng/ml milk peptide, the stimulation of DNA synthesis was increased by 1126 % of control. On addition of 30 ng/ml milk peptide, it was increased by 2072 % of control. However, crude milk was considerably less potent than milk peptide with stimulation of DNA

synthesis. As Figure 3.7b shows the stimulation DNA synthesis was equivalent on addition of 7 ng/ml (7 μl) of milk peptide or 660 μg/ml (100 μl) of crude milk.

SDS PAGE analysis of purification of milk peptide is showed in Figure 3.4. There are two bands with higher molecular weight detected in the bioactive fraction, which is eluted from hydrophobic interaction chromatography. These are not detected in fractions that stimulated DNA synthesis that are eluted from reverse phase-HPLC. It appears that the proteins with high masses eluted from butyl-sepharose were removed during reverse phase HPLC chromatography.

3.1.2.6 General properties of milk peptide

To characterize the action of milk peptide, the stability of the activity of milk peptide was studied during various treatments (Figure 3.8). When the milk peptide was heated at 95°C for 30 seconds, the stimulation of DNA synthesis was decreased only by 9 %. When milk peptide was treated with 1 M HCl, the stimulation of [^{3}H] thymidine into DNA synthesis was decreased by 64 %. The activity was destroyed when treated with 6 M urea.

To investigate if EGF and milk peptide activities were stable to heating and DTT treatments, 1 ng/ml EGF and 20 ng/ml milk peptide were incubated for 2 hours at room temperature with 5mM DTT. As Figure 3.9 shows the level of stimulation of DNA synthesis was not decreased when cells were incubated with heat treated EGF or milk peptide. The level of stimulation of DNA synthesis was decreased when EGF was treated with DTT. However, DTT treatment of milk peptide did not affect the level of stimulation of DNA synthesis even with heating. This indicated that bioactivity induced by milk peptide was not dependent on disulphide bonds. In contrast to milk peptide, bioactivity induced by EGF was correlated with disulphide bonds.

Aliquots of milk peptide were incubated with trypsin for 4 hours at 37 °C. As controls, cells were treated with trypsin alone. The amount of [^{3}H] thymidine incorporated into DNA was reduced to less than 63 % in cells stimulated with milk peptide that had been digested with active trypsin in comparison to cells stimulated

with control milk peptide. The results here suggested that the growth promoting effect of milk peptide stems from peptide rather than protein.

Figure 3.1 Procedure of milk peptide purification

Semi-skimmed milk

Acidification

Centrifugation

Acidified milk

$(NH_4)_2SO_4$ precipitation

Centrifugation

Salted out milk

Dialysis

Filter

Cation Exchange Chromatography

(CM-sepharose)

0.02M NaH_2PO_4,

0.05-0.7M NaCl, pH 6.0

Hydrophobic Interaction Chromatography

(Butyl-sepharose)

0.02M NaH_2PO_4,

4.0-0 M NaCl, pH 6.5

Reverse Phase HPLC

(C_4-aquapore butyl bonded phase)

0.1% TFA,

0.1% TFA/0-60% CH_3CN

Highly pure milk peptide

Figure 3.2 Effect of crude milk on DNA synthesis in Rama 27 cells
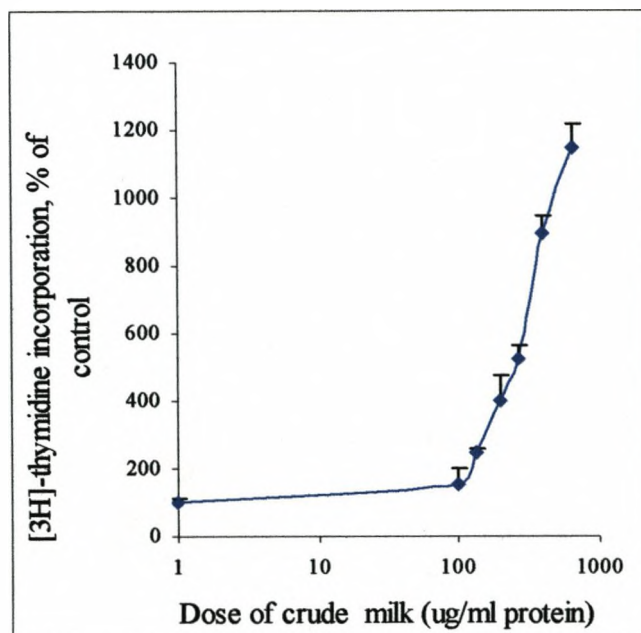
Dose response of DNA synthesis stimulating activity in Rama 27 cells. Crude semi-skimmed milk was added to 0.5 ml serum-free culture medium at the volume indicated above for 18 hours. Control DNA synthesis was based on the serum-free medium with 0.025% BSA. Results are expressed as mean ± SD.

Figure 3.3 Cation exchange chromatography of milk peptide
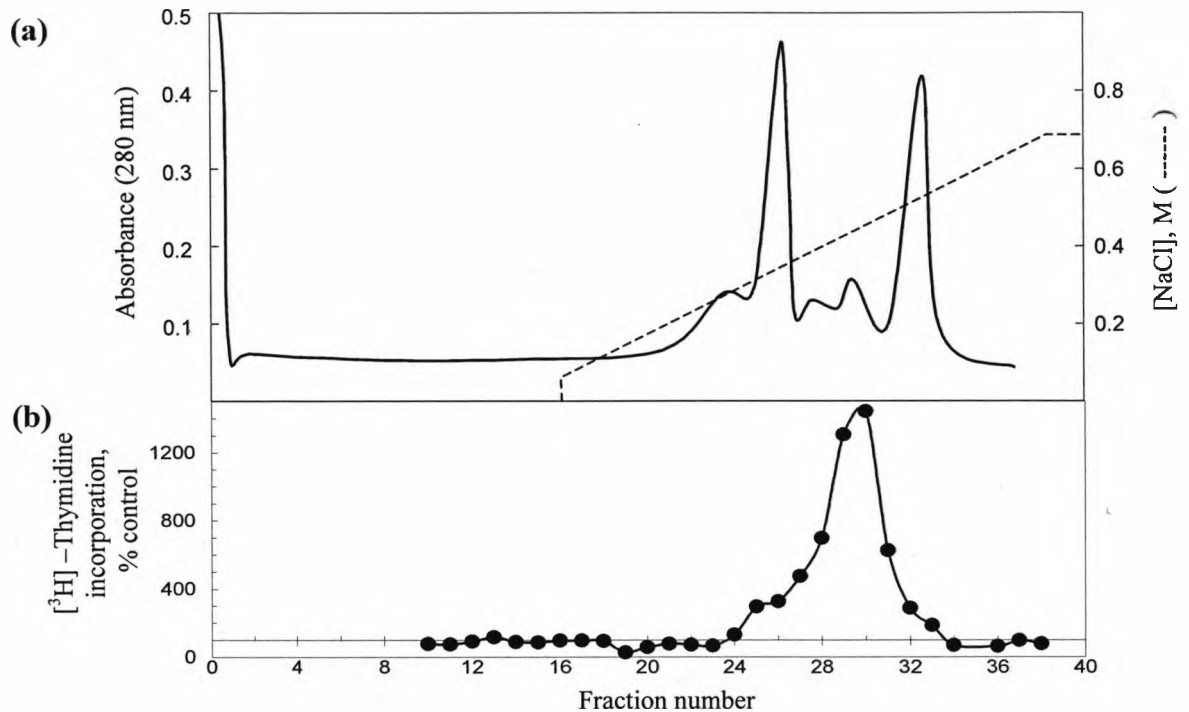
Samples of milk peptide prepared by salting out were applied to cation exchange chromatography (a) on a CM-sepharose column (5x 16 cm) equilibrated with 0.02M NaH_2PO_4, pH 6.0. Column was eluted with a gradient of NaCl in the corresponding equilibration buffer. Fractions of 25 ml were collected at a flow rate of 5 ml /min, measured for absorbance at 280 nm, and tested for the ability to stimulate DNA synthesis in Rama 27 cells. Levels of [^{3}H] thymidine incorporation (b) are shown as the mean of triplicate determination. Standard deviations are not shown for reason of clarity but were within ± 10% of the mean. Control for DNA synthesis was based on serum-free medium with 0.025% BSA.

Figure 3.4 SDS-PAGE analysis of purification of milk peptide from bovine milk
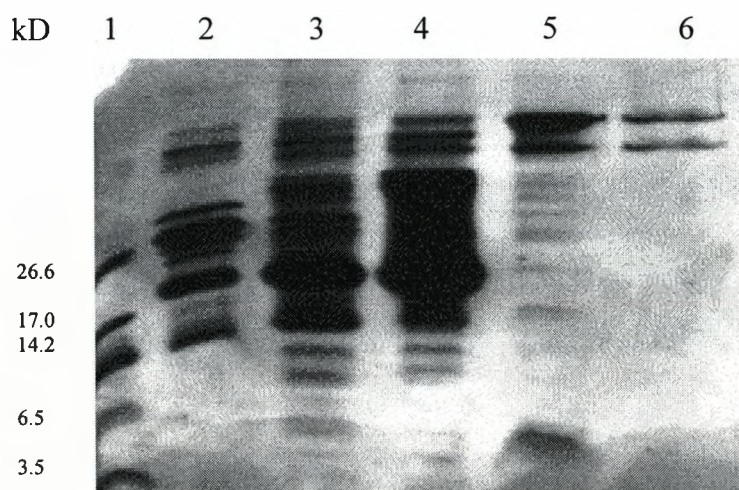
Samples of proteins from each stage of the purification procedure of milk peptide were analysed by SDS-PAGE. The protein were visualised by staining the gel with Silver staining. The molecular weights of standard proteins (Lane1) are shown in Kilodaltons (kDa). The crude semi-skimmed milk (Lane 2) was acidified (Lane 3) followed by precipitation with Ammonium Sulphate (Lane 4). The proteins from dialysis were applied to column of CM-sepharose. A linear salt gradient of NaCl was applied to the CM-sepharose column to remove bound proteins. Eluted proteins were collected as fractions and those fractions with bioactivity (Lane 5) were applied to a column of Butyl sepharose. Proteins that bound to the Butyl sepharose column were eluted with a linear salt gradient of NaCl. The fractions eluted with bioactivity (Lane 6) were subjected to reverse phase HPLC for further purification.

Figure 3.5 Hydrophobic Interaction Chromatography of milk peptide

(a)

(b)
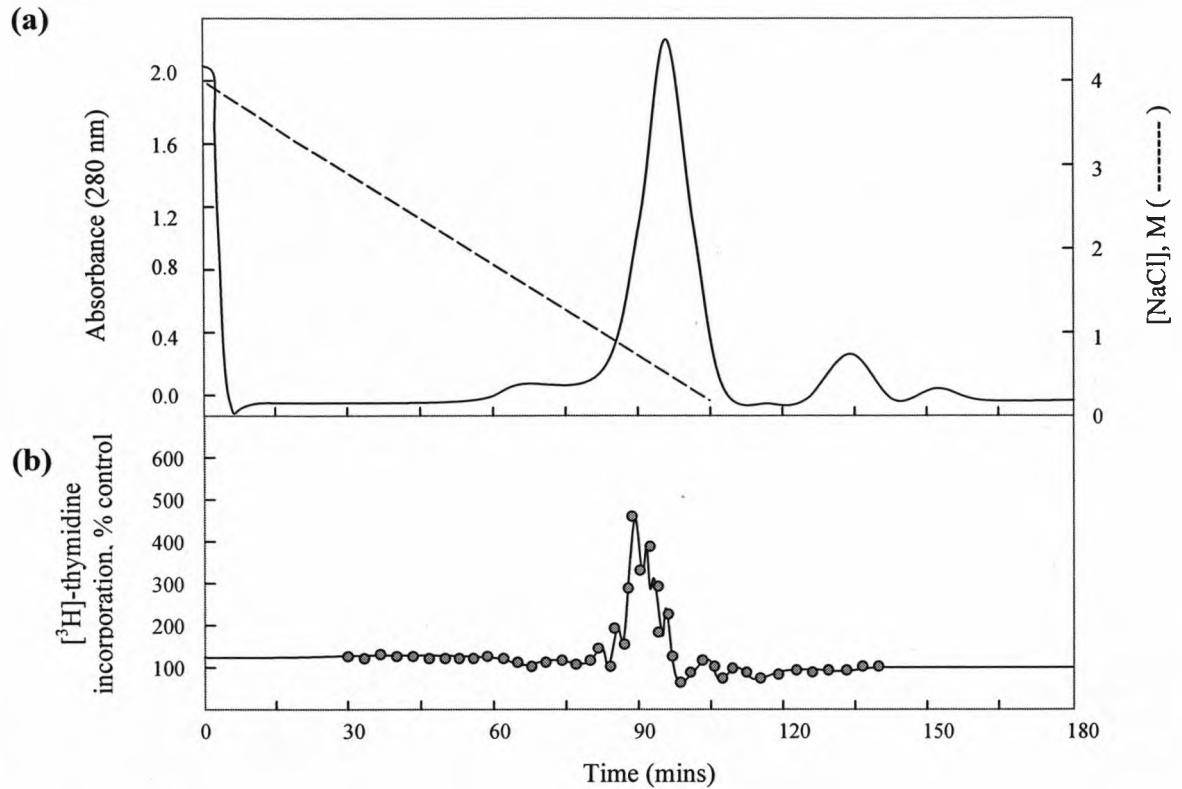
Samples of milk peptide from a CM-sepharose column were applied on Butyl sepharose column (2.5x 112 cm) equilibrated with 0.02M NaH_2PO_4, 4M NaCl, pH 6.0. Column was eluted with a gradient of NaCl in the corresponding equilibration buffer. Fractions of 7 ml were collected at a flow rate of 3.5 ml /min, measured for absorbance at 280 nm, and tested for the ability to stimulate DNA synthesis in Rama 27 cells (low panel). Levels of [^{3}H] thymidine incorporation were shown as the mean of triplicate determination. Standard deviations are not shown for reason of clarity but were within ±10% of the mean. Control for DNA synthesis was based on serum-free medium with 0.025% BSA.

Figure 3.6 Reverse phase HPLC of milk peptide
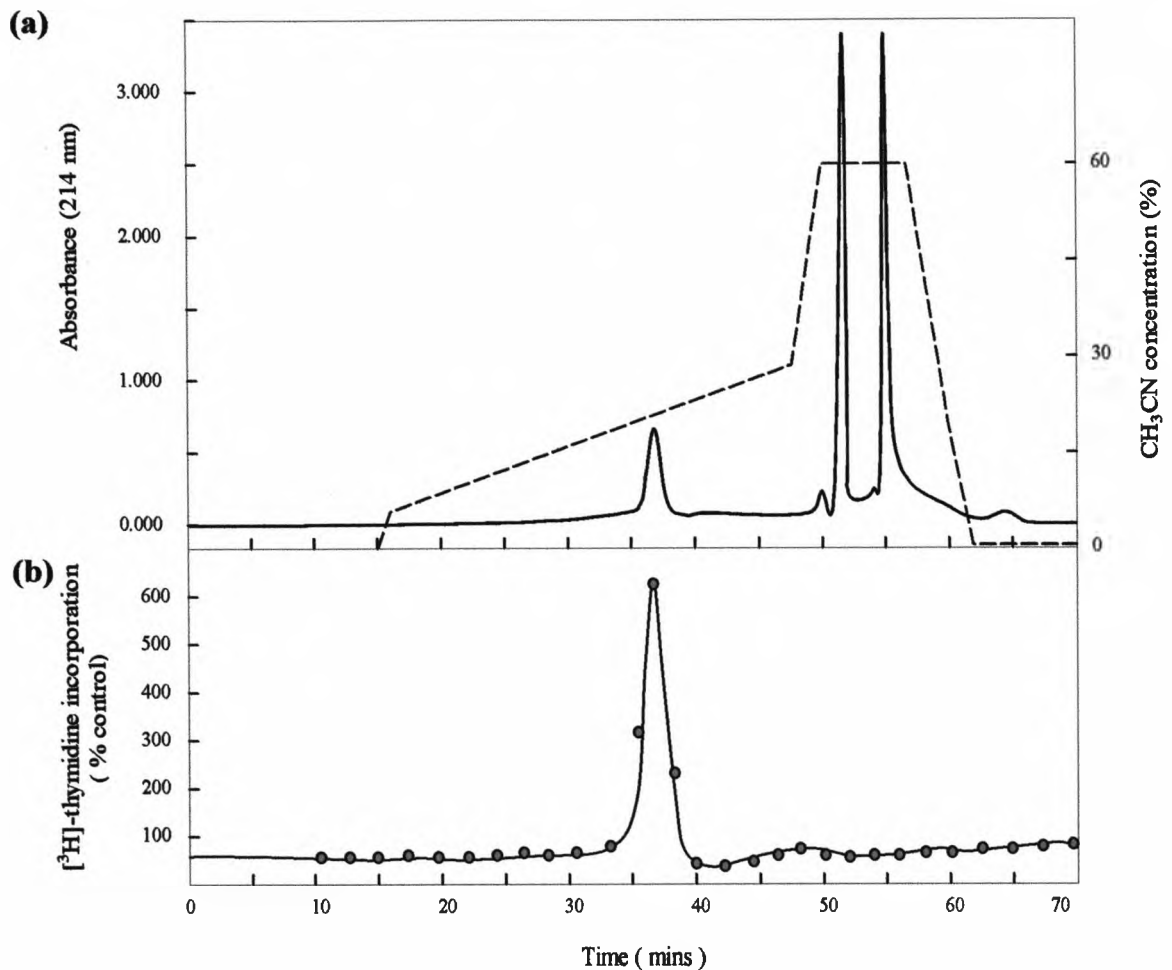
Samples of active fractions eluted from Butyl sepharose were applied to Reverse Phase HPLC C_4 column (upper panel) with 0.1% TFA. Column was eluted with 0 – 60 % gradient of acetonitrile. Fractions of 0.7 ml were collected at a flow rate of 0.7 ml / min, measured for absorbance at 214 nm, and tested for the ability to stimulate DNA synthesis in Rama 27 cells (low panel). Levels of [^{3}H] thymidine incorporation were shown as the mean of triplicate determination. Standard deviations are not shown for reason of clarity but were within ± 10% of the mean. Control for DNA synthesis was based on serum - free medium with 0.025% BSA.

Figure 3.6c Electrospray mass spectrum of milk peptide
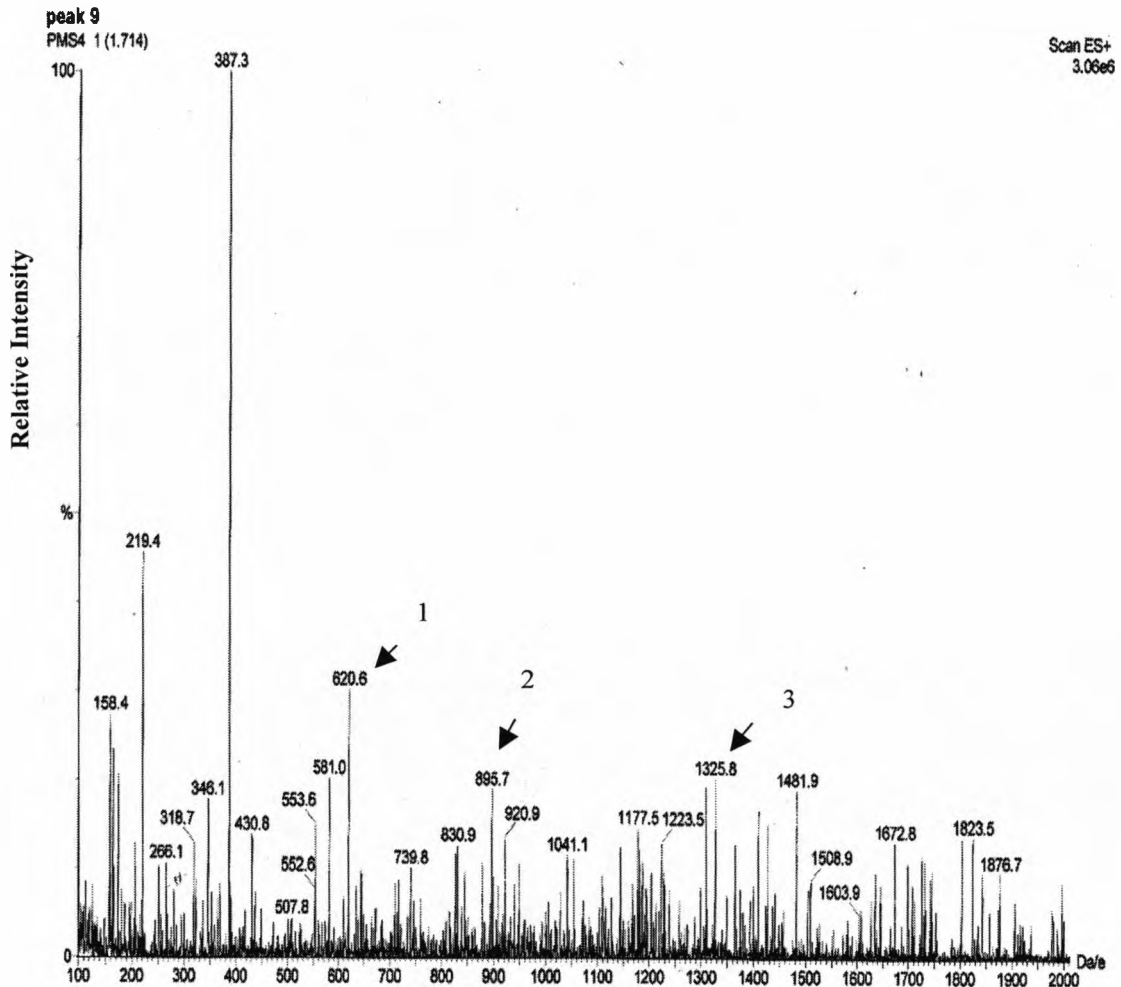
The electrospray mass spectrum for a fraction eluted by HPLC from bovine milk. Three peaks (arrows) are observed in mass spectrum corresponding to the molecular fragments of milk peptide. 1). KVIPY, 2). TVYQHQK. 3). TVYQHQKAMKP.

Figure 3.7 Effect of milk peptide on DNA synthesis in Rama 27 cells

(a)

(b)
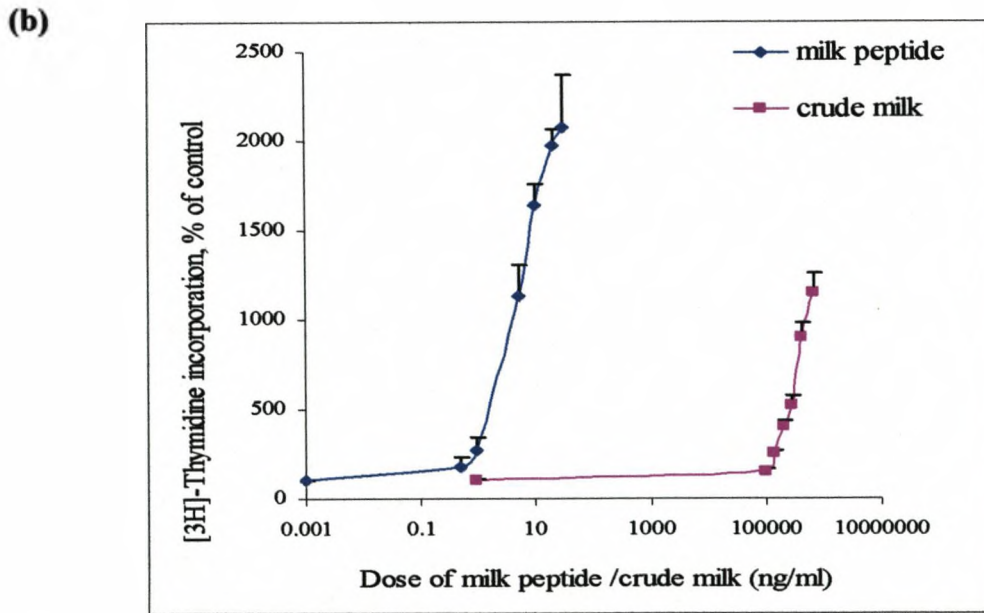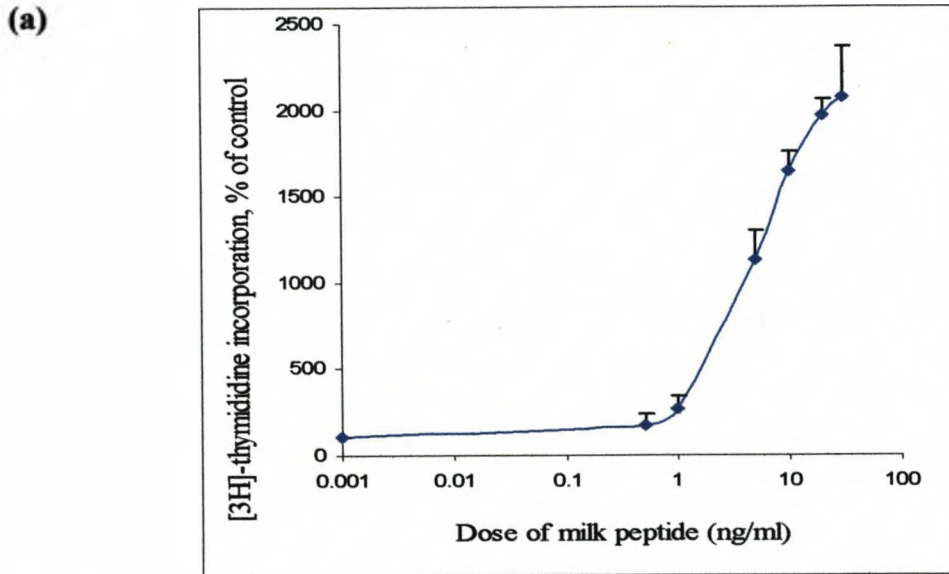
Dose response of DNA synthesis stimulating activity in Rama 27 cells. DNA synthesis was assayed by measuring incorporation of [^{3}H] thymidine at 18 hours. Control for DNA synthesis was based on the serum-free medium with 0.025% BSA. Results are expressed as mean ± SD. (a). milk peptide. (b). Comparison of effect of crude milk with milk peptide on stimulation of DNA synthesis.

Figure 3.8 Effect of milk peptide with various treatments on DNA synthesis in Rama 27 cells
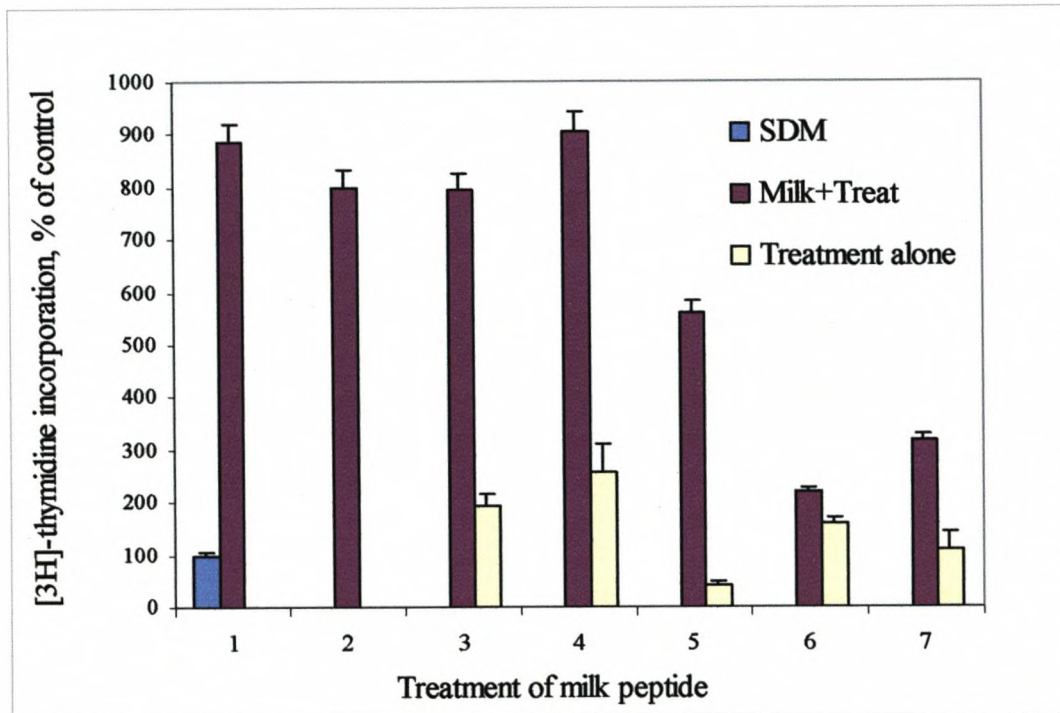
DNA synthesis was assayed by measuring incorporation of [^{3}H] thymidine in 18 hours. Control for DNA synthesis was based on the serum-free medium with 0.025% BSA. Results are expressed as mean ± SD, 'treatment alone' alone indicated cells cultured in the presence of DTT, Urea, HCl, Trypsin and heating in the absence of the peptide.

Samples: 1 –10 ng/ml of milk peptide.

2 – 10 ng/ml of milk peptide, heating 95°C, 30 second.

3 – 10 ng/ml of milk peptide + 5 mM DTT.

4 – 10 ng/ml of milk peptide + 5 mM DTT, heating 95°C, 30 second.

5 – 10 ng/ml of milk peptide + 50 µg/ml Trypsin, at 37°C for 4 hours.

6 – 10 ng/ml of milk peptide + 6 M Urea at 25°C for 2 hours.

7 – 10 ng/ml of milk peptide + 1 M HCl at 25°C for 2 hours

Figure 3.9 Effects of DTT and heating treatments on DNA synthesis stimulating activity in EGF and milk peptide

(a)
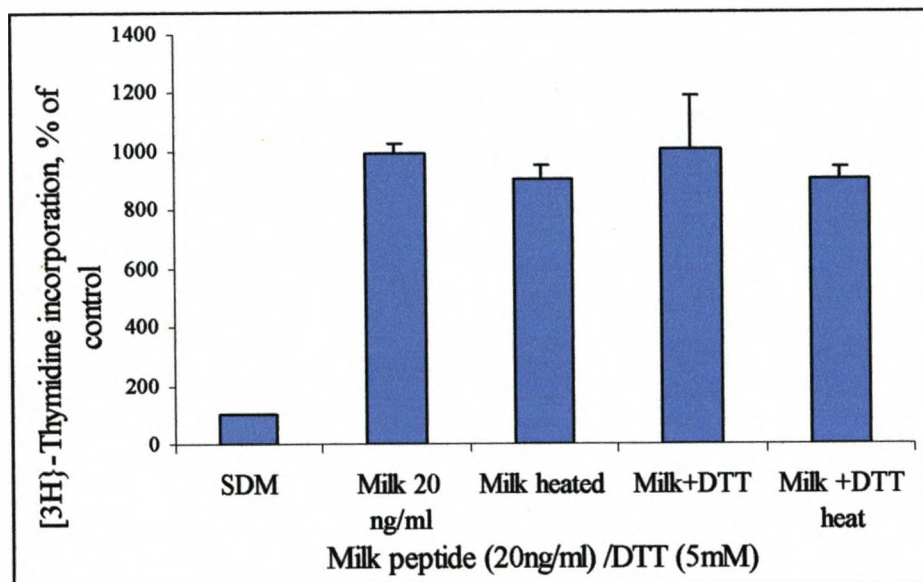
(b)
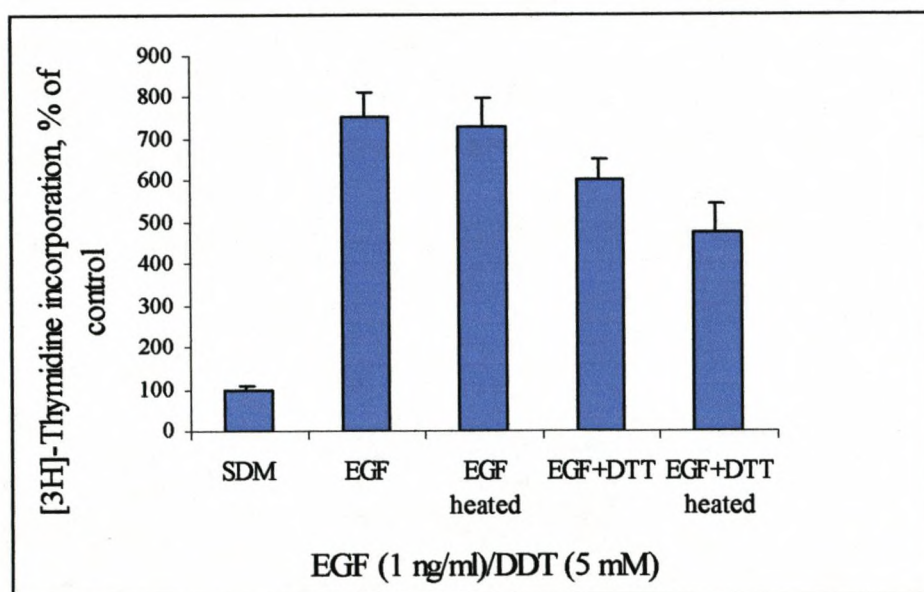
Sample of EGF and milk peptide were exposed for 2 hr at room temperature to the condition of 5 mM DTT or heating treatments, and then assayed on the Rama 27 cells at concentration of 20 ng/ml milk peptide (a) and 1 ng/ml EGF (b). The background incorporation of [^{3}H]-thymidine into DNA by untreated quiescent cells was 100%. Data points represent the means ± S.D.

Table 3.1. Summary of purification of growth promoting activity from bovine milk

Purification step	Total protein (mg)	Activity (units)*	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude milk	148,500	178205	1.2	1	100
Acid extraction	10144	121225	12	10	68
Salt out	4087	41187.6	10.1	8.3	23.1
After dialysis	563.6	20,882.6	37	30.8	11.7
CM-sepharose chromatography	53.2	20335.7	382.3	318.5	11.4
Butyl-sepharose chromatography	0.88	3017.7	3421.4	2851.2	1.6
Reverse Phase-HPLC	0.092	1960	21304.3	17753.6	1.1

One unit of activity is defined as the amount of protein required to produce half maximal stimulation compared with 1% FCS from Rama 27 cells.

3.1.3 Discussion

3.1.3.1 Growth factors in bovine milk

The presence of growth-promoting activity in whole bovine milk is well established (Klagsbrun and Neumann, 1979). More recently, such activity has been demonstrated in the whey fraction. In this context, several mitogenic factors have been identified, including IGF-I, and –II (Francis et al. 1988), FGF-1 and FGF-2 (Rogers et al. 1995), TGF-B (Cox et al. 1991) and PDGF (Shing et al. 1987). It has been shown that addition of a growth factor cocktail from bovine whey milk at the concentrations approximating those detected in mitogenic bovine whey extract achieved a growth response of only 51 % of that observed in mitogenic bovine whey extract (Belford et al., 1997). EGF is considered to be a major growth factor in human milk, but EGF-like polypeptides are absent from bovine milk. The nature of the major growth factor in bovine milk is unclear.

That milk peptide stimulates cell proliferation for Rama 27 cells is well recognised (Liu et al. 1996). Present studies have described the isolation of milk peptide to high purity from bovine milk. This study confirms that bovine milk contains growth promoting activity for Rama 27 cells. This activity is purified following the procedure which was designed before. (Liu et al. 1996).

3.1.3.2 Purity of the milk peptide purified from bovine milk

It has become apparent from this work and others (Liu et al., 1996; Harris et al., 2001) that it is extremely difficult to get the pure milk peptide. Other methods of purification have been tried but none proved successful owing to loss of bioactivity (results not shown). A particular difficulty is that the milk peptide is only a fragment of αS_2-casein, and such a small molecular mass is not easily to be detected by normal techniques at a low concentration.

Table 3.1 summarizes the results of the purification. The overall purification was 1.8 $x10^4$ fold, with a recovery of 1.1%. The actual degree of purification very likely exceeds this value since the total mitogenic activity for Rama 27 cells in the crude

milk homogenate probably contains other mitogens. If, in the other extreme, milk peptide is recovered in 100% yield then it is purified about 16×10^5 fold.

To accurately determine the mechanism of milk peptide, the degree of purification must be such that there is no contamination by any other protein that has the ability to stimulate DNA synthesis in Rama 27 cells.

To ensure the complete removal of other growth factors, the original method for purification of milk peptide was successful (Liu, 2002). The high isoelectric point of milk peptide eluted from cation exchange chromatography should remove most of growth factors with acidic of isoelctric point such as EGF and BTC (betacellulin). (To purify BTC, which is a glycoprotein, affinity chromatography on heparin-sepharose has been used (Dunbar et al., 1999)).

Electrophoresis of fractions eluted from Cation exchange chromatography and Hydrophobic interaction chromatography followed by high sensitivity silver staining do not reveal bands at about 22kDa (BTC) and 6kDa (EGF) (Figure 3.4). Although this system is able to detect about 1 ng of protein per band, it can only be seen at about 1 µg of small peptide per band (data not shown). It was free of bands on a gel, which would have been seen if more than 100ng had been present in 1 µg peptide (Figure 3.4, Lane 5). This evidence also supported the contention that there is no contamination of BTC and EGF in active fraction.

The final step of reverse phase-HPLC led to an increased specific activity of 17753.6 fold in total. It appears that all other proteins from the butyl sepharose column that may bind to C_4 reverse phase HPLC column (such as high molecular weight proteins) were removed during this step as only one peak was active. The bioactive fractions eluted at 23 % acetonitrile. This was also the same as found by Liu (2002). Although SDS-PAGE failured to detect the band from fractions eluted by HPLC even when silver staining was used, mass spectrometry has identified some fragments of milk peptide from the eluted fraction of HPLC.

Highly pure milk peptide gave a remarkable stimulation of DNA synthesis in Rama 27 cells and other cell lines (Chapter 4). Only 5 ng/ml of milk peptide gave DNA

synthesis about 1126% of control. Crude milk gave less potent stimulation of DNA synthesis in Rama 27 cells, although it contains several growth factors.

3.1.3.3 Stability of the milk peptide

The present study suggested that milk peptide has similar properties to other growth factors (EGF, PDGF), such as stability to heat and acid resistance since milk peptide was extracted in acid conditions (pH 3.0). However, the inability of DTT to destroy the bioactivity was the unique property of milk peptide that was different from EGF.

Treatment of milk peptide with DTT does not decrease the stimulation of DNA synthesis suggesting that the molecule is not composed of two peptide subunits held together by interchain disuphide bonds. Similar result has shown that an activity in human milk that stimulates DNA synthesis is resistant to DTT in mouse fibroblasts (Klagsbrun, 1978). In contrast, when "bovine milk growth factor (TGF-β)" was treated with DTT, no activity was detected in eluted samples from gel slices containing the monomer form, but there was activity detected in the sample containing the dimer form without treatment of DTT (Cox et al. 1991). It was also reported that the effect of human milk on DNA synthesis of neonatal hepatocytes was lost competely after reduction with DTT (Kohno et al., 1991). Although it has been reported (Rasmussen et al. 1994) that αS_2-casein has an interchain disulphide bond connecting the two cysteine residues at position 36 and 40, the milk peptide studied here corresponded to residues 192-222 of αS_2-casein in which there are no cysteine residues. That stimulating DNA synthesis with milk peptide was not affected by DTT treatment agrees with the previous results of sequencing.

The results from this study suggested that the milk peptide appears to differ biochemically from EGF which is inactivated by incubation with sulfhydryl-reducing agents, and is digested by trypsin causing reducation of effect.

Besides, some inherent peptides such as the EGF fragment 20-31, (Komoriya et al., 1984), angiotensin II, (Schelling et al., 1979), and vasopressin that had DNA-synthesis stimulating activity has been reported; a casein fragment that can also

stimulate DNA synthesis in BALB/c3T3 cells was also reported (Nagaune et al., 1989). The mechanism for DNA-synthesis stimulation is not known.

3.2 Chemical Synthetic Milk Peptide

In order to demonstrate that the activity was due to the C-terminus of αS_2-casein (192-222), chemically synthesized peptides were made. Subsequently performed bioassays showed that the synthetic peptide also stimulates DNA synthesis in Rama 27 cells (Liu, 2002).

Milk peptides were synthesized by Pepsyn Ltd, using Fmoc chemistry and pentafluorophenyl esters, according to the standard protocol.

3. 3   Recombinant Milk Peptide

3. 3. 1   Introduction

Recombinant milk peptide would facilitate the study of mechanism of milk peptide. Although purification of milk peptide from bovine milk is generally satisfactory, problems may arise in its use for further study. The purification procedures of milk peptide are difficult and also time consuming because milk peptide has such small mass and low concentration that SDS-PAGE is unable to detect it.

To overcome this problem, cloning and expression of milk peptide were investigated in this study. The aim of this part of the study is production of recombinant milk peptide, to confirm that milk peptide is a novel growth factor for fibroblasts, and to produce large quantities of milk peptide.

In this part of the study to produce recombinant milk peptide, recombinant plasmid was constructed, carrying an artificial gene for milk peptide. The suitability of pET expression vector (*p*lasmid for *e*xpression by *T7* RNA polymerase) (Rosenberg et al., 1987) to express milk peptide was established. Recombinant milk peptide was successfully expressed in *E coli*. In addition, isolation of recombinant peptide was attempted. The BL21 cells express a moderate level of the milk peptide as soluble protein.

3.3.1.1 Chemically synthesized gene for milk peptide

Escherichia coli bacteria are a powerful tool for the production of heterologous protein in large quantities. The functional genes coding for polypeptides of interest are introduced stably into *E. coli* bacteria by vector. For many studies monoclonal polypeptides are of great advantage in comparison with protein preparations from natural sources, which may consist of numerous closely related but not identical isoforms. *Escherichia coli* is one of the best-studied organisms and many well-established methodologies used in molecular biology can be applied to modify and handle vectors and coding sequences (Sczakiel et al., 1993).

Since the first successful construction and genetic expression of chemically synthesized gene coding for somatostatin (Itakura et al., 1977), many total human gene syntheses have been reported including growth hormone (Ikehara et al., 1984), insulin (Crea et al., 1978), Leukocyte interferon (Edge et al., 1981), insulin-like growth factors I and II (Mullenbach et al., 1983), and lymph toxin (Gray et al., 1984). Given the amino acid sequence of a peptide hormone or growth factor, it is probably easier to synthesize the gene than to isolate it by cDNA cloning. Also, chemical synthesis permits gene design. Codon usage and restriction sites can be tailored for a specific expression system (Urdea, 1987). Milk peptide is a very short protein, and so it was ideally suited for artificial gene synthesis.

3.3.1.2 The expression vector pET-16b

pET expression vectors, originally developed by Studier et al. (1990) and since expanded, allow regulated expression of foreign genes by bacteriophage T7 RNA polymerase. These vectors typically carry the colicin E1 (colE1) replicon of pBR322 and confer resistance to ampicillin or kanamycin. Their multiple cloning sites allow an inserted coding sequence to be placed under the control of the so-called 'T7lac' promoter, a derivative of natural promoter that has the *lac* operator (*lacO*) placed so that binding of the *lac* repressor blocks transcription initiation (Figure 3.10).

To clone and express milk peptide gene, pET-16b expression vector (Figure 3.15) containing the T7 promoter (IPTG-inducible) was chosen in the present experiments. A major advantage of pET expression vector is that pET system uses the bacteriophage T7 promoter to direct the expression of target genes. Since *E. coli* RNA polymerase does not recognize the T7 promoter, there is virtually no transcription of target genes in absence of a source of T7 polymerase and the cloning step is thus effectively uncoupled from the expression step (Figure 3.10).

3.3.1.3 Host for expression

After plasmids are established in a non-expression host (DH5α), they are most often transformed into a host bearing the T7 RNA polymerase gene (λDE3 lysogen) under *lacUV5* control, and expression is induced by the addition of IPTG. The host used in present study is BL21 (DE3), which has the advantage of being deficient in both *lon*

and *omp*T proteases that can degrade proteins during the purification (Studier et al., 1990). Thus milk peptide might be expected to be more stable in BL21 than in host strains that contain these proteases.

Bacteriophage DE3 is a derivative that has the immunity region of phage 21 and carries a DNA fragment containing the lacI gene, the lacUV5 promoter, the beginning of the lacZ gene, and the gene for T7 RNA polymerase. (Figure 3.10).

3.3.1.4 Overview for the construction of recombinant milk peptide

To constitute an effective expression system for milk peptide, the mode of gene expression should be considered. It affects the location of the proteins produced; the proteins may either be located in the cytoplasm of *E coli* or secreted through the cell membrane. Eukaryotic genes cloned in frame with synthetic or bacterial nucleic acid sequences could be expressed as hybrid products in the cell cytoplasm. Transcription, from bacterial promoters, and translation, yield fusion proteins which include bacterial or synthetic polypeptide sequences in addition to eukaryotic polypeptide (Marston, 1986). Recombinant milk peptide was designed here to be located in the cytoplasm, and expressed in soluble form.

In *E coli* an ATG sequence must precede the gene coding sequence, for initiation of translation. Thus the primary products of translation possess an *N*-terminal methionine residue. *E coli* possesses enzymes which catalyse the efficient removal of the methionine residues from the natural proteins when required, but these enzymes do not appear to work with the same efficiency on recombinant polypeptides. In the present study, if directly expressed milk peptide possessed an unnatural *N*-terminal methionine residue it might affect the activity of milk peptide.

The synthetic genes for the milk peptide were designed to carry an *Nco* I site at the 5'-end facilitating in-frame fusion to encode the four-amino-acid recognition site for Factor Xa, and dual translation stop codons next to the peptide gene as shown in Figure 3.12. Thus, fusion milk peptide could be expressed in a soluble form, and techniques exist to process the fusion peptide and release the desired protein.

3. 3. 2 Experimental and results

3. 3. 2. 1 Preparation of DNA Fragment

The synthetic genes encoding milk peptide were designed based on amino acid sequences of the milk peptide (Liu et al., 1996) according to the codon preference of *E. coli*. An *Nco*I restriction enzyme site, which contains a triplet codon for methionine (ATG), is designed as a starting molecule of the fusion partners in the synthetic milk peptide. The choice the recombinant sequences was that it represented the sequence of the most active single preparation from Liu (2002), and anything smaller would have been too small for this approach. The coding sequence of interest was fused in-frame to the 3' end of a sequence encoding the four-amino-acid recognition site for Factor Xa (Ile/Glu/Gly/Arg) (Nagai and Thogersen, 1984; 1987; Lauritzen et al., 1991; Ohashi et al., 1991). The unique cleavage site of four amino acids recognized by Factor Xa, which is not present in the coding sequence of the milk peptide gene, was linked to the milk peptide to produce a fusion product. Factor Xa cleaves the peptide bond that is carboxy-terminal to the arginine residue in the recognition site (Figure 3.11).

Using the above as a starting molecule, the milk peptide could be separated from the carrier sequences by enzymatic cleavage by Factor Xa (Figure 3.11). The gene for the milk peptide was designed to have an *Nco* I restriction site on the left end, an *Xho* I site at the right end. This was done so that synthetic genes could be cloned in the convenient cloning vehicle pET-16b (Figure 3.12).

3. 3. 2. 2 Preparation of pET 16b vector for Cloning

The pET 16b vector (Figure 3.15) can be used to select an open reading frame in DNA fragments carrying *Nco* I and *Xho* I-compatible sticky ends. To prepare the pET 16b vector for cloning, it was first transformed into DH5α (Method 2.3.2.4) and purified (Method 2.3.2.1.1). After purification it was cleaved with *Nco* I and *Xho* I to produce linear vector (Method 2.3.2.5). The entire sample of DNA was analysed by agarose gel electrophoresis to verify the size of the plasmid by restriction endonuclease digestion prior to subsequent cloning procedures (Figure 3.13). Digested pET-16b vector was purified from an agarose gel and ligated (Method

2.3.2.7) with the synthetic peptide genes. Directional cloning strategy is outlined in Figure3.14.

3. 3. 2. 3 Construction of expression vector

The ligated material was used to transform competent cells of *E coli* strain DH5α as described in materials and methods (2.3.2.3). Twenty colonies of transformed bacteria which were ampicillin-resistant were picked and screened for presence of the desired recombinant plasmid pET-16b using PCR (Method 2.3.2.9). The oligonucleotide primers chosen hybridise to T7 promoter or T7 terminator of pET-16b vector (Figure 3.15b). When used in conjunction in the presence of the recombinant plasmid a PCR product of 240 bp was produced (Figure 3.16). Such PCR analysis identified four colonies (numbers of 1, 3, 11 and12) that could possibly contain recombinant. Plasmid DNA of three colonies (numbers of 1, 3, and12) was prepared (Method 2.3.2.1) and subjected to analysis by *Nco* I and *Xho* I restriction endonuclease digestion (Method 2.3.2.5). The digests were sized by agarose gel electrophoresis (Figure 3.17) and colonies produced restriction fragments with *Nco* I and *Xho* I that correlated with the expected bands of 104 bp. Two restriction endonucleases produced the bands of fragments expected for recombinants, thus confirming the identity of plasmid within colonies 1, 3 as the desired construct. One of the restriction endonuclease digests produced a band of fragment that was smaller than 104 bp (Figure 3.17). Desired constructs that were of correct size were further characterised by nucleotide sequencing.

3. 3. 2. 4 Nucleotide sequencing of the constructed milk peptide gene

To further confirm the desired construct, DNA sequencing of recombinants was carried out using oligonucleotide primer which hybridizes to the pET-16b (Figure 3.15b). DNA sequence from recombinants of colony 1 was identified and 104 bases were read in 5' to 3' direction from this point (Figure 3.18). DNA sequencing revealed that the junction of pET 16b to the milk peptide was correctly formed, and that 5' position of the milk peptide sequenced agreed with the designed sequence. Thus pET 16b-milk peptide appears to have been correctly constructed. The outline of the protein expression procedure is described in Figure 3.19.

3. 3. 2. 5 Induction of protein expression

A consideration when expressing fused gene is whether the fusion product should be in soluble or insoluble form. A large number of soluble fusion proteins have been produced in *E coli*, both intracellularly and secreted (Uhlen and Moks., 1990). In general, recombinant polypeptides accumulate to higher level of total protein when expressed intracellularly than secreted, but many of the poplypeptide products located in the cytoplasm are insoluble and aggregated (Marston, 1986). The expression strategy in present study is expected to produce the fusion peptide in a soluble form. This approach has the great advantage that product with full biological activity can be obtained directly without renaturation.

BL21 (DE3) cells, which contained a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, were transformed with the plasmid pET16b containing the gene of interest (termed pET16b-peptide) (Figure 3.15a), or with pET16b without the cloned gene (termed pET-16b). The latter transformant was used as a control when studying the expression of the cloned gene. Colonies of transformed bacteria containing either pET-16b or pET16b-peptide were incubated at 37°C until the optical density of cultures reached 0.5-0.7 when measured at 600 nm. At this stage, the bacterial cultures were induced with 0.1M IPTG (Method 2.3.2.11). To optimise expression, a pilot experiment was carried out. The conditions of expression which generated the highest levels of protein were determined. The cells from three colonies were harvested after 0, 1, 2, 3, 4 and 22 hours induction with IPTG for analysis (Figure 3.24).

3. 3. 2. 6 Detection of expression of recombinant

SDS –PAGE is the commonest method used to detect protein expression in *E coli*. Milk peptide expression was difficult to detect in this study, because recombinant peptide only has 3.710 kDa mass. Therefore reverse phase HPLC was first choice to detect the expression of milk peptide in *E coli*.

3. 3. 2. 6.1 Detection of expression of recombinant by Reverse Phase HPLC

Reverse phase HPLC techniques have been traditionally applied to the analysis of low molecular weight compounds when even SDS-PAGE fails to detect the small proteins. In the purification of milk peptide from bovine milk, reverse phase HPLC represented a powerful technique (Liu et al., 1996).

To analyse the expression of soluble protein, 20μl of supernatant from sonication of cell lysate was loaded onto RP-HPLC. In the absence of IPTG induction, the expression was low. There is nearly no absorbance peak of peptide eluting at 11% acetonitrile (Figure 3.20a). On IPTG induction for 2 hours, there is a distinct peak eluting at 11% acetonitrile (Figure 3.20b). On IPTG induction for 4 hours, the peak eluting at 11% acetonitrile decreased in amount (Figure 3.20c). There was a significant peak in the sample induced by IPTG for 2 hours. The results suggested that the peak eluting at 11% acetonitrile might be related to expression of the desired product, and that the optimal expression time might be 2 hours.

The results analysed by HPLC indicated that although expression of a peptide product was detected, it is still unclear if the expressed protein is the same mass as that of the expected protein.

3. 3. 2. 6. 2 Detection of expression by gel electrophoresis

To further confirm the expression products SDS-PAGE was used. Since the desired expression peptide is so small (3.71 kDa), normal SDS-PAGE analysis failed to detect the expression of the desired product although a 20% polyacrylamide gel was used (result not shown). The reason is that stacking of small proteins in the presence of SDS is difficult, because small proteins all form complexes of protein and detergent of the 'same' size and charge as the SDS micelle itself (Fish et al., 1970). Glycine as used in the Laemmli system, leads to a stacking even of very large proteins; however, proteins below 20 kDa are only partly or not at all separated from the bulk of SDS. Another reason is that not all small proteins could be stained, because they either eluted completely and /or did not bind the dye (Schagger et al., 1987). To analyse the expression of the recombinant product in this study, Tricine – SDS-PAGE was performed.

A clone transformed with the insert-free pET-16b vector was induced as a negative control. To facilitate comparison, each uninduced sample is loaded alongside the corresponding induced sample. SDS-PAGE analysis demonstrated the presence of a protein with apparent molecular weight of 4 kDa in bacterial cell lysate containing pET 16b-milk peptide. As Figure 3.21 shows, bands at the bottom of the gel (around

4 kDa) were stronger in the induced lysate than uninduced; the same result was observed on the sonicated supernatant (Figure 3.22). It is suggested that recombinant peptide might be expressed as expected molecular size and as soluble proteins under IPTG induction. The band of 3.7 kDa protein should be correlated with peak fraction in 11% acetonitrile from reverse phase HPLC.

3. 3. 2. 6. 3 Detection of expression by DNA assay

To test directly the induced expression of the desired protein, it was assayed by its action in stimulation of DNA synthesis in Rama 27 cells. The incorporation of $[^3H]$-thymidine was first measured after addition of crude cell lysates. No stimulation was observed after the addition of sonicated supernatant from the induced or uninduced samples (data not shown). However, stimulation was observed after the addition of induced and uninduced pellet; uninduced pellet stimulated DNA synthesis more than induced; even the cell pellet from bacteria without pET-16b vector also stimulated DNA synthesis by 1.7 fold (Figure 3.23a).

Since HPLC and SDS-PAGE analysis results showed that the expressed protein is in the sonicated supernatant, the incorporation of $[^3H]$-thymidine into Rama 27 cells was measured after addition of the fraction of supernatant from HPLC containing the peak of peptide. Stimulation of DNA synthesis was observed after addition of the peak fraction. Increasing concentrations of peak fraction added to quiescent Rama 27 cells caused a little increase in DNA synthesis (Figure 3.23b). The maximum stimulation of DNA synthesis was about 200% of control (Data not shown). The results showed that the fraction from HPLC containing the peptide peak caused stimulation of DNA synthesis in Rama 27 cells.

3. 3. 2. 6. 4 Detection of expression by amino acid sequence

The identity of the band from SDS-PAGE was further verified by electroblotting the protein purified by SDS gel electrophoresis onto a Polyvinylidene difluroride (PVDF) membrane, staining with Coomassie brilliant blue and sequencing with automated Edman degradation of the peptide directly. The results of the sequencing are shown as Figure3.25. Three peptides were sent for direct sequencing: The one peptide had the same sequence with designed recombinant product except that an N – terminal methionine was lost; the other two peptides were fragments of recombinant product

with the same C-terminus. The results here confirmed that recombinant milk peptide was correctly expressed in *E coli.*

3. 3. 2. 7 Enhancing the expression of recombinant protein

Before purification, the conditions of the expression which generated the highest levels of protein were determined. Bacterial cultures transformed with pET16b-recombinant plasmid were incubated at 37°C until the optical density of culture was 0.7 when measured at 600 nm. At this point, the bacterial cultures were induced with 0.1 M IPTG for a further 22 hours. At various times during this period, samples from each of time of induction were loaded onto gels for electrophoretic analysis. For each sample removed, the optical density was measured to ensure that the same number of bacterial cells were analysed for each time point (Figure 3.24). The amount of recombinant protein increased with time with the maximum level of production occurring between 1and 3 hours of IPTG induction; after 4 hours the amount of recombinant protein decreased.

Upon lysis of the bacterial cells with lysozyme and also by sonication, the recombinant protein in this study was present in the supernatant. Densitometric scanning of SDS-PAGE gel indicated that BL21 cells expressed milk peptide as 10% of total protein.

3. 3. 2. 8 Recombinant protein isolation

The results described above suggest that the recombinant protein produced in the bacterial culture is the desired insert gene. At this stage, any further characterisation of recombinant products can only be achieved after purification of the protein from bacterial cells. Once the crude lysate has been prepared, the purification of milk peptide can be accomplished by a three-step procedure. The method used for purification of recombinant milk peptide is described in materials and methods (2.3.2.12). The procedure for the isolation of recombinant product is outlined in Figure 3.26.

First cation-exchange chromatography using a column of CM-sepharose equilibrated in a buffer at pH 6.0 was designed to separate the recombinant protein from the bulk of the contaminating *E coli* protein. Proteins that bound to the column under these

conditions were eluted by the application of a linear NaCl gradient. SDS-PAGE analysis of the collected fractions showed the presence of a protein band with the desired molecular weight which eluted from the column in the region of 0.3M NaCl (Figure 3.27a, 3.27b). However, the fractions with the desired band were found to have no effect on stimulation of DNA synthesis in Rama 27 cells (Figure 3.27a).

Those fractions containing the recombinant milk peptide protein (Figure 3.27b) were pooled and subjected to a Butyl-sepharose column which was equilibrated in a salt-containing buffer at pH 6.5. As Figure 3.28a shows only a small amount of protein was washed out, most of protein was bound to the column and then eluted from the column in the region of 3M NaCl by the application of a reducing linear NaCl gradient. SDS-PAGE analysis of the collected fractions showed the presence of a protein band with a desired protein molecular weight. This band seemed not to be enriched, and only a few contaminants were removed during this chromatography (Figure 3.28b). A lack of activity of those fractions was also observed (Figure 3.28a).

For further purification of recombinant milk peptide, reverse phase HPLC was used next. Fractions containing the recombinant milk peptide protein (Figure 3.27b) from CM-sepharose were direct applied to the C_4 reverse phase HPLC (an aquapore butyl bonded phase, Brownlee column). As Figure3.29 shows, those fractions were resolved into several small peaks and one main peak which was eluted at 50% acetonitrile. The fractions from the main peak stimulated DNA synthesis by 170 % of control (Figure 3.29). This step also did not eliminate most of contaminating proteins.

The results here suggested the recombinant milk peptide should be in one of the main peaks because the fractions with stimulation of DNA synthesis were eluted from this peak.

Figure 3.10 Control elements of the pET system
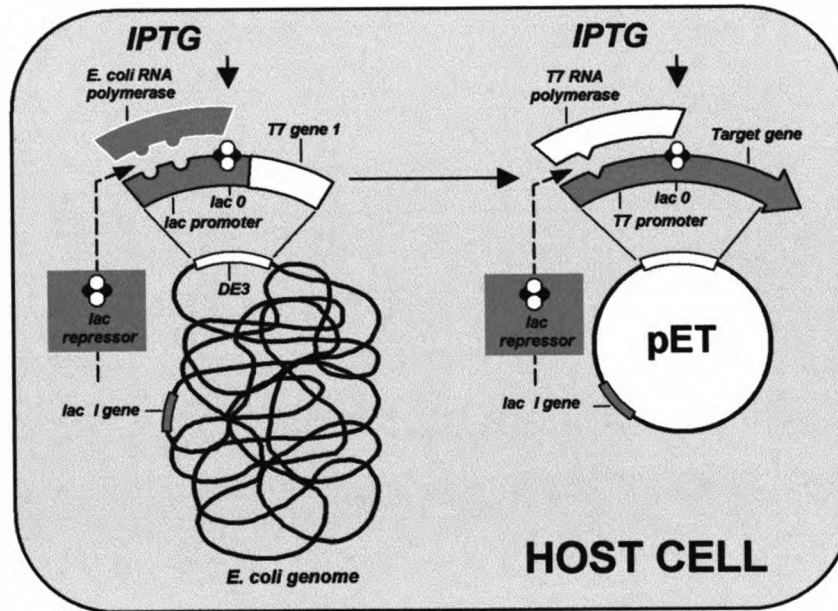
Illustrated here are in schematic form the host and vector elements that are available for control of T7 polymerase levels and subsequent transcription of the a target gene in a pET vector. In λDE3 lysogens, the T7 RNA polymerase gene is under the control of the *lacUV5* promoter. This allows some degree of transcription in the uninduced state and in the absence of further controls is suitable for expression of many genes whose products have innocuous effects on host cell growth. Adapted from Novagen, Inc.

Figure 3.11 Strategy of cleavage of fusion peptide by Factor Xa

Figure 3.12 DNA sequences in the construct and the position of milk peptide – encoding sequence

Figure 3.13 Restriction endonuclease digestion of pET-16b
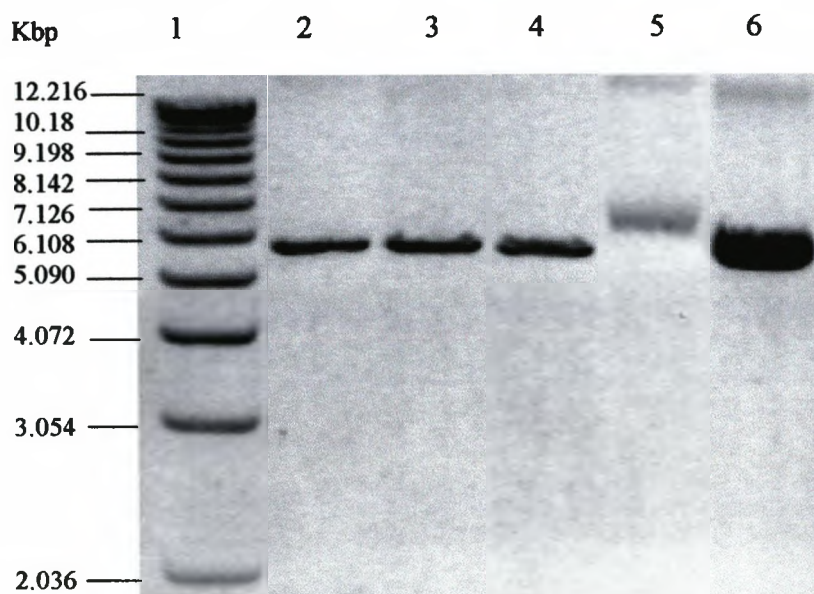
Agarose gel electophoresis of restriction endonuclease digestions.

The molecular sizes of standard nucleic acids are shown in kilo-base pairs (kbp).

Lanes: 1- 1μg 1kb DNA ladder.

2 – 1μg pET 16b digested with *Xho* I.

3 - 1μg pET 16b digested with *Nco* I .

4 - 1μg pET 16b digested with *Nco* I, and *Xho* I.

5 – 1 μg uncut pET 16b for reference.

6 – 5μg pET 16b digested with *Nco* I , and *Xho* I

Figure 3.14 Cloning of the peptide gene into pET-16b

1: Preparation of Vector (pET-16b) for cloning

        a.Preparing competent *E.coli* DH5α

           (Method 2.3.2.3)

        b.Transformation of competent bacterial cells

           (Method 2.3.2.4)

        c. Purification of vector (pET-16b)

           (Method 2.3.2.1)

2: pET-16b digested with *Nco* I and *Xho* I

           (Method 2.3.2.5).

3: Check for linear 5.7 kbp plasmid by agarose gel electrophoresis.

           (Method 2.3.2.6)

           Control: plasmid undigested with enzyme.

4: Extract linear vector from agarose gel.

           (Method 2.3.2.8)

5: Ligate vector and insert gene at a 2:1 molar ratio.

           (Method 2.3.2.7)

6: Transformation into *E.coli* DH5α and plate onto agar with ampicillin for screening

Figure 3.15 Structure of pET-16b-Peptide

(a) Circle map of the pET-16b-Peptide.
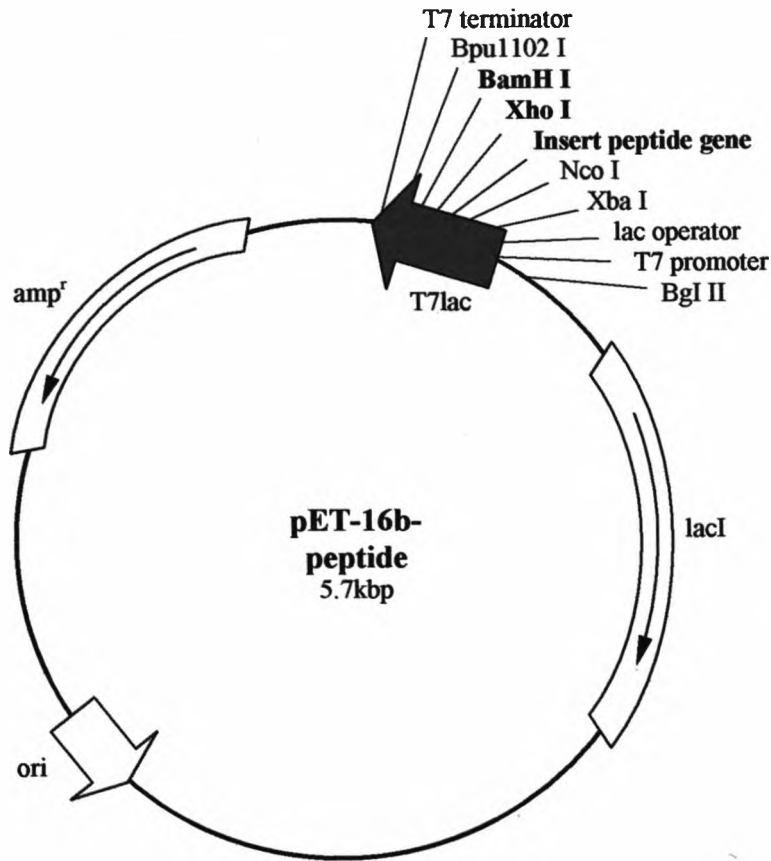
(b) pET- 16b Vector Cloning Region Sequences.

pET-16b-peptide cloning/expression region
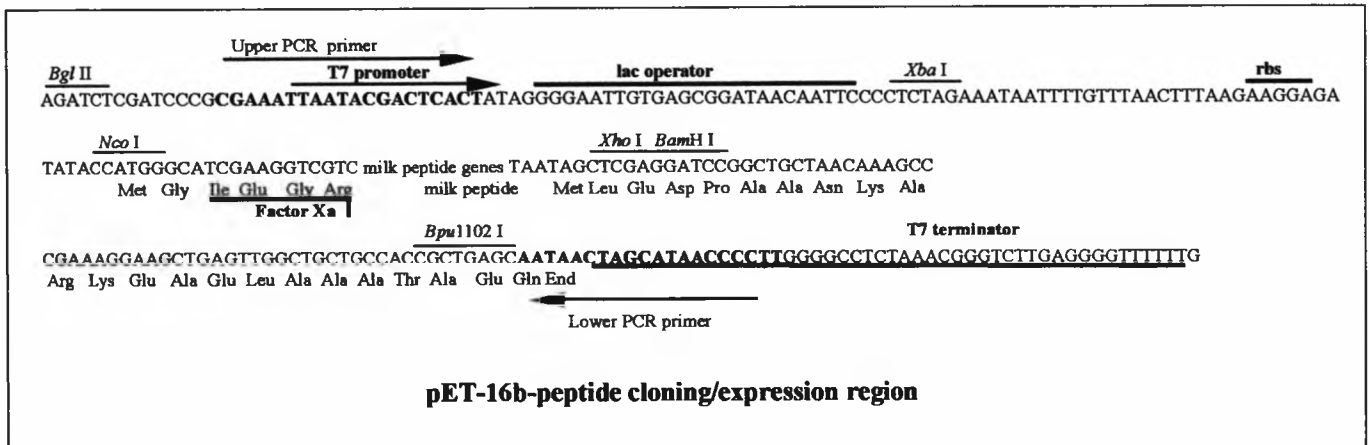
The peptide gene was directionally cloned into pET-16b vector as a 104 bp *Nco* I-*Xho* I (5' to3') fragment (a). The PCR oligonucleotide primers were chosen hybridise to T7 promoter or T7 terminator of pET16b vector (b).

Figure 3.16 PCR analysis of recombinant plasmids
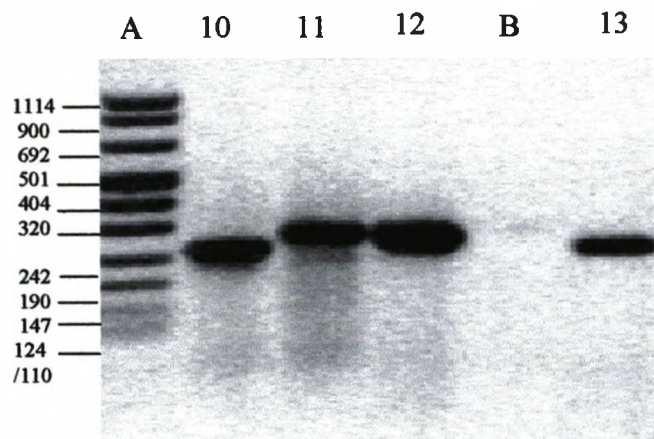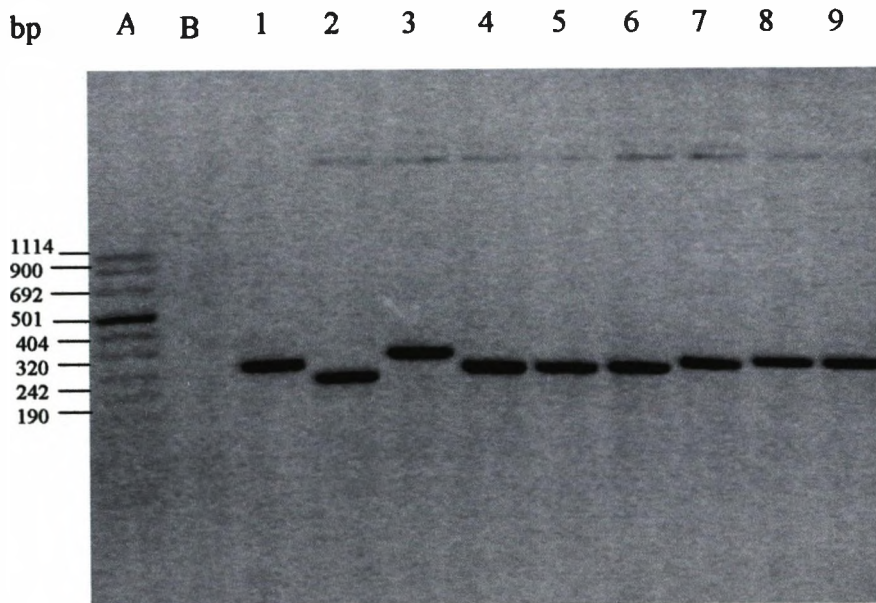
Agarose gel electrophoresis of PCR products. The molecular sizes of standard nucleic acids are shown in base pairs (bp).

Lanes: A – 1µg DNA size markers.
B – PCR negative control.
1 to 13 – PCR products from colonies 1 to 13.

Figure 3.17 Restriction endonuclease digest analysis of recombinant plasmids
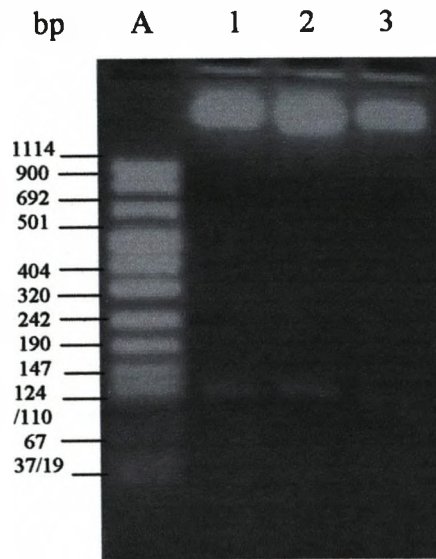
Agarose gel electrophoresis of *Nco* I and *Xho* I restriction endonuclease digests. The molecular sizes of standard nucleic acids are shown in base pairs (bp)

Lanes: A – 1μg DNA molecular weight marker.

1 to 3 – plasmid DNA from colonies 1 to 3 digested with *Nco* I and *Xho* I

Figure 3.18 Nucleotide sequence from cloned synthetic gene

<div>

                                                     <u>CC</u>

```
                    NcoI
 1    ATG GGC ATC GAA GGC CGCACG GTG TAT CAG      30
 1    Met  Gly  Ile  Glu  Gly  Arg  Thr  Val  Tyr  Gln   10

31    CAC CAG AAA GCG ATG AAA CCG TGC ATC CAG      60
11    His  Gln  Lys  Ala  Met  Lys  Pro  Trp  Ile  Gln   20

61    CCG AAA ACG AAA GTG ATC CCG TAT GTG CGC    90
21    Pro  Lys  Thr  Lys  Val  Ile  Pro  Tyr  Val  Arg  30
                                 XhoI
91    TAT CTG TAA TAG CTCGAG
31    Tyr  Leu  End End                          108
                                                  32
```

</div>

Nucleotide sequence from recombinant colony and deduced amino acid sequences. The amino acid sequence of the synthetic gene is underlined, corresponding to the region from ATG in the 5' noncoding region to the stop codons in the 3' noncoding region. The nucleotide sequence and amino acid sequences of milk peptide are shown in bold. Restriction enzyme sites required for insertion into the expression vector are also shown.

Figure 3.19 Expression of Cloned Gene in *E.coli* cells using the Bacteriophage T7 Promoter

1.Selecting positive cloning by PCR. (Method 2.3.2.9)

        Control: plasmid undigested with enzyme.

$\downarrow$

2. Verification of selected clones

a). Minipreparation from each selected clone (Method 2.3.2.1.1)

b). digested with restriction enzymes from purified vectors

c). DNA sequences

$\downarrow$

3. Preparation of host strain for expression

        a.Preparing competent *E.coli* BL21

        b.Transformation of competent bacterial cells

$\downarrow$

4. Testing expression induced by IPTG

        a.   Pilot experiment for optimisation of expression

        b.   Checking expressed recombinant protein (Method 2.3.2.11)

$\downarrow$

5. Detection of expression of recombinants

        a.   SDS-PAGE (Method 2.3.1.5.2)

        b.   Reverse phase HPLC (Method 2.2.3.3)

        c.   Detect growth activity of crude prduct in Rama 27 cells

Figure3.20 Analysis of protein expression by Reverse-Phase HPLC

(a) The sample uninduced by IPTG   **(b)** The sample induced 2 hours by IPTG
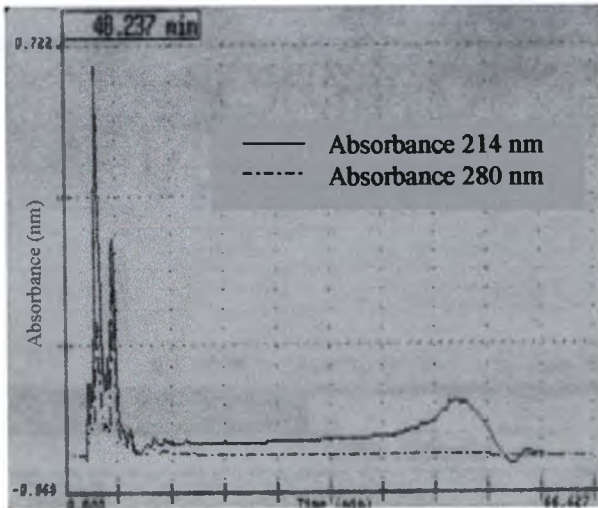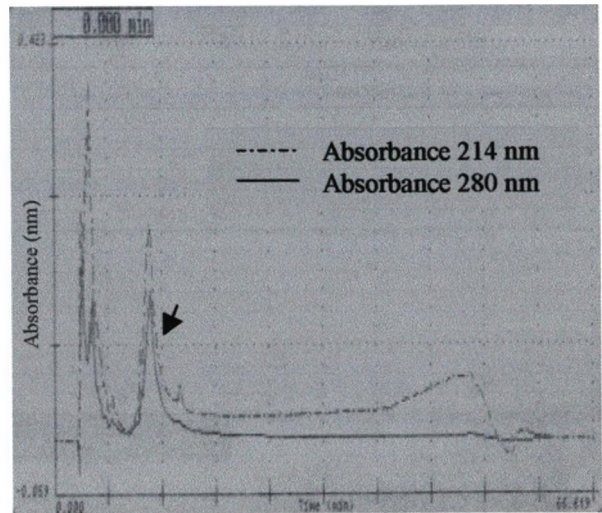
(c) The sample induced 4 hours by IPTG
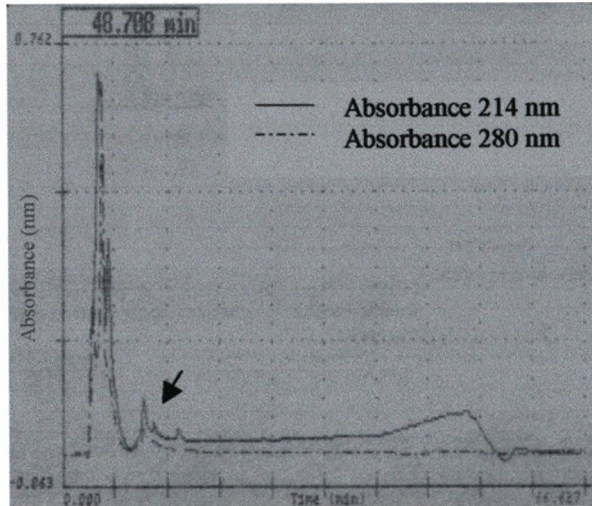
Sonicated supernatants of bacterial lysates were subjected to HPLC on a C4 column with an acetonitrile gradient of 10-30%. Arrows show expressed protein.

Figure 3.21 SDS-PAGE analysis of proteins from lysed *E.coli* strain BL21(DE3)
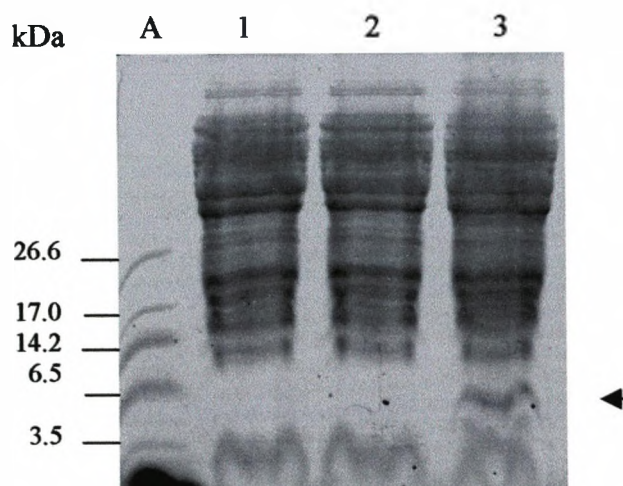
E coli strain BL21(DE3) was transformed with pET-16b-peptide. After 1 hour of induction by IPTG, samples of the bacterial cultures were removed for analysis by gel electrophoresis. Bacterial cells were collected by centrifugation, dissolved in sample buffer (lanes 1, 2, 3). Samples were subjected to SDS-PAGE using Tricine buffer system. Proteins were visualised by staining the gel with Coomassie blue staining.

Lanes: A – Protein standards indicate mass in kilodaltons: Triosephosphate Isomerase (26.6kDa), Myoglobin (17 kDa), α-Lactalbumin (14.2 kDa), Aprotinin (6.5 kDa), Insulin Chain B (3.496 kDa).

Lanes: 1 – whole bacterial extract without pET-16b.

2 - whole bacterial extract with pET-16b-peptide uninduced.

3 - whole bacterial extract with pET-16b-peptide induced.

Figure 3.22 SDS-PAGE analysis of proteins from lysed *E.coli* strain BL21(DE3)
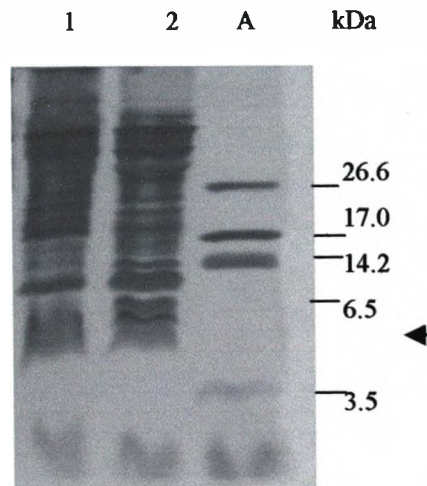
E coli strain BL21 (DE3) was transformed with pET-16b-peptide. After 2 hours of induction by IPTG, samples of the bacterial cultures were removed for analysis by gel electrophoresis. Bacterial cells were collected by centrifugation, suspended in PBS followed by sonication. The supernatants (lanes 1, 2) were subjected to SDS-PAGE using the Tricine buffer system. Proteins were visualised by staining the gel with silver staining. Arrow shows expressed protein.

Lanes: A - Protein standards show mass in kilodaltons. (Ultra-low range markers): Triosephosphate Isomerase (26.6kDa), Myoglobin (17 kDa), α-Lactalbumin (14.2 kDa), Aprotinin (6.5 kDa), Insulin Chain B (3.496 kDa).

Lanes: 1 – lysate supernatant with pET-16b free of insert peptide.

2 - lysate supernatant with pET-16b-peptide induced.

Figure 3.23 Stimulation of DNA synthesis by bacterial lysate in Rama 27 cells

(a) Stimulation of DNA synthesis by 'pellet' in Rama 27 cells.
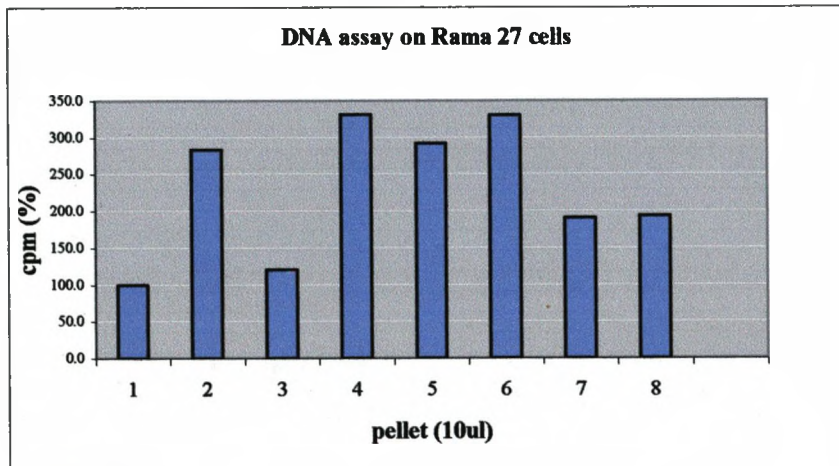
Samples: 1-SDM (serum free medium), 2- pellet from Colony1 uninduced
3- pellet from Colony1 induced, 4- pellet from Colony2 uninduced
5- pellet from Colony2 induced, 6- pellet from Colony3 uninduced
7- pellet from Colony3 induced, 8- pellet BL21without insert

(b) Stimulation of DNA synthesis by freeze-dried fraction of HPLC peak.
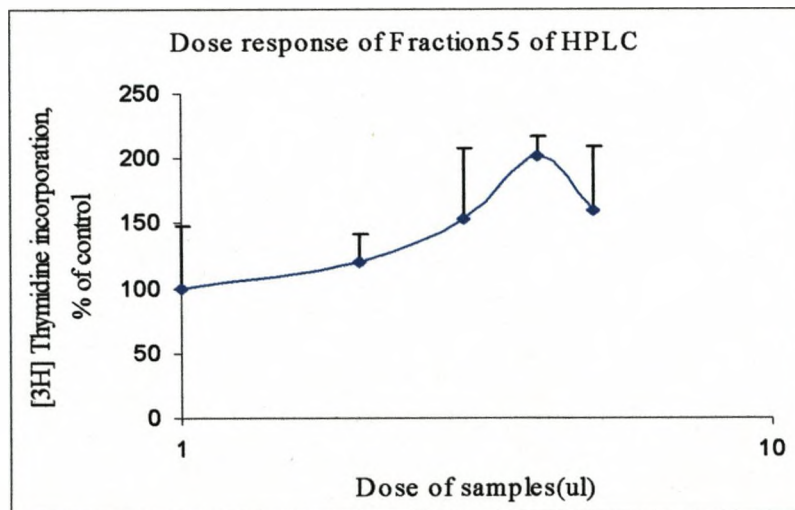
Serum –starved Rama 27 cells were incubated with (a) pellet, (b). peak fraction from HPLC arising when 'supernatant' was loaded on reverse phase HPLC C_4 column for 18 hours. Incorporation of [^{3}H] thymidine into DNA was measured. SDM was used as a negative control (100%). Results are expressed as mean ± SD.

Figure 3.24 Time course of expression of recombinant protein induced by IPTG
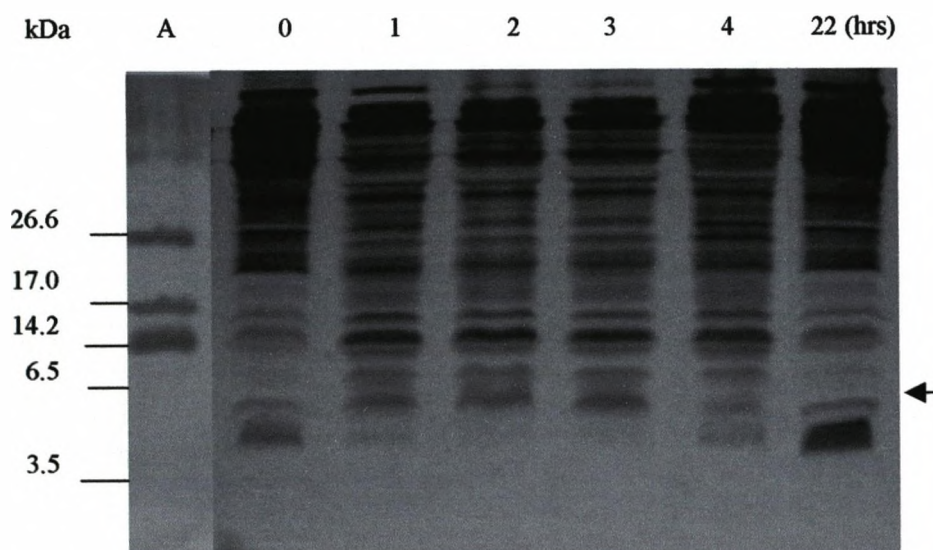
Bacterial cultures of *E. coli* strain BL21(ED3) containing pET-16b-peptide were grown at 37°C until the optical density was 0.5 when measured at 600nm. At this point, the expression of the cloned gene was induced by IPTG. Incubation in these conditions was continued for a further 22 hours. At times during this period, samples of the bacterial culture were removed for analysis by SDS-PAGE. Samples were removed, at 0, 1, 2, 3, 4, 6, 22 hours after the time of induction. To ensure that the same number of bacterial cells were analysed for each time point, the optical density was measured. The bacterial cells were collected by centrifugation, suspended in PBS followed by sonication, and subjected to SDS-PAGE using Tricine buffer system. Proteins were visualised by staining the gel with silver staining. Arrow shows expressed protein.

Lanes: A – Protein standards indicate mass in kilodaltons. Triosephosphate Isomerase (26.6kDa), Myoglobin (17 kDa), α-Lactalbumin (14.2 kDa), Aprotinin (6.5 kDa), Insulin Chain B (3.496 kDa).

0, 1, 2, 3, 4, 22 indicate time (hours) after induction by IPTG.

Figure 3.25 The amino acid sequence of recombinant product

Peptide a

1	G	I	E	G	R	T	V	Y	Q	H 10
11	Q	K	A	M	K	P	W	I	Q	P 20
21	K	T	K	V	I	P	Y	V	R	Y 30
31	L									40

Peptide b

1				G	R	T	V	Y	Q	H 10
11	Q	K	A	M	K	P	W	I	Q	P 20
21	K	T	K	V	I	P	Y	V	R	Y 30
31	L									40

Peptide c

1										10
11		K	A	M	K	P	W	I	Q	P 20
21	K	T	K	V	I	P	Y	V	R	Y 30
31	L									40

The amino acid sequences shown in the figure were derived from expression product in *E coli* with the nucleotide sequence of the cloned synthetic gene.

Figure 3.26 Procedure of recombinant milk peptide isolation

1 litre bacteria (BL21 DE3)

     IPTG, 2hr
     Centrifuge 5000 g, 15min

Pellet

     Lysate buffer, Freeze 30min, (-70°C)

Sonicated

     Centrifuge 12000 g, 20min

Supernatant

     0.02M NaH_2PO_4, pH6.0
     0.02M NaH_2PO_4, NaCl (0-4M), pH6.0

CM-sepharose

     Filter, C4 Hydrophobic column
     0.1%TFA, 0.1%TFA/0-60% CH_3CN

Reverse Phase HPLC

Crude product
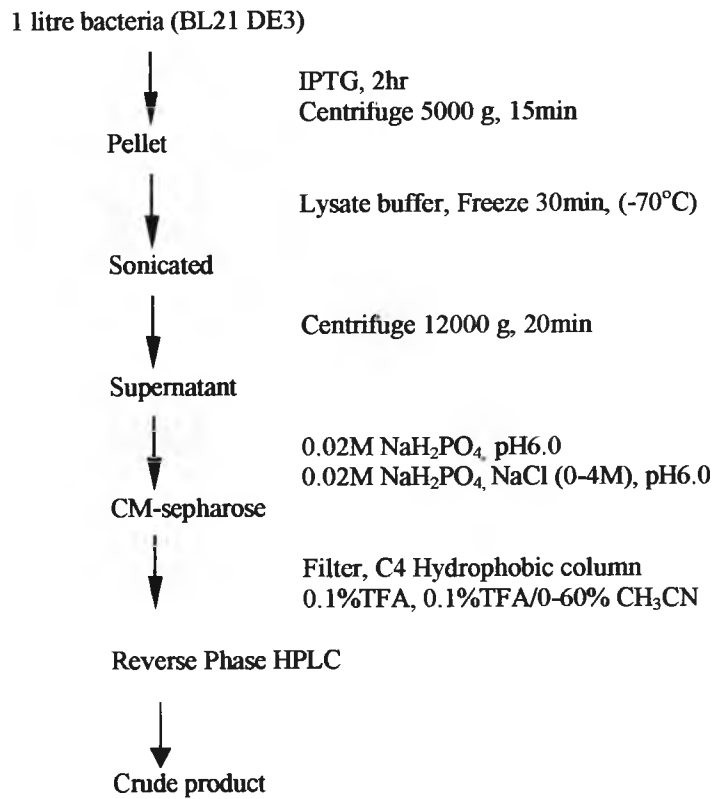
Figure 3.27a. Cation exchange chromatography of the recombinant milk peptide
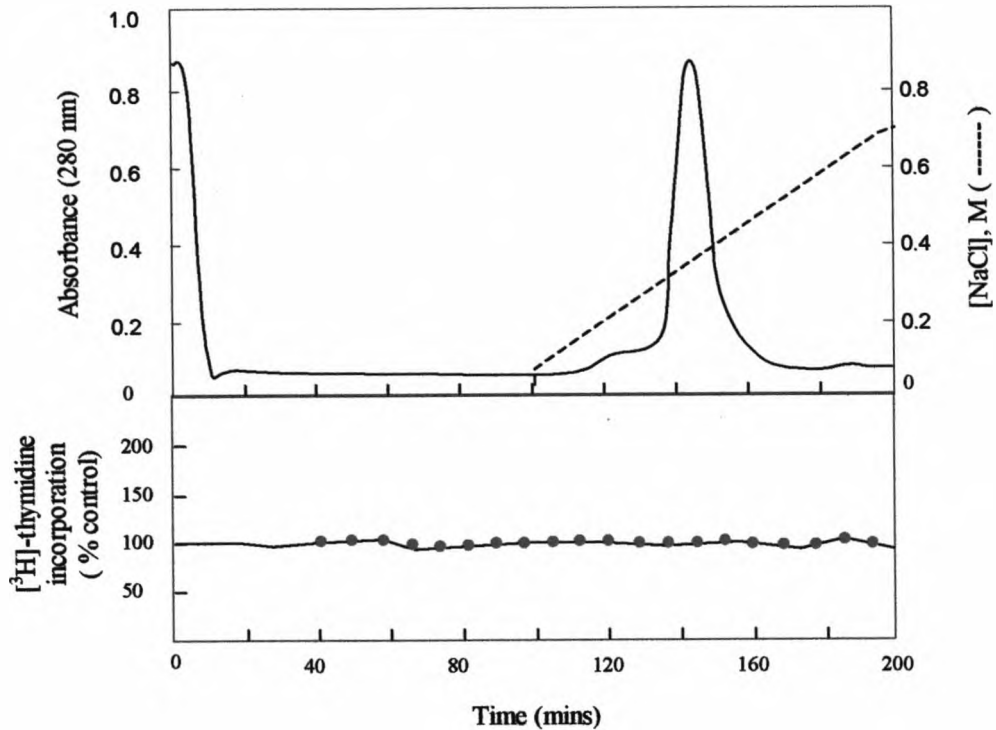
After production of the recombinant protein in bacterial cells, the *E coli* cells were lysed by sonication. Cell debris was removed by centrifugation and the supernatant, which contained the soluble recombinant milk peptide, was applied to cation exchange chromatography on a CM-sepharose column equilibrated with 0.02M NaH_2PO_4, pH 6.0. Column was eluted with a gradient of NaCl in the corresponding equilibration buffer as indicated by dotted line. 10 ml fractions were collected at a flow rate of 5 ml /min, measured for absorbance at 280 nm (upper panel). Fractions were tested for the ability to stimulate DNA synthesis in Rama 27 cells (low panel). Control for DNA synthesis was based on serum - free medium with 0.025% BSA.

Figure 3.27b. Monitoring of purification of recombinant protein by SDS-PAGE
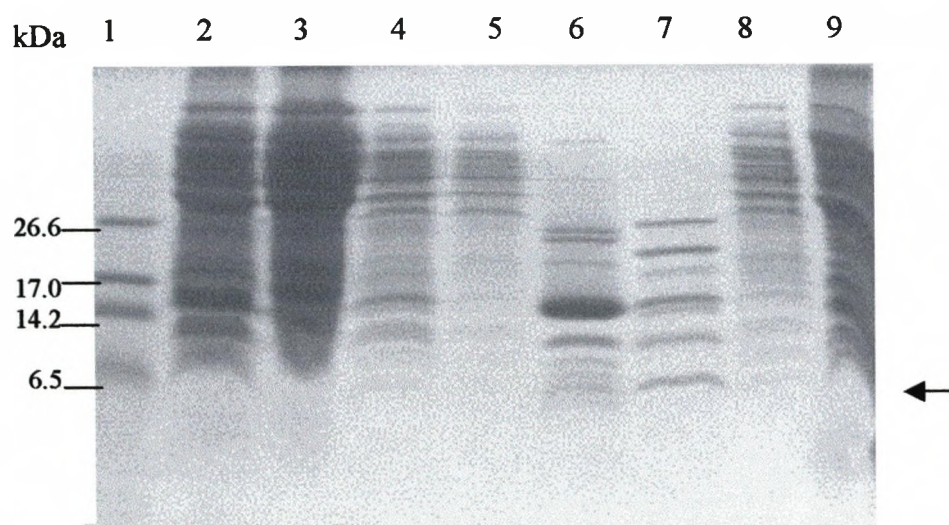
SDS-PAGE analysis of purification of recombinant milk peptide from CM-sepharose column. After production of the recombinant protein in bacterial cells, the E coli cells were lysed by sonication. Cell debris was removed by centrifugition and the supernatant, which contained the soluble recombinant milk peptide, was applied to a column of CM-sepharose equilibrated in a buffer of 20mM NaH_2PO_4, pH6.0. Proteins which eluted from the column during the application of salt gradient were collected as 10ml fractions. Samples of the fractions were analysed by SDS-PAGE. The standard proteins are shown with their molecular mass.

Lanes: 1- low range markers

2- Supernatant before sonication

3- Supernatant after sonication

4- protein loaded on column

5- protein washed out

6- Fraction from gradient 1 (F20)

7- Fraction from gradient 2 (F29)

8- Load through

9- Pellet

Figure 3.28a Butyl sepharose chromatography of the recombinant milk peptide
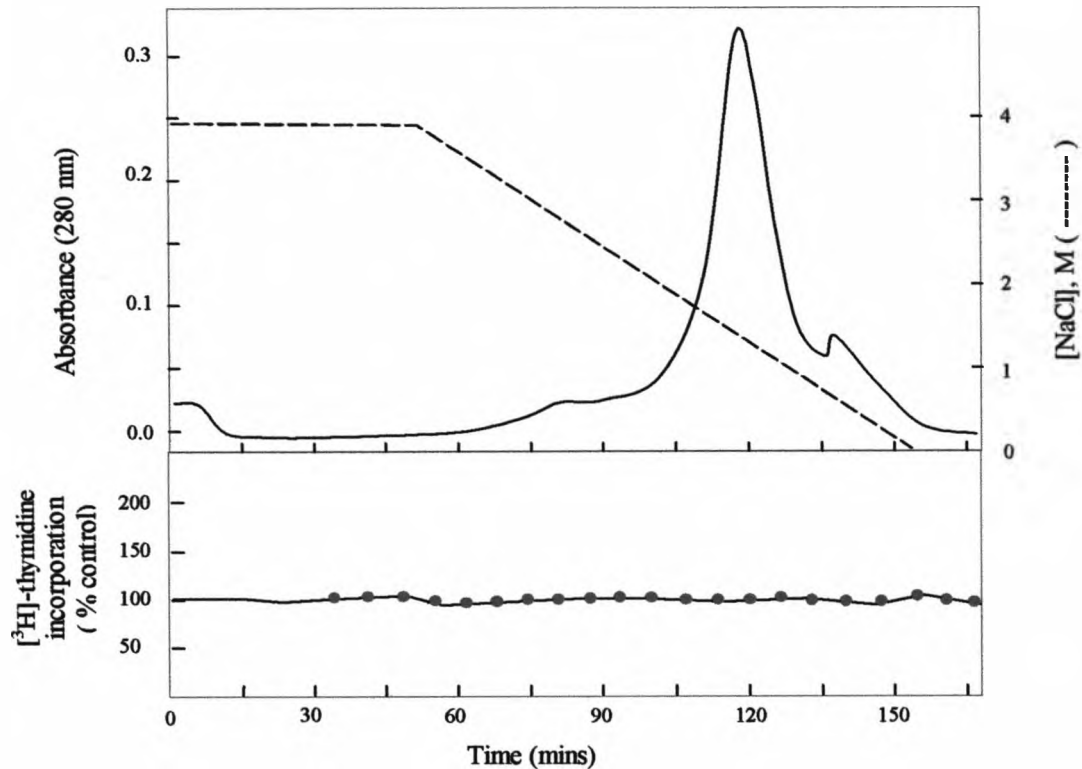
Samples of eluate of recombinant milk peptide from CM-sepharose column were pooled and applied to a Butyl sepharose column equilibrated with 20 mM NaH_2PO_4, pH 6.5 containing 4 M NaCl. Column was eluted with a gradient of NaCl in the corresponding equilibration buffer as indicated by dotted line. 5 ml fractions were collected at a flow rate of 2.5 ml /min, measured for absorbance at 280 nm (upper panel). Fractions were tested for the ability to stimulate DNA synthesis in Rama 27 cells (low panel). Control for DNA synthesis was based on serum - free medium with 0.025% BSA.

Figure 3.28b. Monitoring of purification of recombinant protein by SDS-PAGE
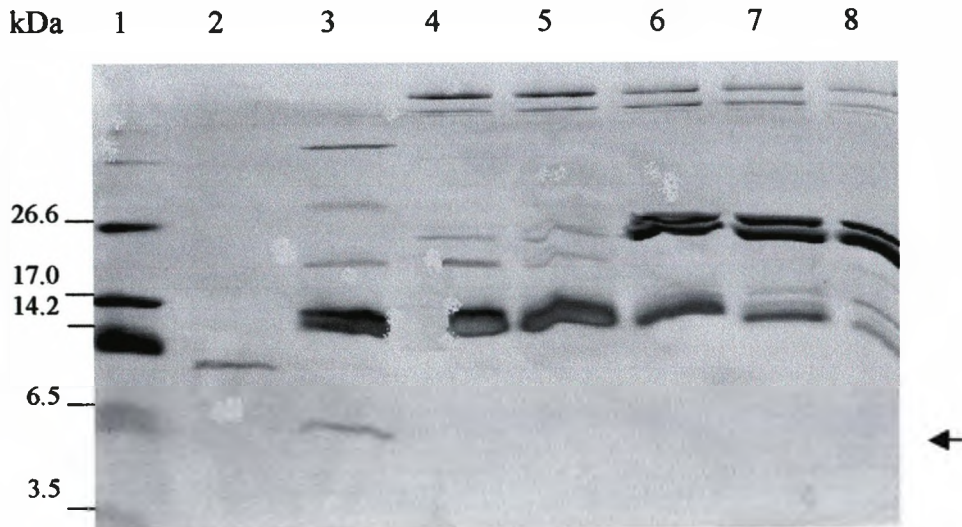
After elution from the CM- sepharose column eluted fractions with the recombinant milk peptide were pooled and applied to a column of Butyl sepharose equilibrated in a buffer of 20mM NaH_2PO_4, pH6.0 containing 4 M NaCl. Proteins which eluted from the column during the application of a reducing salt gradient were collected as 5ml fractions. Samples of the fractions (fraction numbers) were analysed by SDS-PAGE. The standard proteins are shown with their molecular mass. Arrow shows the recombinant protein.

Lanes: 1- Low range markers

2- Fraction 42.

3- Fraction 54 (with recombinant milk peptide).

4- Fraction 61

5- Fraction 62.

6- Fraction 63.

7- Fraction 64.

8- Fraction 66

Figure 3.29 Purifying recombinant milk peptide by Reverse Phase HPLC
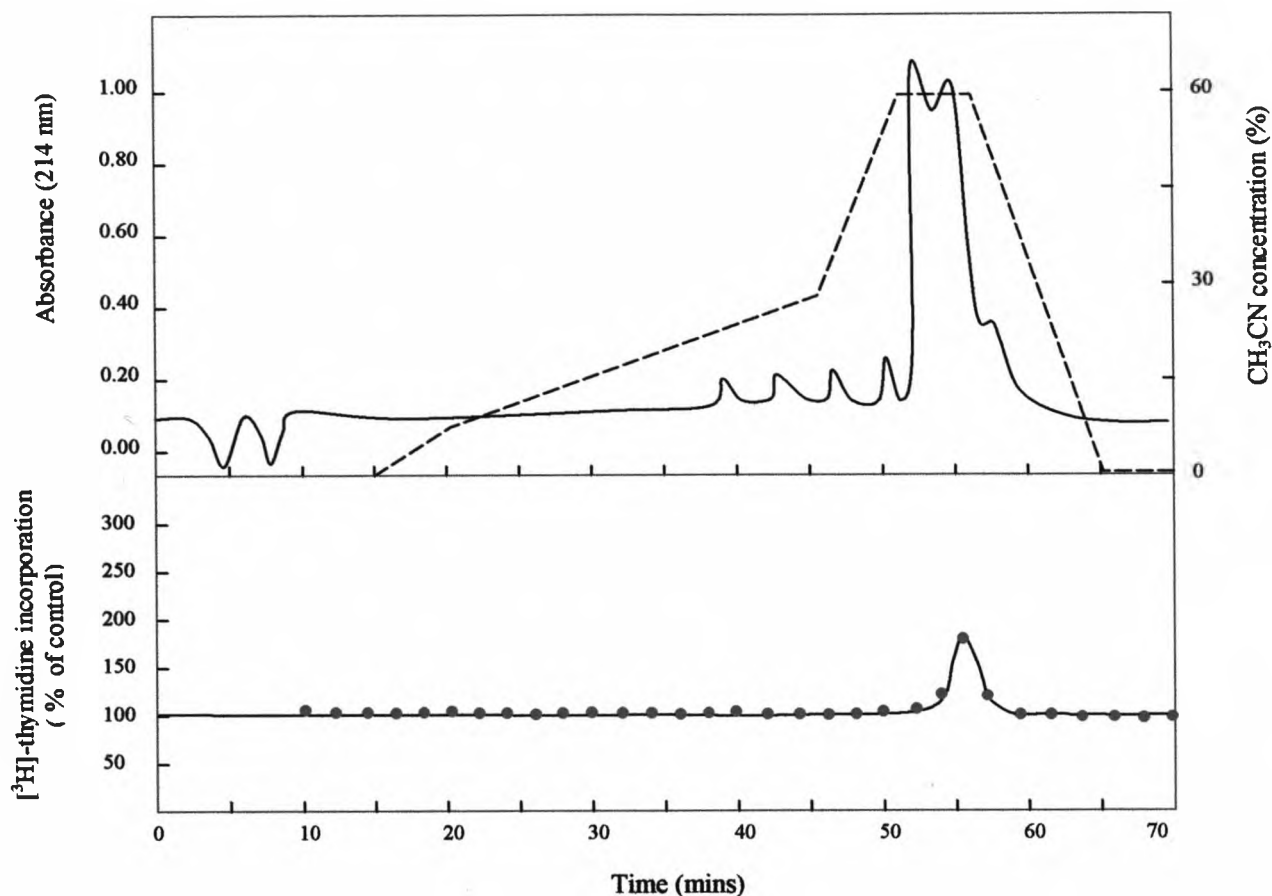
Reverse phase HPLC of recombinant milk peptide. Sample of fraction eluted from CM-sepharose with recombinant milk peptide were applied to Reverse Phase HPLC C_4 column (upper panel) with 0.1% TFA. Column was eluted with $0-60\%$ acetonitrile. Fractions were collected at a flow rate of 0.7 ml / min, measured for absorbance at 214 nm, and tested for the ability to stimulate DNA synthesis in Rama 27 cells (lower panel). Control for DNA synthesis was based on serum - free medium with 0.025% BSA.

3. 3. 3 Discussion

As the earlier part of this chapter described (Section 3.1), milk peptide purified from bovine milk is present at a very low concentration. SDS-PAGE failed to detect the band of milk peptide at any step of purification. The approach of recombinant milk peptide was used hereto overcome this problem and to confirm that milk peptide is a new mitogenic growth factor for Rama 27 cells.

The successful expression of recombinant milk peptide has been confirmed by amino acid sequence. Apart from the loss of the initiating methionine the amino acid sequences determined for the recombinant protein matched that of the synthetic milk peptide gene which had been derived from the nucleotide sequence of cloned gene. The partial amino acid sequence obtained for the recombinant milk peptide also matched the sequence derived from the nucleotide sequence of gene.

3. 3. 3.1 Expression of soluble milk peptide in *E coli*

The recombinant milk peptide was demonstrated from the sonicated supernatant. It was expressed as soluble protein in *E coli*, just like human INF-α (Staehelin et al., 1981; De Maeyer et al., 1982), Bovine INF-α (Grosfeld et al., 1985), human lymphotoxin (Gray et al., 1984), human TNF (Pennica et al., 1984) and murine TNF (Pennica et al., 1985). Expression levels varied from < 0.1%-25% of total protein. Expressed level of milk peptide was estimated at least 10% of total protein. Due to the small peptide eluting during the staining and not binding the dye, it is only possible to estimate the expression level of the recombinant product by SDS-PAGE roughly.

There are no obvious common features to explain why these proteins are soluble. Some, but not all, authentic proteins are naturally glycosylated. The fact that none of these proteins contain large numbers of disulphides may be significant (Marston, 1986). In agreement with this, milk peptide does not contain disulphides and this might be one reason why milk peptide was expressed in the soluble form.

3. 3. 3.2 Degradation of recombinant product during the expression and purification

When recombinant milk peptide was expressed it was observed that degradation of protein was very fast, the maximal expressed milk peptide was at 1-2 hours. In addition, degradation of recombinant milk peptide was also observed during storage although BL21, which was used as the bacterial host for expression, should be deficient in the *lon* protease and also lack the *omp*T outer membrane protease that can degrade proteins during the purification (Studier et al., 1990). It was reported that the intracellular accumulation of eukaryotic polypeptide expressed directly in *E coli* may be limited, because the protein is recognized as foreign and degraded. This is particularly apparent with small polypeptides (Marston, 1986). The same result is also described by others (Thompson et al., 1991). Thompson et al. (1991) reported that with storage at 4°C in buffer, the structure of the recombinant bFGF had been observed to change so that less of the protein eluted from the chromatography as the same peak. Recombinant Urogastrone (human epidermal growth factor) in *E coli* has been rapidly degraded. Its stability and purification properties are improved when a modified gene is used to produce urogastrone as an N-terminal fusion protein with the TrpE protein and C-terminal fusion of six arginine residues (Allen et al., 1985).

The amino acid sequences of recombinant milk peptide determined by automated Edman degradation of protein, which were obtained directly from electroblotting the protein purified by SDS gel electrophoresis onto a PVDF membrane, indicated three homologous peptides in the desired band. The sequencing of the three peptides from one sequencing is based on the fact that the full length sequence is far more abundant than the other two, hence, the sequence is easily readable as normal, the other sequences are relatively minor compounds, especially the middle one. An explanation might be that as the fragment was of low molecular weight, Tricine SDS-PAGE used here failed to separate well those peptides, therefore only one band was visualised on the electrophoreses. Heating the protein in SDS-PAGE sample loading buffer might have caused partial degradation of recombinant product. A similar observation was also reported by Thompson et al., (1991) in which recombinant bFGF degraded partially to lower molecular weight fragments after heating in SDS-containing buffer. Degradation of other proteins after heating in SDS-containing buffer has also been reported by Rittenhouse and Marcus (1984).

3. 3. 3.3 Description of the purification of recombinant milk peptide

Since recombinant milk peptide only has four extra amino acids at the *N*-terminal of natural milk peptide, ideally purification procedure should be tried by the same procedure as that of the natural milk peptide first.

As described in the early part of this chapter, purification of milk peptide from bovine milk was based on three chromatographic procedures. It included using ion-exchange chromatography, hydrophobic interaction chromatography followed by reverse phase HPLC. In this study, purification of recombinant milk peptide followed this procedure.

Cation exchange chromatography was described as a good method to purify the positively charged recombinant protein because most bacterial proteins are acidic and are negatively charged at pH 5-6 (Marston, 1986). During purification of recombinant milk peptide, it was showed this is an effective purification step. It is illustrated by SDS-PAGE showing that most of the contaminants pass through the column without binding, because the pH value chosen is above the pI of most bacterial proteins.

The protein eluted from the Butyl-sepharose column by a gradient NaCl elution was not observed to improve much the degree of purification. Therefore, C_4 reverse phase was used for the second step in the purification. Reverse phase HPLC has been previously established as an efficient tool in purification of milk peptide from bovine milk (Liu et al., 1996), and in this procedure it resulted in good purification although the protein was not well separated. It was observed that a fraction eluted from this step could stimulate DNA synthesis in Rama 27 cells.

The present study has indicated that procedure for purification of natural milk peptide is not exactly suitable for recombinant milk peptide, since recombinant milk peptide has four extra amino acids at the *N*-terminus and theoretical pI is 10.38 (natural milk peptide pI is 10.12). Also recombinant milk peptide is derived from *E coli* rather than bovine milk so the impurities present are different. Therefore, the procedure of purification for recombinant milk peptide needs to be developed in the future.

3.3.3.4 Stimulation of DNA synthesis on Rama 27 cells by recombinant milk peptide

Although purified intact fusion protein has been demonstrated to contain biological activity of the Fc portion of IgE (Liu et al., 1984) and crude bacterial product has also been reported to have bioactivity, the failure to detect activity in the early stage of purification recombinant peptide might have been caused by the dilution of recombinant peptide with inactive material, which made concentration of active material too low. Alternatively the presence fusion sequence might have abrogated the activity. In the present study, stimulation of DNA synthesis was observed in Rama 27 cells after reverse phase HPLC purification of recombinant milk peptide. Since the recombinant milk peptide was expressed from synthetic gene of milk peptide, which was confirmed by amino acid sequencing to match the synthetic milk peptide gene, the stimulation of DNA synthesis for Rama 27 cells observed here was definitely derived from recombinant milk peptide.

The level of stimulation of DNA synthesis by recombinant peptide was lower than that of natural milk peptide. It is therefore possible that the fraction from HPLC with activity was not pure. Another possibility might be that the extra four amino acids at *N*-terminal sequence had not been cleaved, and that *N*-terminal fusion interferes with protein folding, which might begin there. The reason for not cleaving with factor Xa was that an active material was obtained without it, and there was no overriding reason to take it further within the scope of the thesis.

The mechanism by which proteins fold *in vivo* is still unknown. From studies *in vitro* it is evident that the amino acid sequence of each protein contains the information required for folding, but it is not apparent which residues specify folding information (Marston, 1986). Another consideration is what influence, if any, the chemical environment within the cell has on protein folding. In the absence of such information it is only possible to speculate why some eukaryotic polypeptides failed to fold correctly in *E. coli* (Marston, 1986).

For proteins which contain disulphide bonds, formation of these bonds may be an essential component of the folding process. The inability to form disulphide bonds in the reducing environment of the *E. coli* cytoplasm may thus prevent folding. However this may not be the only reason why these proteins do not fold correctly,

and certainly does not explain why eukaryotic proteins which lack disulphide bonds can also fail to fold in *E. coli* (Marston, 1986).

Although recombinant milk peptide does not contain disulphide bonds, the folding of milk peptide might be the main reason to affect the stimulation of DNA synthesis in Rama 27 cells; the same situation also has been seen in the chemical Solid Phase Synthetic milk peptide (Liu, 2001). To recover more activity and further characterize the recombinant peptide, more purification steps need to be developed and the fusion peptide needs to be cleaved in the future.

3. 3. 4 Summary of work

The evidence that we have obtained correctly expressed peptide from chemically synthesized genes for milk peptide can be summarised as follows:

1). DNA sequences obtained after cloning and plasmid construction have been directly verified to be correct as designed.

2). The correct amino acid sequences were directly obtained after purification by SDS-PAGE onto a PVDF membrane. Because the protein sequences were obtained, translation must be in phase.

3). The *E coli* products, after purification on a small scale by ion-exchange chromatography and Reverse Phase HPLC, can stimulate DNA synthesis in Rama 27 cells, although the fusion peptide has not been cleaved.

Taking together, that results have been shown in this chapter confirmed that milk peptide is a novel growth factor for Rama 27 cells. The mechanism for DNA-synthesis stimulation is not known. To explain further the mechanism for DNA-synthesis stimulation by this small peptide, the intracellular signal events and some pathway of this peptide will be investigated in the next two chapters. Since the recombinant milk peptide had not yet been produced, and the synthetic peptide did not display the same degree of activity, all studies on mechanism in the following chapter were carried out with the factor purified from milk.

Chapter 4

Investigation of Stimulation of DNA Synthesis by Small Peptide Growth Factors and the Activation of Intracellular Signaling Pathways in Fibroblasts

4.1 Introduction

As described in chapter 1, milk peptide is a growth factor involved in the stimulation of DNA synthesis and proliferation of rat mammary fibroblast cells (Liu et al. 1996). Whether this novel growth factor would stimulate DNA synthesis in other cells, and the signaling pathways by which milk peptide mediates growth in these cells were unknown. The present study was designed to examine the ability of a selection of peptide growth factors to stimulate DNA synthesis in various permanent cell lines, and also to identify intracellular events necessary for milk peptide induced cell proliferation.

Firstly, some cell lines were chosen to assess the role of milk peptide in stimulating DNA synthesis and to select suitable cell lines which may be used for investigation of the activation of intracellular signaling pathways of milk peptide. In addition, several small peptides and peptide growth factors were used to compare the mechanism by which they stimulated DNA synthesis.

Secondly, the effect of milk peptide activating the phosphorylation of tyrosine on proteins in Rama 27 cells and Swiss 3T3 cells was explored. The aim was to find which signaling pathways of milk peptide are used for stimulation of DNA synthesis in fibroblast cells.

Thirdly, from the introduction (Chapter 1) it is clear that PLC - γ pathway is not used for mitogenesis so I have looked at other two pathways, that is Ras/MAP kinase and PI3 kinase pathways. The effect of milk peptide treatment on activation of MAP kinase pathway was investigated in Rama 27 cells. Chemical inhibitors of signaling pathways, PD 098059 and wortmannin, were used to block DNA synthesis stimulated by milk peptide and other growth factors in Rama 27 cells.

4.1.1 The effect of growth factors on various cell lines

It is well known that milk peptide stimulated DNA synthesis in Rama 27 cells (Liu et al. 1996). Rama 27 cells were isolated from the fast-sticking fraction of normal rat mammary gland culture and are fibroblastic in character. But, whether milk peptide stimulates DNA synthesis on other cell lines is unknown. Since milk peptide stimulated DNA synthesis in rat fibroblast cells, it was important to test the effect of milk peptide on a different type of fibroblast cells. Swiss 3T3 cells, which are mouse embryo fibroblast cells, were used in this study.

Besides, in the present study the effect of several small peptides was investigated on different cloned cell lines, including Rama 27, Rama 29, Rama 37, Rama 37E8. Rama 27, Rama 37 and Rama 29 are epithelial-derived cell lines, being cuboidal, epithelial, and elongated, myoepithelial-like cells, respectively; Rama 37E8 is a cell line intermediate between epithelial and myoepithelial-like; they were all obtained from a DMBA-induced mammary tumour growing in a Sprague-Dawley rat (Bennett et al., 1978; Rudland et al., 1977).

4.1.2 Small peptide growth factors

Since milk peptide is only a fragment of αS_2-casein (192-222), it would be ideal to find other small peptides that stimulate DNA synthesis in the same cells, to look for similarity in the structure or in physicochemical properties among them. In this chapter, several small peptides were studied, including:

Milk peptide which is isolated from bovine milk.

'KVIPY" represents KVIPYVRYL which is a chemically synthesized peptide based on the sequences of milk peptide; It has been shown to stimulate DNA synthesis in Rama 27 cells (Liu et al. 1996).

'VREKS' represents VREKSDPHIKGGC which is a chemically synthesized peptide of bFGF fragment 52-64; It has been shown to stimulate DNA synthesis in Rama 27 cells as well.

Bombesin, EQRLGNQWAVGHLM was isolated from the skin of the European frog. It has been shown to stimulate DNA synthesis in Swiss 3T3 cells (Rozengurt, 1986). Most peptide growth factors are really small proteins that work via tyrosine kinase receptors such as EGF, Insulin, PDGF and bFGF. However, Bombesin stimulates DNA synthesis and cell proliferation via a G-protein linked receptor. The mitogenic signaling pathways of these growth factors have been well studied. As reference compounds, EGF, bFGF and Bombesin were used to study the mitogenic signaling pathways activated by milk peptide.

4.1.3 Tyrosine phosphorylation after treatment with growth factors

The first step in the interaction of many polypeptide growth factors with their target cell is binding of the factor to specific, high-affinity receptors, which upon occupancy undergo rapid phosphorylation, redistribution in the plane of the membrane, and endocytosis (Carpenter, 1985). The binding of growth factors promotes the generation of early signals in the membrane and cytosol. Within minutes, the mitogenic signal is propagated into the nucleus. These early events are followed in parallel sequences by multiple molecular and cellular responses, which eventually converge into a common final path leading to synthesis of DNA and cell division. Since the initiation of DNA synthesis is a late event, occurring 10 to 15 hours after the addition of mitogens, attention has focused on the initial cellular responses associated with the interaction of mitogenic factors with the cells in the expectation that the early events will provide clues to the primary regulatory mechanism (Rozengurt, 1986).

In this context, the early phosphorylation events following treatment of Rama 27 cells and Swiss 3T3 cells with milk peptide and other growth factors were investigated.

4.1.4 MAPK pathway

To attempt to determine 'Critical Signaling Events', which of these signaling components are needed for cell proliferation and differentiation has been much studied. It has been shown that activation of the ERK family of mitogen- activated protein kinases (MAPKs) is a critical event in signal transduction from receptor tyrosine kinases (Marshall, 1995). In the present study the activation of $p42^{MAPK}$ and $p44^{MAPK}$ by milk peptide, EGF and bFGF were tested on Rama 27 cells. The role of activated MAP kinases in milk peptide induced DNA synthesis of Rama 27 cells was also studied.

4.1.4.1 PD 098059 inhibits MAPK kinase (MEK)

In order to dissect MAPK pathways and to elucidate their physiological roles, PD 098059 was used. It was discovered at Parke-Davis in a directed screen for compounds that blocked ERK activation in an in vitro system (Dudley et al, 1995). MEK has been shown to be responsible for the phosphorylation of the MAP kinase p42 and p44 at both threonine (Thr) and tyrosine (Tyr) residues, leading to the activation of $p42/p44^{MAPK}$ (Cano et al., 1995; Garrington et al., 1999). It has been reported that PD 098059 exerted its effects not through direct inhibition of the kinase activity of MEK1, but by itself binding to MEK1 and thus preventing its activation by upstream molecules such as Raf. The activation of Raf is suppressed and its inactivation is accelerated by a downstream component(s) of the MAP kinase pathway (Alessi et al., 1995). In this study, PD 098059 was used to try blocking DNA synthesis and activation of $p42/p44^{MAPK}$ in Rama 27 cells.

4.1 5 Wortmannin inhibits PI3-kinase

Phosphatidylinositol (PI) 3-kinase, a heterodimer of 85-kDa (p85) adaptor subunit and a 110-kDa (p110) catalytic subunit (Hiles et al., 1992, Hu and Schlessinger, 1994), is activated by most growth factors and has been implicated as a critical factor in the control of cell proliferation and cell survival. PI 3-kinase phosphorylates the D-3 position of inositol ring of phosphoinositides, which in turn act as second messengers (Whitman et al., 1988). The p85 subunit contains two Src homology 2 (SH2) domains, which bind to tyrosine-phosphorylated receptors after stimulation of cells with growth factors and in this manner recruit p110 into the complex at the cell membrane. The region between the two SH2 domains, the iSH2 region, mediates the association with p110, and this interaction is required for the enzymatic activity of p110 (Klippel et al., 1994). Phosphorylation of the p85 subunit of PI 3-kinase upon VEGF stimulation (Guo et al., 1995) was reported. Thakker et al., (1999), demonstrated the importance of PI 3-kinase as a necessary signaling component of VEGF-mediated cell cycle progression.

In the present study, Wortmannin, an inhibitor of PI 3-kinase, was used to test whether PI 3-kinase is involved in stimulation of DNA synthesis in Rama 27 cells by milk peptide and other growth factors.

4.2   Experimental and Results

4.2.1   Effect of peptide growth factors on DNA synthesis

4.2.1.1 Response of quiescent Rama 27 cells to growth factors

As Figure 3.7 shows, stimulation of DNA synthesis by the milk peptide was dose dependent. To determine if the other small peptide growth factors stimulated DNA synthesis in a dose dependent manner, increasing concentrations of EGF, milk peptide, VREKS, KVIPY and Bombesin were added to quiescent Rama 27 cells.

In the case of EGF, an increase in the rate of DNA synthesis occurred between concentration of 0.00017 nM and 0.5 nM of EGF (Figure 4.1a). Milk peptide has the same potential to stimulate DNA synthesis in the same cells (Figure 3.7, Figure 4.1c). KVIPY was considerably less potent than milk peptide in stimulating DNA synthesis; the maximal stimulation was at 90 µM (Figure 4.1b).

With VREKS treated cells, an increase in the rate of DNA synthesis was observed, and the maximal stimulation was observed at 21 µM (Figure 4.1d). In contrast to these growth factors, Bombesin did not stimulate DNA synthesis in this cell line (Figure 4.1e). This suggested stimulation of DNA synthesis by the milk peptide might be similar to that by EGF or VREKS, but different from that by Bombesin.

4.2.1.2 Response of quiescent Swiss 3T3 cells to growth factors

To determine whether the milk peptide stimulated other fibroblast cells, growth factors described above were added to Swiss 3T3 cells. EGF and milk peptide alone had an effect on DNA synthesis in Swiss 3T3 cells (Figure 4.2a, 4.2c). In contrast to the effect on Rama 27 cells, EGF and milk peptide alone only stimulated DNA synthesis moderately, the level of DNA synthesis by milk peptide was only increased by 4 fold compared to control cells. VREKS seems not to stimulate DNA synthesis in Swiss 3T3 cells (Figure 4.2d). Nevertheless, Bombesin had a great effect in stimulating of DNA synthesis in Swiss 3T3 cells (Figure 4.2 e). The presence 0.5 nM Bombesin caused a 25-fold increase in DNA synthesis compared to that of control cells. The results here strongly suggest that milk peptide stimulates DNA synthesis via different pathways than Bombesin.

4.2.1.3 Response of quiescent mammary cells to growth factors

To determine whether the peptide growth factors have the same effect on other mammary cells, different cells were treated by these growth factors. In the case of Rama 37 cells (Figure 4.3a), milk peptide seemed not to affect DNA synthesis in Rama 37 cells (P> 0.05, from the student t test), EGF only had a little effect on these cells. VREKS was more potent than EGF in stimulation of DNA synthesis. In contrast to Rama 37 cells (Figure 4.3b), EGF and milk peptide had a significant

effect in Rama 37 E8 cells ($P < 0.05$, from the student t test). However, VREKS, KVIPY and Bombesin had no effect on Rama 37E8 cells.

On Rama 29 cells (Figure 4.3c), all the growth factors including Bombesin stimulated DNA synthesis; Milk peptide had more effect on DNA synthesis.

4.2.1.4 Summary of the effect of growth factors on different cell lines

Milk peptide stimulated DNA synthesis in different cell lines (Table 4.1), the most sensitive cells being Rama 27 cells. KVIPY only had significant effect on Rama 29 cells. Milk peptide activity is much greater than that of KVIPY. VREKS had an effect on Rama 27, Rama 29 and Rama 37, which is greater than that of KVIPY. EGF had the effect on Rama 27, Swiss 3T3 cells and Rama 37E8. Finally, Bombesin only stimulated Swiss 3T3 cells and Rama 29 cells.

The experiments present here do not support the idea that milk peptide acts through bombesin receptors. The peptide VREKS also stimulates DNA synthesis in Rama 27 cells. The results so far obtained do not allow a clear decision as to whether it acts in the same way as milk peptide.

4.2.2   Analysis of rapid tyrosine phosphorylation of protein induced by growth factors

Previous chapters detailed attempts to isolate milk peptide from bovine milk, using as assay Rama 27 cells, in which milk peptide had a great effect on DNA synthesis. This cell line would have contained enough milk activity receptors for us to attempt to identify a protein tyrosine kinase activity associated with its receptor in this way. The stimulation of the autophosphorylation of the EGF and FGF receptor tyrosine kinases present on the surface of Rama 27 fibroblasts has been demonstrated by immunoblot analysis (Chen, 1995). To investigate if milk peptide induced proliferation via tyrosine kinase receptors, the rat mammary fibroblast cell line Rama 27 was employed. In addition, mouse embryo fibroblast cells (Swiss 3T3) were also used since they express Bombesin receptor that works via G-proteins.

4.2.2.1 Growth factors and milk peptide induced tyrosine phosphorylation on Rama 27 cells

To examine whether milk material stimulated tyrosine phosphorylation of any Rama 27 cell membrane proteins, serum-starved cells were treated with milk peptide, EGF and bFGF for 15 minutes and cell lysates were electrophoresed by SDS-PAGE (Method 2.3.1.5) employing a 7.5% (v/v) acrylamide resolving gel to increase the solution of proteins in the high molecular weight range. The gels were electroblotted onto Hybond-C membrane and incubated with primary antibody PY-20, a mouse monoclonal anti-phosphotyrosine antibody (which was raised against phosphotyrosine) at a dilution of 1:1000. Bound primary antibody was detected using an anti-mouse horseradish peroxidase-conjugated secondary antibody (Section 2.3.1.5) at a dilution of 1:1000.

In the cell lysate samples of Rama 27 cells an increase in the amount of an immunoreactive protein of 180 kDa was detected. Even in absence of growth factors, the cell lysate possessed an abundant amount of tyrosine phosphorylation on a polypeptide of 180 kDa. The level of phosphorylation on tyrosine of this band increases markedly when the cells were stimulated by EGF (Figure 4.4a) and thus it is likely to represent the EGF receptor (Figure 4.5a). The EGF receptor was identified as 180 kDa phosphoprotein (Figure 4.5a) in cell lysates of A431 cells as a positive control. The 180 kDa EGF receptor was detectable in milk peptide-treated cells, which increased markedly as for EGF treated cells. The levels of protein tyrosine phosphorylation were quantified densitometrically from the blot and results were expressed relative to amount of phosphorylation detected in the absence of growth factors (Figure 4.5b). The intensity of the band of 180 kDa EGF receptor is more than 4 times higher than control when EGF is added. The density of 180 kDa protein when milk-derived growth factor is used is 431%. Additionally a protein of 85 kDa which was specific to bFGF was detected. This protein of 85 kDa in bFGF-treated cells is increased by 618% compared with untreated cells (Figure 4.5b). A protein of 45 kDa was preferentially found in EGF-treated cells (Figure 4.5a). In all the mitogen-treated samples, proteins of 120 and 90 kDa were observed, which were absent or less intense in control samples. These results suggest that milk

peptide induced tyrosine phosphorylation in Rama 27 cells is similar to that of EGF rather than bFGF in Rama 27 cells.

4.2.2.2 Tyrosine phosphorylation induced by growth factors in Swiss 3T3 cells

To examine the phosphorylation events following treatment of intact cells by milk peptide and other growth factors, we used Swiss 3T3 cells which are bombesin-responsive. These cells also have high-affinity receptors for PDGF and EGF (Isacke et al., 1986) and respond mitogenically to these factors.

Cells were cultured for 7 days until they were quiescent and dishes were treated with Bombesin, EGF, bFGF and milk peptide then subjected to Western blotting with anti-phosphotyrosine Ig (Figure 4.6a). The 180 kDa EGF receptor was strongly detectable in EGF-treated cells, but was also seen less strongly in cells treated with milk peptide. The protein of 120 kDa was readily detected; in EGF-treated cells the intensity of staining was less than with milk peptide. It was also detected in Bombesin and bFGF-treated cells. In cells treated with bFGF, proteins of 150, 85 kDa were seen of which the bFGF receptor may be the 150 kDa phosphoprotein (Figure 4.6a). In all the mitogen-treated samples proteins of 38 and 66 kDa were observed, which were less intense in control samples. However, there were no striking bands, which were unique to extracts of bombesin-treated cells. Levels of protein tyrosine phosphorylation were quantified densitometrically from the blot and results were expressed relative to amount of phosphorylation detected in the absence of growth factors. Band intensity of 180 kDa EGF receptor is 1168% higher than control; Milk peptide-treated density of 180 kDa protein is 976% (Figure 4.6b). Band intensity of 85 kDa bFGF-treated is 2800% (100ng/ml) or 2900% (500ng/ml) higher than control. Results above suggest milk peptide induced tyrosine phosphorylation is similar to that of EGF, but different from bFGF and Bombesin in Swiss 3T3 cells.

4.2.3   EGFR activation in response to growth factors in Rama 27 cells

Although experiments of phospho-tyrosine have shown that milk peptide induced the proteins of mass 180 kDa are similar to that EGF, whether these proteins are

EGF receptor is not clear. To test the possibility that milk peptide used the same receptor as EGF in Rama 27 cells, whole lysates from control and factor-treated cells were incubated with different growth factors, then immunoblotted with anti-phospho EGF receptor antibody. As expected, milk peptide exhibited activation of protein 180 kDa of EGF receptor as did EGF (Figure 4.7). On the basis of the results presented here, we deduced that the milk peptide stimulation of DNA synthesis was mediated by EGF receptors. This needs to be further investigated to see if major signal pathways of milk peptide are similar to other growth factors especially to EGF.

4.2.4 MAP Kinase activation in response to growth factors in Rama 27 cells

4.2.4.1 Effect of PD-098095 on growth factor-stimulated DNA synthesis

The activation of ERK is closely correlated with the stimulation of DNA synthesis by growth factors (Meloche et al, 1992, Kahan et al, 1992). To explore the role of the ERK pathway in the induction of DNA synthesis by milk peptide and other GFs, the effect of PD-098059, an inhibitor of MEK, was tested in Rama 27 cells. PD-098059 was found to potently inhibit DNA synthesis in Rama 27 cells induced by EGF, bFGF and milk peptide at a concentration of 50μM (Figure 4.8). It was found that 50μM PD 098059 reduced EGF, bFGF and milk peptide-induced DNA synthesis to the same level, which was approximately 50% that of unstimulated cells (Figure 4.8). These data suggest that milk peptide stimulates DNA synthesis in Rama 27 through MAPK activation like other growth factors. The activation of MEK is a critical pathway to the induction of DNA synthesis in Rama 27 cells.

In order to establish a dose-response relationship for inhibition by PD 098095 of DNA synthesis induced by growth factors, increasing concentrations of PD-098059 were added to quiescent Rama 27 cells for 15min before the GFs were added (Figure 4.9a, b, c). In the case of EGF (Figure 4.9a), when 10μM PD-098095 was used, it inhibited 60% of the stimulation of DNA synthesis, 50μM PD-098095 inhibited 90% of the stimulation. Therefore, treatment of cells with PD-098095, inhibited EGF-induced DNA synthesis in Rama 27 cells, and the effect is dose dependent. Similar results were also seen in bFGF and milk peptide-treated cells

(Figure 4.9a, b, c). The results suggested that PD-98095 had a significant inhibitory effect on DNA synthesis stimulated by growth factors.

4.2.4.2 Effect of growth factors on the phosphorylation of p42/p44^{MAPK}

Both p44 and p42 MAP Kinase (Erk 1 and Erk 2) function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation. Activation of MAP Kinase occurs through phosphorylation of threonine 202 and tyrosine 204 of human MAP Kinase (Erk1) at the sequence T*EY* by a single upstream MAP kinase kinase (MEK). In order to investigate the activation of phosphorylation of p42/p44^{MAPK} by milk peptide and other growth factors, an anti-phospho-p44/42 MAP Kinase monoclonal antibody was used. Chemiluminescent detection of antibody-bound proteins revealed two bands at 42 and 44 kDa in the lysates of Rama 27 cells stimulated with milk peptide, EGF, bFGF, VREKS and FCS (Figure 4.10). These two bands correspond to the molecular weight of p42/p44^{MAPK}, especially EGF.

To determine whether MEK inhibitor PD-098095 blocked the ability of growth factors to activate the phosphorylation of p42/p44^{MAPK}, 50 µM PD-098059 was added to quiescent Rama 27 cells for 15min before the GFs were added. After 15 mins treatment with growth factors, the cells were lysed and analyzed by western blotting as described in Section 2.3.1.5 using an anti-phospho-p44/42 MAP Kinase monoclonal antibody. As shown in Figure 4.11a, there were two bands of p42/p44 visible in lysates treated with growth factors, which were nearly abolished by PD-098095.

When PD-098095 was removed from the medium after 15 minutes treatment, quiescent Rama 27 cells were treated with growth factors for 15 mins; Analysis of lysates by Western blotting showed that the effect of PD-098095 in blocking the ERK phosphorylation disappeared (Figure 4.11b). Thus it would appear that in Rama 27 cells, treatment of cells with PD-098095 prevented activation of p42/p44^{MAPK}, and the effect of PD-098095 blocking the activation of ERK phosphorylation is reversible.

4.2.5 PI3-kinase activation in response to growth factors in Rama 27 cells

4.2.5.1 Effect of Wortmannin on growth factor-induced DNA synthesis

To assess whether PI 3-kinase was involved in DNA synthesis of Rama 27 cells, Wortmannin, an inhibitor of PI 3-kinase, was added to quiescent Rama 27 cells (Figure 4.12). Wortmannin was found to reduce DNA synthesis to 78 % of the basal level in untreated cells. There was also a similar level of reduction of DNA synthesis in response to both EGF and VREKS (84% of that stimulated by EGF, and VREKS respectively). However, in the case of bFGF and milk peptide treated cells, the level of DNA synthesis was only reduced to 95% and 97% of that stimulated by bFGF, and milk peptide respectively. The results here suggested that Wortmannin did not block the DNA synthesis in Rama 27 cells in these conditions.

4.2.5.2 Effect of Wortmannin on growth factor-induced the phosphorylation of p42/p44^{MAPK} in Rama 27 cells

To assess the role of PI 3-kinase in MAP kinase activation by Milk peptide and other growth factors, Wortmannin was added to quiescent Rama 27 cells for 15 minutes. After 15 minutes treatment with growth factors, the cells were lysed and analyzed by Western blotting as described in Section 2.3.1.5 using an anti-phospho-p44/42 MAP Kinase monoclonal antibody. As shown in Figure 4.13, there were two bands of p42/p44^{MAPK} visible in lysates treated with growth factors. These two bands were not abolished with Wortmannin treatment (Figure 4.13).

Thus it would appear that in Rama 27 cells, wortmannin did not block activation of the phosphorylation of p42/p44^{MAPK} by milk peptide and growth factors in the present study. Curiously, wortmannin alone appeared to induce some phosphorylation of p42/p44^{MAPK}.

Figure 4.1 Stimulation by growth factors of incorporation of [³H]-thymidine into DNA of Rama 27 cells
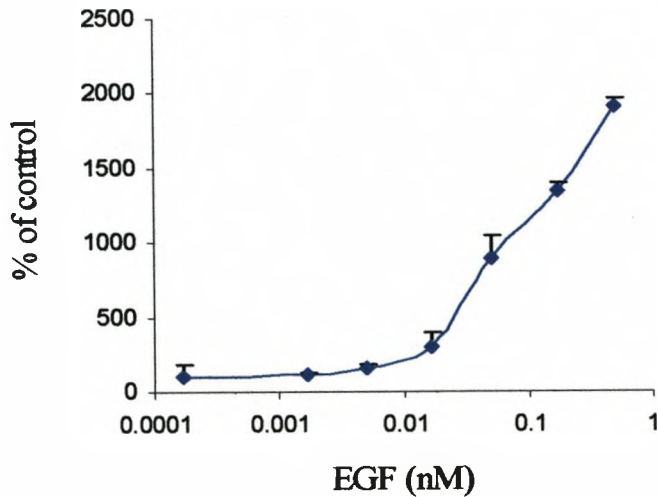
(a) The cells were treated with EGF as described in Method 2.3.1.3.
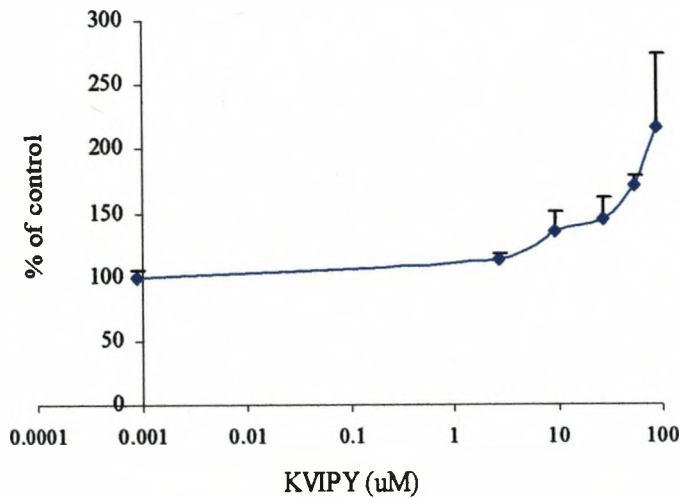
(b) The cells were treated with KVIPY as described in Method 2.3.1.3.
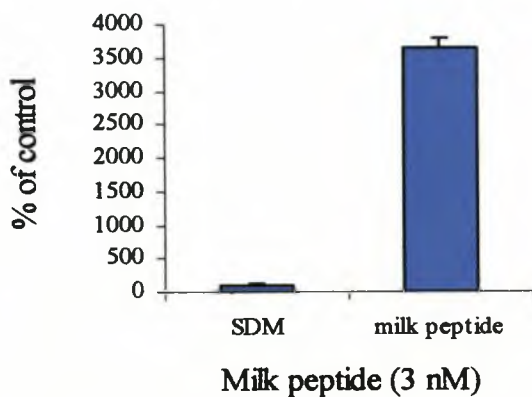
(c) The cells were treated with milk peptide as described in Method 2.3.1.3.
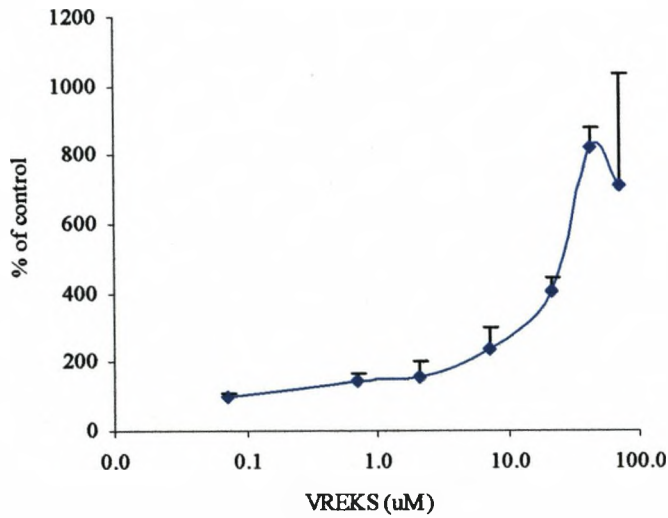
(d) The cells were treated with VREKS as described in Method 2.3.1.3.
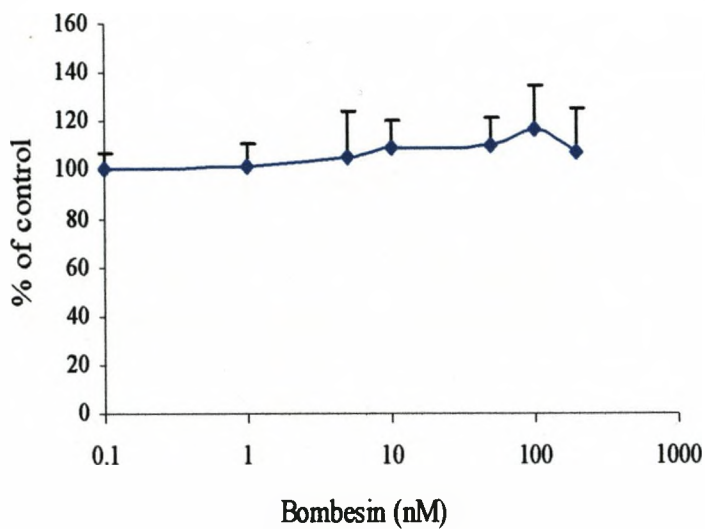
(e) The cells were treated as described in Method 2.3.1.3.

The Rama 27 cells were treated as described in Method 2.3.1.3. Results are expressed as the mean percentage of the control $[^3H]$-thymidine incorporation (100%) observed in the presence of 0.025 % BSA ±SD

Figure 4.2 Stimulation by growth factors of incorporation of [^{3}H]-thymidine into DNA of Swiss 3T3 cells
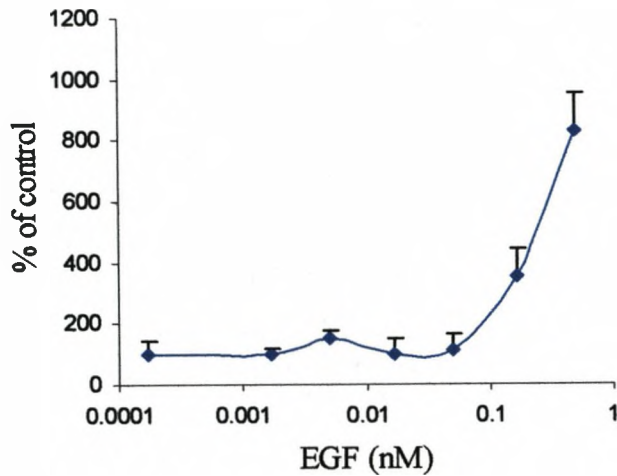
(a) The cells were treated with EGF as described in Method 2.3.1.3.
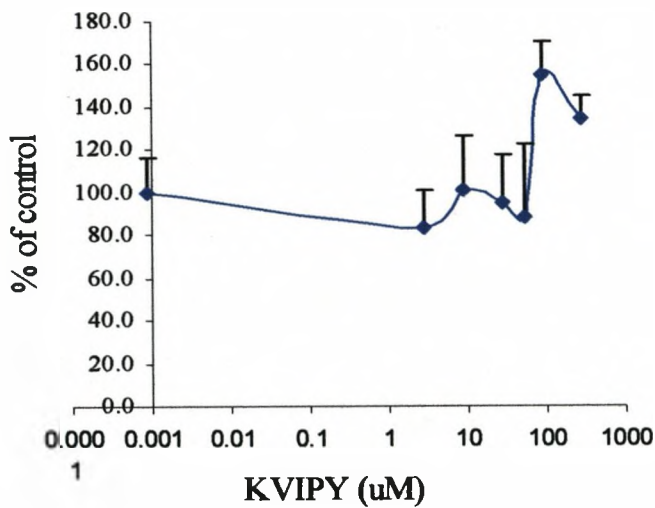
(b) The cells were treated with KVIPY as described in Method 2.3.1.3.
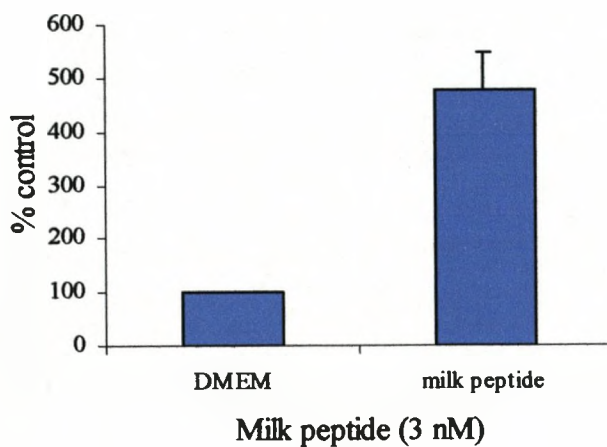
(c) The cells were treated with milk peptide as described in Method 2.3.1.3.
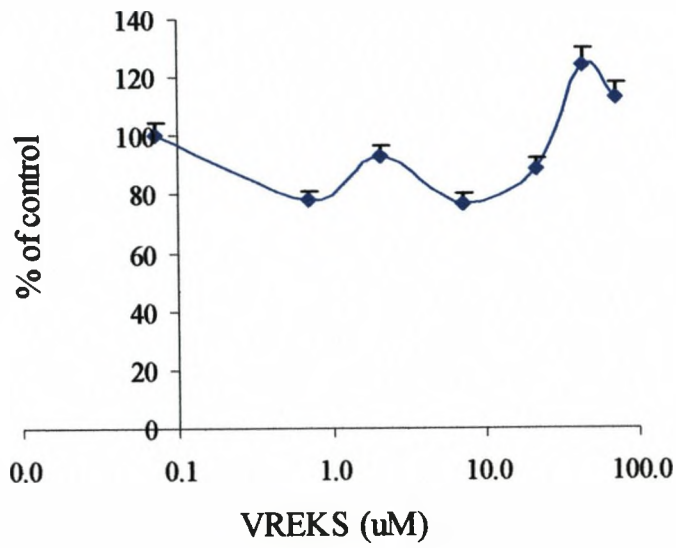
(d) The cells were treated with VREKS as described in Method 2.3.1.3.
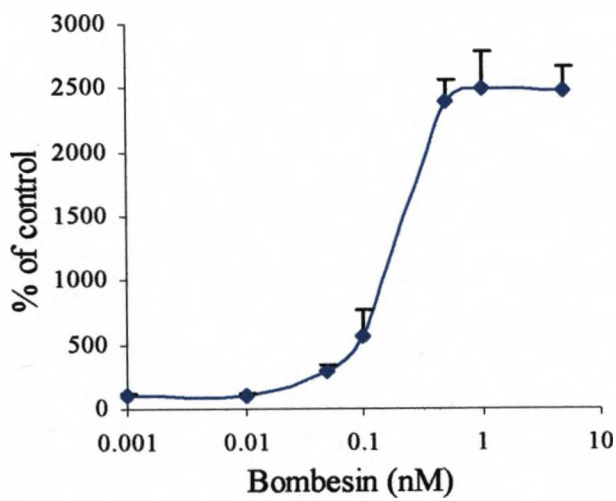
(e) The cells were treated bombesin as described in Method 2.3.1.3.

The cells were treated as described in Method 2.3.1.3. Results are expressed as the mean percentage of the minimal stimulation observed on DMEM (100%) ± SD.

Figure 4.3 Stimulation by growth factors of incorporation of [^{3}H]-thymidine into DNA of other cell lines

The rat mammary cells were treated as described in Method 2.3.1.3. Results are expressed as the mean percentage of the minimal stimulation (100%) observed in the presence of 0.025 % BSA ± SD.

Rama 37 cells

(a)
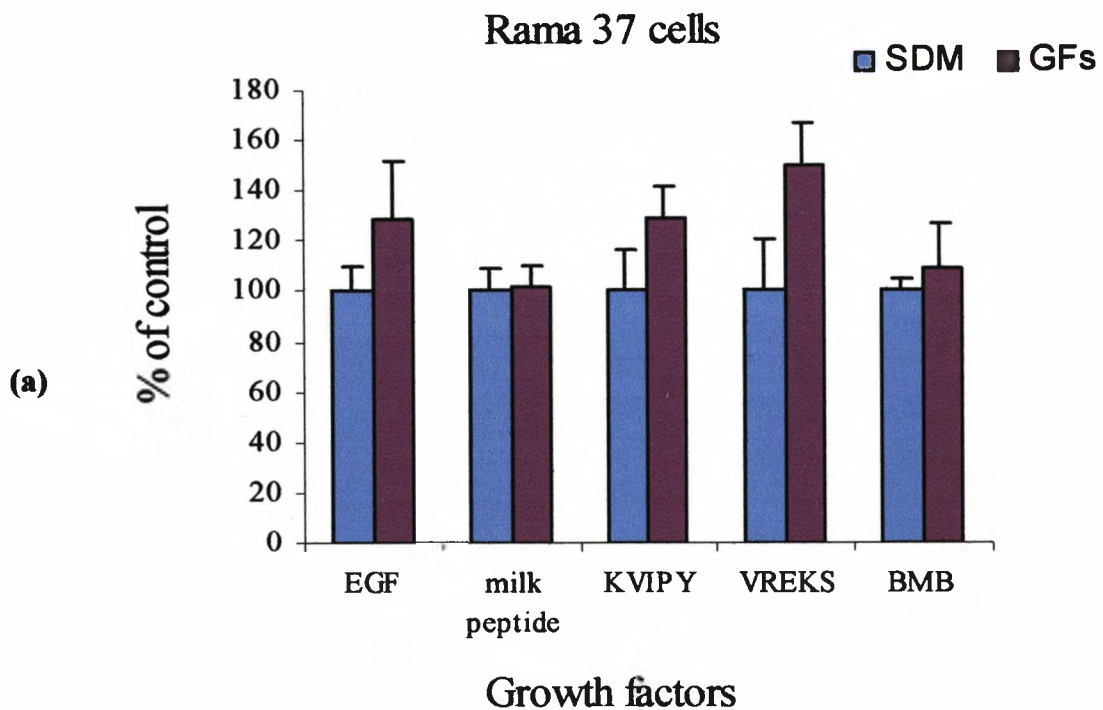
Growth factors

The maximal stimulation of DNA synthesis by growth factors in Rama 37 cells. For the EGF (0.5 nM), milk peptide (3 nM), KVIPY (27 µM), and Bombesin (0.1 nM), significant differences were not proven (P ≥0.05, from the student t test). For the VREKS (21 µM), difference was significant (P≤ 0.05, from the student t test).

Rama 37E8 cells

(b)
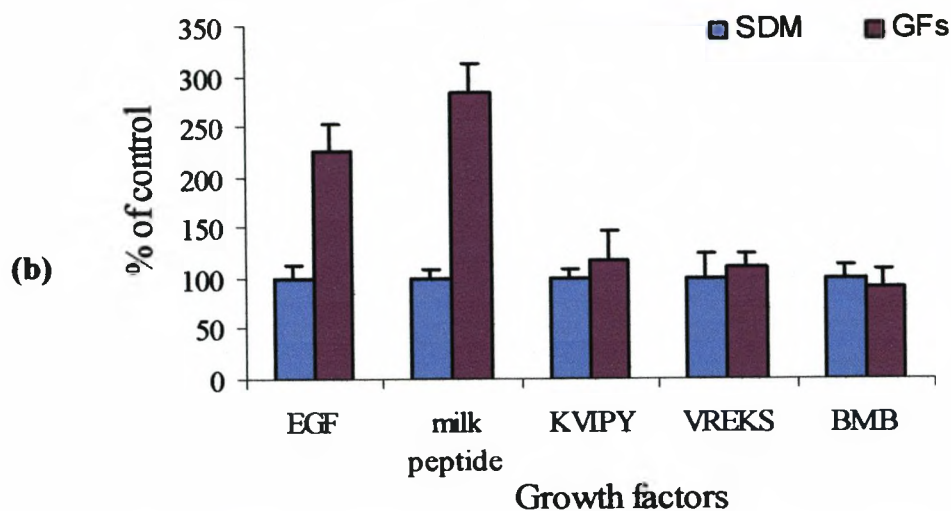
The maximal stimulation of DNA synthesis by growth factors in Rama 37 E8 cells. For the EGF (0.17 nM) and milk peptide (3 nM), difference was significant (P ≤ 0.05, from the student t test). For the KVIPY (27 µM), VREKS (2 µM), and Bombesin (5 nM), significant differences were not proven (P ≥0.05, from the student t test).

Rama 29 cells

(c)
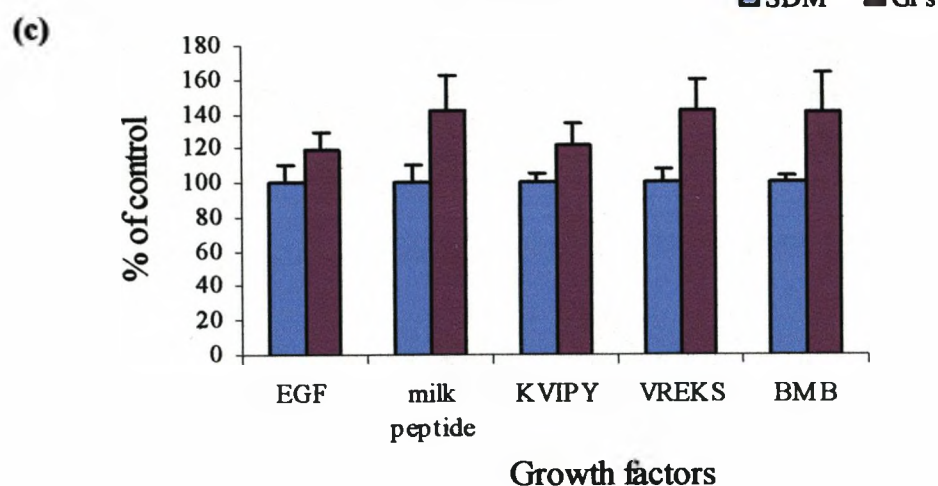
The maximal stimulation of DNA synthesis by growth factors in Rama 29 cells. For the EGF (0.5 nM) significant differences were not proven (P ≥0.05, from the student t test). For the milk peptide (3 nM), KVIPY (27 µM) and Bombesin (5 nM), difference was significant (P ≤ 0.05, from the student t test). For the VREKS (42 µM), difference was highly significant (P ≤ 0.01, from the student t test).

Figure. 4.4 Detection of tyrosine phosphorylation of protein in Rama 27 cells by immunoblot analysis
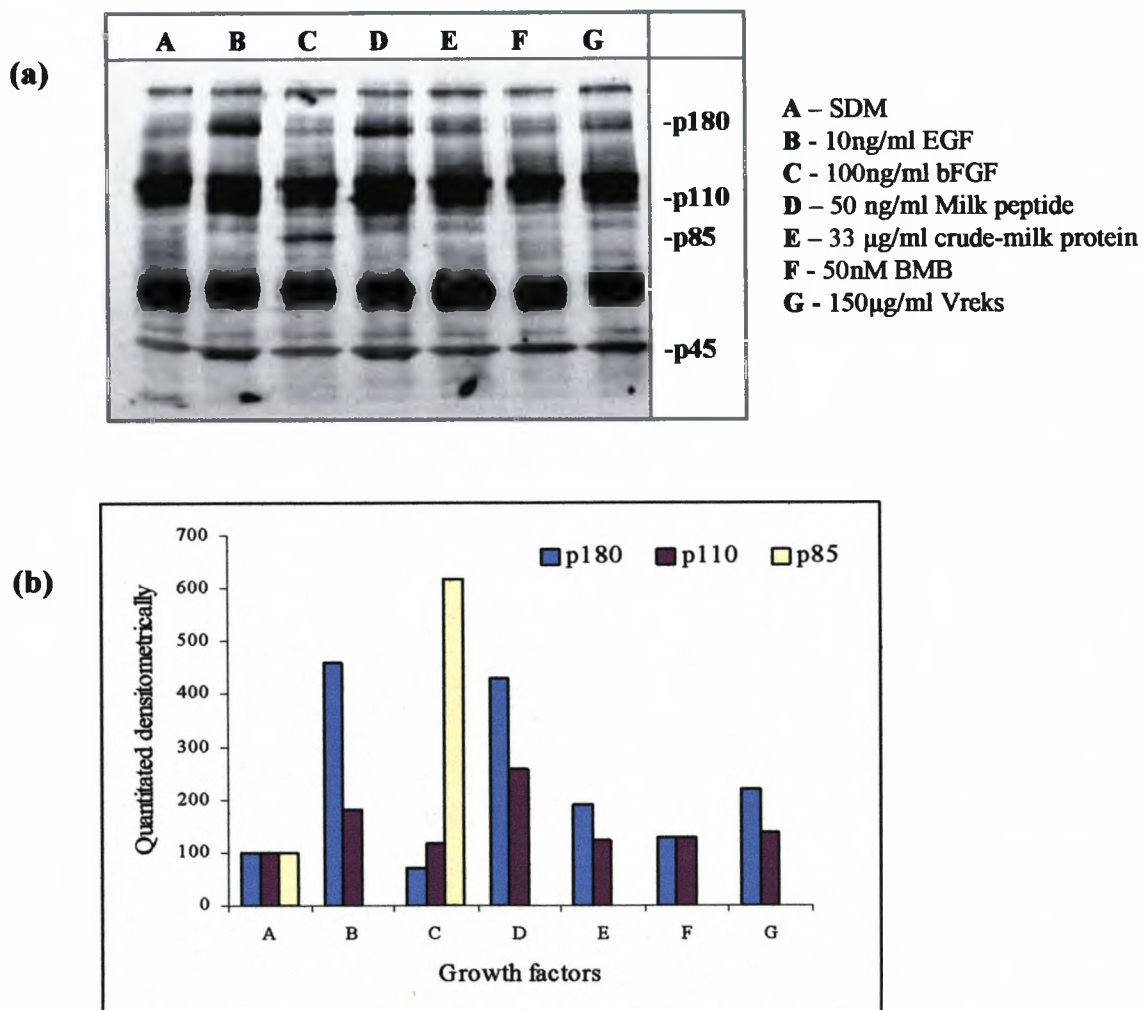
(a)

A – SDM
B - 10ng/ml EGF
C - 100ng/ml bFGF
D – 50 ng/ml Milk peptide
E – 33 µg/ml crude-milk protein
F - 50nM BMB
G - 150µg/ml Vreks

(b)

(a) Serum-starved cells were incubated for 15 min and lysed in lysis buffer. Cells were harvested, and aliquots of the cell lysates were load onto SDS-PAGE and immunoblotted with anti tyrosine phosphate antibody (PY20). Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5).

(b) Tyrosine phosphorylation, expressed as a percentage of basal values, which was calculated from quantitative, two-dimensional densitometry of the immunoblot.

Figure 4.5 Tyrosine phosphorylation in response to EGF in Rama 27 cells
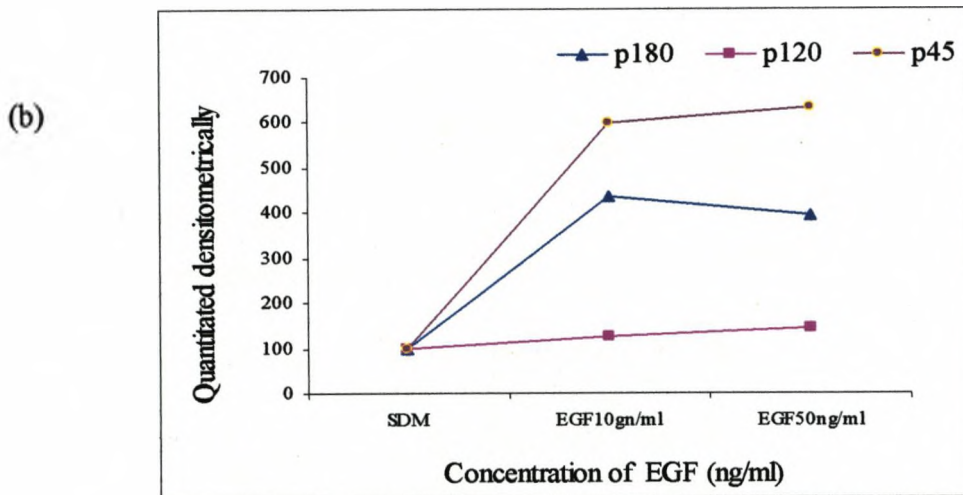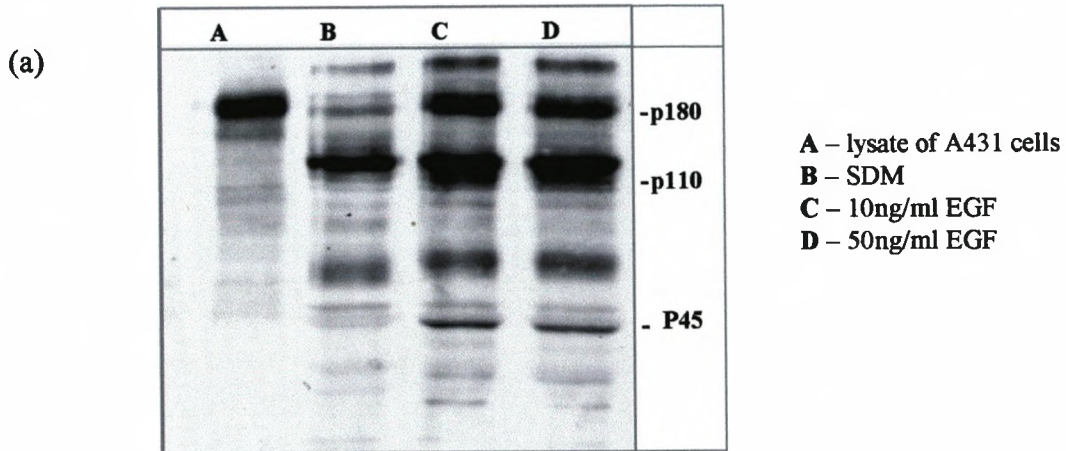
(a)

A – lysate of A431 cells
B – SDM
C – 10ng/ml EGF
D – 50ng/ml EGF

(b)

(a) Serum-starved cells were incubated for 15 min and lysed in lysis buffer. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with anti tyrosine phosphate antibody (PY20). Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5).

(b) Tyrosine phosphorylation, expressed as a percentage of basal values, which was calculated from quantitative, two-dimensional densitometry of the immunoblot.

Figure. 4.6 Detection of tyrosine phosphorylation of protein in Swiss 3T3 cells by immunoblot analysis
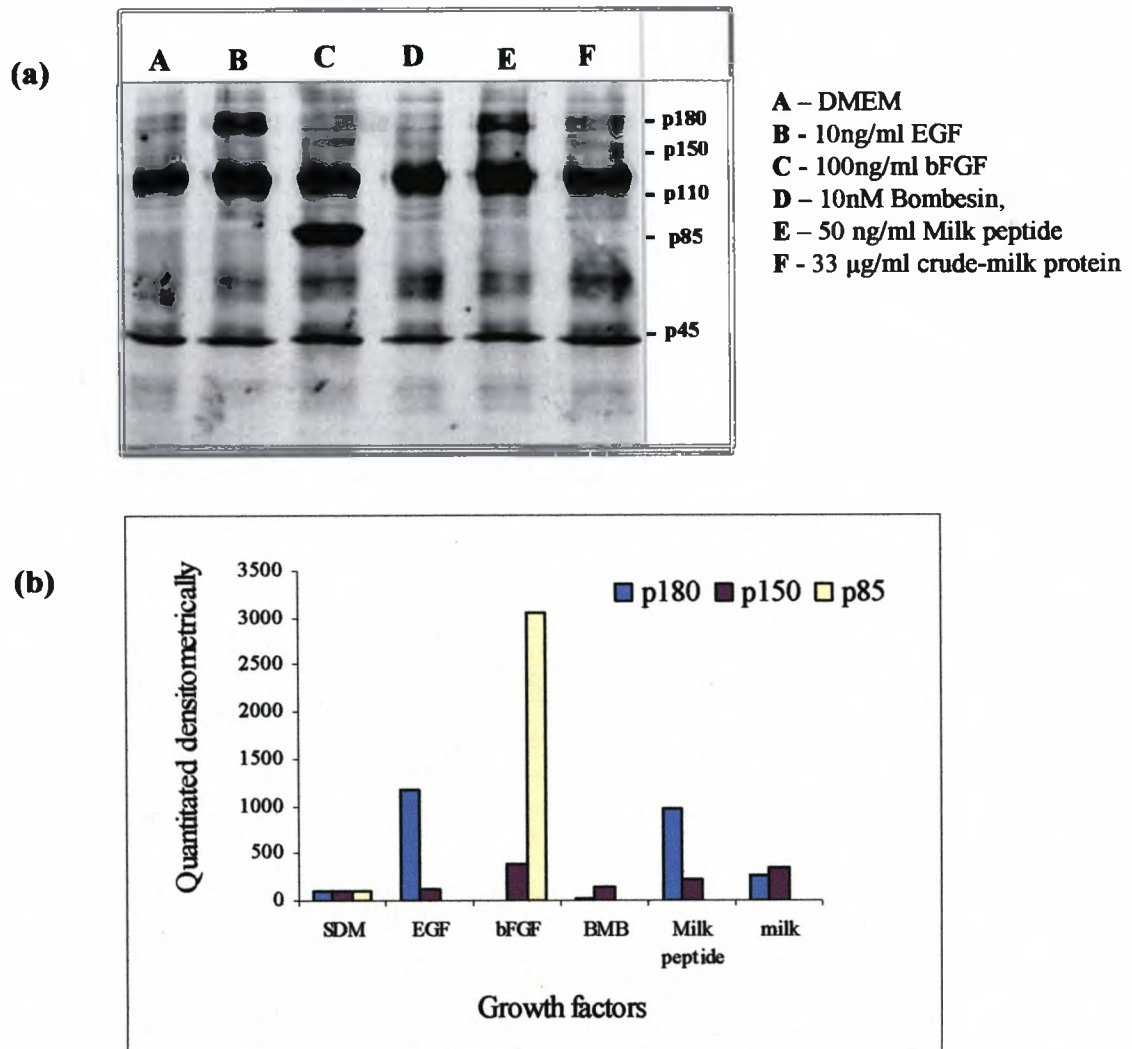
(a). Serum-starved cells were incubated for 15 min and lysed in lysis buffer. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with anti tyrosine phosphate antibody (PY20). Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5).

(b). Phosphotyrosine activation , expressed as a percentage of basal values (untreated cells), which was calculated from quantitative, two-dimensional densitometry of the immunoblot.

Figure 4.7 Effect of milk peptide on phosphorylation of EGFR in Rama 27 cells
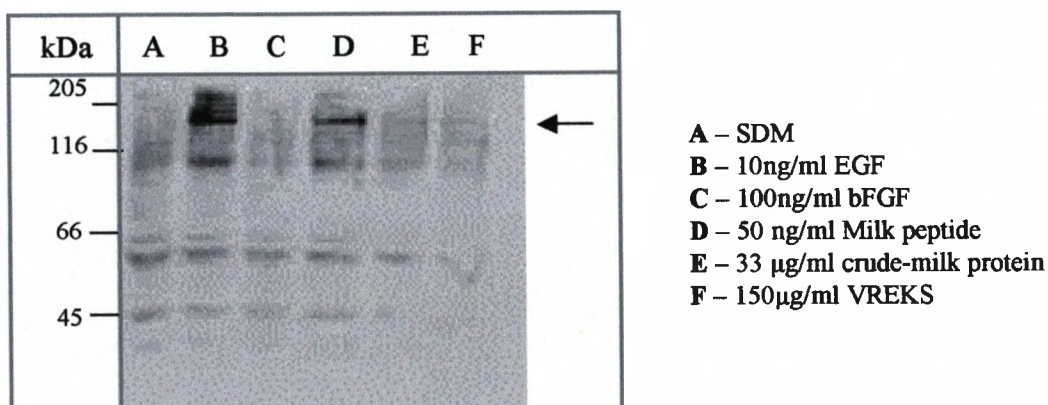
A – SDM
B – 10ng/ml EGF
C – 100ng/ml bFGF
D – 50 ng/ml Milk peptide
E – 33 µg/ml crude-milk protein
F – 150µg/ml VREKS

Serum-starved cells were incubated for 15 min and lysed in lysis buffer. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with anti Phospho-EGFR antibody. Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5). Arrow shows the phosphorylation of EGFR.

Figure 4.8 Effects of PD-098095 on growth factor induced-DNA synthesis
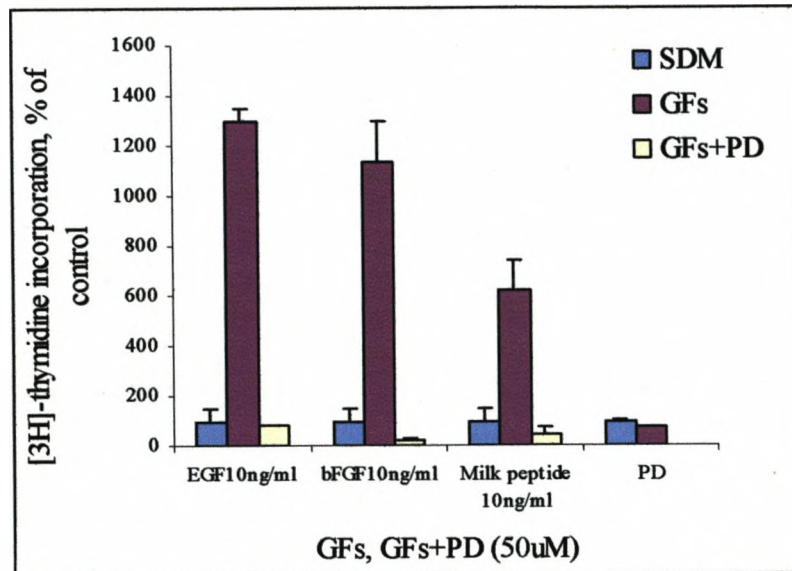
Serum-starved Rama 27 cells were incubated for 15 min with DMSO carrier PD-098095 (50 µM) before an 18-h incubation in growth factors as indicated. Incorporation of [^{3}H] thymidine into DNA was determined as described in Methods (2.3.1.3). Results are expressed as the mean percentage of the minimal stimulation (100%) observed in the presence of 0.025 % BSA ± SD.

Figure 4.9 Dose response of PD 098095 on DNA synthesis with growth factors in Rama 27 cells

(a)

(b)

(c)
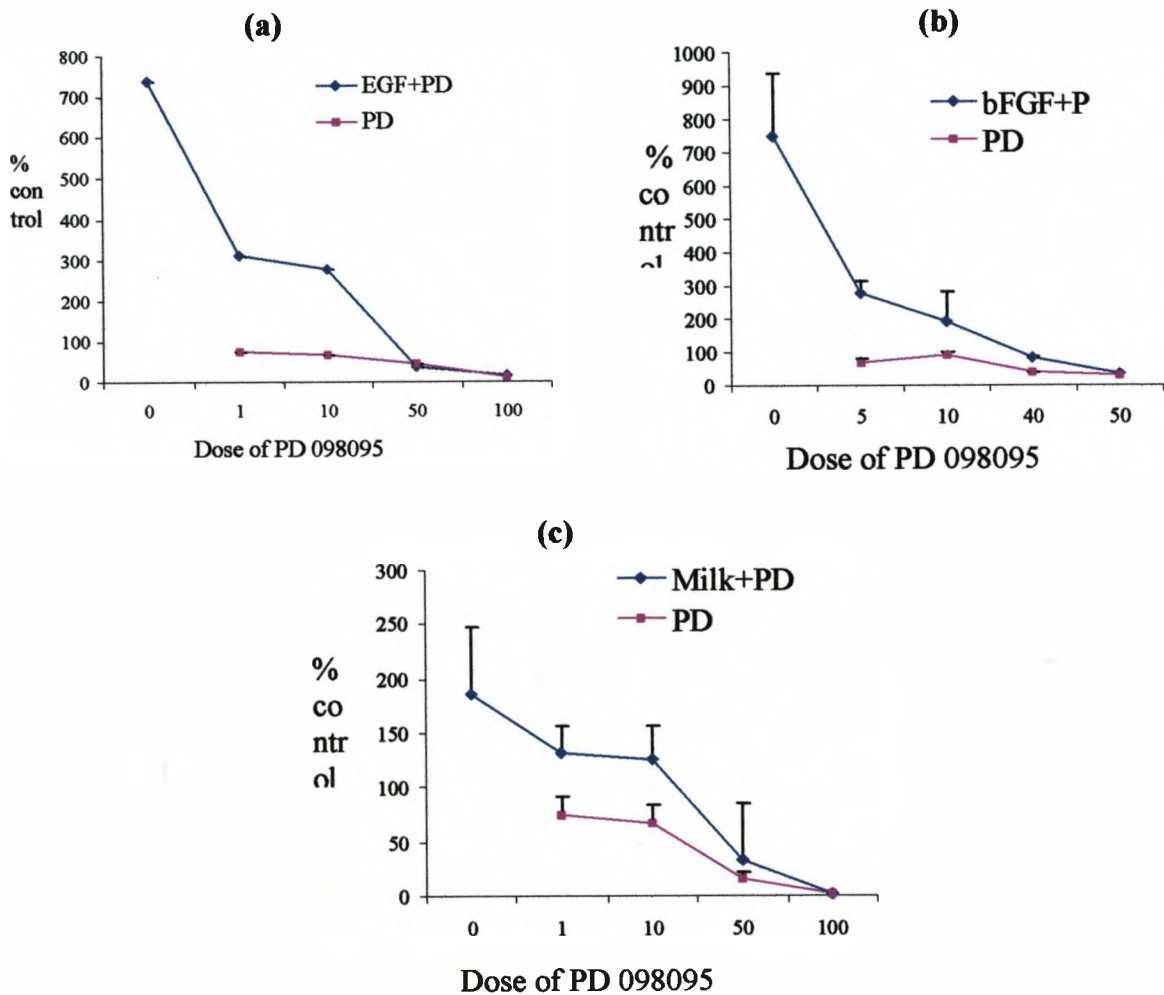
Serum-starved Rama 27 cells were incubated for 15 min with indicated concentration of PD-098095 before an 18-h incubation in

(a) 1ng/ml EGF,

(b) 10ng/ml bFGF and

(c) 10 ng/ml milk peptide.

Incorporation of [^{3}H] thymidine into DNA was determined as described in Method (2.3.1.3). Results are expressed as the mean percentage of the minimal stimulation (100%) observed in the presence of 0.025% BSA ± SD.

Figure 4.10 Effect on ERK of growth factors in Rama 27 cells
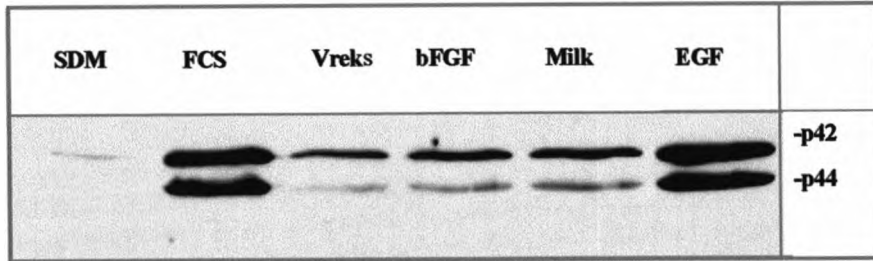
Serum-starved cells were incubated with: SDM, 2% FCS, 30μg/ml Vreks, 10ng/ml bFGF, 10 ng/ml Milk peptide, 2ng/ml EGF, for 15 min and lysed in lysis buffer. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with anti phospho MAPK antibody.

Figure 4.11 PD-098095 inhibits GFs stimulated ERK activation in Rama 27 cells
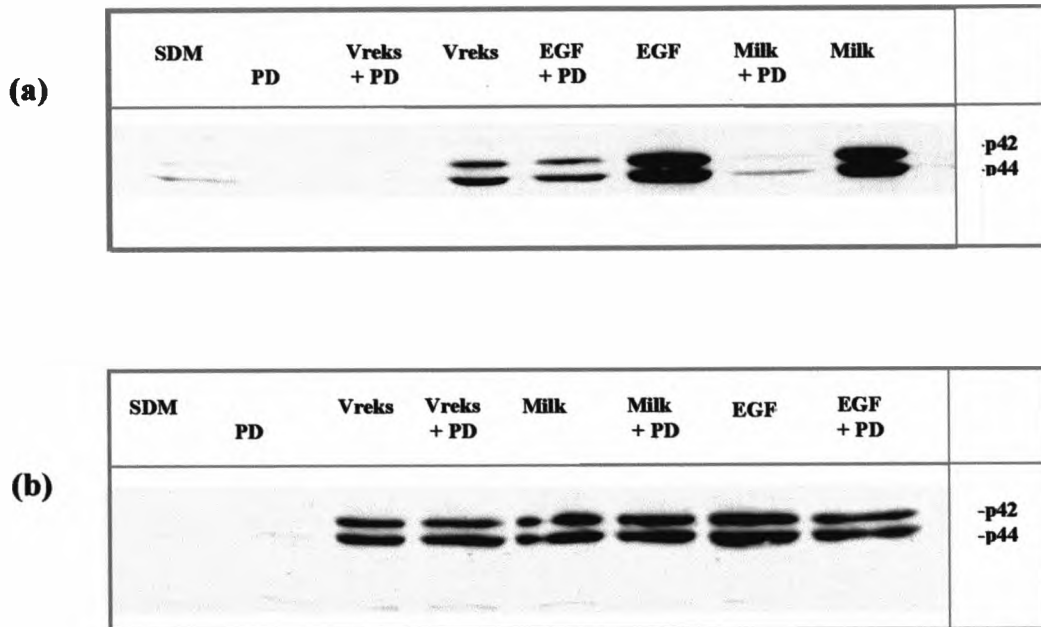
(a) Serum-starved Rama 27 cells were either untreated or pretreated with PD-098095 at 50μM for 15 min and stimulated with GFs at indicated concentration (30μg/ml VREKS, 10 ng/ml milk peptide and 2ng/ml EGF) for 15 min. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with phosphospecific anti MAPK antibody.

(b) Serum-starved Rama 27 cells were either untreated or pretreated with PD-098095 at 50μM for 15 min. Cells were then cultured with control SDM, 30μg/ml VREKS, 10 ng/ml milk peptide and 2ng/ml EGF 15 min **without PD-098095**. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with phosphospecific anti MAPK antibody.

Figure 4.12 Effect of Wortmannin on growth factor induced DNA synthesis in Rama 27 cells
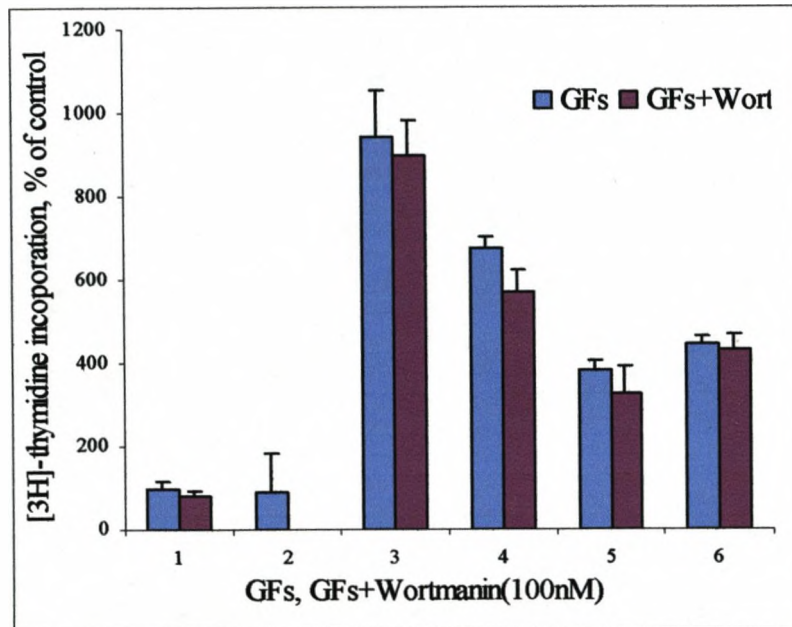
Serum-starved Rama 27 cells were incubated for 15 min 100 nM Wortmannin before an 18-h incubation with indicated concentration of growth factors. Incorporation of [^{3}H] thymidine into DNA was determined as described in Method (2.3.1.3). Results are expressed as the mean (of % control) ± SD. Samples: 1-SDM, SDM+wortmannin; 2-DMSO; 3-10 ng/ml bFGF; 4-1 ng/ml EGF; 5-30µg/ml Vreks; 6-10 ng/ml milk peptide.

Figure 4.13 The effect of Wortmannin on growth factor stimulated ERK activation in Rama 27 cells

SDM	WT	Vreks +WT	Vreks	EGF +WT	EGF	Milk +WT	Milk

-p42
-p44
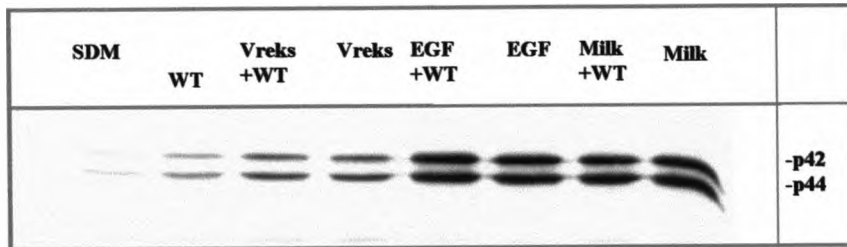
Serum-starved Rama 27 cells were either untreated or pretreated with Wortmannin at 100nM for 15 min and stimulated with GFs at indicated concentration (30µg/ml VREKS, 10 ng/ml Milk peptide and 2ng/ml EGF) for 15 min. Cells were harvested, and aliquots of the cell lysates were loaded onto SDS-PAGE and immunoblotted with phosphospecific anti MAPK antibody.

Table 4.1 Summary of effect of stimulation of DNA synthesis by growth factors on the different cell lines

	VREKS	KVIPY	Milk peptide	BOMBESIN	EGF
Rama 27	++++ **	++	+++++ **	+	++++ **
Rama 29	+ **	+ *	+ *	+ *	+
Rama 37	+ *	+	+	+	+
Rama 37 E8	+	+	++ *	-	++ *
Swiss 3T3	+	+	++++ **	+++++ **	++++ **

Different cell lines of cells were grown to confluence in 24-well plates, serum-starved and stimulated with growth factors as indicated. After 17 hours the cells were treated with [^{3}H]-thymidine for an additional 1 hour. (-) represented stimulation of DNA synthesis increased < 1 fold than control (SDM); (+) represented increased 1-2 fold; (++) increased 2-3 fold; (+++) increased 4-10 fold and (+++++) increased >10 fold. * differences were significant (P ≤ 0.05, from the student t test). ** differences were highly significant (P ≤ 0.01, from the student t test).

4.3 Discussion

Milk peptide isolated from bovine milk has been shown to act as a growth factor in Rama 27 fibroblasts (Liu and Smith, 1996). In this chapter, it was demonstrated that milk peptide was a mitogen for several cell lines. Since the signaling pathway(s) by which milk peptide mediates growth in these cells is (are) unknown, the present study was designed to identify the necessary intracellular events.

4.3.1 Peptide growth factors: detection using the incorporation of [^{3}H] thymidine into the DNA of Rama 27 fibroblast cells

Rama 27 fibroblast cells were growth arrested in G_0 of the cell cycle by culturing them in serum free medium. Growth arresting the cells in G_0 allowed observation of the incorporation of [^{3}H] thymidine into the DNA to be carried out 18 hours after stimulation, because maximum DNA synthesis occurred at this time, being at the peak of the S phase of the cell cycle. Rama 27 fibroblasts responded to FCS by increasing DNA synthesis. The half maximal stimulation occurred at 1.2% FCS. FCS has previously been shown to stimulate an increase in the number of Rama 27 fibroblasts (Smith et al., 1984), therefore an increase in DNA synthesis as measured by [^{3}H]-thymidine incorporation can be used as measure of cell growth. EGF also stimulated cell growth, half maximal DNA synthesis occurring at a concentration of 60pg/ml (McAndrew, 1993). In this study, milk peptide was also shown to have potent effect on DNA synthesis (Figure 3.7, 4.1c).

The milk peptide is a DNA synthesis stimulating peptide from as_2-casein in bovine milk (Liu et al., 1996). The present study describes how some small peptides with growth promoting activity including milk peptide induce DNA synthesis in some cell lines. Some stimulation of DNA synthesis by milk peptide was found for a number of cell lines, including rat mammary fibroblast cells, mouse embryo fibroblast cells, and

myoepithelial-like cells, in addition to human colon cancer cell lines (Data not shown) and the most sensitive cell line is rat mammary fibroblast cells. In Rama 27, the maximal stimulation of DNA synthesis obtainable with milk peptide was 37 fold higher than control which was significant difference (P<0.001 from the student t test), but with synthetic 'KVIPY' was 2 fold higher than control in which significant difference was not proven (P>0.05, from the student t test). In this study, the milk peptide had a much higher potency (3 nM) than synthetic peptides containing the sequence obtained from bioactive peptides (90 µM). The reason may be due to the conditions of production of synthetic peptides, such as pH, temperature, ions, urea, guanidinum and buffers. Alteration of one of above conditions might induce increased activity (Liu, 2002). Alternatively, 'KVIPY' may constitute only part of the observed natural activity, or it might not be folded well *in vitro*.

In contrast, Rama 37 cells did not respond to milk peptide (Figure 4. 3.b) and only slightly responded to EGF in which significant difference was not proven from the student t test (P>0.05) (Figure 4.3 a). It has been shown by others that the proliferative response of cells to EGF depends on their state of growth and differentiation. In rat intestinal epithelial (RIE)-1 cells, the effect of EGF on cell proliferation depends on the cell population density, being stimulatory in dense cultures and inhibitory at low population densities (Blay and Brown, 1986). Senescence of cultured cells is associated with decreased responsiveness to EGF (Matsuda et al., 1992). Rama 37 cells, which are rat mammary epithelial cells, as used here seem to be not easy to stop growing, and in this state the cells might not respond well to growth factors.

4.3.2 Identification of signaling pathways involved in the response to milk peptide

4.3.2.1 Milk peptide stimulates DNA synthesis via tyrosine kinase receptors

Growth factor receptors, such as the EGF receptor, which have associated protein kinase activity are phosphorylated on tyrosine in presence of their ligand. None of techniques employed so far were designed specifically to detect autophosphorylation of putative milk peptide receptor. In the absence of specific anti-receptor antibodies, autophosphorylation of growth factor receptors can be detected using anti-phosphotyrosine antibodies either by immunoprecipitation of membrane preparations phosphorylated *in vitro* by [γ^{32}P]ATP in the presence of ligand or by western blotting of whole cell exacts following treatment with ligand. Although the milk peptide receptor has not been characterized at the molecular level, Rama 27 cells contain enough milk activity receptor for us to attempt to identify a protein-tyrosine kinase activity associated with its receptor in this way since the milk peptide was isolated using this cell line as an assay.

In examining phosphorylation events induced by milk activity and other growth factors in rat fibroblast (Rama 27 cells), the most obvious changes are those which can be attributed to the activation of tyrosine kinase. Here we examined the effect of milk activity on the cellular events and found that milk activity activated tyrosine phosphorylation in a similar manner to EGF (Figure 4.4a) in Rama 27 cells and Swiss 3T3 cells (Figure 4.6a). The experiment of Western blotting with anti-phospho-EGF receptor, which does not interact with non-phosphorylated EGF receptor or other unrelated phosphotyrosine proteins, has shown that milk peptide activated EGF receptor in Rama 27 cells, thus, it gave a direct evidence that milk peptide induced DNA synthesis via the same receptor as EGF. Therefore, it is demonstrated that the mechanism of induction of DNA synthesis by the milk peptide is different from that of either bFGF or Bombesin. Although the 180 kDa band seen in the Western blot represents specifically EGFR, it does not prove the presence or absence of other member of the Erb family of receptors.

4.3.2.2 Milk peptide stimulates DNA synthesis via MAP kinase pathways

Several lines of evidence suggest that MAP-kinase plays an important role as a signaling enzyme regulating cell replication: 1). MAP-kinase integrates diverse signaling pathways activated by growth factor tyrosine kinase receptors, tumor promoting phorbol ester and G-protein coupled receptors. 2). Activated MAP-kinase phosphorylates substrates implicated in cell proliferation, such as S6-kinase, and the nuclear transcription factors, c-fos, c-jun, c-myc and $p62^{TCF}$ (Pulverer et al., 1991; Gille et al., 1992; Blenis, 1993, Schorb, 1995). 3). MAP-kinase has been showed to be activated in M-phase in Xenopus oocytes and $G_0/$ G_1 and M-phase in mammalian cells (Rossomando et al., 1989; Gotoh et al., 1991a,b; Posada et al., 1991). The reports demonstrating nuclear localization of MAP-kinase and translocation from the cytoplasm to the nucleus in response to serum support the hypothesis that MAP-kinase regulates nuclear targets (Chen et al., 1992).

In this study, it is demonstrated that predominantly p44 and to a lesser degree p42 MAP kinase are activated by milk peptide and other growth factors in Rama 27 cells. This effect was confirmed to be mediated through activation of MEK by use of PD-098059.

In many cells, activation of PI 3-kinase leads to activation of MAPK/ ERKs (King et al., 1997; Grammer et al., 1997) and PI 3-kinase inhibitors block VEGF-mediated activation of MAPK/ERKs (Thakker et al., 1999). Such cross-talk has been observed in HGF (Hepatocyte growth factor/Scatter factor)-stimulated migration of Rama 27 cells (Delehedde et al., 2001). It would be interesting to examine the effect of Wortmannin on $p42/p44^{MAPK}$ phosphorylation in milk peptide-stimulated Rama 27 cells. Growth factors induced significant activation of MAK/ERKs which was abolished by PD98059 in present study, but was resistant to PI 3-kinase inhibitors (Wortmannin) (Figure 4.13) in Rama 27 cells. In addition the stimulation of DNA synthesis by milk peptide, EGF, and bFGF in Rama 27 cells was also resistant to Wortmannin (Figure 4.12). These data indicated that, in contrast to VEGF-mediated signal transduction (Thakker, 1999), Hepatocyte growth factor/Scatter factor stimulating migration of Rama 27 cells (Delehedde et al., 2001), activation of

MAPK/ERK in Rama 27 cells with growth factors under the present experimental conditions is PI 3-kinase independent.

4.4 Conclusion

Milk peptide is a mitogen for several cell lines. Milk peptide stimulated cell proliferation by activation of rapid tyrosine phosphorylation, by activation of EGF receptor and the phosphorylation of p42/p44^{MAPK} in Rama 27 cells. Milk peptide induced DNA synthesis is dependent on MAP kinase activation, which can be blocked by PD98095; but is PI3 kinase independent. Whether milk peptide only activates EGF receptor or other receptor for its action will be studied in the chapter 5.

Chapter 5

Comparison of Biological Properties of EGF and Milk Peptide

5.1 Introduction

As described in the previous chapter, milk peptide, as well as EGF, stimulated DNA synthesis in rat mammary fibroblast cells (Rama 27 cells) and other cell lines. The mechanism of milk peptide stimulation of DNA synthesis appears to be via tyrosine kinase receptor, EGF receptor, rather than G- protein –coupled receptors. The milk peptide stimulated DNA synthesis pathway is similar to that of EGF. The final chapter of this thesis sets out to compare in more detail the stimulation of DNA synthesis by milk peptide and EGF, and also compare the pathway of stimulation of DNA synthesis invoked by them. The objective of this chapter was to find some clues of whether milk peptide stimulated DNA synthesis using EGFR or another receptor; if they used the same receptor, whether those two growth factors used different signalling pathways or used the same pathways with different sensitivities to stimulate DNA synthesis.

5.1.1 Receptor tyrosine kinases (RTKs)

Most peptide growth factors work via RTKs. RTKs comprise an extracellular portion which binds polypeptide ligands, a transmembrane helix and a cytoplasmic region which possesses catalytic activity and sites for protein – protein interactions (described in chapter 1). The protein tyrosine kinases constitute a family of enzymes that are thought to play a critical role in the regulation of cell proliferation (Comoglio et al., 1990). These enzymes catalyse the transfer of γ-phosphate of adenosine triphosphate (ATP) to the side-chain hydroxyl group of tyrosine residues in protein substrates which include the receptors themselves (autophosphorylation) (Hubbard, 1999). The interaction of growth factors with their cognate RTK triggers a series of

events that culminate in cell division. Comparison of the amino acid sequences within the tyrosine kinase domain of members of the RTK family have revealed a high degree of homology due to the conservation of residues essential for the catalytic function, especially within the ATP binding site.

5.1.2 EGFR family

Erb B proteins belong to subclass I of the superfamily of receptor tyrosine kinases (RTKs). There are four receptors termed the Erb B receptors as described in chapter1. Family members have in common an extracellular ligand-binding domain, a single membrane-spanning region and a cytoplasmic protein tyrosine kinase domain. A family of ligands, the EGF-related peptide growth factors, bind the extracellular domain of Erb B receptors leading to the formation of both homo- and heterodimers. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of the specific tyrosine residues within the cytoplasmic domain. These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular signal cascades (Olayioye et al., 2000).

5.1.3 Signaling to downstream effectors

Tyrosine phosphorylation of specific residues of the EGFR family members creates binding sites for Src-homology 2 (SH2) and phosphotyrosine - binding (PTB) - domain – containing proteins. Some of these proteins are enzymes that are tyrosine phosphorylated and thereby activated, such as Src, phospholipase Cγ (PLCγ) and phosphatidylinositol 3 – kinase (PI3K), whereas Shc, Grb2, Grb7 and Nck are adaptor molecules that link receptor tyrosine kinases (PTKs) to downstream signaling pathways. The recruitment and phosphorylation of p46/52 Shc isoforms and Grb2 represent receptor-proximal steps towards the activation of ras – MAP kinase pathway and subsequent regulation of gene transcription (Hackel et al., 1999).

5.2 Experimental and Results

5.2.1 Comparison of effect of EGF/ milk peptide on DNA synthesis

Confluent monolayers of Rama 27 cells can be stimulated to synthesize DNA by addition of EGF or milk peptide (chapter 4). The stimulatory activities of milk peptide and EGF were compared (Figure 5.1). Milk peptide, as well as EGF, stimulated DNA synthesis of rat mammary fibroblast cells at concentrations of 0.5 - 30 ng/ml. The profile of dose- dependent stimulatory activity by milk peptide was similar to that for EGF. The stimulation was increased by concentration of milk peptide up to 30 ng/ml. When 5 ng/ml milk peptide was added on Rama 27 cells, the level of stimulation of DNA synthesis was nearly that reached by 1ng/ml of EGF. Addition of 30 ng/ml of milk peptide induced the level of stimulation of DNA synthesis by 20 fold. (Figure. 5.1). Therefore, milk peptide is another potent mitogen known for rat fibroblasts.

EGF displayed higher potency as a mitogen for mouse fibroblast Swiss 3T3 cells; when 3 ng/ml EGF was added the level of stimulation of DNA synthesis was increased by 8 – fold. However, milk peptide induced only an approximately 5 - fold increase at 10 ng/ml (Figure 5.2). Therefore, the stimulation by milk peptide was less potent than EGF in Swiss 3T3 cells. However, milk peptide seems to have higher potency than EGF at the same concentration in R37 E8 (myoepithelial-like) and Rama 29 (myoepithelial-like) cells. On the other hand, milk peptide had no significant effect on the DNA synthesis of Rama 37 (epithelial) cells, likewise EGF had little effect on Rama 37 cells (Figure 5.2). Results here suggested that while milk peptide is a mitogenic factor for several cell lines that respond to EGF, the concentrations of milk peptide required for maximal stimulation were higher than those for EGF.

5.2.2 Comparison of autophosphorylation in fibroblast cells by EGF/milk peptide

As an assay for detecting receptor interactions, patterns of tyrosine phosphorylation were tested in Rama 27 cells and Swiss 3T3 cells. To this end, cells were stimulated with EGF and milk peptide, and whole-cell lysates were analyzed by immunoblotting with anti-phosphotyrosine antibodies. This analysis indicated that the EGF increased the tyrosine phosphorylation of a 180 kDa protein, which corresponds to Erb B-1, in Rama 27 cells, as well as in Swiss 3T3 cells (Figure 5.3). Likewise, milk peptide was able to stimulate the same protein. As Figure 5.3 shows the induction of tyrosine phosphorylation by 50 ng/ml milk peptide was similar to that of 10 ng/ml EGF. In addition, bands around 45 kDa were strongly detected in EGF and milk peptide – treated Rama 27 cells (Figure 5.3a). However, those bands were less strongly detected in EGF- and milk peptide – treated Swiss 3T3 cells (Figure 5.3b).

To examine whether the observed band of 180-kDa protein is time dependent during milk peptide and EGF activation, whole cell lysates were prepared after treatment of the cells with EGF and milk peptide for various times. Cell lysates from EGF treated serum-starved Rama 27 cells exhibited a time dependent increase in the tyrosine phosphorylation of the EGF receptor p180, which is maximal in 5 min and was retained to a lesser extent up to 60 min (Figure 5.4a). Cell lysates from milk peptide treated serum-starved Rama 27 cells also exhibited a time dependent increase in the tyrosine phosphorylation of the EGF receptor p180, which is maximal in 5 min and was retained to a lesser extent up to 30 min (Figure 5.5a). Relative levels of protein tyrosine phosphorylation were quantified densitometrically from the immuno-blot and results were expressed relative to amount of phosphorylation detected in the absence of growth factor (Figures 5.4b and 5.5b).

Comparison of the kinetics of Rama 27 cells treated with EGF or milk peptide revealed both milk peptide and EGF evoked a time–dependent tyrosine phosphorylation of an 180 kDa protein as detected in whole cell lysates (Figure 5.4, 5.5). Anti-phosphotyrosine analysis of lysates from Rama 27 cells displayed that the

kinetics of milk peptide activated tyrosine phosphorylation of an 180 kDa protein was similar to those induced by EGF.

5.2.3 Competition of EGFR by milk peptide

As described in chapter 4, milk peptide activated p180 EGF receptor (Erb B 1). To further examine if a high-affinity, specific binding site is expressed on Rama 27 cells that mediated the biological effects of milk peptide and EGF, preliminary experiments of binding of milk peptide to the EGF receptor were undertaken. As shown in the Figure 5.6a, unlabeled EGF competed with ^{125}I – EGF binding to Rama 27 cells in a dose-dependent fashion at concentrations above 10 ng/ml to 500 ng/ml. Fifty percent inhibition of binding of ^{125}I – EGF was observed at 100 ng/ml (17 nM). To ascertain if milk peptide can interact with EGF receptors on Rama 27 cells, the effect of milk peptide in the binding of ^{125}I – EGF to Rama 27 cells was investigated (Figure 5.6b). Although 85 nM (500 ng/ml) unlabeled EGF nearly completely competed out binding of ^{125}I – EGF to Rama 27 cells, the unlabelled purified milk peptide exhibited only about 15 % competition at 1.5 nM (P=0.3 from the student t test) and about 20 % competition at 3 nM milk peptide (P=0.1 from the student t test) (Figure 5.6b). Data presented here suggested that significant differences were not proven (P> 0.5 from the student t test) by using milk peptide to compete for binding to EGFR although the experiment was limited by amount of material available.

5.2.4 Signalling pathways of milk peptide and EGF display similarities and differences

To provide an initial mechanistic basis for stimulation of DNA synthesis by EGF and milk peptide, we analyzed one of the major signalling pathways that funnel many extracellular signals into the nucleus. This is the mitogen-activated protein kinase (MAPK)- pathway.

As described in chapter 4, milk peptide like other growth factors activates the MAP kinase (ERK) pathway. In order to investigate the time course for ERK activation by milk peptide and EGF, a series of time course experiments were analysed by Western blotting (Figures 5.7 and 5.8) with a phospho-p44/42 MAP Kinase (Thr

202/Tyr 204) E10 Monoclonal Antibody. EGF (2ng/ml) induced a rapid phosphorylation of p42/p44^{MAPK}, which was detectable starting from 5 min. The maximal level of phosphorylation of p42/p44^{MAPK} was seen at 15 min up to 100% (Figure 5.7b) and then slowly decreased to 78% of maximum at 30 min and 65% at 60 min (Figure 5.7b). Similarly, with milk peptide (10 ng/ml), the maximal level of phosphorylation of p42/p44^{MAPK} was seen at 15 min up to 100% (Figure 5.8a) and then quickly decreased to 56% of maximum at 30 min, 31% at 60 min (Figure 5.8b). The phosphorylation of p42/p44^{MAPK} was completely abolished by PD-098095 at 5 min. The level of phosphorylation of p42/p44^{MAPK} at 15 min was inhibited 90% with EGF induced, 85% with milk peptide-induced phosphorylation.

Comparison of the kinetics of stimulation of MAP-kinase, ERK, was analyzed in EGF or milk peptide- treated cells. Evidently, the presence of milk peptide was sufficient to enable the activation of ERK. Although similar, MAP-kinase activation by EGF displayed subtle differences (Figure 5.7, 5.8). The activation of ERK decreased more slowly in EGF-treated Rama 27 cells than milk peptide–treated cells. These observations suggest the possibility that the differences between the growth signals of EGF and milk peptide are caused, at least in part, by receptor-specific signalling pathways.

5.2.5 Effect of milk peptide on stimulation of DNA synthesis in CHO/CB1 cells

To further investigate if milk peptide-stimulated DNA synthesis used the same receptor as EGF, CHO and CB1 cells were used in this study. Chinese hamster ovary (CHO) cells offer the advantage of having no endogenous expression of EGF receptors and low background of Erb B-2. In addition, these cells were sensitive to Insulin, so they offer a sensitive system for detection of growth regulatory signals (Tzahar et al., 1996).

To determine the pathway of milk peptide stimulated DNA synthesis in CHO cells, [^{3}H] thymidine incorporation into cells was measured in CHO cells. As Figure 5.9a shows, EGF did not stimulate DNA synthesis in CHO cells although high concentrations of EGF were used. In contrast, Insulin had high potency to stimulate DNA synthesis in CHO cells. When 50 nM Insulin was added to CHO cells, the level

of DNA synthesis was increased 34 fold (Figure 5.9c). Surprisingly, when 5 ng/ml milk peptide was added to CHO cells, the level of DNA synthesis was increased 9 fold.

The results here suggested milk peptide stimulation of DNA synthesis used other receptor(s) as well as EGF receptors. To clarify which receptors milk peptide are used for stimulation of DNA synthesis more study will be needed in the future.

Figure 5.1 Concentration dependence of the stimulation of DNA synthesis in Rama 27 cells by EGF and milk peptide
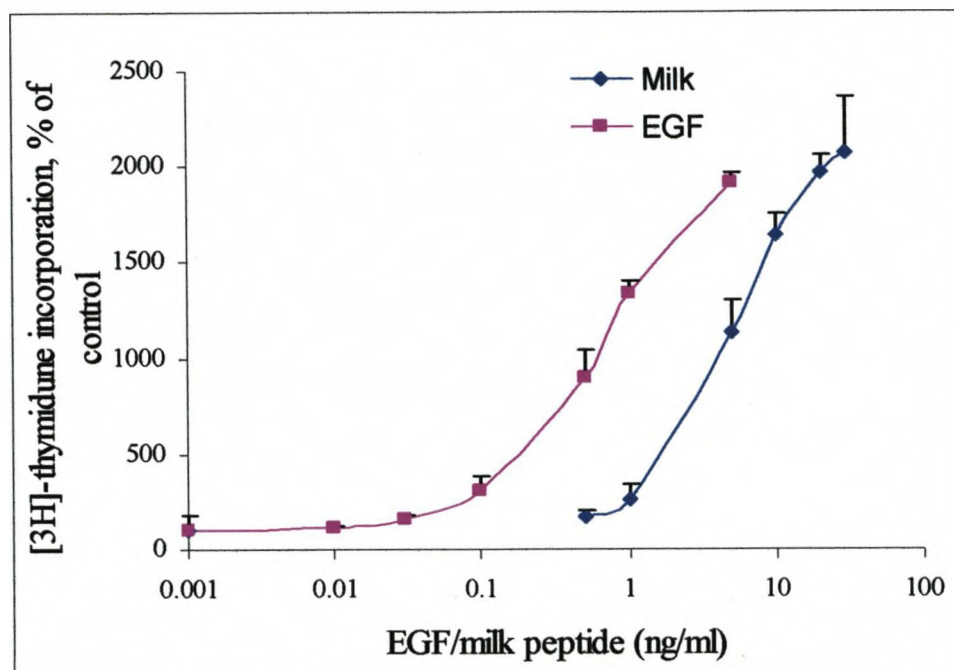
Rama 27 cells were grown to confluence in 24-well plates, serum-starved and stimulated with EGF and milk peptide. After 17 hours the cells were treated with [^{3}H]-thymidine for an additional 1 hour. The background incorporation of [^{3}H]-thymidine into DNA by untreated quiescent cells was 100%. Data points represent the means ± SD. (This figure is a compilation of figure 3.7 and figure 4.1.)

Figure 2] Concentration dependence of the stimulation of DNA synthesis in Rama 27 cells by EGF and milk peptide

Rama 27 cells were grown to confluence in 24-well plates, serum-starved and stimulated with EGF and milk peptide. After 17 hours the cells were treated with [³H]-thymidine for an additional ... hour. The radioactive incorporation of [³H]-thymidine into DNA by untreated quiescent cells was 100%. Data points represent the mean ± SD. This figure ...

Figure 5.2 Effect of milk peptide/ EGF on DNA synthesis in different cell lines
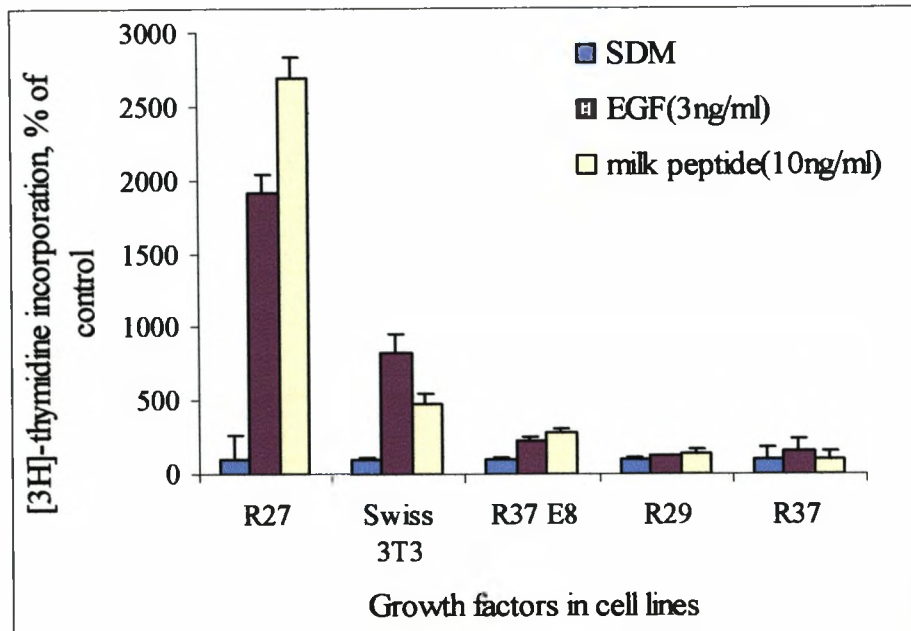
Different cell lines of cells were grown to confluence in 24-well plates, serum-starved and stimulated with EGF (3 ng/ml) /milk peptide (10 ng/ml). After 18 hours the cells were treated with [^{3}H]-thymidine for an additional 1 hour. The background incorporation of [^{3}H]-thymidine into DNA by untreated quiescent cells was 100%. Data points represent the means ± S.D.

Figure 5.3 Ligand-stimulated tyrosine phosphorylation of proteins in fibroblast cells

(a)

(b)
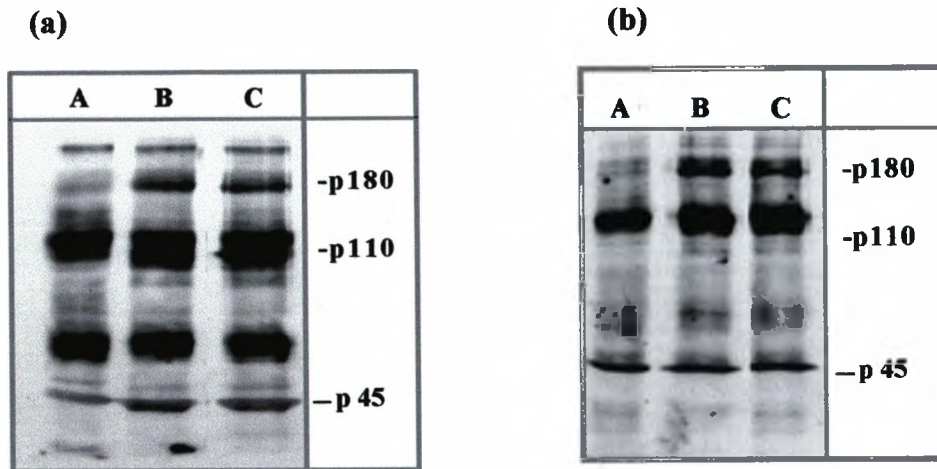
A – SDM
B – 10ng/ml EGF
C – 50ng/ml Milk

Cell monolayers of Rama 27 cells (a) and Swiss 3T3 (b) were serum-starved and stimulated with either EGF or milk peptide for 15 min at 37°C. Whole cell lysate was fractioned in a 7.5% SDS-PAGE gel and analyzed by Western blotting for the presence of phosphotyrosine using anti tyrosine phosphorylation antibody (PY20). Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5). Figure 5.3(a) consists of lanes A, B and D from figure 4.4; figure 5.3(b) consists of lanes A, B and E from figure 4.6.

Figure 5.4 Time course of EGF induced tyrosine phosphorylation in Rama 27 cells

(a)

(b)
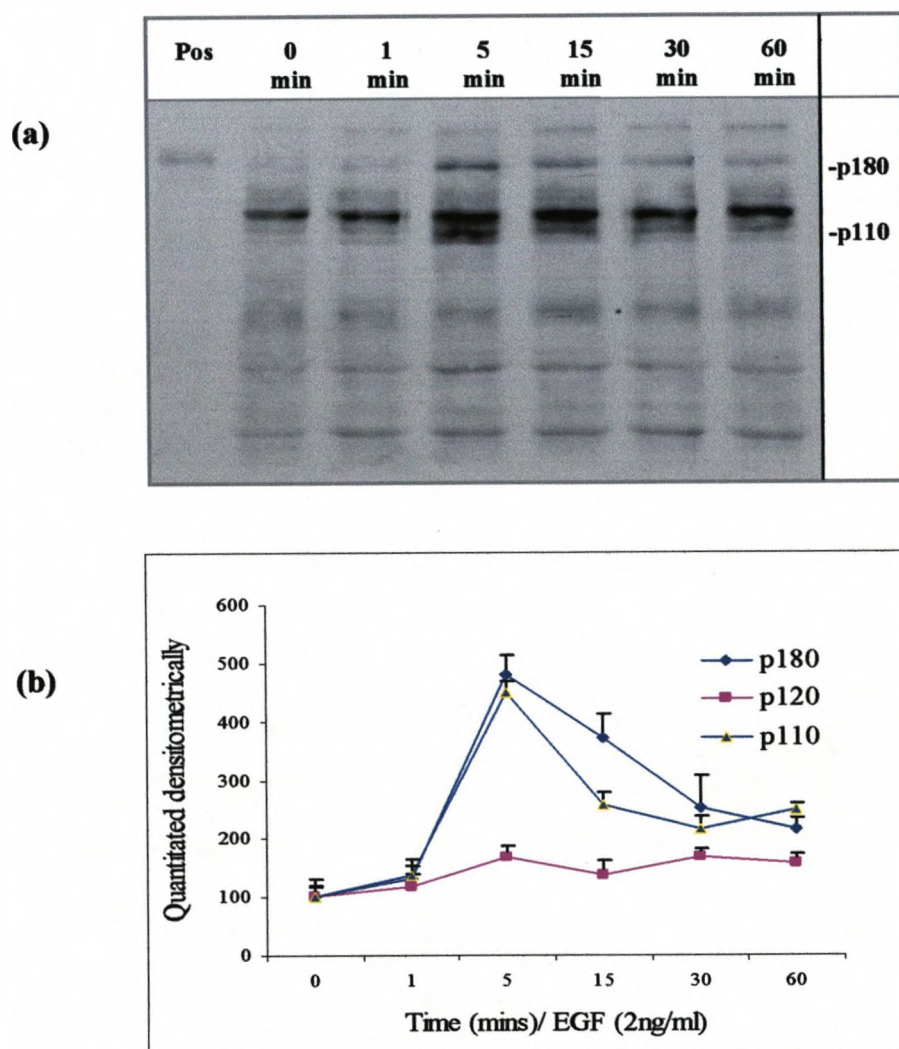
(a) Time course of EGF-activated tyrosine phosphorylation in Rama 27 cells. A representative immunoblot of Rama 27 cell lysates incubated with anti-phosphotyrosine antibody. Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5).

(b) Tyrosine phosphorylation, expressed as a percentage of basal values (100%), which was calculated from quantitative ± SD, two-dimensional densitometry of the immunoblots.

Figure 5.5 Time course of milk peptide induced tyrosine Phosphorylation in Rama 27 cells
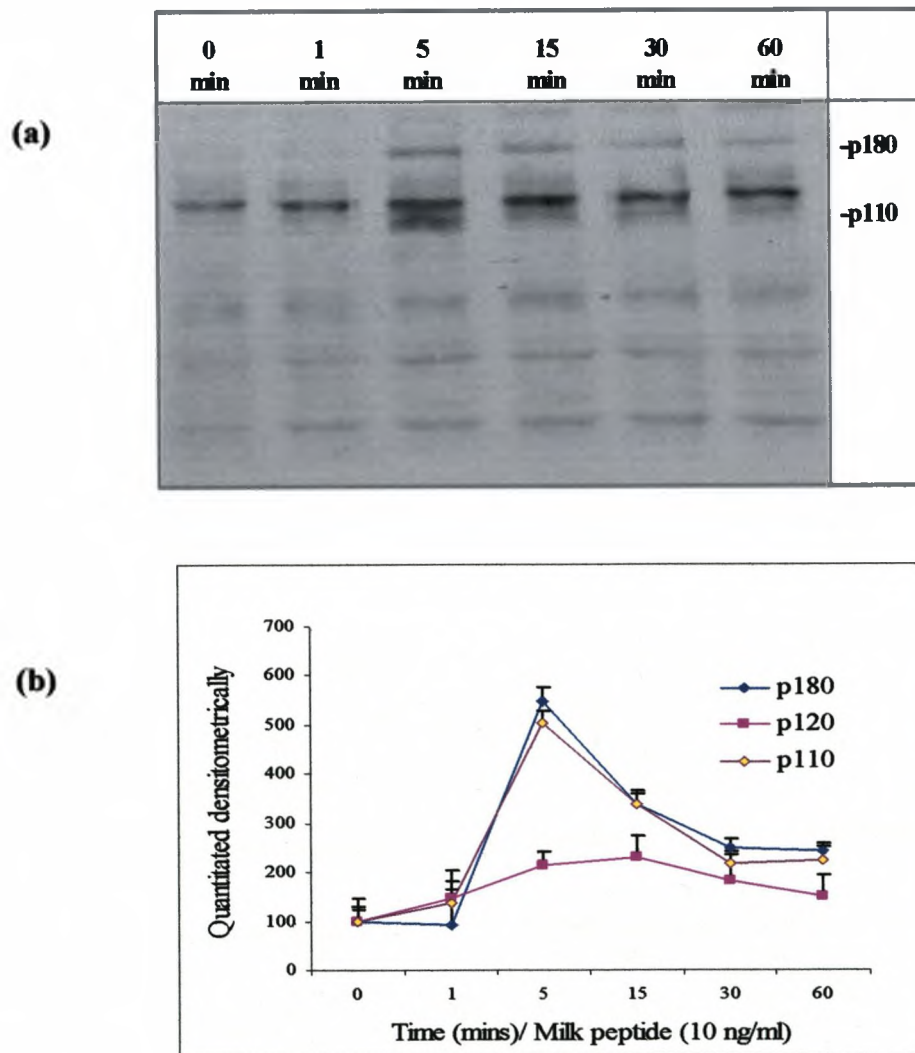
(a) Time course of milk peptide-activated tyrosine phosphorylation in Rama 27 cells. A representative immunoblot of Rama 27 cell lysates were incubated with anti-phosphotyrosine antibody. Second antibody bound to immunoblots was detected by ECL (Method 2.3.1.5).

(b) Tyrosine phosphorylation , expressed as a percentage of basal values ± SD, which was calculated from quantitative, two-dimensional densitometry of the immunoblots.

Figure 5.6 Competition with ^{125}I-EGF in binding to Rama 27 cells

(a) Dose response of EGF
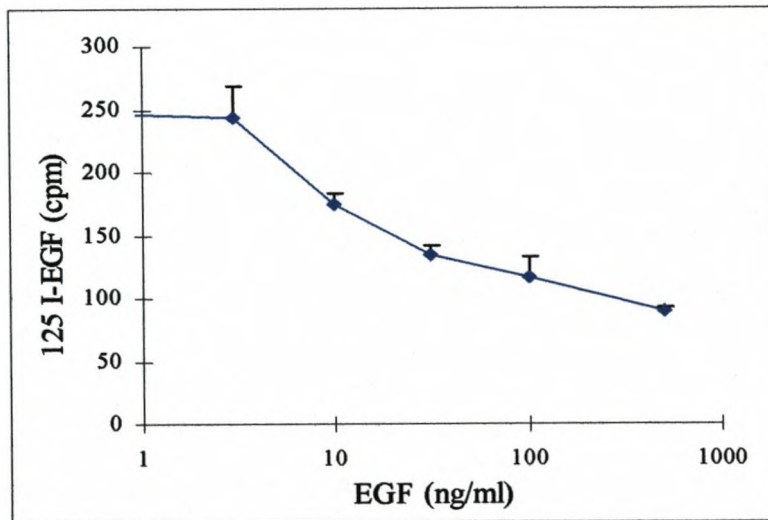
(b) Competition of [125] I-EGF with milk peptide and KVIPY
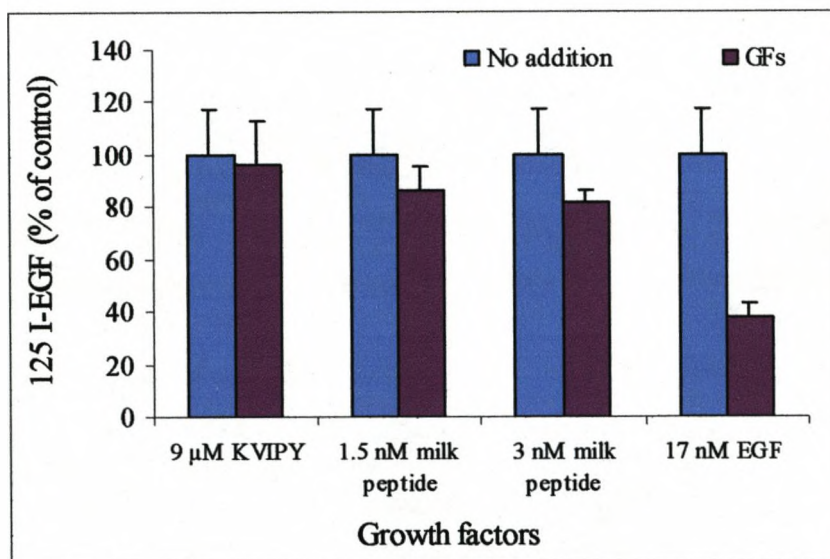
^{125}I-EGF (0.34 nM, 10^5cpm) was added to the confluent Rama 27 cultures with the indicated amounts of growth factors. Control was based on no addition of growth factors. The cells were treated as described in Method 2.3.1.6. Results are the mean ± SD. For the KVIPY and milk peptide, the significant differences were not proven between their competition for binding to EGFR and control ($P > 0.05$ from the student t test). For the 17 nM EGF there was a significant difference in the competition for binding to EGFR ($P < 0.05$ from the student t test).

Figure 5.7 The time course of MAPK activity stimulated by EGF on Rama27 cells
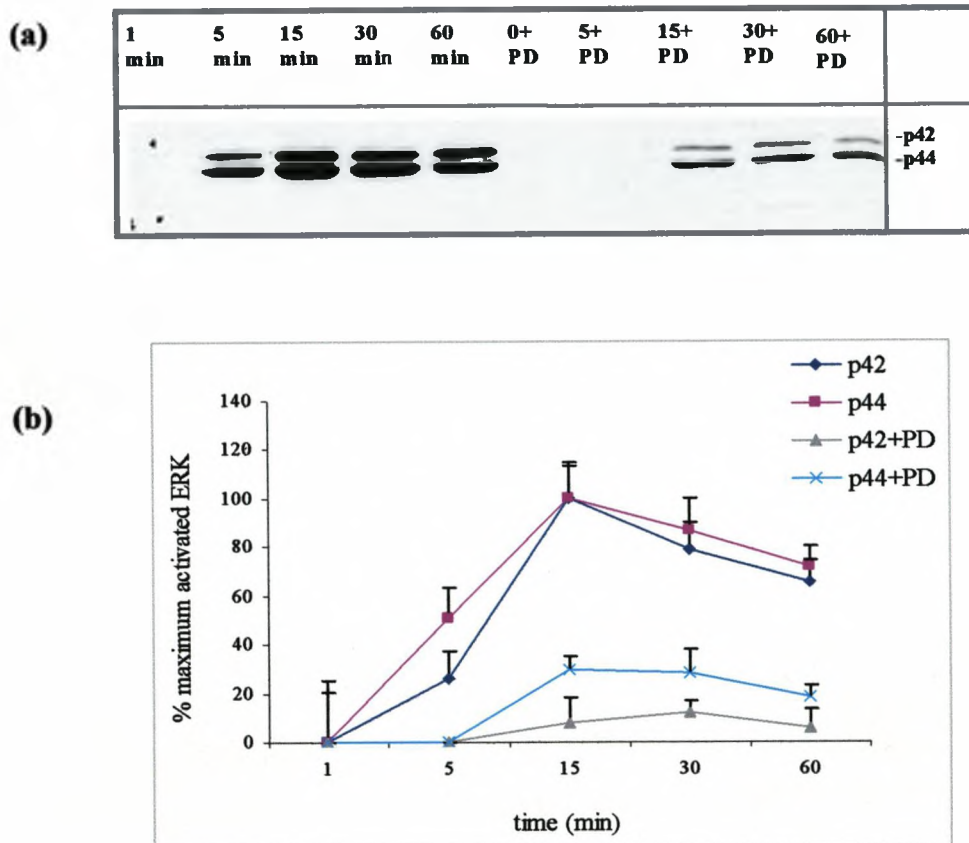
(a) Serum-starved cells were incubated with 2ng/ml EGF for indicated times. A representative immunoblot of Rama 27 cell lysates incubated with anti-phospho-ERK antibody. PD098095 (50μM) was added as a pretreatment 15 min before 2ng/ml EGF administration.

(b) Densitometric quantification of the level of immunoreactive dually phosphorylated p42/p44^{MAPK} as a percentage of the maximum phosphorylation observed ± SD at time indicated after addition of 2 ng/ml EGF.

Figure 5.8 The time course of MAPK activity stimulated by Milk peptide in Rama27 cells
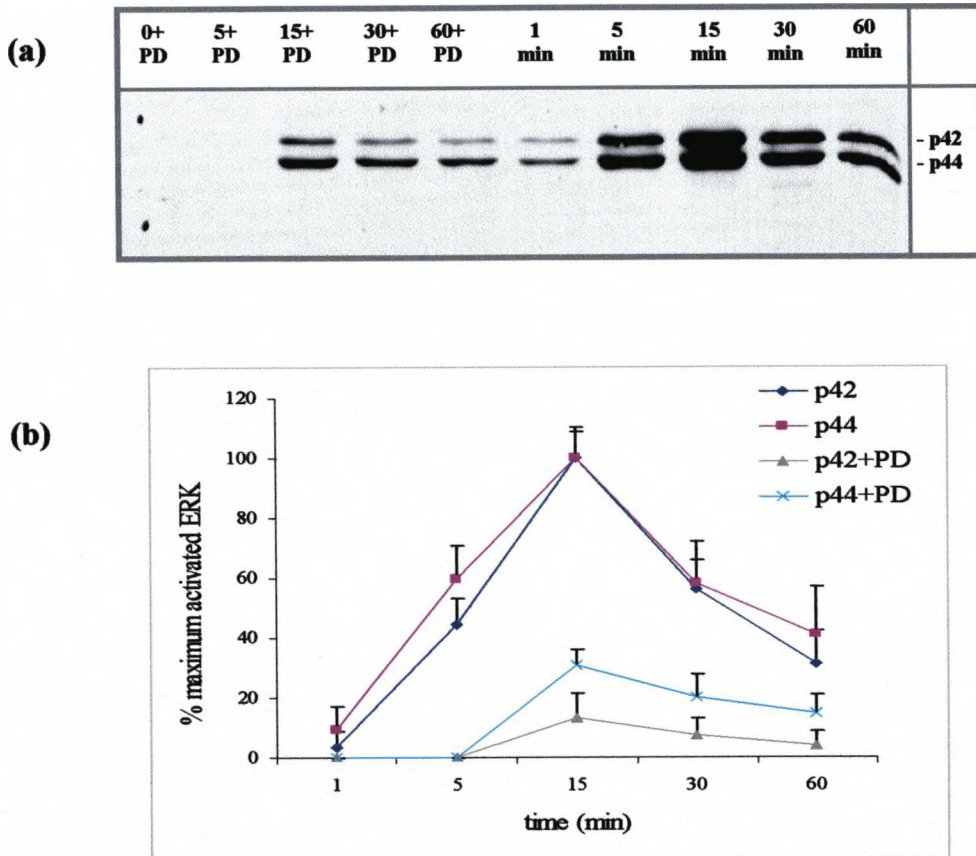
(a) Serum-starved cells were incubated with 20 ng/ml milk peptide for indicated times. A representative immunoblot of Rama 27 cell lysates incubated with anti-phospho-ERK antibody. PD098095 (50μM) was added as a preteatment 15 min before milk peptide administration.

(b) Densitometric quantification of the level of immunoreactive dually phosphorylated p42/p44 MAPK as a percentage of the maximum phosphorylation observed ± SD at time indicated after addition of 20 ng/ml milk peptide.

Figure 5.9 Effect of growth factors on DNA synthesis in CHO parent cells

The cells were treated as described in Method 2.3.1.3. Results are expressed as the mean percentage of the minimal stimulation (100%) observed in the presence of DMEM ±SD.

(a) Dose response of EGF
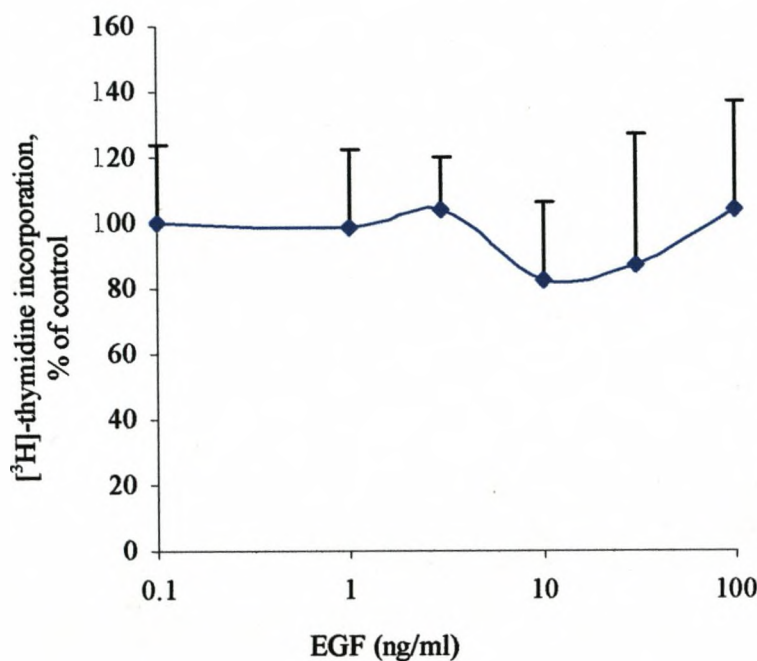
(b) Dose response of insulin
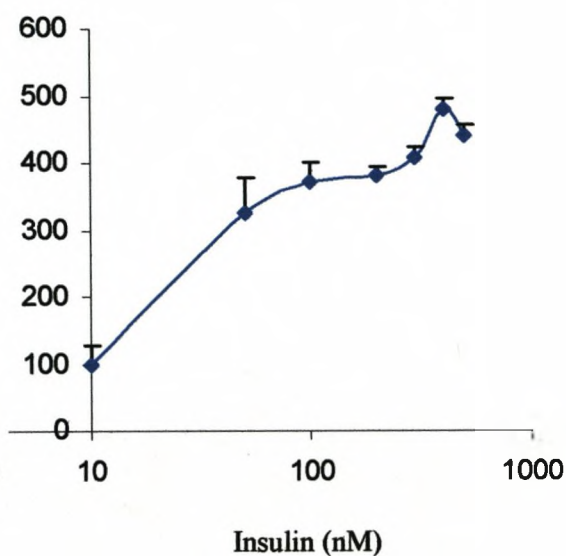
(c) Growth factors stimulated DNA synthesis
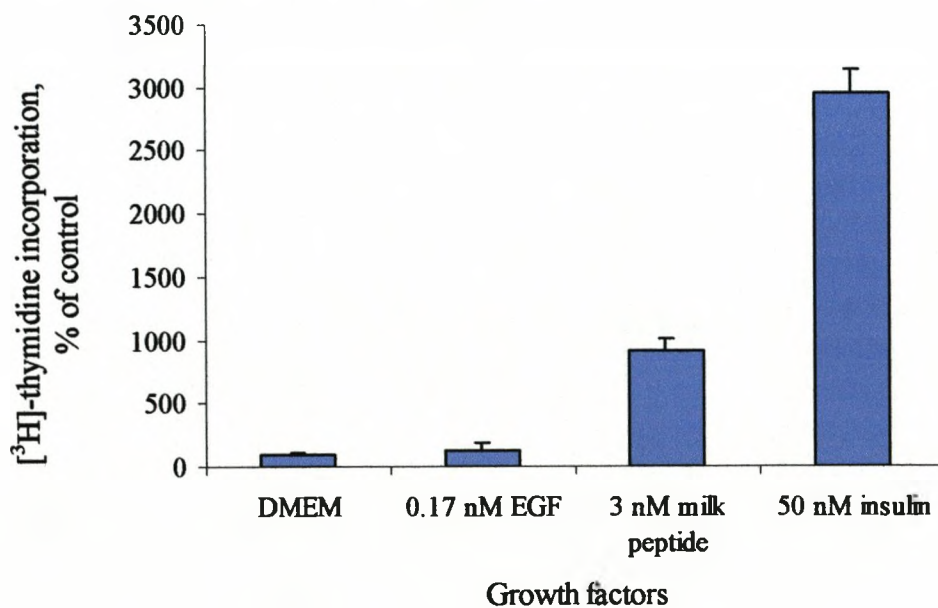
5.3   Discussion

In addition to nutrients, vitamins and minerals, milk contains a variety of growth factors thought to be important in the regulation of growth and secretory functions of maternal mammary tissue and, in the newborn, regulation of growth, development, and maturation of the gut and immune system (Grosvenor et al., 1992). In human milk, EGF has been identified as a major growth promoting factor (Carpenter, 1980), whereas there seems to be very little, if any, EGF in bovine milk (Iacopetta et al., 1992). Instead, the major growth –promoting factors identified in bovine milk seem to be PDGF (Shing and Klagsbrun, 1987) and IGF-I and IGF-II (Francis et al., 1988). In the previous chapter, it has been shown that milk peptide purified from bovine milk is a novel growth factor for fibroblast cells and other cell lines. Milk peptide stimulated DNA synthesis in fibroblast cells via RTKs, EGF receptor. Pathways by which milk peptide stimulated DNA synthesis in fibroblast cells seemed to be similar to those for EGF.

The aim of this chapter was to find molecular basis for the differential signalling by milk peptide and EGF. Unlike EGF, milk peptide did not require disulfide bonds to exert its effect on DNA synthesis in Rama 27 cells (as described in the chapter 3). Milk peptide can stimulate DNA synthesis in CHO cells, whereas EGF did not. Besides, it was observed that there was a small difference in the kinetics of activation of MAP kinase ERK.

Rat fibroblast cells contain a significant level of phosphotyrosine in protein of 180 kDa when treated with milk peptide. This protein corresponds to Erb B-1. Cell lysates prepared from Swiss 3T3 cells also exhibited milk peptide-stimulated phosphotyrosine similar to that stimulated by EGF. It is therefore possible that stimulation of DNA synthesis in fibroblast cells occurs through RTKs, EGF receptor. It is interesting to note that fibroblast cells contain a significant level of phosphotyrosine in proteins of mass 45 kDa when treated with milk peptide or EGF. Whether this is another EGF receptor in fibroblasts or other substrate of signaling pathway is not clear.

Under the assay conditions used in this study, milk peptide and EGF-stimulated phosphorylation of tyrosine in protein 180 kDa is rapid for approximately 5 min. The essentially immediate stimulation of tyrosine phosphorylation upon addition of EGF and milk peptide suggests that activation of tyrosine – directed protein kinase occurs very rapidly after binding of the growth factor to its receptor. Thus, the initial response to EGF/ milk peptide may occur at the level of the plasma membrane and may not require growth factor – receptor complex internalization.

A purified preparation of milk peptide did not exhibit statistical significant ($p > 0.05$ from student t test) competition with ^{125}I – EGF in the binding to Rama 27 cells in which the EGF receptor was expressed. There may be several reasons for the inability of milk peptide to compete for EGF receptor in this study. Firstly, to compete EGF receptors fully, at least 10 times higher concentration of growth factors would be needed. It was difficult to get such high concentrations of milk peptide for this experiment so far. Another reason might be that milk peptide might bind to the EGF receptor with low affinity. To further investigate if a high-affinity, specific site is expressed on Rama 27 cells more studies need to be done in the future.

The exact mechanism whereby milk peptide stimulates tyrosine phosphorylation remains to be elucidated. Binding of EGF to its receptor on the cell surface is essential for the cellular response, but the specificity of this response may crucially depend on the type of cell and the physiological conditions. Stabilized complexes of EGF and its receptor on the cell surface can stimulate RNA synthesis but not mitogenesis, suggesting that internalization and processing of EGF–receptor complex is necessary to produce the mitogenic signal (Wakshull and Wharton, 1985). It is reported that in EGF – sensitive cells, EGF – induced phosphorylation of the EGF receptor may be necessary but insufficient in itself to trigger mitogenesis (Bishop et al., 1985). Similarly, Chana and Smith (1991) showed that removal of EGF caused no DNA synthesis in fibroblasts even through the early signals would be complete. Thus, stimulation of early tyrosine phosphorylation by milk peptide would be necessary to trigger mitogensis in fibroblasts.

That activation of the ERK pathway is sufficient is strengthened by the finding that expression of constitutively activated forms of MEK induces mitogenesis and transformation in fibroblasts as well as differentiation of PC12 cells (Cowley et al., 1994; Mansour et al., 1994). When the cells were exposed to milk peptide for 1min both the p44 and p42 MAP-kinase isoforms demonstrated an increased intensity on SDS PAGE indicative of phosphorylation, reaching the maximum level of phosphorylation at 15 min. The amount and time course of p44 and p42 phosphorylated in response to milk peptide was similar to that observed when cells were exposed to 2ng/ml EGF. A small difference was observed in the kinetics of the p42/44 [MAPK] phosphorylation by milk peptide and EGF in Rama 27 cells. This may imply that there was a different signaling pathway between the EGF and milk peptide. Kramarski et al., (1996) reported that all of the combinations of three Erb B proteins employed the MAP-Kinase pathway, with the exception of Erb B –3 homodimers. The kinetics of MAP-kinase activation through each kind of receptor complex differed. This may determine the potency of the resulting growth signals, by analogy with the results obtained in p12 cells, indicating that the duration of ERK activation is critical for the signaling decision (Marshall, 1995). What is exactly different in the signaling pathway between EGF and milk peptide is still unknown.

Since the amount of milk peptide is limited, the second phase of MAP-kinase was not tested in the present study. Schorb et. al., (1995) reported a biphasic activation of MAP-kinase when cardiac fibroblasts were exposed to [Sar[1]] Angiotensin II. After a transient peak at 2 min, the kinase activity returned to near baseline values followed by second sustained phase of activity observed 120-150 min after stimulation. PDGF-BB produced a transient peak of MAP-kinase activity which was followed by sustained phase of activation, while carbachol, in contrast to [Sar[1]] Angiotensin II and PDGF-BB, failed to induce a secondary phase of kinase activity and also had no effect on DNA synthesis. This finding supported the hypothesis that prolonged activation of MAP-kinase is necessary to trigger DNA synthesis. Schorb et. al., (1995) also suggested that both phases of MAP-kinase activation contribute to *c-jun* transcription activity and cell proliferation. Based on the experiment of this study, since milk peptide stimulated DNA synthesis in Rama 27

cells, it was assumed that milk peptide stimulated transient and sustained activation of MAP kinase, therefore stimulated DNA synthesis in Rama 27 cells as does EGF.

Results from this study indicated that milk peptide stimulated DNA synthesis in CHO cells. This cell line does not respond to EGF and is sensitive to insulin. Although it was shown that insulin had great effect on CHO cells, there was variability of the responses seen in these cell lines. It would suggest that although it activated EGF receptor in Rama 27 cells, milk peptide has another receptor in CHO cells. The requirements of HC11 mouse mammary epithelial cells for both EGF and insulin for growth under-restricted condition allowed another comparison of biological activities of the peptides. While all the EGF agonists and Neu differentiation factor (NDF or neuregulin) were able to replace EGF in the growth medium, none of the EGF agonists was able to replace insulin. Only NDF could partially replace insulin, thereby cooperating with EGF for the stimulation of HC11 cells growth. Thus, it seems that NDF has a dual EGF-and insulin –like activity enabling it to stimulate signaling pathways that normally require two separate growth factors (Beerli and Hynes, 1996). Whether milk peptide, like NDF, uses insulin receptor in CHO cells will need more study in the future.

Taken together, study of this chapter demonstrated that milk peptide and EGF both had similar properties in stimulating DNA synthesis in the different cell lines. EGF and milk peptide stimulated the kinetics of activation of phosphotyrosine in a similar manner. However, EGF and milk peptide stimulated the kinetics of activation of MAP kinase somewhat differently. In addition to EGF receptor, milk peptide uses another receptor for its action.

Chapter 6

Discussion

6.1 General Discussion

The overall aim of this thesis was to verify the milk peptide as a novel growth factor for rat mammary fibroblast cell line (Rama 27) and to examine its cell specificity and its mechanism of action and to produce active peptide in E coli.

Successfully purified milk peptide from bovine milk was initially important to ensure that there were enough materials for later study of its mechanism. In order to identify the mechanism of milk peptide, the level of contaminating other growth factors in active fractions needs to be reduced to a concentration such that only a single activity was obtained. Most growth factors, such as EGF and Betacellulin identified in bovine milk have acidic isoelctric points, whereas milk peptide has pI 10.12. Hence cation exchange chromatography combined with hydrophobic interaction chromatography could be utilized to adsorb and elute the milk peptide under conditions in which major proteins and unwanted growth factors were not adsorbed (Liu, 2002). The eluted bound protein from the final C_4 reverse phase HPLC had a single active peak, which gave a remarkable stimulation DNA synthesis in Rama 27 cells.

The mitogenic activity from highly purified milk peptide is stable to DTT, a characteristic that is different from that of EGF and BTC (see below). The mitogenic activity from highly purified milk peptide was observed in CHO cells, which lacks endogenous EGF receptor. The evidence here strongly indicated that stimulation of DNA synthesis by highly purified milk peptide could not be due to a contaminant of other growth factors in the preparation.

In addition, the milk peptide that stimulates DNA synthesis appears to be extremely stable. Treatment of milk peptide with DTT did not affect its activity strongly suggesting that there were not disulfide bonds in the milk peptide. To further confirm this result, after heating milk peptide with DTT which might be expected to denature the sample, the activity still was not destroyed. It indicated that the effect of milk peptide was due to a peptide without disulfide bonds. In contrast with milk peptide, betacellulin which is present in bovine milk (Dunbar et al. 1999) includes a characteristic six-cysteine consensus motif within the EGF-like domain. Treatment of EGF with DTT decreased its the activity because the central fragment of the EGF structure has three intrachain disulfide bonds.

To further verify the milk peptide as a novel growth factor, recombinant technology was used in this thesis. In trying to achieve expression of milk peptide in *E coli*, a synthetic milk peptide gene was made. Use of a synthetic gene is a very efficient step for producing recombinant milk peptide compared with getting cDNA from cells, in which it is not universally present.

The successful expression of the milk peptide was due to constituting an effective expression system for milk peptide. The strategy of using a fusion partner was intended to increase the expression of the milk peptide by avoiding proteolysis of the expressed milk peptide in *E. coli*. Enhanced expression of proinsulin in *E. coli* by the N-terminal addition of short homo-oligopeptides has been described by others (Sung et al. 1986). The expression of soluble milk peptide shown in this thesis might easily allow the recovery of activity of recombinant milk peptide. The major problem that was encountered here was in detecting the expression of the protein in *E coli*. Since the expressed peptide only has a mass of 3.7 kDa, normal SDS-PAGE was unsuitable for its detection in the absence of antibody to detect the expressed protein. The combination of reverse phase HPLC with Tricine electrophoresis made it possible to detect the expression of protein.

The incorporation of a methionine residue at the amino terminus of each nascent polypeptide is a universal translation signal used by both eukaryotes and prokaryotes. In prokaryotes, the methionine residue carried by the initiator tRNA is formylated prior to its incorporation. *E. coli* possesses an aminopeptidase which catalyses the

efficient removal of formyl methionine from recombinant polypeptides is variable (Liang et al. 1985; Hirel et al. 1989; Dalboge et al. 1990). The loss of the methionine residue from the amino terminus of recombinant milk peptide produced in *E.coli* might be due to aminopeptidase catalysis of its removal from recombinant peptide. The evidence that the correct amino acid sequences were directly obtained after purification by SDS-PAGE onto a PVDF membrane confirmed that fused milk peptide was successfully expressed.

Although the *E coli*. products, after purification by ion-exchange chromatography and reverse phase HPLC, could stimulate DNA synthesis in Rama 27 cells, the activity was less potent than that of natural milk peptide. The most likely explanation for this would be several reasons: although purification of recombinant milk peptide was attempted in this thesis, it was shown that the peak fraction that eluted from reverse phase HPLC was not pure. Although purified intact fusion proteins have been used in the development of vaccines (Kleid et al., 1981; Pilancinski et al., 1984; Jacob et al., 1985) and in the development of diagnostic kits for the AIDS retrovirus and to demonstrate biological activity of the Fc portion of IgE (Liu et al., 1984), the 'fusion' amino acids on the *N*-terminus of milk peptide were not cleaved in this thesis, and this might affect the milk peptide's binding to its receptor and cause the low activity. This is in agreement with the observation that the major determinant of NDF binding residues is at its *N*-terminus (Tzahar et al.1997). Deletion of the first two amino acids of NDF severely reduced binding of biregulin to NDF receptors, whereas individual replacements of any one of the next three residues did not impair receptor binding (Barbacci et al. 1995). In addition, the structure of the folding milk peptide may also be important if differences in the structure resulted in the proteins being recognized as foreign.

Once it had been confirmed that milk peptide is a novel growth factor for Rat mammary fibroblast cells (Rama 27 cells), it was necessary to find the possible reason for milk peptide as a growth factor.

Milk peptide is only part of α_{s2}-casein, but it was shown to have high growth promoting activity. Investigation into the mechanisms of physiologically functional proteins have revealed relatively or extremely short peptides with biological

functions such as angiotension, neurotensin and enkephalin. These findings suggest that an oligopeptide which is part of a functional protein could be a minimum unit for this functionality.

Milk peptide is derived from casein, but intact casein has no growth promoting activity. It has been reported that caseins release some bioactive peptides in the course of digestion in the gastrointestinal tract. Also some bioactive peptides would be produced during the storage of bovine milk because milk contains endogenous proteinases (Liu, 2002).

It has been reported (Cox et al.1991) that acid treatment of the normally secreted latent TGF-β forms produces the biologically active molecule (Lawrence et al., 1984). They reported that exposure to the low pH of stomach activates latent forms which may be present in the milk (Cox et al., 1991). They have already observed that acid treatment of whole milk results in an increased level of activity in the fibroblast-migration assay than produced by an equivalent volume of the same, but untreated, milk sample, and the mildly acidic conditions used in the purification experiments would presumably explain why they managed to obtain the active mature form of the milk growth factor molecule. The high degree of stability of such proteins in an acid environment might also facilitate their absorption into the circulation thereby directing their actions to other sites which are adjacent to or distant from the gastrointestinal tract (Cox et al., 1991). It might be another possibility that when milk peptide was exposed to the low pH of stomach or acidified, α_{s2}-casein was broken to small peptide fragments which could stimulate DNA synthesis. Whether milk peptide is absorbed into circulation is not clear.

The mechanisms whereby milk peptide stimulates DNA synthesis are unknown. That milk peptide stimulated DNA synthesis in fibroblast cells by receptor tyrosine kinase was demonstrated in this thesis. The early events of phosphotyrosine in fibroblast cells were investigated by immunoblotting. When cells were treated with milk peptide, phosphorylation on tyrosine was significantly enhanced on the 180kDa protein in Rama 27 cells, which was similar with that caused by EGF, but different from that by bFGF, or bombesin. This was consistent with bFGF and bombesin having different mechanisms of action from EGF, and from milk peptide too.

The presence of such a tyrosine –phosphorylation site in the milk peptide treated cells revealed that the receptor of milk peptide belongs to the receptor tyrosine kinase family. Whether tyrosine phosphorylation is necessary for the long-term growth-promoting effects of milk peptide is unclear. In A431 cells, the elevated levels of phosphotyrosine are maintained for a longer period than that during which EGF must be continuously present to initiate DNA synthesis in responsive cells (Hunter and Cooper, 1981). The effect of EGF on the level of phosphotyrosine is evidently reversible, since the increment of phosphotyrosine is lost quite rapidly upon removal of EGF with anti-EGF anti-serum. It is possible that increased phosphorylation of tyrosine is simply an early and transient event in the response of cells to EGF. Nevertheless, phosphorylation of specific target proteins during this phase might trigger a series of events that would ultimately increase the probability of a cell initiating DNA synthesis and proceeding to cell division (Hunter and Cooper, 1981).

Furthermore, the kinetics of phosphorylation on tyrosine by milk peptide and EGF were shown to be similar. The fact that these changes were extremely rapid, occurring within one minute of addition of EGF, or milk peptide, suggests that the phosphorylation of tyrosine may be the primary signal. This leads one to consider whether milk peptide uses EGF receptor to stimulate DNA synthesis. The experiments of immunoblotting with anti-phospho EGF receptor antibody showed that when cells were treated with milk peptide, as when they were treated with EGF, the EGF receptor was phosphorylated. However, bFGF and Vreks did not affect EGF receptor. Thus, it is clear that milk peptide stimulated DNA synthesis, at least part, via EGFR (Erb B-1); it might interact with EGF receptor in the membrane or with its substrates. Monospecific antibodies to EGF identified two non-overlapping regions within the EGF molecule (residues 22-32 and 33-53) as essential for receptor binding (Katsuura and Tanaka, 1989). Likewise, in TGF-α, two groups of ligand-inhibitory peptides were identified (residues 22-34 and 36-50) (Richter et al. 1992). Analysis of single- and double-site mutants (Campion et al. 1993) identified specific residues within these two groups as necessary for receptor recognition (Groenen et al. 1994). However, not all members of the EGF family bind to Erb B family; the human cripto-1 (CR-1) gene (also known as teratocarcinoma-derived growth factor-1 (TDGF-1)), another new member of EGF family, can bind to a unique receptor that

is not the EGFR, erb B2, erb B3, or erb B4 (Kannan et al. 1997). CR-1 was found to enhance the tyrosine phosphorylation of the SH2-adapter protein, Shc, and to promote the association of Grb2-mSOS intracellular signalling complex with tyrosine-phosphorylated Shc. These events subsequently related to the down stream activation of the $p42^{erk-2}$ MAP kinase isoform. Although milk peptide has a different structure from the EGF family, the results in this thesis suggested that it acts via EGFR. What the essential residues are for milk peptide recognition of EGF receptor is unclear.

Purified milk peptide and synthetic KVIPY both exhibited much weaker competition than EGF with ^{125}I-EGF in the binding to Rama 27 cells. It is possible that milk peptide might bind to the EGF receptor with low affinity, as do Epiregulin and Amphiregulin (Shoyab et al. 1989; Toyoda et al. 1995). Alternatively, the other receptors which bind EGF-related ligands, erb B2, erb B3, and erb B4, may serve as receptors for milk peptide.

This raises a question whether milk peptide action is only through EGFR, Erb B-1, or is another protein involved. By using CHO cells which lack endogenous EGFR, it was shown that insulin had great effect on CHO cells. EGF seemed not to affect CHO cells although high concentrations of EGF were administrated. However, milk peptide under special conditions, that is only cells well stepped down when serum-starved, did have an effect on CHO cells, but the effect was less than that of insulin. These results implied that although the receptor of EGF (Erb B-1) was activated by milk peptide for its action, there really is another receptor for milk peptide action. Whether this is a novel receptor, or is another ErbB family receptor is unclear. The evidence in this thesis did not support the idea that milk peptide uses insulin receptor because insulin always gave a good stimulation of DNA synthesis in CHO cells and also Rama 27 cells do not respond to insulin (Liu et al. 1996, Mc Andrew 1993). However, milk peptide had great potency to stimulate DNA synthesis in Rama 27 cells and can stimulate DNA synthesis in CHO cells only under the special conditions. It was reported that NDF has a dual EGF-and Insulin-like activity enabling it to stimulate signalling pathways that normally require two separate growth factors. Activated insulin and insulin-like growth factor-1 receptors induce the rapid tyrosine phosphorylation of insulin receptor substrate-1, which contains

multiple binding sites for the p85 subunit of PtdIns 3-kinase (Myers et al. 1994) (Beerli and Hynes, 1996). Thus, it seems that NDF is the best activator of ErbB-3, the only Erb B receptor involved in efficient recruitment of p85 (Soltoff et al. 1994; Fedi et al. 1994). Thus, it possible that the ability to efficiently activate PtdIns 3-kinase is responsible for NDF's insulin-like activity. Whether milk peptide like NDF has dual activity in CHO cells is unknown. To answer these questions, more investigations are needed in the future.

The differential requirement for DNA synthesis of CHO cells for EGF and milk peptide signalling implies a certain degree of ligand-specific diversity in the signal transduction mechanisms of the EGF receptor. To study in detail the signal transduction pathways underlying milk peptide mediated DNA synthesis, another main finding in this thesis is the demonstration that MAP kinase/ERK is necessary for efficient mitogenic signalling by milk peptide for Rama 27 cells, and also by EGF. As shown in this study, an inhibitor of this signalling pathway results in blocking the level of DNA synthesis stimulated in response to milk peptide in Rama 27 cells. Furthermore, phosphorylation of $p42/44^{MAPK}$ was shown to take place in response to milk peptide in Rama 27 cells, as to EGF. In the presence of PD-098095, a MEK-1 inhibitor, this phosphorylation was abolished. The results here were in agreement with the idea that MAP kinase is on the critical signalling pathway (Marshall, 1995) for cell proliferation.

In contrast, Wortmannin, a PI3 kinase inhibitor, had no statistically significant effect on milk peptide-induced, or EGF induced-DNA synthesis in Rama 27 cells. The lack of an effect of Wortmannin on DNA synthesis induced by milk peptide and EGF in this study might suggest that PI3 kinase appears not necessary to stimulation DNA synthesis induced by milk peptide and EGF. Analysis of the role of PI3-kinase in cell proliferation and differentiation has been difficult because specific inhibitors have not been available. Wortmannin contains a rather unstable group, and it is possible that it degraded in the medium. Kimura et al. (1994) reported Wortmannin was effective for 3 hours; it became less effective after 5 hours. There might be another explanation that milk peptide induced Rama 27 cell proliferation following the degradation of Wortmannin in the medium. However, in the case of primary rat hepatocytes, Wortmannin reversed insulin- and EGF- induced [^{3}H] thymidine

incorporation into DNA (Band et al. 1999). PD 098095 was without significant effect on insulin- and EGF- induced [^{3}H] thymidine incorporation into DNA. It might be another possibility that Wortmannin had significant effect only on the cells in which initial PI3 kinase activation is important to trigger DNA synthesis.

Thakker et al. (1999) have demonstrated a link between PI3-kinase and the downstream activation of MAP kinase in VEGF stimulated cells by significant inhibition with wortmannin, or by blocking MAP kinase activation with a domain negative p85 mutants. They also demonstrated the critical role of PI3 kinase activation in generating a maximal mitogenic response to VEGF. However, the failure of wortmannin to block the dual phosphorylation of p42/44^{MAPK} response to milk peptide and EGF in Rama 27 cells was observed in the present study. This suggested that activation of PI3 kinase immediately after stimulation with milk peptide and EGF was not required for the dual phosphorylation of p42/44^{MAPK} in these cells. This data indicated that in contrast to VEGF-mediated signal transduction (Thakker et al. 1999), milk peptide activated MAPK/ERK is PI3 kinase independent.

Another phosphotyrosine protein of mass around 45 kDa was also enhanced when Rama 27 cells were treated with EGF and milk peptide; whether this might be p46 Shc, which bind to EGF receptor and can associate with the Grb2-mSOS signaling complex, is not clear. Although these proteins were not characterized in the present study, it might be assumed that these were SH2 domain-containing, tyrosine-phosphorylated proteins which potentially couple to the activation of Ras-MAP kinase pathway. EGF activation of MAP kinase is by ras- raf- MAP kinase kinase (Section 1.3.2.4.3). Kinetics of phosphorylation of p42/44^{MAPK} in response to milk peptide were shown to be similar to those in response to EGF in Rama 27 cells. This might lead to the idea that milk peptide activated MAP kinase by EGFR- and Ras-dependent pathway in Rama 27 cells. However, in rat liver epithelial cells, it was shown that angiotension II could activate ERK-MAP kinase by two different pathways (Li et al., 1998). The downstream pathway of milk peptide stimulated DNA synthesis in CHO cells is unclear.

In conclusion, the main findings of this study were as follows: 1). Milk peptide was confirmed as a novel growth factor for fibroblast cells by purifying milk peptide

from the bovine milk, and by production of recombinant of milk peptide in *E coli*; 2). Milk peptide was shown to act through tyrosine kinase receptor, at least partly by through EGF receptor (Erb B-1) in Rama 27 cells; 3). MAP kinase is a major pathway for milk peptide stimulated DNA synthesis which is PI3 kinase independent.

6.2 Future Investigation

Since milk peptide is a novel growth factor, there clearly remain many questions to be answered.

To further identify the receptor of milk peptide, it would be good idea to determine if a high affinity specific binding site is expressed on Rama 27 cells that have been shown to respond to the biological effect of milk peptide in the present study and others (Liu et al., 1996). To achieve this, the Rama 27 cells with the highest number of receptors will be used for competition studies with known growth factors such as other members of the EGF family or insulin family to establish whether the peptide is using a known receptor. Rama 27 cells will be incubated with ^{125}I-milk peptide (either natural milk peptide or purified recombinant milk peptide or chemically synthesized peptide) in absence or presence of increasing concentrations of unlabelled milk peptide. To further examine whether milk peptide is or is not directly binding to EGFR, competition for EGF receptor, will be attempted using anti-EGFR antibody which is sufficient to impede ^{125}I-EGF (Kannan et al., 1997) to see if this leads to any change in the specific binding of ^{125}I-milk peptide to Rama 27 cells. The same method will be used on other members of the Erb B family and insulin like growth factor family.

There remain many other aspects that need to be studied, including purification of the recombinant milk peptide followed by factor Xa cleavage. Since the milk peptide structure was determined, antibodies and nucleic acid probes will be generated. Anti-milk peptide antibody will be employed to demonstrate milk peptide in tissues by Western blotting and immunohistochemistry. Similar techniques may be employed to localize cell surface receptors, and receptor distribution can also be demonstrated using autoradiography- a technique based on the application of radiolabeled ligand to tissue section.

In the intact animal insight into the effect of milk peptide may be obtained by administering it, or by blocking its action with antibody. More recently, molecular

technology has made it possible to develop animal models with upregulated endogenous growth factor expression (transgenic aminals) or impaired growth factor or receptor expression ("knock-out" animals) (Murphy, 1998).

Chapter 7

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