ANALYSES OF ALTERNATIVE CELL SIGNAL TRANSDUCTION PATHWAYS

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ABSTRACT

Living cells keep sensing the changes in their environments, mostly, via cell surface receptors for different ligands. Attachment-dependent cells are sensitive to alterations in extracelluar matrix (ECM). ECM is not only required for cell survival, but also prerequisite for epidermal growth factor (EGF) to stimulate cell proliferation. The receptors for the majority of ECM components are integrins and the receptor for EGF is EGF receptor (EGFR). When bound by their ligands, integrins and EGFR induce signal transduction cascades composed of alternative pathways. A quantitative assessment of relative contributions of alternative pathways to one final cell signaling will help understand designing principles of the network. Unfortunately, a methodology for such assessment is still not available, partly because of lack of relatively mature mathematical models. On the other hand, in most biochemical cascades, existence of alternative pathways increases the complexity and thus the robustness of networks. The relationships between the topology and robustness of large-scale biochemical networks have been studied intensively recently. In small-scale networks, while feedback has been revealed as an important contributor for adaptation and robustness, the quantitative correlation between the topology/pathway redundancy of small networks and their robustness remains unknown.

In this thesis, apoptosis of bovine mammary gland epithelial cells was demonstrated to be induced when fibronectin, one of the major components of ECM, was degraded by overexpressed tPA via two potential ways: deprivation of attachment and the effects of fibronectin fragments. Secondly, a mathematical model for EGFR activation of

the MAPK cascade, in which alternative pathways exist, was explored and it was found that the Shc-dependent pathway is both redundant and dominant. We hypothesize that the Shc-dependent pathway is important for EGFR to compete with other receptors, which need Shc to transduce cell signals; and this pathway is not aimed to increase the robustness of the EGFR cascade. Finally, for the general importance of alternative pathways to the network topology and robustness, several concepts have been proposed to decompose and quantitatively characterize the networks. We demonstrate that the pathnet score is a better assessment for robustness than the molecular connectivity.

RÉSUMÉ

Les cellules perçoivent en permanence les changements de leur environnement, principalement via les récepteurs des ligands de la surface cellulaire. Les cellules dépendant des attachements sont sensibles aux altérations de la matrice extracellulaire (MEC). Outre la survie de la cellule, la MEC est aussi nécessaire à la stimulation de la prolifération cellulaire par les facteurs de croissance épidermique (FCE). Les récepteurs de la majorité des composants de la MEC sont des intégrines ; celui du facteur de croissance épidermique est le récepteur FCE (RFCE). Lorsqu'ils se lient à leurs ligands, les récepteurs induisent des cascades de transduction de signaux composées de voies alternatives. Une évaluation quantitative des contributions relatives des voies alternatives à la signalisation cellulaire pourrait aider à comprendre les principes d'établissement du réseau. Malheureusement, la méthodologie nécessaire à une telle évaluation n'est pas encore disponible, du fait du manque de modèles mathématiques aboutis. D'autre part, en général, l'existence de voies alternatives augmente la complexité et la robustesse des réseaux. Les relations entre la topologie et la robustesse de réseaux biochimiques de grande taille ont récemment été étudiées intensivement. Dans les réseaux de petite taille, tandis que le feedback est important pour l'adaptation et la robustesse, la corrélation quantitative entre la topologie et la redondance des voies des petits réseaux, et leur robustesse, reste inconnue.

Dans cette thèse, il a été montré que l'apoptose des cellules épithéliales des glandes mammaires bovines est induite lorsque la fibronectine, un composant majeur de la MEC, est dégradée par la sur-expression de tPA via la privation des attachements et

les effets des fragments de la fibronectine. Ensuite, un modèle mathématique de l'activation par le RFCE de la cascade MAPK a été étudié, et il a été trouvé que la voie Shc-dépendante est redondante et déterminante. L'hypothèse a été faite que la voie Shc-dépendante est importante pour que le RFCE puisse être en compétition avec les autres récepteurs, qui ont besoin du Shc pour transcrire les signaux ; cette voie n'a pas pour but d'augmenter la robustesse de la cascade RFCE. Enfin, concernant l'importance générale des voies alternatives dans la topologie et la robustesse du réseau, plusieurs concepts ont été proposés pour décomposer et caractériser quantitativement les réseaux. Nous démontrons que le score *pathnet* donne une meilleure évaluation de la robustesse que la connectivité moléculaire.

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Dr. Mustafa and Dr. Zadworny from McGill University provided me great help during the preparation of the tPA project. Dr. Wade from McGill University made it possible for me to use the departmental server at the beginning of my research on computational biology. Babara Stewart from McGill University has been a great assistance of my graduate study in McGill. The computer science teachers in John Abbott College, Vanier College and Dawson College, provided me great courses on Web/Programming, Database and Networking. Drs Ezhou Long and Jeiwei Lee, helped me get familiar with the lab environment when I was starting my lab work. Stephanie Portet and Farah Juma from Mount Sinai Hospital helped me with the French version of the abstract.

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Last but not least, I am grateful to my hard-working wife and lovely son and daughter.

CONTRIBUTIONS TO KNOWLEDGE

1. It was demonstrated that bovine mammary gland epithelial cells carrying and expressing constructs of active tPA showed a significantly higher rate of apoptosis. This was caused by direct degradation of one ECM component, fibronectin, which is expressed by the cells and secreted in the culture media. This is the first time to reveal the apoptosis induction by tPA degradation of fibronectin.

2. A protocol for assessment of relative contribution of alternative pathways in the cell signal transduction cascades was proposed, using an existing biochemical simulator, GEPASI, published previously by another group. This is the first effort to do such assessment based on the flux of individual reactions in the cascade.

3. With the protocol mentioned in item 2, the Shc-dependent pathway during the MAPK activation by EGFR was revealed to be both redundant and dominant. Its dominance was not reported before as either experimental or computational results. Also, the hypothesis proposed in this thesis, that its dominance is important for the competence of this signaling cascade, is novel.

4. To decompose the cell signaling networks, the concepts of pathnet and pathwome were proposed. These concepts, together with the quantities pathway score (PWS), pathnet score (PNS) and pathwome size, also proposed in this thesis, make it possible to quantitatively assess the network.

5. With the concepts and quantities mentioned in item 4, it was demonstrated that network robustness could be quantitatively represented as PNS, and it is a more accurate assessment for robustness than molecular connectivity.

FORMAT OF THESIS

This thesis has been written in the format of manuscripts submitted to scientific journals in accordance with the "**Guidelines for Thesis Preparation, C: Manuscript-based Thesis**", which states,

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. *a table of contents;*
2. *a brief abstract in both English and French;*
3. *an introduction which clearly states the rational and objectives of the research;*
4. *a comprehensive review of the literature (in addition to that covered in the introduction to each paper);*

5. *a final conclusion and summary;*

6. *a thorough bibliography;*

7. *Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.*

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

CONTRIBUTIONS OF AUTHORS

Xin Zhao 1) The work described in chapter 2 was supported by the grants to Dr. Xin Zhao; 2) Proposal of the possibility that ECM degradation by tPA could be responsible for the cell loss via apoptosis during the cell cultures; 3) Involved in the discussion with Yunchen Gong on the project and heavily involved in the editing the manuscripts of all the chapters.

Yunchen Gong 1) Implementing of the experiments described in chapter 2; 2) Proposal of the protocol for assessment of the alternative pathways in cell signaling cascades; 3) Implementation of the assessment of Shc-dependent and -independent pathways contributions to MAPK activation by EGFR, which resulted in revealing of the dominance of the Shc-dependent pathway, and the consequent hypothesis that this dominance is important for the competence of EGFR cascade; 4) Proposal of the concepts pathnet, pathwome, pathway score and pathnet score, and the protocol for assessing the network robustness, using these concepts; 5) Construction of a random artificial pathnet space, implementation of above mentioned protocol on this space, with the results that pathwome size and pathnet score are linearly correlated, the latter is a better index for robustness than the molecular connectivity.

C. Karatzas and A. Lazaris-Karatzus **from Nexia Biotechnologies Inc .** 1) Preparation of the active and mutated tPA gene constructs; 2) Preparation of cell lines transformed and continuously expressing active or inactive tPA.

TABLE OF CONTENT

LIST OF ABBREVIATION

Abl	abelson murine leukemia viral oncogene homolog
Akt	v-akt murine thymoma viral oncogene homolog
AP1	activator protein 1
ATF	activating transcription factor
ATF	amino-terminal fragment
Bad	Bcl2 antagonist of cell death
Bax	Bcl2-associated X protein
Bcl-2	B-cell lymphoma
BH3	Bcl-2 homology region 3
Bmf	Bcl2-modifying factor
BSA	bovine serum albumin
C3G	Crk SH3-binding GEF
cAMP	cyclic adenosine mono phosphoate
Cas	CrK-associated substrate
Cbl	Cas-br-M murine ecotropic retroviral transforming sequence homolog
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
c-Flip	flice inhibitory protein
c-Fos	v-fos fbj murine osteosarcoma viral oncogene homolog
CH	calponin homology domain
ILKBP	ILK binding protein
c-Jun	v-jun avian sarcoma virus 17 oncogene
Cln	ceroid lipofuscinosis, neuronal
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
CREB	cAMP-responsive element-binding protein
Crk	v-crk avian sarcoma virus ct10 oncogene homolog
CS-1	connecting segment 1
CS-5	connecting segment 5
Dab	disabled, drosophila, homolog of
DAG	Diacylglycerol
DMEM	Dulbecco's Modification of Eagle's Medium
Dok-R	(Dok: docking protein)
DRAL	downregulated in rhabdomyosarcoma lim protein
DT	diphtheria toxin
EAST	EGFR-associated protein with SH3 and TAM domains
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Elk	Ets-like transcription factor
Eps-8	epidermal growth factor receptor pathway substrate 8
ErbB	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
Erk	extracellular signal regulated kinase
FAK	focal adhesion kinase
Fas	apoptosis antigen 1 (apt1),fas antigen, surface antigen apo1 (apo1), cd95

FF	fibronectin fragments
FGF	Fibroblast growth factor
FKHR	forkhead in rhabdomyosarcoma
FN	Fibronectin
FP	Fibrinogen peptide
Fyn	fyn oncogene related to src, fgr, yes
GAP	GTPase-activating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GEPASI	a biochemical simulator
GF	growth factor
GFR	growth factor receptor
Grb2	growth-factor-receptor-bound protein2
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HBSS	Hanks Balanced Salt Solution
HGF	hepatocyte growth factor
HMG	high mobility group
HRP	Horseradish Peroxidase
HTML	hypertext markup language
IAP	inhibitor of apoptosis
IBP	integrin-binding protein
ICAP	integrin-cytoplasmic-domain-associated protein
IDAPS	Ile183-Asp184-Ala185-Pro186-Ser187 ?
IGF	insulin-like growth factor
ILK	integrin-linked kinase
ILKAP	integrin-linked kinase-associated serine/threonine phosphatase
INOS	inducible NOS
IP3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
LEF	lymphocyte enhancing factor
LH	containing luteinizing hormone
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MEC	mammary epithelial cell
Mek	MAP kinase/ERK kinase
MEKK	MEK kinase
MIBP	muscle-specific β1-integrin-binding protein
MLC	myosin light chain
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
Mnk	MAPK interacing protein kinase
MSK	mitogen- and stress-activated protein kinase
Nck	a novel cytoplasmic protein
NF	nuclear factor

NGF ..... nerve growth factor
p53 ..... tumor protein p53
PA ..... plasminogen activator
PAGE ..... Polyacrylamide Gel Electrophoresis
PAI ..... plasminogen activator inhibitor
PAK ..... p21-activated protein kinase
PDK ..... phosphoinositide-dependent kinase/PI3K-dependent kinase
PHP ..... Preprocessor Hypertext Protocol
PI3K ..... phosphoinositide 3-OH kinase
PIP3 ..... phosphatidylinositol-3,4,5-trisphosphate
PKB ..... protein kinase B
PKC ..... protein kinase C
PLA ..... phospholipase A
PLC ..... phospholipase C
PLD ..... phospholipase D
PNS ..... pathnet score
PTB-1B ..... protein tyrosine phosphatase 1B
PtdIns ..... phosphatidylinositol
PWD ..... pathway distance
PWS ..... pathway score
Raf1 ..... v-raf-1 murine leukemia viral oncogene homolog 1
Ral ..... v-ral simian leukemia viral oncogene homolog
Rap ..... ras-related protein
Ras ..... Rous sarcoma virus
RGD ..... Arg-Gly-Asp
Rho ..... Ras homologous
Rsk ..... ribosomal S6 kinase p90rsk
p70S6K ..... p70 ribosomal S6 kinase
SAP-1 ..... SRF accessory protein 1/stress-activated protein 1
SDS ..... Sodium Dodecyl Sulfate
SFPF ..... serum-free and protein-free
SH2 ..... Src homology 2
Shc SH2-domain-containing α2-collagen-related
SHIP ..... SH2-containing inositol phosphatase
SHP ..... SH-domain-containing protein tyrosine phosphatase
SOCS ..... Suppressor of cytokine signaling
SOS ..... son of sevenless
Src ..... v-src avian sarcoma (schmidt-ruppin a-2) viral oncogene
SRF ..... serum-response factor
STAT ..... signal transducer and activator of transcription
TF ..... transcription factor
TGF ..... transforming growth factor
TNF ..... tumor necrosis factor
tPA ..... tissue-type plasminogen activator
uPA ..... urokinase-type plasminogen activator
uPAR ..... urokinase-type plasminogen activator receptor

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CHAPTER I. GENERAL INTRODUCTION AND REVIEW OF LITERATURE

GENERAL INTRODUCTION

Tissue plasminogen activator (tPA) is a protease and catalyzes activation of plasminogen into plasmin. In the transgenic goats carrying active tPA gene and overexpressing in the milk the active tPA product, milk production was declined earlier and quicker than non-transgenic goats (Nexia Biotechnologies Inc., unpublished result). A high concentration of plasminogen exists in the milk. The activated product, plasmin, degrades a wide range of the protein substrates, including extracellular matrix (ECM) component fibronectin. Most of mammalian cells survive only when they attach to the proper ECM, and most of the ECM components are able to bind to different members of the cell surface protein family, the integrins. Degradation of ECM components deprives the cells of the media to attach on, thus inducing cell death. Milk reduction in the tPA-transgenic animals could be induced by two means: a direct effect of increased plasmin on death of the mammary epithelial cells (MECs) or an indirect effect on degradation of fibronectin by tPA and subsequent effects of fibronectin fragments on MECs. However, there is no report that the bovine mammary gland epithelial cell death is induced by tPA in the latter way.

ECM is one type of extracelluar environment. Living cells are always sensing the changes in extracellular components, soluble such as epidermal growth factor (EGF), or insoluble including ECM. Responses of the cells to these changes are fulfilled with the transmembrane sensing molecules, the receptors, which are exposed to extracelluar

environment. Upon activated (usually bound by extracellular ligands), these receptors initiate signal transduction pathways and activate or inhibit activities of intracellular molecules and expression of some specific genes, consequently changing the cellular function. Activation of one cell surface receptor, such as one specific integrin, usually induces more than one signal transduction pathways. These pathways could be parallel, cross-talking and affecting the same or a different set of intracellular molecules. Thus, the overall of these pathways is also called the signal cascade. This also holds true for EGF receptor (EGFR) (and other growth factor receptors). For example, during EGFR activation of mitogen activated protein kinase (MAPK), at least two pathways are involved, the Shc-dependent and the Shc-independent pathways. Furthermore, the pathways induced by integrins and EGFR have cross-talk, and this may be the mechanism of cell attachment as a prerequisite for EGFR to stimulate cell proliferation. A detailed understanding of this mechanism of the co-operation between integrins and EGFR needs a complete model for the signal cascades of the two types of receptors and their cross-talks. Unfortunately, such a model is currently unavailable. However, a few mathematical models have been proposed for EGFR cascade. Among them, a relatively mature one was published in 2002 (Schoeberl et al., 2002). This model can be the basis for future studies of the co-operative mechanisms between EGFR and other receptors. It is also a test-bed to study the features of the cell signalling cascade itself, such as the relative contributions of the alternative pathways and the relationship between existence of the alternative pathways and the robustness (resistence of the system to perturbation) of the signalling cascade. As one of the important characteristics of biochemical networks, robustness has been studied in both large and small scales. Interesting results

include the scale-free structure and the feedback loops as important sources of robustness in large- scale and small-scale networks, respectively. However, the relationship between small network topological structure and the network robustness has not received much attention.

REVIEW OF LITERATURE

1. The Plasmin/Plasminogen Activators (PAs) System

1.1. Components of the system

The plasmin/plasminogen activators (PAs) system is composed of pasminogen, plasmin, plasminogen activators, PA inhibitors (PAIs) and uPA receptor (uPAR).

The single-chain bovine plasminogen is able to efficiently bind fibrin in the blood system (Andreasen et al., 1990; Heegard et al., 1994a) and casein in milk (Heggard et al., 1994a; White et al., 1995). Upon cleavage of plasminogen by PAs, plasmin is formed (Schaller et al., 1985), which consists of two chains held together by disulfide bonds. Plasmin has a trypsin-like specificity (Politis et al., 1989a; Politis, 1989; Politis et al., 1989b).

There exist inhibitors of plasmin in vivo. The most important ones are α2-antiplasmin and α2-macroglobulin, two normal components of plasma (Dano et al., 1985; Saksela and Rifkin, 1988). α2-Macroglobulin is a non-specific inhibitor for almost all known proteases (Dano et al., 1985; Saksela and Rifkin, 1988). In contrast, α2-antiplasmin is a specific plasmin inhibitor and reacts rapidly to inactivate plasmin.

PA exists in two forms in mammals: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Dano et al., 1985; Saksela and Rifkin, 1988). Both enzymes catalyze the cleavage of the same peptide bond in plasminogen, but they are antigenically distinct and are products of different genes. The EGF-like domain and the kringle structure make up the amino-terminal fragment (ATF) of uPA (Dano et al., 1985; Saksela and Rifkin, 1988), which mediates binding of uPA to a specific receptor present in numerous cell types. Unlike uPA, tPA has a high affinity for fibrin which is attributed to its finger domain and two kringle structures (Dano et al., 1985; Saksela and Rifkin, 1988). Fibrin enhances tPA (but not uPA) activity by decreasing the Michaelis constant of tPA for plasminogen (Zamarron et al., 1984).

The activity of PAs can be controlled by specific PAIs (Andreasen et al., 1990). Both PAI-1 and PAI-2 form equimolar complexes with uPA and tPA (Dano et al., 1985; Saksela and Rifkin, 1988; Andreasen et al., 1990). PAI-3 was isolated from urine and inhibits both uPA and tPA, but more slowly than PAI-1 and PAI-2 (Dano et al., 1985).

uPAR is a plasma membrane protein which binds uPA with high affinity (Andreasen et al., 1991; Ellis and Dano, 1991; Kariko et al., 1993; Kratzschmar et al., 1993; Nguyen et al., 1992; Nusrat and Chapmen, 1991; Reuning et al., 1993; Stopelli et al., 1986; Saksela and Rifkin, 1988). The primary function of uPAR is to localize plasminogen activation at the cell surface by enhancing the activity of bound uPA (Ellis and Dano, 1991). The cell membrane-associated plasmin is less sensitive to α2-antiplasmin than in solution (Ploug et al., 1991). The receptor-bound uPA can complex with PAI-1 and be internalized, the uPA and PAI-1 are then degraded, and the uPAR is recycled to cell membrane (Ellis and Dano, 1991; Nguyen et al., 1992; Nusrat and Chapmen, 1991). This

provides a mechanism for changing the proteolytic activity localized on the cell membrane.

1.2. Plasmin/PA system in bovine mammary epithelial cells and milk

Several bovine mammary epithelial cell lines have been established: MAC-T and its subclones MACT-UV1 and MACT-UV2, and BME-UV. uPA was detected in the medium of MAC-T cells (Heegard et al., 1994b), and uPA mRNA was detected in BME-UV cells (Politis, 1996). The majority of uPA was bound to the cell membrane (Politis, 1996). Low concentrations of PAI-1 were detected in the MACT-UV1 and MACT-UV2 (Heegard et al., 1994b), and BME-UV cell cultures (Heegard et al., 1994b). PAI-1 formed complexes with uPA (Heegard et al., 1994b). uPAR mRNA was detected in BME-UV cells (Heegard et al., 1994b). PAs was found in milk and convert plasminogen to plasmin. PA in milk was mainly associated with the casein fraction, and the major type was tPA (Heegard et al., 1994a).

1.3. Enzymatic activities of plasmin/PAs

1.3.1. plasmin activities

Plasmin participates in a plethora of biological phenomena, including thrombolysis, issue remodeling, and cell migration (Dano et al., 1985; Saksela and Rifkin, 1988). The primary effect of plasmin in tissue remodeling events is the breakdown of matrix and basement membrane proteins, such as laminin (Tran-Thang et al., 1994), fibronectin (Salonen et al., 1985) and type V collagen (Liotta et al., 1981).

Plasmin also activates other degradative enzymes such as the matrix metalloproteinases (MMPs) (Murphy et al., 1991). The MMP family includes three major enzyme categories: collagenases, including MMP-1, MMP-8 and MMP-13; gelatinase, including MMP-2 and MMP-9; and stromelysins, including MMP-3, MMP-10 and MMP-7 (for reviews see Murphy et al., 1991; Werb, 1997; Cox et al., 1999). MMPs are secreted as inactive pro-MMPs in most cases (Nagase and Woessner, 1999). Plasmin was demonstrated to activate collagenase and stromelysin (He et al., 1989; Berton et al., 2000; Pins et al., 2000; Murdoch, 1998) and gelatinase (Wong et al., 1992).

Plasmin is also involved in releasing of several cytokines, including tumor necrosis factor α (TNFα) (Murdoch et al., 1999), transforming growth factor β (TGF-β) (Lyons et al., 1988; Miyazono and Heldin, 1989; Brown et al., 1990; Lyons et al., 1990; Taipale et al., 1992; Falcone et al., 1993) and basic Fibroblast growth factor (bFGF) (Saksela and Rifkin, 1990; Falcone et al., 1993).

Plasmin in milk hydrolyses caseins (including αs-casein, β-casein and κ-casein) (Politis, 1989; Berton et al., 2000; Politis et al., 1989a, 1989b; Gordon et al., 1972; Dumas et al., 1972; Eigel, 1977; Kaminogawa et al., 1972; Eigel et al., 1977; Eigel et al., 1979; Aimutis and Eigel, 1982; Aslam and Hurley, 1997), lactoferrin (Aslam and Hurley, 1997) and milk fat globule membrane (MFGM) (Hofmann et al., 1979). Elevated plasmin activity during mammary involution may be primarily responsible for the concurrent hydrolysis of milk proteins in mammary secretions (Aslam and Hurley, 1997).

1.3.2. tPA and uPA activities

Besides activating plasminogen, tPA was also reported to degrade fibronectin and fibrinogen. Plasminogen-independent ECM degradation due to tPA activity has been observed during the process of neurite outgrowth (Sumi et al., 1992). Direct degradation of purified fibronectin by tPA was also observed (Marchina and Barlati, 1996). tPA has also a thrombin-like effect on fibrinogen and releases fibrinopeptides A and B (FPA, FPB) (Weitz et al., 1988).

In comparison with tPA, uPA has a broader range of substrates, including fibronectin (Quigley et al., 1987; Gold et al., 1989; Marchina and Barlati, 1996), MMP-2 (Keski-Oja and Vaheri, 1982; Keski-Oja et al., 1992), fibrinogen (Weitz and Leslie, 1990; Sumi et al., 1983; Rijken et al., 1986; Naldini et al., 1991), pro-HGF (Naldini et al., 1992; Naldini et al., 1995) and diphtheria toxin (DT) (Cieplak et al., 1988).

1.3.3. plasmin/PAs during mammary gland involution

After parturition, milk production in dairy cows continues to increase for about 3 to 6 weeks, and gradually declines after the peak is attained (Bath et al., 1985). During early lactation, mammary cell numbers increase continuously, thereafter the rate of cell loss exceeds the rate of cell division (Bath et al., 1985). Thus the udder contains substantially fewer cells, and produces less milk at the end of lactation than at the beginning.

Plasmin is the major protease in bovine milk (Eigel 1979; Fox, 1982). At late lactation plasmin activity was high (Politis et al., 1989a; Politis et al., 1989b), and during the early dry period it increased in mammary secretions of cows (Athie et al., 1997). It

has been suggested that the plasminogen and plasmin system is involved in involution of bovine mammary tissue (Politis et al., 1989a), as had been observed for the mouse (Lund et al., 1996; Ossowski et al., 1979). Increased milk plasmin is the result of increased activation of plasminogen as lactation progresses (Politis et al., 1989a) and can potentially cause protein degradation (Fox, 1982).

PA activity was higher in late lactation than in early or mid lactation (Gilmore et al., 1995). In mice, uPA activity was correlated with initiation of mammary involution (Ossowski et al., 1979). tPA mRNA was detected three days after involution (Talhouk et al., 1992), and uPA mRNA was detected after day two of weaning in mice (Strange et al., 1992). In bovine, PA activities were higher on day 7, 14, and 21 of involution than on day 7 post calving (Aslam and Hurley, 1997). The co-occurrence of cell loss and high PA/plasmin activities during late lactation and involution implies the correlation between them.

1.3.4. plasmin/PAs and apoptosis

It was recently reported that tPA alone at very high concentrations triggers neuronal apoptosis in vitro (Flavin et al., 2000). However, the pathway(s) for this effect of tPA is unknown.

In sheep, a model is proposed of temporal interactions of gonadotropin, ovarian cell types, PA/plasmin, collagenases, and TNFα in the breakdown of the apical follicular wall during ovulation. The following activation or degradation events occur in a sequence: LH → PA → plasmin → collagenase and TNFα; collagenase → fibril network

and baement membrane; TNFα → apoptosis and collagenolysis; finally resulting in follicular rupture (Murdoch et al., 1999).

In bovine milk, TNFα levels increased steadily after drying-off onset, reflecting its role in remodeling/apoptosis during mammary gland involution (Rewinski and Yang, 1994). On the other hand, the plasmin/PA system could induce MEC apoptosis via fibronectin degradation. Mammary fibronectin (FN) protein and its fragmentation levels were observed to peak between 4 and 6 days post-weaning, coincident with the peak in epithelial cell death (Schedin et al., 2000). FN fragmentation was also associated with apoptosis of MECs deprived of lactogenic hormones in vitro (Schedin et al., 2000). Furthermore, there is a dose- and time-dependent decrease in cell number with the FN fragments (FN120) treatment (Schedin et al., 2000). The molecular mechanism resposible for the cell death from fibronectin deprivation is not known. However, it was recently reported that MAPK pathway signaling was essential for ECM determined MEC survival, and inhibition of the ECM-induced MAPK pathway led to apoptosis in MECs (Finlay et al., 2000). Alternatively, fragments of FN may be able to induce apoptosis directly without binding to integrin (Buckley et al., 1999). The fibronectin/integrin pathways will be reviewed in the next section (1.2.2).

Putting them together, the possible paths of apoptosis induced by plasmin/PAs are illustrated in Figure 1.1.

2. Fibronectin and Integrin Signalling Cascades

Fibronectin and integrin signalling cascades have been well reviewed in literatures (Zamir and Geiger, 2001; Miranti and Brugge, 2002; Danen and Yamada,

2001; Schwartz and Ginsberg, 2002; Coppolino and Dedhar, 2000; Damsky and Llic, 2002; Brown, 2002; Guan, 1997a; Wu and Dedhar, 2001; Aplin et al.., 1998; Schwartz and Assoian, 2001; Liu et al.., 2000; Turner, 2000; Guan, 1997b; Hanahan and Weinberg, 2000; Flier and Sonnenberg, 2001; Plow et al.., 2000). The major steps involved in these cascades are briefly introduced in this section.

2.1. Fibronectin as a ligand for integrins

Fibronectin acts as a ligand for the following integrins: $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 4\beta 7$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$ and $\alpha IIb\beta 3$ (for review, see Plow et al., 2000). The CS-1, CS-5 and IDAPS domains of fibronectin mediate its interaction with $\alpha 4\beta 1$ integrin. CS-1 domain also mediates its interaction with $\alpha 4\beta 7$ integrin. Other integrins interact with fibronectin through its RGD (Arg-Gly-Asp) sequence.

2.2. Integrin cascade

The cytoplasmic proteins associating with integrin cytoplasmic domains have been listed elsewhere (Flier and Sonnenberg, 2001). They were categorized as five types: cytoskeletal-associated proteins, adaptor and signalling proteins, protein kinases, chaperone/calcium binding proteins and nuclear/cytoplasmic proteins, and transcription (co-)factors. Currently, there are 34 different cytosolic proteins in total, which have been demonstrated to be capable of binding with integrin. These interactions reflect the variety of cell functions the integrins are possibly involved in.

The main pathways for downstream of integrins have been well recognized and reviewed. Basically, integrins affect metabolism, gene expression, cell migration,

proliferation, survival and cytoskeletal changes via interaction with focal adhesion kinase (FAK) (Guan, 1997a, 1997b). They affect actin cytoskeleton and signalling complexes via integrin-linked kinase (ILK) (Wu and Dedhar, 2001) and proliferation and gene expression via paxillin (Turner, 2000). A network of integrin signalling cascades is schemed in Figure 1.2, which is assembled with the information from several review papers listed above. The potential alternative pathways for integrins to activate MAPK will be illustrated together with EGFR-MAPK pathways in a later section.

Two major pathways of anchorage regulation of apoptosis have been described (Aplin et al.. 1998). One involves FAK, PI-3K, and the Akt kinase. The other involves Bcl-2 and Caspases (see Figure 1.2). Two key control points are regulating pro- or anti-apoptotic proteins of the Bcl2 family (e.g. Bcl2 and Bax) and regulating activation of caspases, a family of pro-apoptotic proteases. The third control point could be regulation of death ligands (see Figure 1.3A). Several proofs for these control points are listed below. Gilmore et al. (2000) showed that detachment of mammary epithelial cells triggered rapid translocation of pro-apoptotic Bax from the cytoplasm to mitochondria and exposure of its death-promoting BH3 domain. This could be suppressed by activated PI3K and Src and induced in adherent cells by expression of dominant-negative FAK. Cytochrome c release from mitochondria and cell death occur after a long lag time. In endothelial cells, matrix adhesion suppresses Fas-mediated apoptosis by a mechanism requiring both reduced Fas expression and enhanced expression of c-Flip, an antagonist of caspase-8 (Aoudjit and Vuori, 2001). Also in endothelial cells, Stupack et al. (2001) has shown that the presence of excess unligated $\alpha V\beta 3$ integrin in otherwise adherent cells could recruit caspasse-8 to the membrane to be activated. Production of excessive

(unligated) α5β1 integrin has also been suggested as a mechanism by which the p16INK4a tumor suppressor might promote apoptosis and suppress tumor growth (Plath et al., 2000). The idea of Bmf, a Bcl-2 member, as an intracellular sensor of the cytoskeleton and the state of ECM attachment, is illustrated in Figure 1.3B.

3. Cross Talk between Integrin and EGFR Cascades

3.1. EGFR cascade

EGFR is one of the most studied growth factor receptors. Two ligands were revealed to bind the extracellar domain of EGFR, EGF and TGFα and they bind to the EGFR in the same mode (Garrett et al., 2002; Ogiso et al., 2002). Uopn binding, the (ligand-EGFR)2 complex forms on the cell surface (Sako et al., 2000). This results in activation of the EGFR, ie the phosphorylation of its intracellular domains in its C-terminus. Consequently, acitvated EGFR can be associated with different signaling proteins, including Grb2, Shc, Nck, Crk, Dok-R, PLC-P, p120RasGAP, PTB-1B, SHP-11, Src and Abl (for review, see Jorissen et al., 2003). The pathways downstream of these proteins are different. The most extensively studied pathways are MAPK activation downstream of Grb2 and Shc. Association of Grb2 to EGFR directly, or indirectly via Shc, induces the Ras/MAPK pathway. The activated Erk1 and Erk2 in turn translocate to nuclear and phosphorylate nuclear transcription factors (Johnson and Vaillancourt, 1994). Grb2 and Shc are also involved in other pathways, such as cytoskeletal reorganization for Grb2 (Schlaepfer et al., 1999) and JNK pathway activation for Shc (Pomerance et al., 1998). Other examples of pathways downstream of EGFR activation are JAKs and

STATs pathways and phospholipid metabolism pathways. The incomplete EGFR signaling cascade is illustrated in Figure 1.4.

3.2. Integrin dependent cell attachment is required for EGFR stimulation of cell proliferation

The required attachment of most animal cells for survival and proliferation stimulated by growth factor serves as a clue for cooperation of the integrin and growth factor pathways. As a matter of fact, normal signalling induced by growth factor does not occur if the cells are not addherent to the ECM through integrins (Schwartz and Ginsberg, 2002). The molecular basis for this cooperation could be that integrin and EGFR signaling have multiple convergent points. For example, these convergent points are recruitment of RasGAP and downregulation of Ras activity, activation of Raf, activation of Mek and nuclear translocation of Erk in the MAPK pathway (Schwartz and Ginsberg, 2002). After comparing Figure 1.2 and Figure 1.4, several possible intersection points of the two cascades are illustrated in Figure 1.5A. A general discussion of the potential ways of co-operation between cell surface receptors has been made (Miranti and Brugge, 2002) and modified here in Figure 1.5B.

3.3. Computational models of EGFR signalling

As in the case of the electronic circuits, the wiring of the electronic parts, the transsistors, is the basis for analysis of input-output relationship, but this analysis is not possible without the parameters of the components. The networking of extracellular signalling, as illustrated in Figure 1.2 and Figure 1.4, has been sketched with

accumulation of the known signalling molecules and the interactions between them. With more and more biochemical parameters of the molecular interactions measured, it is possible to construct quantitative models for biochemical processes, including cell signal transduction. Such models are important for explanation and understanding of the biochemical or cellular phenomenon, providing of testable predictions, and engineering of the biochemical networks. However, available models are currently very limited.

In the big family of cell surface receptors, EGFR is probably the best-understood system. Its quantitative modelling, coupled with the parallel development of experimental technologies for the testing and refinement of the models, has played an important role during its elucidation (Wiley et al., 2003). The early modelling for EGFR system focused on EGF binding and internalization of ligand-bound or -unbound EGFR and resulted in the enzymology-based models (Wiley and Cunningham, 1982; Wiley et al., 2003). Other models for EGFR system included the interactions between EGFR-family members (Worthylake et al., 1999; Waterman et al., 1998), autocrine signalling (Shvartsman et al., 2001), and transactivation and positive feedback (Gechtman et al., 1999; Dong and Wiley, 2000; for review, see Wiley et al., 2003). These models of EGFR were relatively local in that they did not cover a complete signalling pathway, such as the MAPK activation pathway, in kinetic details.

The induction of MAPK pathways as the major consequence of the EGFR activation was recently modeled quantitatively as a whole. The EGFR-MAPK model established by Brightman and Fell in 2000 was aimed to interpret the quantitative differences in EGF and nerve growth factor (NGF) signalling in PC12 cells as the differential feedback regulation of the MAPK cascade. This was achieved by the

construction of the basic model and then the variation of the values of kinetic parameters governing a number of signalling events (Brightman and Fell, 2000). The possibility proposed in that study was that EGF and NGF induced the phosphorylation of SOS by different kinases at distinct phosphorylation sites, resulting in different effects on Shc-Grb2-SOS complex regulation (Brightman and Fell, 2000). A more recent EGFR-MAPK model was published in 2002 (Schoeberl et al., 2002). This model was focused on the comparison of the signalling induced by cell surface and internalized EGFRs and deemed to be the most comprehensive by including the fullest range of dynamic processes and making several interesting, significant and experimentally validated predictions (Wiley et al., 2003). The common result from both models is that the EGFR activation of MAPK is transient, although they differed in several modelling details. For example, the Schoeberl model adopted more intermediate complexes than the Brightman model. Another difference is that both Shc-dependent and –independent pathways were included in the Schoeberl model but not in the Brightman model, which took into account the Shc-dependent pathway only. Inclusion of the alternative pathways makes the Schoeberl model possible to be used to assess the relative contributions of the distinct pathways to the final signalling. The results of this assessment could answer questions such as if an individual pathway is redundant or all the alternative pathways are needed for the final signalling. Furthermore, the biological importance of the individual pathway could be inferred. However, this was not addressed in the Schoeberl model.

4. Robustness: A Feature of Cell Signalling Networks

Besides some common signalling molecules (such as Src, Grb and Shc etc.) utilized in both integrin and EGFR cascades (see Figure 1.2 and Figure 1.4), their topological structures also have a feature in common, that is, both contain multi-pathways. As a matter of fact, this feature also characterizes most of the currently established signalling transduction cascades. Furthermore, more signalling cascades are being revealed to possess multi pathways for signalling transducing. The question as to why alternative (and possibly redundant) pathways exist for the same signalling has not been thoroughly answered yet. One possibility is that all pathways are needed for maximum amplification of the final signal. Before the complete mathematical models are established and the importance of the alternative pathways could be inferred via quantitative analysis, it has been believed that multipathways are one of the fundamentals for the robustness, ie the resistance to random genetic mutations, of the biological systems. In another word, the alternative pathways are backup of the dominant pathway (if any) and will compensate the functional loss of the main pathway caused by random gene mutation.

The term robustness could mean the system's ability to adapt to the environment (adaptation), to tolerate damage by preventing functional degradation, or to operate normally under a range of parameters (parameter insensitivity) in different contexts (reviewed by Kitano, 2002). Robustness may be depicted as the system's ability to buffer any kinds of perturbation exerted to the system (from the environment), or arising from the system itself (such as the deletion of a node in biochemical network resulting from the mutation of the coding gene). Perturbations could be roughly categorized into two

classes, quantitative perturbation which changes the values of the system parameters (such as reaction rate and component concentration), and qualitative perturbation which changes the structure of the system (for example, removal of a node from the protein network due to gene mutation). However, in some cases quantitative perturbation can also be seen as qualitative, for example, when parameters or concentration is changed into zero. As early as 30 years ago, it was hypothesized that robustness was an essential property of some genetic networks, in which variation of component quantities did not change the functioning (Savageau, 1971; Savageau, 1975). Recent studies, both theoretical (Yi et al., 2000; Barkai and Leibler 1997) and experimental (Alon et al., 1999; Eldar et al., 2002), demonstrated that intracellular networks exhibited robustness under variation of biochemical parameters. It is now believed that robustness has become a fundamental concept characterizing the dynamical stability of biological systems (Aldana and Cluzel, 2003).

Robustness could be attained in various ways (for review see Kitano, 2002). First, robustness is attained via feedback and feed-forward control, as in the case of bacterial chemotaxis (Yi et al., 2000). Second, a structurally stable network makes the system converge to the desired state in the presence of various noises, as illustrated with the circuit for lambda phage fate decision (McAdams and Shapiro, 1995). Third, redundancy plays an important role in attaining robustness of the system, for example, Cln1, Cln2 and Cln3 have redundant functions in activating Cdc28 activation (component redundancy results in pathway redundancy). Finally, a modular design contributes to robustness, as seen in the separation of p53 pathway and metabolic pathways. This classification is arbitrary in that a network structure is believed to be the fundamental source responsible

for robustness. This is due to the fact that feedback, redundancy and module can all be reflected in the level of network structure/topology.

The correlation between the network topology and robustness has been studied on both large-scale and small-scale/simple networks. A network is usually represented by a directed or undirected graph, in which the nodes are network components (bio-molecules in the case of biochemical networks) and the edges are interactions between the nodes (material or signal flow, or simply binding/association, in the case of biochemical networks). Based on the distribution of the connectivity (the number of connections with other nodes) of all the nodes, the large-scale networks are roughly classified into two categories, one with random topology, the other with scale-free topology (possessing a power law distribution). Scale-free topology has been demonstrated to be characteristic of several real-world networks, ranging from Hypertext Markup Language (HTML) document networks and the journal article citing networks, to molecule networks (Albert et al., 2000; Barabasi and Albert, 1999; Cohen et al., 2000; Jeong et al., 2000). Such networks are robust and error tolerant (Albert et al., 2000). The dynamical robustness of scale-free biochemical networks were further confirmed by other authors (Fox and Hill, 2001; Aldana and Cluzel, 2003). In contrast, either Poisson or delta function networks were shown to be less ordered (chaotic dynamics) than scale-free network (Fox and Hill, 2001), and in networks with homogeneous random topologies, fine-tuning of their internal parameters was required to sustain stable dynamical activity (Aldana and Cluzel, 2003). In small-scale/simple biochemical networks, more local topology was assessed related to robustness. With a simple two-state model, which involved a feedback loop, it was found that in bacterial chemotaxis network, the network's connectivity was

responsible for the adaptation property and ensured that the fine-tuning of parameters was not required (Barkai and Leibler, 1997).

In the above-mentioned robust systems, for both large- and small-scale networks, alternative pathways were not taken into account as the direct source of robustness. This issue was partly addressed in a recent publication (Kitami and Nadeau, 2002). The redundancy in metabolic networks was implied to provide more genetic buffering than does gene duplication. This is based on the fact that genes with a redundant network evolved more quickly than did genes without redundant networks, but no significant difference was detected between single-copy genes and gene families (Kitami and Nadeau, 2002). It is assumed here that, an investigation of the network features based on the quantities of alternative pathways could provide new evidence for the topology-robustness relationship.

5. Objectives of This Research

As reviewed in previous sections, PA/plasmin system is correlated with cell death and mammary gland involution in vivo, when milk production is decreased. This notion is further supported by the observation that active tPA-transgenic goats experience an earlier and faster decreasing of milk production than control animals. As the fibronectin in the milk of the tPA-transgenic goats were found to be degraded more intensively (Zhao et al., unpublished results), we hypothesized that direct degradation of fibronectin by tPA induced bovine MEC death via pathways of attachment deprivation and the direct effect of fibronectin fragments. Therefore, the first objective of our study was to prove this hypothesis, using a cell culture model.

The possible dual (or more in vivo)-pathways for fibronectin degradation-induced cell death illustrates the multiple-pathway style of cellular functions. This is also true for intra-cellular signaling cascades, where multiple alternative pathways could be induced downstream by a single activation of the cell surface receptor, as reviewed previously for integrins and EGFR. A quantitative assessment of the relative contributions of the alternative pathways to a final cell signaling would be interesting and help understand the designing principles of the bio-molecular cascades/networks. However, the protocol for this assessment has not been reported up to now. Therefore, the second objective of our study was to propose such a protocol, to implement it with a published mathematical model of a well-investigated cascade, the MAPK activation by EGFR, using the biochemical simulator, GEPSI, and to infer the biological importance of the alternative pathways.

The exact reason for existence of the alternative pathways available for a single receptor to transduce signals remains unknown. A widely accepted idea is that this increases the complexity of the cell signaling cascades, consequently increases its robustness. Like other networks, the bio-molecular networks have topological structures (how the nodes are connected). While a quantitative characterization of the topological structure could help understand the designing principle of bio-molecular networks, we still lack the proper concepts to this end. Therefore, the third objective of our study was to propose several concepts and evaluate their usability in assessment of the network robustness.

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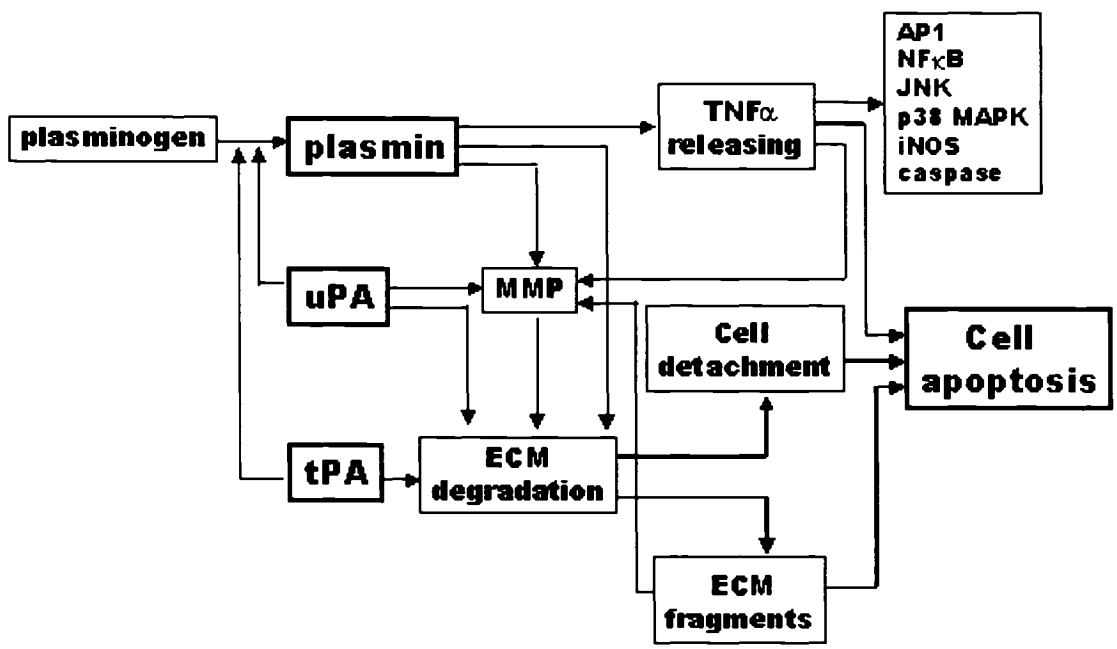


Figure 1.1. Potential pathways of apoptosis induced by plasmin/PAs (plasminogen activators).

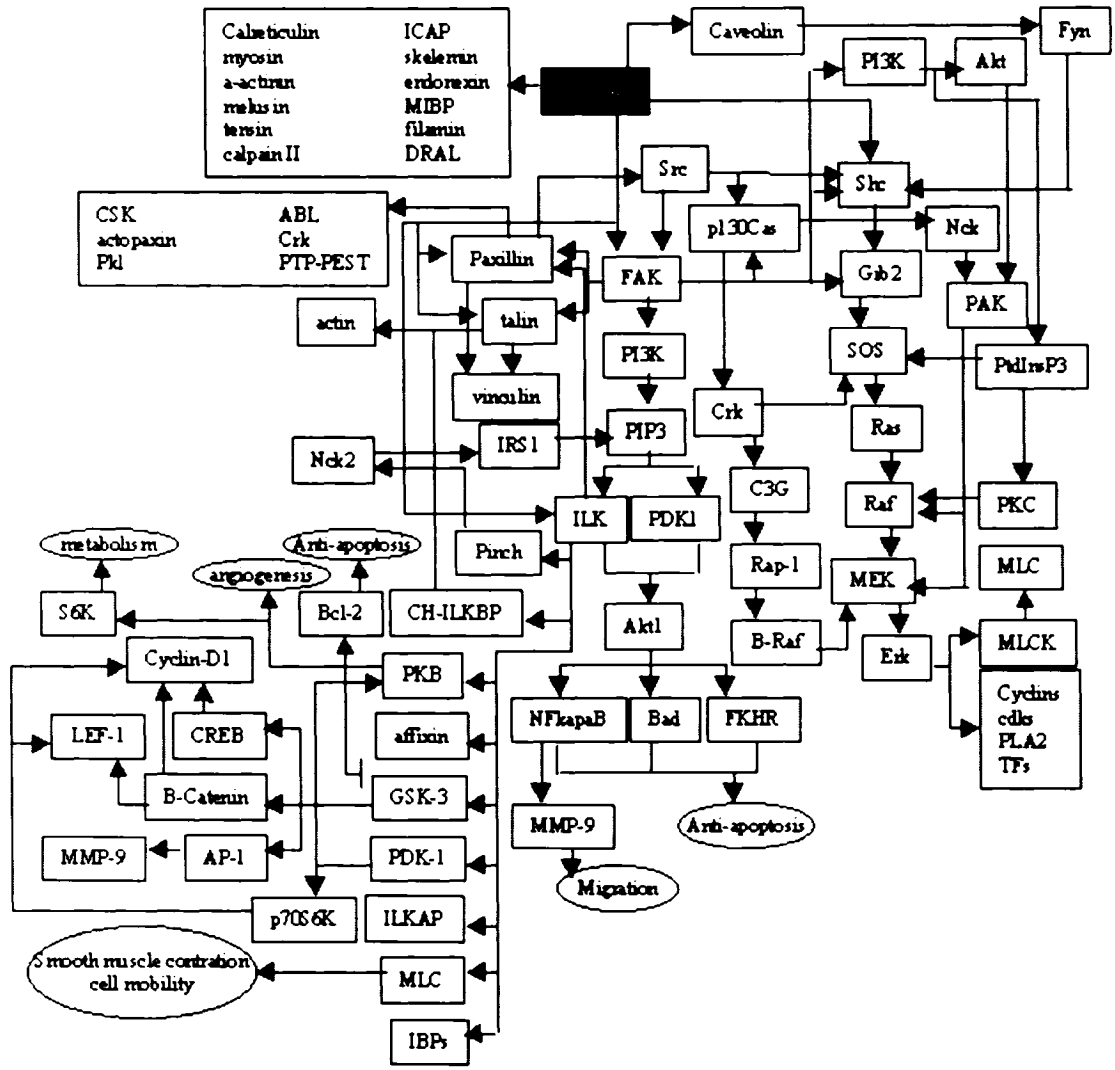
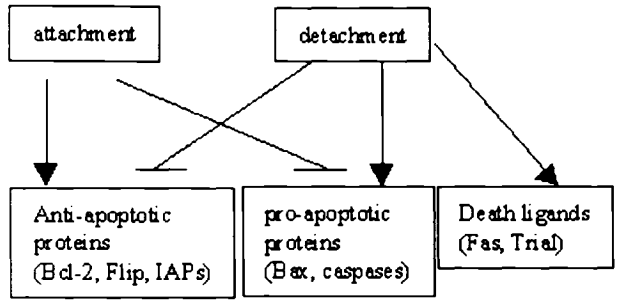


Figure 1.2. Schematic integrin signaling cascade.
Modified from Di-Poi et al., 2002; Carragher and Frame, 2002; Danen and Yamada, 2001; Wu and Dedhar, 2001; Coppolino and Dedhar, 2000; Liu et al., 2000; Turner, 2000; Yoganathan et al., 2000; Aplin et al., 1998; Guan, 1997a. Notes: One component displayed in this scheme could include more than one isoforms. This scheme does not include a complete description of the interactions/signaling of all the components appearing here. Arrows could represent direct or indirect activation, binding or other interactions (eg catalysis). Complexes formed via the interactions are not shown.

A. Effects of cell attachment status on apoptotic machinery



B. Bmf as intracellular sensors of the cytoskeleton and the state of ECM attachment

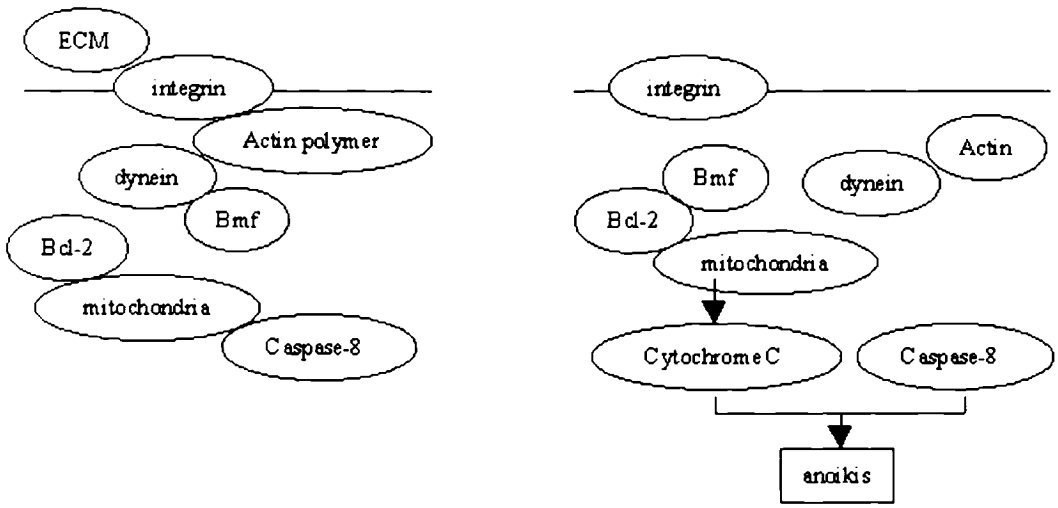


Figure 1.3. Cell attachment status and apoptosis.
Modified from Miranti and Brugge, 2002; Frisch and Screaton, 2001.

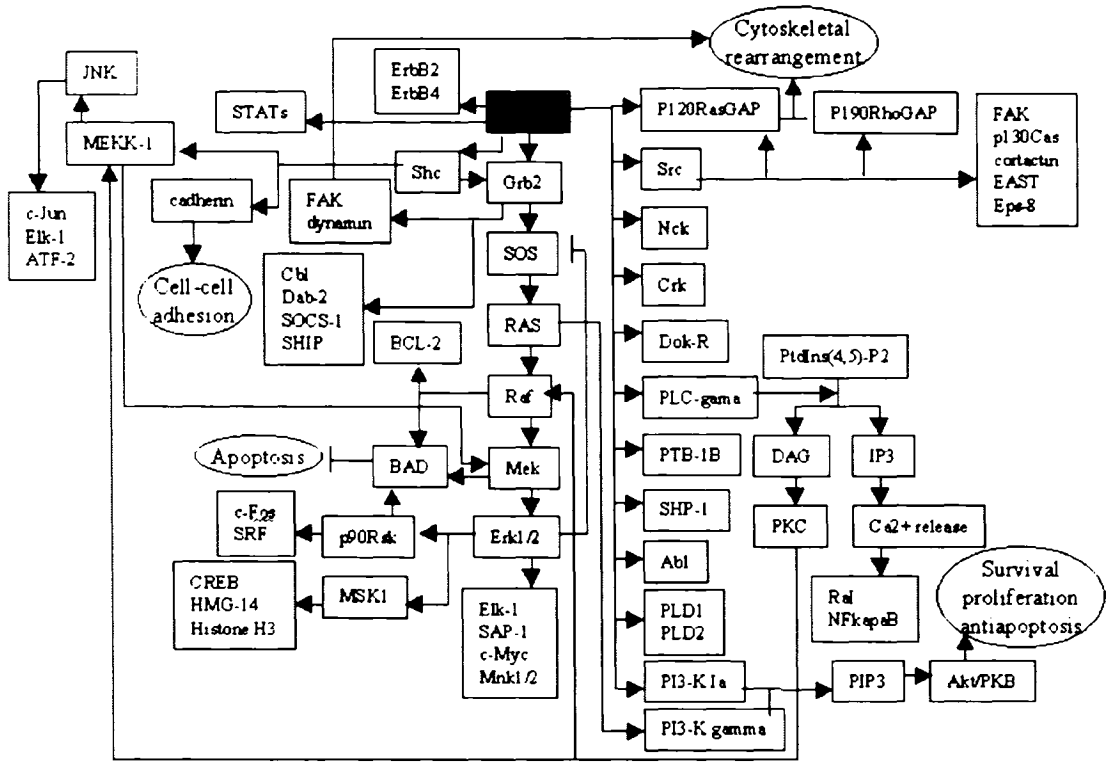


Figure 1.4. Scheme of EGFR signaling cascade.
Modified from Filardo, 2002; Franklin and McCubrey, 2000; Denhardt, 1996; Jorissen et al., 2003; Brightman and Fell, 2000; Hazzalin and Mahadevan, 2002. Notes: One component displayed in this scheme could include more than one isoforms. This scheme does not include a complete description of the interactions/signaling of all the components appearing here. Arrows could represent direct or indirect activation, binding or other interactions (eg catalysis). Complexes formed via the interactions are not shown.

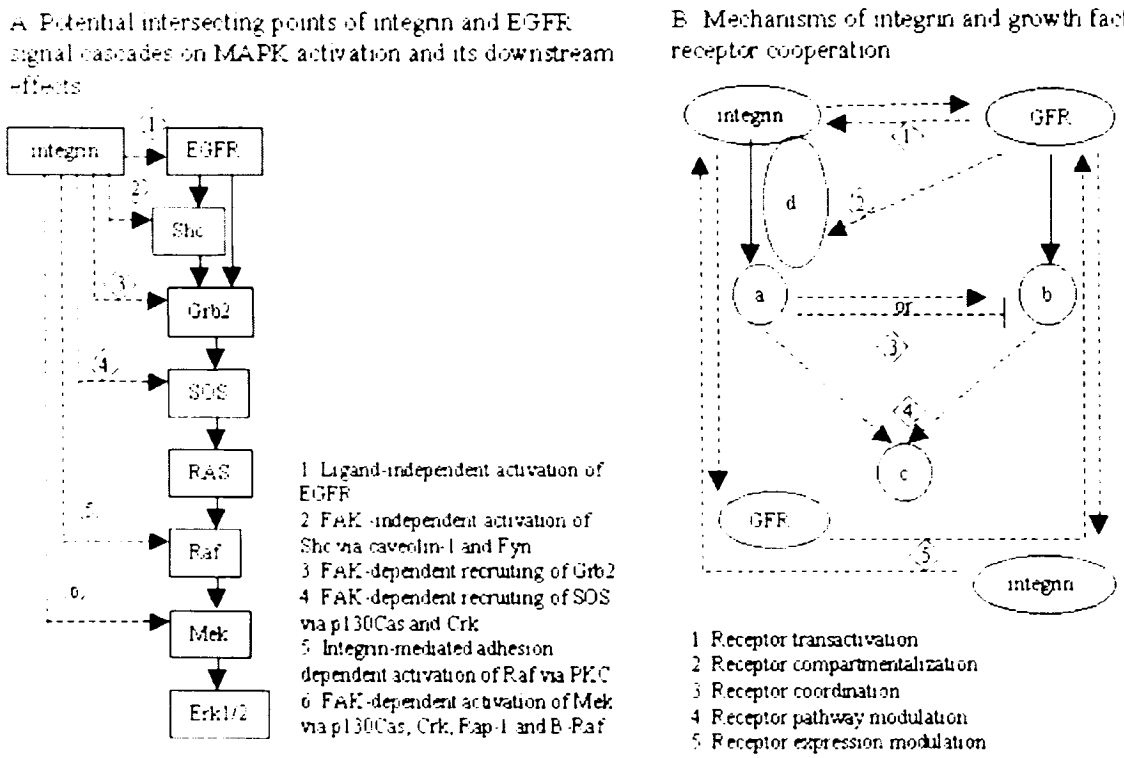


Figure 1.5. Cooperation of integrin and EGFR.
Modified from Figure 1.2; Figure 1.4; Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002.

CHAPTER II. tPA INDUCES APOPTOSIS ON BOVINE MAMMARY GLAND EPITHELIAL CELLS VIA DEGRADATION OF FIBRONECTIN

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ABSTRACT

The Plasmin/Plasminogen activator system is involved in tissue remodeling during development and involution. However, its possible role in inducing cell death has not been well studied. Here we report that, after being introduced with active tPA genes, bovine mammary epithelial cell MAC-T overexpressed tPA. Consequently, cell numbers in the cultures were significantly lowered ($P<0.001$). The percentages of apoptosis in these cell cultures were significantly elevated ($P<0.01$). Western blots revealed degradation of fibronectin in these cell cultures. Also, supplemented fibronectin fragments decreased the numbers of the cells introduced with inactive tPA gene or vector ($P<0.05$). These suggest that tPA induced apoptosis via degradation of fibronectin. Furthermore, supplementation of exogenous plasminogen resulted in high plasmin activity which was absent previously and the detachment of cells from the plastic substrate in active tPA-expressing cell lines but not in other cells. Our in vitro results suggest that cell death caused by increased tPA in gradual involution result from both direct degradation of ECM component and indirect activation of plasminogen.

Key Words: bovine, mammary gland, epithelial cell, gradual involution, tissue-type plasminogen activator, fibronectin, apoptosis.

INTRODUCTION

Milk production changes through the lactation period of cows. At parturition milk production commences at a relatively high rate, and the amount secreted continues to increase for about 3 to 6 weeks (Bath et al, 1985). After the peak is attained, milk production gradually declines, which is commonly referred to as gradual involution (Bath et al, 1985; Politis, 1996). Gradual involution is characterized by a decrease in the number of secretory cells (Politis, 1996). When the lactation has ended, post-lactation involution begins; this involution is characterized by a further marked decrease in secretory tissue mass (Knight and Wilde, 1993).

The components in mammary gland and/or milk, which induce decrease in the number of the secretory cells, have not been identified. However, the physiological importance of proteolysis by plasmin/plasminogen activator during tissue remodeling has been well documented (Dano et al, 1985; Saksela and Rifkin, 1988). For example, microglial tPA triggered neuronal apoptosis in vitro (Flavin et al, 2000). Plasmin caused programmed cell death of ovarian surface epithelial cells by cleaving tumor necrosis factor α exodomain from follicular endothelium (Murdoch et al, 1999). Also, the plasmin/plasminogen activator system in bovine milk was revealed to correlate with gradual involution (Politis, 1996). There are several lines of evidence to suggest possible involvement of the plasmin/plasminogen activator system in mammary gland remodelling. First, both plasmin/plasminogen and plasminogen activator activities increased during gradual involution (Politis et al, 1989; Gilmore et al, 1995). Second,

plasmin was the most significant protease in total proteolytic activity (Eigel, 1977; Fox, 1982). Third, plasminogen activators were found in milk and they converted plasminogen to plasmin. Nevertheless, the importance of plasmin/PA in regulating mammary epithelial cell death and viability has not fully confirmed. Here we report that over-expression of tPA in bovine mammary epithelial cell (MAC-T) increased the tPA activity in medium and induced cell apoptosis. Degradation of fibronectin as well as the absence of significant plasminogen/plasmin activities in these cultures implied the effect of over-expressed tPA on cell apoptosis via breaddown of fibronectin. This was further supported by negative effect of fibronectin fragments on cell growth. Furthermore, increased plasmin activity, which resulted from supplementation of exogenous plasminogen in cell lines with over-expressed tPA, induce large-scale detachment of the cells from the substrate. All together, our in vitro results suggest the possible mechanism for tPA/plasmin roles in gradual involution of mammary gland and support in vivo observation.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium (DMEM), fetal bovine serum and gentamicin were from Life Technologies (Rockville, MD). Serum-free and protein-free hybridoma medium and streptomycin were from Sigma (St. Louis, Mo). Hygromycin B was from Boehringer Mannheim Corporation (Indianapolis IN). Plasmin substrate Spectrozyme, uPA, plasminogen and fibrin fragment Desafib were from American Diagnostica du Canada (Montreal, QC). Acridine orange was from Fisher Scientific (Nepean, ON). Nine-

cm tissue culture dishes were from Nunclon (Rochester, NY). Twenty four-well tissue culture plates were from Sarstedt, Inc. (Newton, NC). Ninety six-well flat-bottomed microtiter plates were from Evergreen Scientific (Los Angeles, CA).

Cell Lines

Parent MAC-T cells (Huynh et al., 1991) were transfected with human tPA gene, goat tPA gene, mutated human tPA gene and vector alone to produce cell line called B, C, E and PC, respectively. These cell lines were provided by Nexia Biotechnologies, Inc.

Cell Culture

All cells were cultured in DMEM supplemented with 5% of fetal bovine serum, 10 μg/ml of Gentamicin, 10 μg/ml of streptomycin and 50 μg/ml of Hygromycin B. Cells (1×10^4 in 1 ml DMEM) were seeded per well in 24-well plates. DMEM was replaced with serum-free and protein-free medium at the second day. At day six, medium was collected for measuring enzyme activities and for Western blotting, and cells attached on the substrate were lysed with 0.1N NaOH and the optical density at 260nm (A260) was measured with Milton Roy Spectronic 601. For reading the cell numbers, a standard curve was plotted for cell numbers against A260.

Assays for Plasmin, Plasminogen, uPA and tPA Activities

All activities were measured in 100mM Tris-HCl buffer, pH8.0, containing 0.5mM Spectrozyme. For plasminogen measurement, 100IU/ml uPA was supplemented. For uPA measurement, 50μg/ml plasminogen was supplemented. For tPA measurement,

50μg/ml plasminogen and 20μg/ml Desafib (tPA activator) was supplemented. Assays were carried out in 96-well microtiter plates with total volume of 250μl. Reaction was progressed at 37°C and absorbance at 405nm (A405) was read using an ELISA plate reader (Labsystems Multiskan MCC/340) every half an hour for at least 3 hours. The curves were plotted, and the slope was calculated over the linear part of the curve with Microcal Origin program. One unit of the enzymes was the amount of enzyme that induces a change in absorbance of 0.001 A405 in one min at 37°C (Aslam and Hurley, 1977). Plasmin activity was just the slope of the curve. Plasminogen activity was the slope for plasminogen curve subtracted with plasmin activity. uPA activity was the slope for uPA curve subtracted with plasmin activity. tPA activity was the slope for tPA curve subtracted with the slope for uPA curve. The activities in unit volume of medium were total activities divided by the volume of the sample being assayed.

Detection of Apoptosis

To measure the percentage of the apoptotic cells, attached cells were stained with 4μg/ml acridine orange in HBSS and fixed with 4% formadehyde. Nuclei were observed under fluorescent microscopy and pictures were taken. The cells with de-fragmented or condensed nucleus were considered apoptotic. At least 420 cells for each cell line were observed and apoptotic and total cell numbers were counted.

Fibronectin Western Blot

Equal amounts of conditioned medium from the cultures of four cell lines were electrophoresed with SDS-PAGE (6% separation gel). The prestained SDS-PAGE

standards from Bio-Rad containing myosin (205kDa), β-galactosidase (118kDa), BSA (85kDa) and ovalbumin (47kDa) were used for molecular weight estimation. Both first antibody (rabbit anti fibronectin antibody) and second antibody (mouse anti rabbit IgG, HRP conjugated) were purchased from Sigma. Blocking buffer was 5% nonfat dry milk in 10mM Tris (pH7.5), 100mM NaCl and 0.1% Tween20. Blocking was conducted at 37°C for 30min then room temperature 1hr. Reaction with 1^{st} antibody (1:1000 in blocking buffer) was conducted at 37°C for 30min then room temperature 6~7hr. After washing with 10mM Tris (pH7.5), 100mM NaCl and 0.1% Tween20 for three times (10min), reaction with 2^{nd} antibody (1:1000 in blocking buffer) was conducted at 37°C for 30min then at 4°C overnight. After washing as before, membranes were developed as described in the HRP Color Development Reagent manual (Bio-Rad).

Effects of Plasmin on Cell Attachment

To estimate the effects of plasmin on cell attachment, plasminogen and fibrin fragment Desafib (for activation of tPA) was added to cultures of the tPA-expressing cells. The measurement of the attached cells was conducted as described previously.

Statistical Analysis

All cell cultures and assays described were performed in triplicate. All experiments were repeated 3 times. Mean values and standard deviations were calculated in Microsoft Excel. t-test was also carried out with Excel assuming equal variances of two-samples.

RESULTS

Cell lines were established by transfecting human tPA gene (line B), goat tPA gene (line C), mutated human tPA gene (line E) and vector alone (line PC) (Nexia Biotechnology, Inc., unpublished), respectively. Activities of different components of plasmin/PA system were measured. Plasmin/plasminogen/uPA activities were absent or very limited in all conditioned media (data not shown). The tPA activities in the conditioned medium of the cell lines B and C after 6 days culture were 1505 units/ml and 1060 units respectively. They were significantly higher ($P<0.001$) than the activities in conditioned media of cell lines E and PC which were very limited (37 units/ml and 60 units/ml, respectively) (Figure 2.1). The result confirmed that the expression of tPA genes were stable in B and C cells.

To assess the possible effects of overexpressed tPA on the growth of the cells, cell numbers were assayed using the UV method based on the quantification of DNA contents in NaOH-lysed cultures. The cell lines transfected with different genes exhibited different growth behavior. After six-day culture in serum-free and protein-free medium, cell numbers for the four cell lines were significantly different (Figure 2.2). Basically, the cell numbers in the cultures of cell lines B and C were significantly lower than those of cell lines E and PC.

One possible explanation for the lowered number of cells in the presence of tPA is the cell loss during culturing. Cell loss could be due to necrosis usually occurring under non-physiological conditions, or programmed cell death (apoptosis) occurring under physiological conditions. In order to determine whether the cell death observed

previously is due to necrosis or apoptosis, the attached cells was stained with fluorescent DNA dye, acridine orange. Apoptotic cells were found in the cultures of all the four cell lines determined by the existence of fragmented or condensed nucleus after fixing and acridine orange staining (Figure 2.3.A). The percentage of the apoptotic cells was calculated after counting the number of these cells and all cells in several microscopic fields. The B cell lines had a significantly higher percentage of apoptotic cells than other cell lines after six-day culture (Figure 2.3.B). No significant differences were found among other cell lines (Figure 2.3.B).

Besides plasminogen, the component of ECM, fibronectin, is also substrate of tPA. To test if fibronectin was produced by the cells used in this study and possibly degraded by tPA produced by the tPA-overexpressing cell lines, Western blot experiments was conducted using a fibronectin specific antibody. Cell culture medium samples were taken at day six of culture. Western blot results showed that the large fragment of fibronectin (244.5kD) was degraded in the culture medium of cell lines B and C (Figure 2.4), but not in the culture medium of cell line E and PC.

Fibronectin fragments have been reported to induce apoptosis in mouse mammary epithelial cells. To test the effect of the fibronectin fragments produced via its degradation on the cell proliferation, a commercial available fibronectin fragment product (Peninsula Lab; Belmont, CA) was used to treat cell line E and PC. The cell number after six-day culture in serum-free, protein-free medium in the presence and absence of the fibronectin fragment were compared. Results showed that treatment with fibronectin fragments significantly reduced the cell number after six-day culture in both E and PC cell lines (Figure 2.5).

The plasmin/plasminogen activities were absent in the conditioned medium of the four cell lines even in the presence of fibrin fragment (data not shown). This situation was contrast to the in vivo one, where plasminogen exists in a considerable amount in the milk. To mimic the in vivo situation, the B cell line culture medium was supplemented with both fibrin fragments and plasminogen. Consequently a large-scale detachment of the cells from the substrates was observed one day after the supplementation (Figure 2.6).

DISCUSSION

In this study, bovine MEC cell lines were used to examine the possibility that tPA could induce cell apoptosis and detachment. Our results indicated that this effect was through the direct degradation of a component of ECM, fibronectin.

Western blots in this study proved that MAC-T cells produced fibronectin in culture. This is in agreement with the previous observation in cultured epithelial cells enzymatically digested from human mammary tissues (Stampfer et al, 1981) and epithelial cell line derived from a dimethylbenzanthracene-induced rat mammary tumor (Ormerod et al, 1983). However, another group, by indirect immunofluorescence using antisera specific for fibronectin, reported the absence of fibronectin in both normal epithelial cells palleted from human breast milk and malignant epithelial cells released from primary human breast carcinomas by enzymatic digestion (Yang et al, 1980). The divergent source of the cells could be the reason for these different results. Neveretheless, it appears that cultured bovine mammary epithelial cells express fibronectin.

It has been previously reported that human breast epithelial cells in culture secreted tPA (Electricwala and Atkinson, 1985; Electricwala and Griffiths, 1986; Griffiths and

Electricwala, 1987). However, in another study using bovine epithelial cell line MAC-T, it was found that tPA was not present in the culture medium (Heegard et al, 1994). In the culture medium of subclonal lines (MACT-UV1 and MACT-UV2), fibrin caused a small but significant increase in PA activity, indicating the presence of low concentrations of tPA (Heegard et al, 1994). Our result on measurement of tPA activity showed the production of a limited amount of tPA by the MAC-T cells introduced with inactive tPA gene or vector itself (Figure 2.1), indicating the cells used in this study were similar to the subclones of MAC-T (Heegard et al, 1994).

Although the basic function of tPA in vivo is activation of plasminogen (Madison et al, 1995) into plasmin, a more effective protease with broader spectrum of substrates including ECM components and other proteins, direct degradation of fibronectin by tPA was also reported (Marchina and Barlati, 1996). In our study, overexpression of tPA was coupled with degradation of fibronectin. Considering the absence of plasmin and uPA activities in the cell cultures (data not shown), this degradation is supposed to be the direct effects of tPA on fibronectin. In contrast to the previous results, in which fibronectin fragments of 30 and 220-250 kDa were generated by tPA treatment, the fragment of ~245 kDa completely disappeared in the culture medium of active tPA-expressing cell lines in our experiments (Figure 2.4).

Epithelial cell viability dependents on attachment to appropriate substrate, detachment-induced apoptosis had been observed in primary mouse mammary cells (Gilmore et al, 2000). Bovine mammary epithelial cells used in this study underwent apoptosis (0.6% for cell lines E and PC, 1.4% for cell line B and 0.9% for cell line C). At the same time, the attachment of the cells was significantly decreased in cell lines B and

C (Figure 2.2), with more rounding cells which was detaching or had detached from the substrate (data not shown). Thus, tPA could directly degrade fibronectin and induce cell detachment and apoptosis. Supplement of the cell cultures with a fibronectin fragment also caused cell loss, supporting the effects of the fibronectin fragments on the cells. We failed to observe the small fibronectin fragments on the Western blot from the cell cultures, possibly because that the amount of them was too small to be detected. Besides detachment, apoptosis was reported to be induced by fibronectin fragments in mouse mammary epithelial cells (Schedin et al., 2000). Taking consideration of those, we suggest that, in the in vitro systems of our experiments, there are at least two ways in which fibronectin degradation by overexpressed tPA induces apoptosis: first, via detachment of the cells; second, via the effects of fibronectin fragments.

Plasmin/PA system has long been thought to be involved in mammary gland involution after cessation of milking (initiated involution) in mice or cow (Politis et al, 1989; Ossowski et al, 1979). In mice, tPA was detected by day four of involution, and maintained expression until at least day ten (Talhouk et al, 1992). Compared to in vitro system, these in vivo conditions are more complex, because of the existence of plasminogen and its activated product, plasmin. Compared to tPA, plasmin has a broader spectrum of substrates, including fibrin (Kang and Triantaphyllopoulos, 1977), laminin (Goldfinger et al, 1998), fibronectin (Salonen et al, 1985), procollagenase and prostromelysin (He et al, 1989), β-casein (Eigel, 1977), αS1-casein (Eigel, 1977), κ-casein and lactoferrin (Aslam and Hurley, 1997), basic fibroblast growth factor (Falcone et al, 1993), transforming growth factor β (Taipale et al, 1992) and tumor necrosis factor α (Murdoch et al, 1999). Our plasminogen supplement experiment partly mimicked the

in vivo situation. The subsequent complete detachment of the cells from the substratum is consistent with the much stronger protease activity of plasmin, and probably the subsequent activation of other proteases, for degradation of multiple ECM components.

Cell loss is a feature of initiated involution and gradual involution in bovine (Wilde et al, 1997). Although the proportion of apoptotic cells in involuting mammary tissue is 4.8%, in lactating tissue, there were still 2.4% of the cells undergoing apoptosis (Wilde et al, 1997). It is implicated that extracellular proteolysis may dissociate individual cells from stabilizing basement membrane components, with resultant induction of apoptosis (Boudreau et al, 1995; Pullan et al, 1996). Also, bovine mammary apoptosis during involution appeared to be localized predominantly in the areas defined histologically by loss of alveolar integrity (Wilde et al, 1997). Our results in this study confirmed the role of tPA and plasmin in inducing the cell detachment and death via apoptosis. Although our results don't rule out the other systems possibly involved in gradual involution, it is a step closer to understanding the cell loss during gradual involution in which the tPA/plasmin activities increase continuously. Inhibition of these enzyme activities may be a way in which the milk production loss due to gradual involution can be attenuated in a considerable extent.

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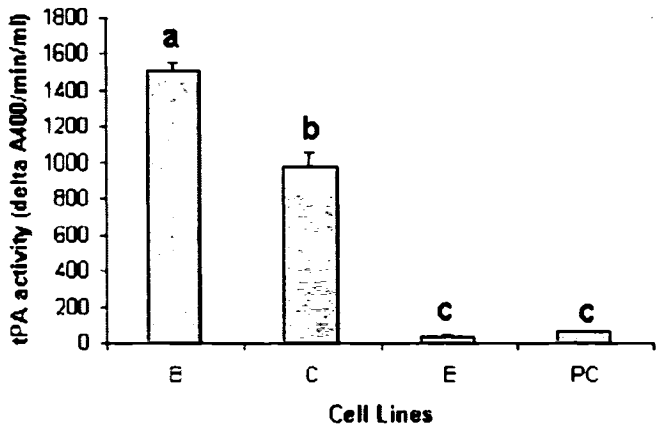

Figure 2.1. tPA activities in the medium after six day culture.
Cells were seeded at the density of 1×10^4 cells/ml/well in 24 well plates with DMEM medium supplemented with 5% fetal bovine serum. One day after seeding, DMEM was replaced with serum-free and protein-free medium (Sigma) (SFPF). After six days in SFPF, culture media were collected and snap frozen at -20°C until measurement of enzyme activities. Displayed are means and standard deviations. a, b, c: means with different letters are significantly different ($P<0.001$).

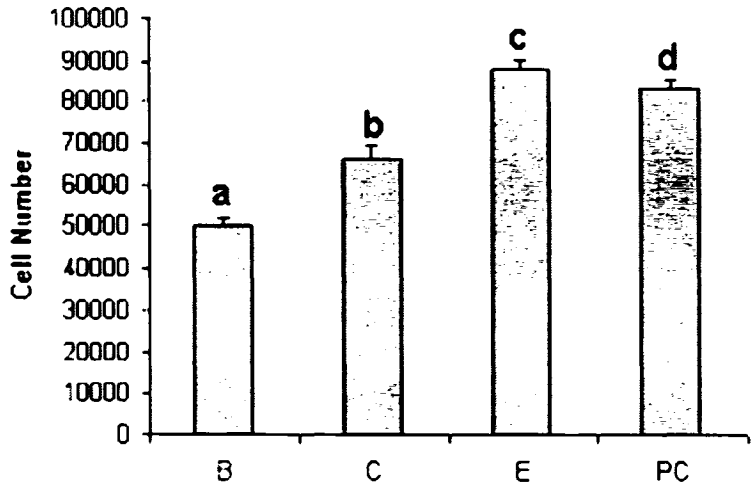

Figure 2.2. Cell numbers after six-day culture.
Seeding and culture conditions were the same as described in Figure 2.1. Displayed are means and standard deviations. a, b, c, d: means with different letters are significantly different ($P<0.001$, $P<0.005$ or $P<0.05$).

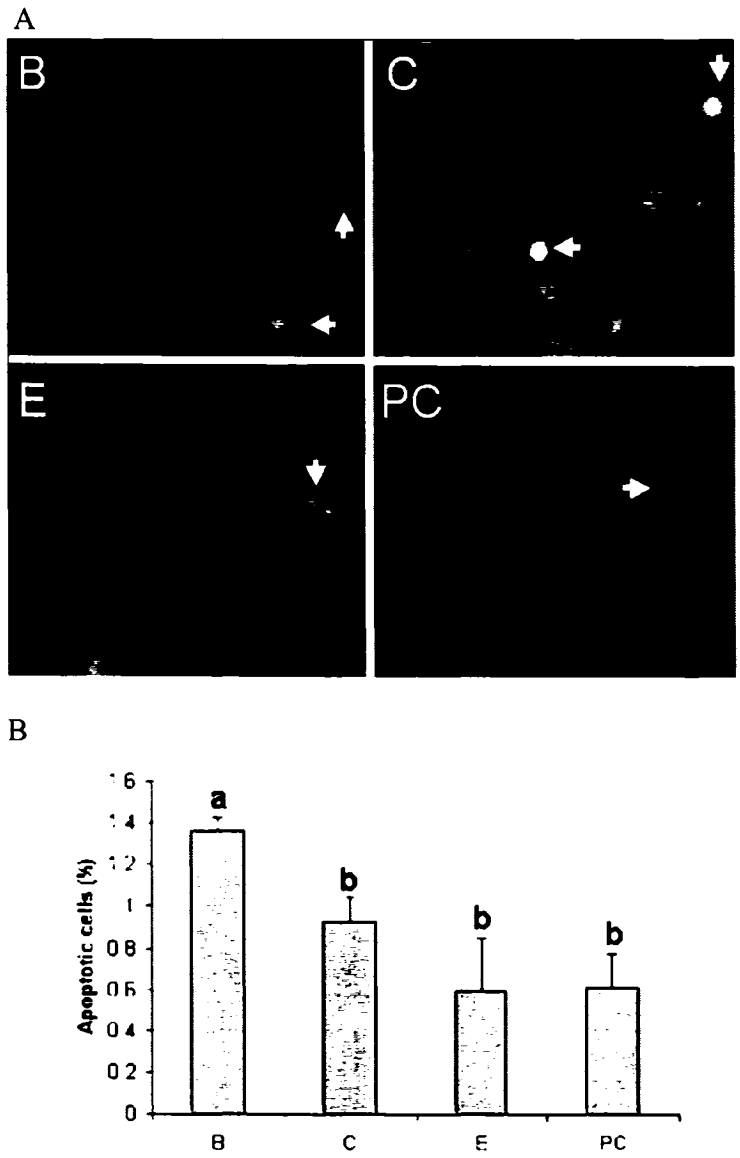

Figure 2.3. Apoptosis in the four cell lines.
A. Pictures of the cells taken from cell culture fixed with formaldehyde and stained with acridine orange after six day culture in SFPF medium. Arrows points to the condensed or fragmented nucleus (apoptotic cells). B. Percentage of the apoptotic cells in four cell lines. Pictures of the cells were taken from several different microscopic fields. The numbers of apoptotic cells and total cells were counted and the percentages were calculated. a, b: means with different letters are significantly different ($P<0.005$ or $P<0.01$).

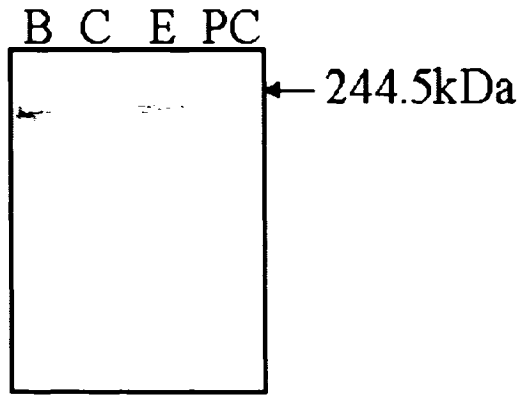

Figure 2.4. Degradation of fibronectin in tPA-transgenic cell lines.
The culture condition was the same as described in Figure 2.1. SDS-PAGE, blotting, and detection of fibronectin proteins and fragments were described in the section of "Materials and Methods".

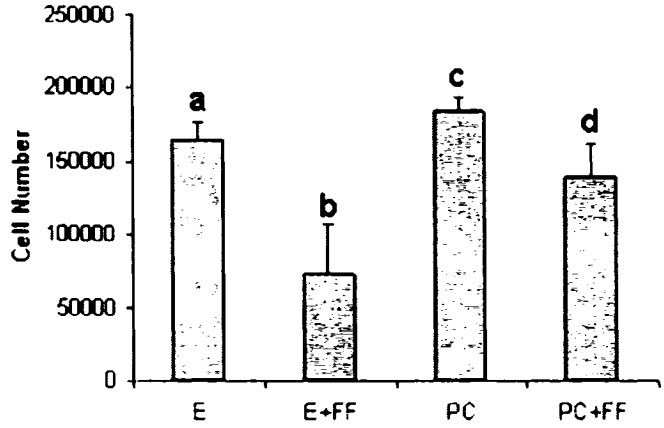

Figure 2.5. Effects of fibronectin fragments on cell number after six-day culture.
E: E cell line alone; E+FF: E cells treated with fibronectin fragments; PC: PC cell line alone; PC+FF: PC cells treated with fibronectin fragments. 2×10^4 cells were seeded in 24 well plates in DMEM supplemented with 5% fetal bovine serum. After one day, DMEM was replaced with SFPF and fibronectin fragments (20ug/ml) were added. Cell counting after six days was performed. a, b: means with different letters are significantly different ($P<0.05$). c, d: means with different letters are significantly different ($P<0.05$).

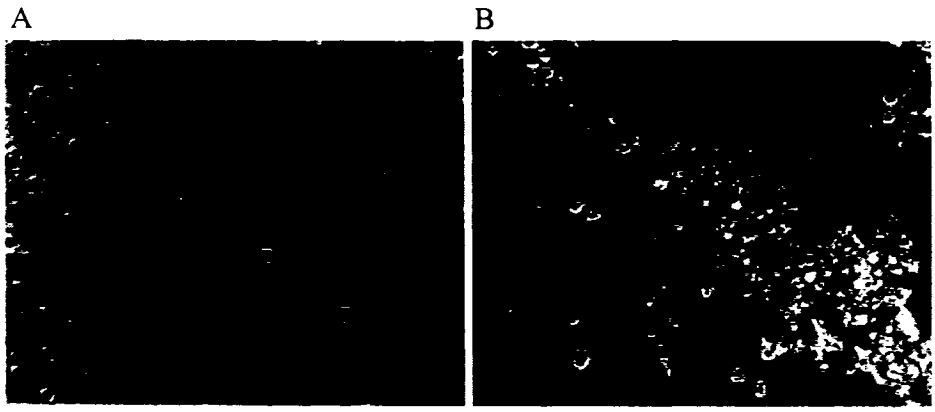

Figure 2.6. Plasmin induces detachment of cells from substrate.
Cells are seeded in a density of 5×10^4 cell/well in 24 well plate in DMEM medium supplemented with 5% fetal bovine serum. After one-day attachment, DMEM was replaced with SFPF medium with or without fibrin/plasminogen. After another day culture, cells were photographed. A. B cells in the absence of fibrin/plasminogen. B. B cells in presence of fibrin/plasminogen.

CONNECTING STATEMENT I.

In cell cultures, ECM is required for cell attachment and survival. Degradation of ECM components such as fibronectin results in cell death by deprivation of cell attachment as exemplified in chapter 2. Cell attachment is necessary not only for cell survival, but also for growth factor to efficiently stimulate cell proliferation. Without cell attachment to proper substratum, growth factor had very limited proliferation-stimulating effects, as reviewed in chapter 1.

The integration of signaling cascades induced by different enviromnental components of the cells, such as ECM and EGF, is an important topic in current cell biology. Very few qualitative models have been proposed for this integration. Meanwhile, a few mathematical models for signal transduciton of a single receptor have been proposed and continuously improved. One of the relatively mature models is for EGFR induction of MAPK cascade. The mathematical models are fundamental for further analysis of signal integration such as the relative contribution of alternative pathways. In chapter 3, a reported model was further explored to make such assessment. Further, the biological importance of the results was proposed.

CHAPTER III. Shc-DEPENDENT PATHWAY IS REDUNDANT BUT DOMINANT IN MAPK CASCADE ACTIVATION BY EGF RECEPTORS: A MODELING INFERENCE

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ABSTRACT

In cell signaling cascades, one stimulus often leads to various physiological functions by multiple pathways. Perturbation of one pathway by blocking or overexpressing one of its components will result in changes in multiple pathways and multiple cell functions. Thus, it is important to reveal the relative contribution of each pathway to each function in order to assess the consequence of perturbations (e.g. drug delivery). By exploring an established mathematical model, the Shc-dependent pathway is found to be both redundant and dominant during activation of the MAPK cascade by epidermal growth factor receptor (EGFR). Its dominance results from the majority consumption of the common precursor ((EGF-EGFR*)2-GAP) by this pathway. The key steps for the dominance are the binding and phosphorylation of Shc. In conclusion, cells may prefer the long Shc-dependent pathway to the short Shc-independent pathway.

Key words: epidermal growth factor receptor; Shc; cell signal transduction; biochemical simulation.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is one of the tyrosine kinase receptors and is involved in various physiological or pathological phenotypes including embryonic and postnatal development (Sibilia et al., 1998), progression of tumors (Kim and Muller, 1999), and transactivation processes (Hackel et al., 1999). Upon being bound by the ligand EGF at the extracellular domain, EGFR forms a dimer and phosphorylates

itself at the intracellular domains. Phosphorylated EGFR then recruits a series of signaling molecules and induces activation of MAP kinase cascade (Morrison and Cutler, 1997). The activated MAP kinase then regulates several cellular proteins and nuclear transcription factors (Marshall, 1995). There are two pathways used by EGFR to activate MAPK cascade, Shc-dependent and Shc-independent (Batzer et al., 1995; Buday and Downward, 1993). Although Shc mediated MAPK activation has been well established (Bonfini et al., 1996; Lai and Pawson, 2000), dominant negative Shc or loss of Shc expression often results in only a partial loss of MAPK activation (Ravichandran, 2001). It remains unclear how much Shc contributes to MAPK activation. Recently a computational model for the dynamics of MAPK cascade activation by EGFR was proposed (Schoeberl et al., 2002), which provided an insight into signal-response relationships between the ligand binding and activation of downstream proteins in the signaling cascade. However, the report did not address the issue as the relative contribution of Shc-dependent and -independent pathways. In this study, we further explore the model to quantitatively assess the relative contribution of the Shc-dependent and Shc-independent pathways to MAPK activation by EGFR. We report here that the Shc-dependent pathway is both dominant and redundant during activation of MAPK by phosphorylated EGFR. The redundancy is supported by several experimental results in the literature, and the key step responsible for the dominance is revealed in this study. Furthermore, the sub-pathways within the Shc-dependent pathway are dissected mathematically and their contributions to MAPK activation are discussed. As more and more signaling cascades are revealed to utilize multiple pathways (see the review by Liebmann, 2001), the protocol demonstrated in this study for assessing their relative

contributions will have a broad usage in simulating cell signal transduction and inferring the biological importance of alternative pathways.

MODEL AND SIMULATION

The model described by Schoeberl et al. (2002) was used in this study. The scheme of the EGFR-induced MAPK cascade was modified so that each molecule species appears only once (Figure 3.1). The Shc-dependent and Shc-independent pathways were separated graphically so that the components in each pathway were readily identified. The two pathways share the same events at the beginning, i.e., binding of EGF to EGFR, dimerization and phosphorylation of EGFR, and recruiting of GAP (not shown in Figure 3.1). They diverge at the point of (EGF-EGFR*)2-GAP. Instead of Grb2, Shc is recruited in the Shc-dependent pathway. After phosphorylation of Shc, Grb2 is recruited to form (EGF-EGFR*)2-GAP-Shc*-Grb2, which is the equivalent of (EGF-EGFR*)2-GAP-Grb2 in the Shc-independent pathway (Figure 3.1). The following common events are SOS binding and then Ras-GDP binding. The two pathways converge at the point of Ras-GTP production, and thereafter follow the same track to sequentially activate Raf, MEK and ERK (not shown in Figure 3.1).

Simulation was conducted using the biochemical simulator GEPASI version 3.21 (Mendes, 1993; Mendes, 1997; Mendes and Kell, 1998). The reactions, their kinetics parameters and the initial quantities of the components were inputted into GEPASI using its GUI (graphical user interface). Totally there were 125 reactions and 94 components, as described by Schoeberl et al. (2002).

For assessing the relative contributions of the two or more pathways, the concentrations of the components are of limited values. Molecules may be produced and consumed at the same time. If both processes occur in a same strength, the concentration of a molecule remains constant. More importantly, the concentration is the component (molecule species) specific and does not reflect contribution of a single reaction. Thus, it is more meaningful to observe production and consumption of each component dynamically via each reaction in which it is involved for the purpose of this study. To do so, the fluxes (velocity of the molecular flow, which is the reaction specific) of the reactions were traced. This function is one of the features of the software GEPASI. The amounts of the molecular flow (which is the reaction specific, accordingly) were calculated with integration over the simulation time, by using the following formula: molecular flow = Σ ($\Delta t \times F_t$). Where Δt is the small segment of time (typically 1 second in this study) and F_t is the flux at time t (in the unit of M/s). With molecular flows, it is easy to dissect the ratio of a component produced by different reactions, or the flows of a component into different reactions.

For reversible reaction steps, unidirectional arrows are used in the scheme for assignment of the forward and reverse reactions. The flux value can be positive or negative. When the value of the flux is positive, the flow of the molecule has the same direction as the arrow. If the value of the flux is negative, the molecular flow has a reverse direction to the arrow.

RESULTS

It has been modeled previously that EGFR could be internalized before or after activation, and the internalized receptors transduce signal as well (Schoeberl et al., 2002). However, internalization of the receptors had very limited effects on production of Raf, MEK-PP and ERK-PP under the simulation condition used in this study (with 50ng/ml EGF) (data not shown), which was in agreement with the published results (Schoeberl et al., 2002). Therefore, the effects produced by both cell surface and internalized receptors were combined for the simulation in this study.

The relative contribution of the Shc-dependent and Shc–independent pathways to MAPK activation was first assessed. The index of contribution was evaluated by the molecular flow to Ras-GTP from its direct precursors ((EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP and (EGF-EGFR*)2-GAP-Grb2-Sos-Ras-GDP for the Shc-dependent and Shc–independent pathways, respectively). The fluxes of the reactions were traced up to 60 minutes and production of Ras-GTP was calculated. As shown in Figure 3.2, the Shc-dependent pathway was the dominant one used by EGFR to produce Ras-GTP, while only 2% of the total Ras-GTP was produced by the Shc-independent pathway, when the initial concentration of EGF was set at 50ng/ml and the concentration of Shc at the reported value (Schoeberl et al., 2002).

To further determine the extent to which the Shc-dependent pathway was dominant and also whether it was redundant as indicated by several experiments, the effects of initial Shc concentration on Ras-GTP production by the two pathways were tested, using the component concentration perturbation scanning, a function provided by GEPASI. The total production of Ras-GTP only had a small reduction (less than 5%) when the initial

Shc concentration was set to 0. Under this condition, all of the Ras-GTP was produced via the Shc-independent pathway (Figure 3.3). This result suggested that the Shc-dependent pathway was redundant in the MAPK activation by EGFR. Nevertheless, existence of a small amount of Shc (<12% of the reported value) would restore the Ras-GTP production almost to a normal level (>99%). Furthermore, this small amount of Shc caused the Shc-dependent pathway produce more than 83% of the total Ras-GTP. When the Shc level was half of the reported value, this ratio was as high as 95% (Figure 3.3), indicating that a low level of Shc was enough to make this pathway dominate the MAPK activation.

There are at least two possible scenarios for the dominance of the Shc-dependent pathway. One is the crosstalk between the pathways (see Figure 3.1). The early components in the Shc-independent pathway may be produced in a considerable amount, but consumed by the cross-talk paths between the pathways, instead of leading to production of Ras-GTP via their own pathway. Alternatively, most of the common precursor of the two pathways ((EGF-EGFR*)2-GAP) is consumed by the Shc-dependent pathway at the presence of even a very small amount of Shc. To test these two possibilities, consumption of the common precursor ((EGF-EGFR*)2-GAP) by the two pathways was compared by tracing the fluxes from the common precursor and calculating the molecular flows. As shown in Figure 3.4, a majority of the precursor (99.9%) flowed to the Shc-dependent pathway. To rule out the possibility of the cross-talk, production of the intermediates equivalent in two pathways ((EGF-EGFR*)2-GAP-Shc*-Grb2 vs. (EGF-EGFR*)2-GAP-Grb2, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos vs. (EGF-EGFR*)2-GAP-Grb2-Sos, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GTP vs. (EGF-EGFR*)2-

GAP-Grb2-Sos-Ras-GTP, and (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP vs. (EGF-EGFR*)2-GAP-Grb2-Sos-Ras-GDP; also see Figure 3.1) were also compared. There was no significant production of the intermediates in the Shc-independent pathway in comparison with their counterparts in the Shc-dependent pathway (data not shown). Thus it is concluded that existence of Shc caused the common precursor ((EGF-EGFR*)2-GAP) flow to the Shc-dependent pathway, consequently leading to the dominance of the Shc-dependent pathway in Ras-GTP production.

A question remains as which step(s) along the Shc-dependent pathway determines the majority flow of the common precursor to this pathway and subsequently production of most Ras-GTP. A systematic scanning of the parameters of all the reactions involved in the Shc-dependent pathway was conducted, taking advantage of the power of GEPASI for parameter perturbations. The results showed that changes in 3 of the parameters could significantly affect production of Ras-GTP by the Shc-dependent pathway. They were v22k1 (forward rate constant of the reaction v22), v23k1 and v26k1 (Figure 3.5a, b and c; also see Figure 3.1 for their locations). Changes of parameters in all other forward reactions and all reverse reactions did not affect the differential production of Ras-GTP (data not shown). v22 is the first step from the common precursor in which Shc is bound, and v23 is the subsequent step in which Shc is phosphorylated. Both v22 and v23 are specific to the Shc-dependent pathway. v26 is the step in the Shc-dependent pathway, in which the Ras-GTP precursor is produced and which has an equivalent in the Shc-independent pathway (v18). Both v26 and v18 have same values for forward and reverse rate constants, suggesting that the key steps determining the flow of the common precursor are v22 and v23. Furthermore, as low as 0.05% of the reported v23k1 value, or

2% of the v22k1 reported value, resulted in that the two pathways produced the same amount of Ras-GTP (Figure 3.5a and 3.5b). Further, The effects of changes in v22K1 and v23K1 on the dynamics of the downstream ERK signal are also different. When v22k1 or v23k1 was set to 0, the total Ras-GTP production was almost unchanged (97.2%) or dropped to 63.3% of full amount, respectively (Figure 3.5a and 3.5b). Interestingly, when v26k1 was set to 0, the total Ras-GTP production was reduced by 88% (Figure 3.5c), suggesting that production of Ras-GTP by the Shc-independent pathway did not compensate the block of this step in the Shc-dependent pathway. It was hypothesized that in this situation most common precursor flowed to the Shc-dependent pathway and accumulated as the intermediates in this pathway during the blockage of reaction v26. The accumulation could be fulfilled via, for example, the loop: v22-v23-v37-v36-v22. This was confirmed by tracing each reaction in this loop, where their fluxes were significantly increased when v26k1 was set to 0 (data not shown).

After quantitative dissection of the Shc-dependent and Shc-independent pathways, the sub-pathways in the Shc-dependent pathway were further dissected. In the Shc-dependent pathway, there are two sub-pathways that possibly lead to production of Ras-GTP. They are (also see Figure 3.1):

1. (EGF-EGFR*)2-GAP → (EGF-EGFR*)2-GAP-Shc → (EGF-EGFR*)2-GAP-Shc* → (EGF-EGFR*)2-GAP-Shc*-Grb2 → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP → Ras-GTP;
2. (EGF-EGFR*)2-GAP → (EGF-EGFR*)2-GAP-Shc → (EGF-EGFR*)2-GAP-Shc* → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP → Ras-GTP.

Instead of reactions v24 and v25, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos is produced directly from (EGF-EGFR*)2-GAP-Shc* and Grb2-Sos via reaction v41 in the second sub-pathway. This is independent of the first sub-pathway, because Grb2-Sos is produced from pre-existing Grb2 and Sos. A flux-tracing experiment showed that, at first, both sub-pathways were used to produce Ras-GTP. Reactions v24 and v25 were reversed after 270 s and 126 s, respectively (Figure 3.6). Thereafter, only the second sub-pathway was used (see the top curve on Figure 3.7A). (EGF-EGFR*)2-GAP-Shc* is the common precursor of the two sub-pathways. Its flow to two sub-pathways depends on the value of v41k1 (Figure 3.7). At low v41k1, flow to the first sub-pathway was always prevalent. At considerable v41k1 value, at first there was part of the common precursor flowed to the first sub-pathway, then all the flow was through the second sub-pathway. At high v41k1, flow to the second sub-pathway was always prevalent (Figure 3.7). However, as previously demonstrated, change in single kinetic parameter of reactions v24 or v25 or v41 did not affect the Ras-GTP production by the Shc-dependent pathway. Consequently, blockage of either of the sub-pathways would not affect the dynamics of the Ras-GTP production by EGFR.

DISCUSSION

Shc is an adaptor protein, its tyrosine is phosphorylated by several different types of receptors (growth factor receptors, antigen receptors, cytokine receptors, G-protein coupled receptors and hormone receptors, for review see (Ravichandran, 2001)). The real role Shc plays in signal transduction is not clear. Two possibilities have been proposed: Shc provides a central and universal component of intracellular signaling, or, the

phosphorylation of Shc is a bystander effect (Ravichandran, 2001). In MAPK activation by EGFR, Shc provides an alternative pathway (Batzer et al., 1995; Buday and Downward, 1993). Existence of the Shc-dependent pathway increases the complexity of signal transduction networking. Although networking could contribute to genetic buffering (Kitami and Nadeau, 2002), i.e., the robustness of the biochemical networking, the Shc-dependent pathway in this currently used model might not be aimed at this, based on the fact that this pathway shares all other signal molecules with the Shc-independent pathway.

In this study we revealed computationally the redundancy and dominance of the Shc-dependent pathway during MAPK activation by EGFR. The structure basis for the redundancy is the ability of phosphorylated EGFR to be bound by both Shc (via Shc SH2 and PI domains) and Grb2 (via Grb2 SH2 domain) as well as the binding of Grb2 to phosphorylated Shc (via Grb2 SH2 domain) (Batzer et al., 1995). The redundancy of Shc in the EGFR signaling has been observed in several types of cells, e.g., chicken DT40 B cell lines in which Shc was knocked out (Hashimoto et al., 1999), and rat pheochromocytoma cell line PC12 where Shc inhibitor TRK-Y490P was used (Basu et al., 1994). Shc redundancy was also observed in several other situations. For example, when proliferation or viability signaling was activated by granulocyte-macrophage colony-stimulating factor, the tyrosine phosphorylation of Shc was not necessary (Durstin et al., 1996). In addition, Raf1 was activated by erythropoietin via the Shc-independent pathway (Barber et al., 1997) and 8-Bromo-cAMP induced a proliferative response independent of Shc (Barge et al., 1997). In contrast, Shc was non-redundant in thymic T cell development (Zhang et al., 2002). Also, the mice embryos with homozygous mutant

at ShcA locus died at day 11.5 with severe defects in heart development (Lai and Pawson, 2000). Obviously, the situations at the cellular or whole animal levels are much more complex than the model used in this study. The complexity comes, at least, from the multi pathways Shc may be involved in. Besides the MAPK activation, Shc is also related to phosphatidylinositol 3-kinase/Akt pathway (Gu et al., 2000), c-Myc activation, (Gotoh et al., 1996), and JNK signaling pathway (Hashimoto et al., 1999). Besides the EGF cascade, Shc was also reported to regulate cascades of other growth factors, cytokines or hormones such as estrogen (Song et al., 2002), IGF-1 (Sasaoka et al., 2001), and interleukin 2 (Ravichandran et al., 1996). While our results suggest that Shc is redundant in the EGF-EGFR stimulation of MAPK, they can not easily be extrapolated to other systems. However, it is safe to say that Shc is redundant in the cases in which there exists alternative pathway(s) for the execution of the observed cell functions; and it is non-redundant in the cases in which there is not alternative pathway(s) for the cell to use for expressing specific phenotype(s). The perturbation or knockout experiments could provide clues on the existence of alternative pathways involved in the relevant cellular phenotypes.

The dominance of the Shc-dependent pathway during activation of MAPK was unexpected, considering that the Shc dependent pathway is longer than the Shc independent pathway. Also, this dominance, to our knowledge, has not yet been observed experimentally and its biological meaning remains unknown. While one possibility is that activation of MAPK via the Shc-dependent pathway could be more efficient (i.e., resulting in larger amplification of the signal) than via the Shc-independent pathway, the result of Shc concentration scanning experiments (Figure 3.3) does not support this. The

real roles Shc plays in physiological and pathological processes can only be figured out with larger scaled and more detailed models. However, our hypothesis is that, the importance of its dominance might not reside in EGFR activation of Ras-GTP itself. Rather, it might increase the competition of this activation with other receptors induced pathways in which Shc is also involved. Besides, an interesting conclusion implied by this result is that, during MAPK activation by EGFR, the longer pathway (Shc-dependent), instead of the shorter pathway (Shc-independent), is preferentially used. Further experimental results are needed to confirm this computational inference. A possible experimental strategy is to measure the contents of the molecular species with the newly developed techniques such as mass spectrometry-based proteomics. In a recent study, this methodology was applied to analyze the proteins from the HeLa cells treated with EGF, and absorbed by immobilized Grb2 fusion protein (Blagoev et al., 2003). Although increases in proteins absorbed by Grb2 SH2 domain were observed, similar yet more quantitative (e.g. put the labeled proteins as inner references) experiments in a time series will be more informative.

As demonstrated, the key steps determining the dominance of Shc-dependent pathway are Shc binding and phosphorylation by EGFR (rate constants v22k1 and v23k1). Three isoforms of Shc (46 kDa, 52 kDa and 66 kDa) could be expressed in the cells, and the major substrates for EGFR are 46 kDa and 52 kDa Shc (phosphorylated at similar extent), very limited 66 kDa Shc was phosphorylated by EGFR (Okada et al., 1995). The parameters in the model used in this study were based on the kinetics of 46 and 52 kDa Shc isoforms (Schoeberl et al., 2002). Thus, if the 66 kDa Shc is the predominant or exclusive isoform, utilization of the two pathways could be different from

the modeling result, and it is possible for them to be utilized at a similar extent. However, more accurate modeling demands the differentiation of the Shc isoforms in kinetic level.

While there might be more sub-pathways in the Shc-dependent pathway, via v37, v39 and v32, they are not considered important under the current modeling situation. First, all of them need phosphorylated Shc (Shc*) or complexes containing Shc* and thus they are dependent on v22 and v23. Second, flux-tracing results showed that they were all involved in the dissociation, instead of formation, of the intermediates of the Shc-dependent pathway (data not shown). Interestingly, in some tumor cells, Shc was constitutionally phosphorylated, because of the constitutionally activated tyrosine kinases (Pelicci et al., 1995). Under those circumstances, the alternative sub-pathways might be used in Ras-GTP production. However, detailed modeling needs extra and/or renewed initial state variables.

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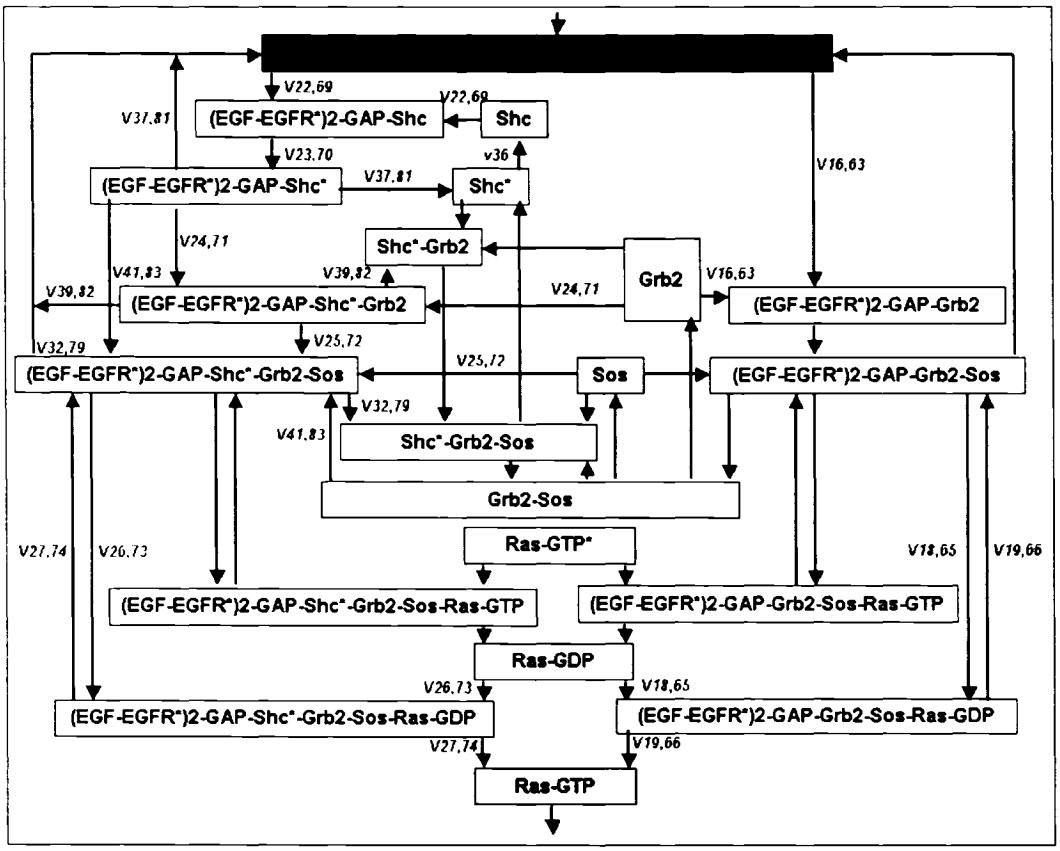

The common precursor of Shc-dependent and Shc-independent pathways.
The convergent point of Shc-dependent and Shc-independent pathways.
The components specifically in Shc-dependent pathway.
The components specifically in Shc-independent pathway.
Several precursors of the system.
Possible cross-talk between Shc-dependent and Shc-independent pathways.

(to be continued)

(continued from last page)

Figure 3.1. Partial scheme of the EGF receptor-induced MAP kinase cascade.
This figure was aimed to show the Shc-dependent and Shc-independent pathways. The Shc-dependent pathway is located on the left part, whereas the Shc-independent pathway is located on the right part. The common precursor of, convergent point of, possible crosstalks between the two pathways, and several initial components were located in the middle of the scheme. Each component label represents a molecular species at cell surface, and internalized as well, if applicable. Arrows represent the reaction direction. In the case of reversible reaction, the direction of the arrows determines the forward and reverse reaction parameters, which is not shown in the scheme. The IDs for the reactions begin with letter v and are close to the arrows representing the reaction. For simplification, only the IDs of the reactions being discussed are displayed. In the case of the two IDs, the second one represents the reaction occurred with the internalized component. (modified from [Schoeberl et al., 2002])

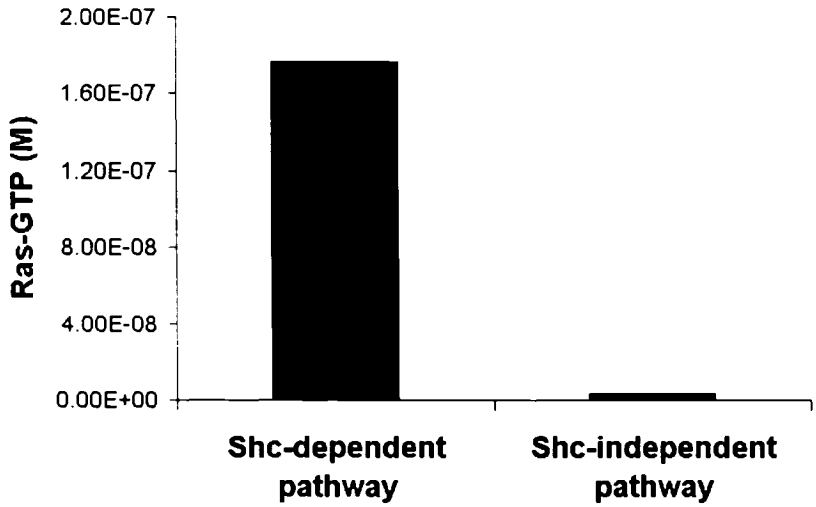

Figure 3.2. Relative contributions of the Shc-dependent and Shc-independent pathways to production of Ras-GTP.
The y-axis represents the molecular flow to Ras-GTP. The simulation was conducted using the same set of reaction parameters and initial concentrations as described (Schoeberl et al, 2002). EGF concentration was 50ng/ml. The time course of fluxes for reactions v27(74) (in the Shc-dependent pathway) and v19(66) (in the Shc-independent patyway) were traced for 60 min with 1 second for each segment. The fluxes from reactions v27 and v74, and from reactions v19 and v66, were combined, respectively. Molecular flows were then calculated as the sum the fluxes at each time point.

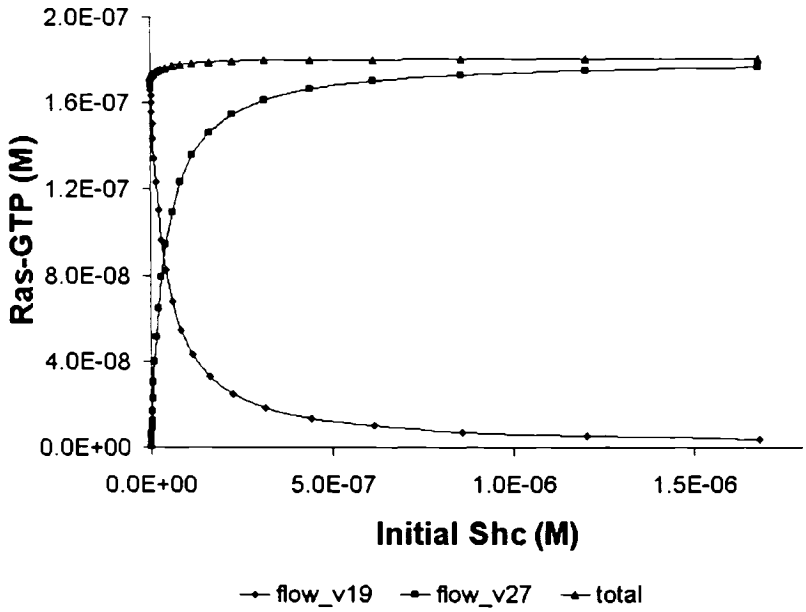

Figure 3.3. Relative contributions of the Shc-dependent and the Shc-independent pathways to production of Ras-GTP at varied initial Shc concentrations.
The y-axis represents the molecular flow to Ras-GTP. The simulation condition and calculation was the same as described in Figure 3.2, except that the Shc concentration was scanned. The values of initial Shc concentrations were selected logarithmically, spanning from 0 to 1.68E-6 M.

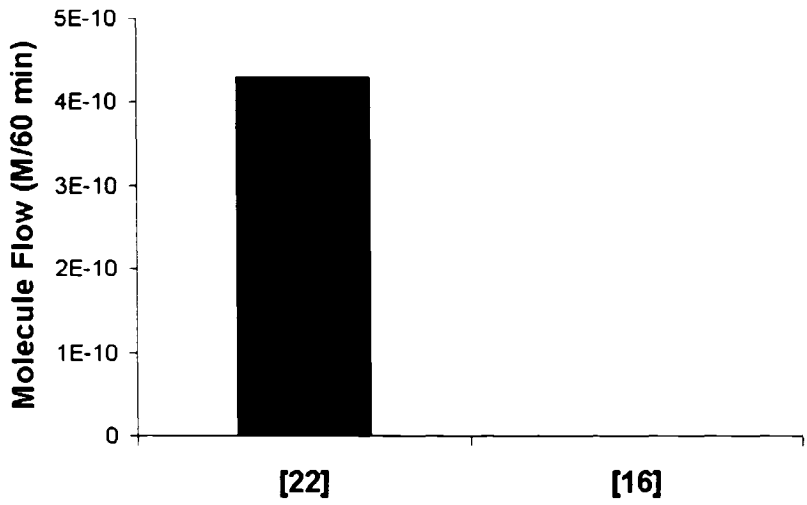

Figure 3.4. Amount of the common precursor, (EGF-EGFR*)2-GAP, flowing to Shc-dependent pathway via reactions v22 and v69 (combined and labeled as [22] in this figure), and to the Shc-independent pathway via reactions v16 and v63 (combined and labeled as [16] in here). The simulation condition was the same as described in Figure 3.2.

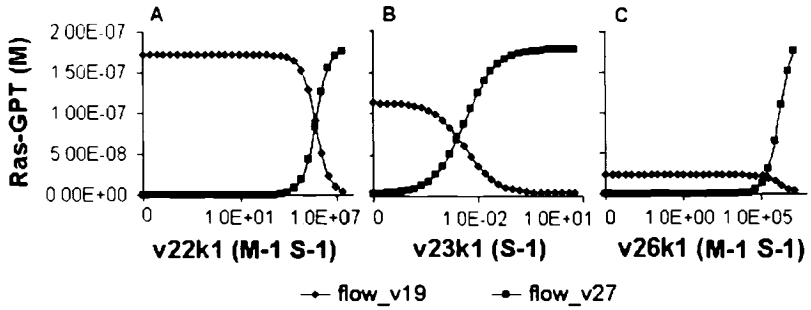

Figure 3.5. Relative contributions of the Shc-dependent and Shc-independent pathways to production of Ras-GTP at varied values of rate constants.

The y-axis represents the molecular flow to Ras-GTP. The simulation condition was the same as described in Figure 3.3, except the rate constants were scanned instead of initial Shc concentration. Rate constant values were selected, and the x-axis values were displayed logarithmically. A. rate constant k1 of reaction v22 was scanned; B. rate constant k1 of reaction v23 was scanned; C. rate constant k1 of reaction v26 was scanned. flow_v19 and flow_v27 represent the contribution of the Shc-independent and Shc-dependent pathways, respectively. The effects of cell surface and internalized receptors were combined.

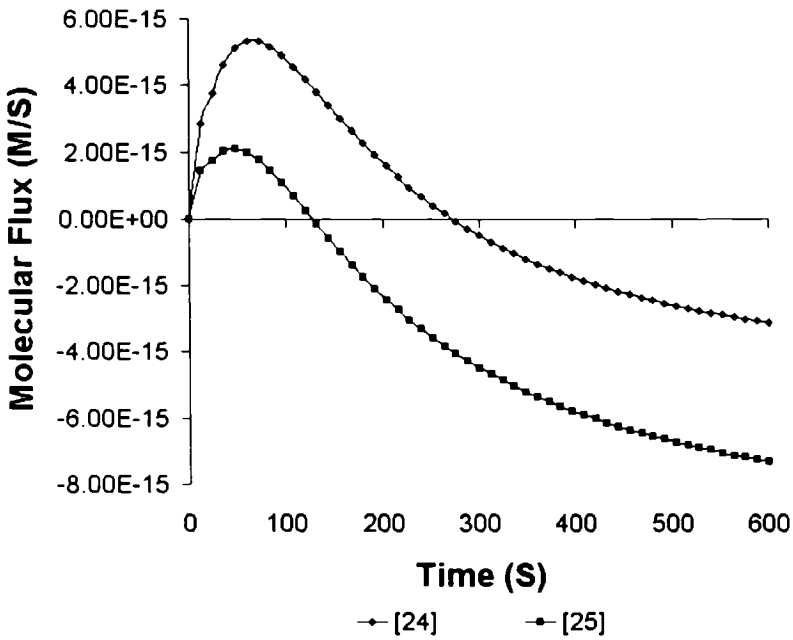

Figure 3.6. Time courses of the fluxes of reaction v24 (including v71) and v25 (including v72).
The simulation condition was the same as described in Figure 3.2 except fluxes for reactions v24 (and v71) and v25 (and v72) were traced. At the beginning of the simulation, the molecular flows were towards the Ras-GTP production. Then fluxes were reversed and these reactions negatively contributed to Ras-GTP production.

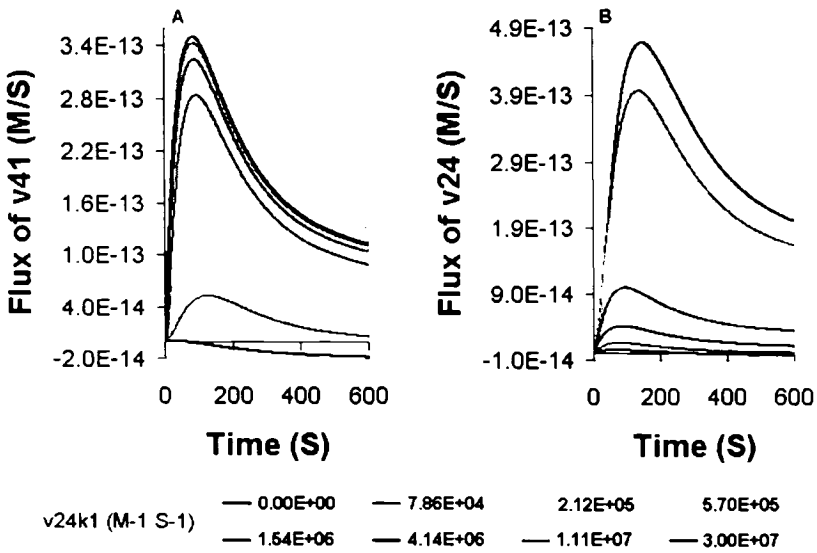

Figure 3.7. The level of the rate constant v41k1 determines the usage of two sub-pathways in the Shc-dependent pathway for producing Ras-GTP.
Time course of fluxes of reaction v24 and v25 (not shown) and v41 in 10 min at different values for rate constant k1 of reaction v41. Simulation condition was the same as described in Figure 3.5, except the v41k1 was scanned and the fluxes of the reactions v24 and v41 were traced. Note that at different v41k1 values, different sub-pathways could be used in the Shc-dependent pathway.

CONNECTING STATEMENT II.

Many cell signaling cascades are composed of alternative pathways (such as integrin and EGFR cascades reviewed in chapter 1). The hypothesis for the alternative, longer but dominant Shc-dependent pathway during EGFR activation of MAPK, based on the modeling inference in chapter 2, is the competence of this activation to the other receptor activated signaling cascades in which Shc is also involved. However, the existence of the alternative pathways is, in most cases, believed to provide the signaling transduction with robustness.

While alternative pathways are ubiquitous, different signaling cascades have different connections between the involved molecules, resulting in different topologies of the networks or the abstract graphs. Thus, a question was raised whether there are correlations between the network topologies and their robustness. If yes, is it possible to quantitatively assess the correlation. In chapter 4, a model for assessment of the robustness of small networks based on the network topologies was proposed, using the herein proposed concepts of pathnet, pathwome, the pathway score and pathnet score. The topological space for a fixed number of nodes was used to test the assessment.

CHAPTER IV. A NOVEL MODEL FOR ASSESSMENT OF ROBUSTNESS OF CELL SIGNALLING NETWORKS

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ABSTRACT

Decomposition of biological networks is important for their analysis. In this study, the concepts of pathnet and pathwome were proposed to decompose the cell signal transduction networks. Both terms deal with the direct and/or indirect interactions between pairs of molecules and were used to analyze the features of the signal transduction between them. As one usage of the concepts, the pathnet was quantified based on the features of the pathways constituting the pathwome. The quantities computed were the pathwome size (the number of the pathways in the pathwome) and pathnet score (PNS) (the sum of the pathway scores, PWSs). Using hypothetical networks, we showed that PNS represented the robustness of the pathnets. With a random artificial pathnet space, the relationship between PNS and node connectivity was revealed. Also, PNS seems to be a more accurate measurement of robustness than the connections of the nodes in the networks.

Key words: biological networks, robustness, pathnet, pathwome, pathway score, pathnet score

INTRODUCTION

Bio-organisms are complex systems. Physiological and pathological functions can be traced at the cellular and molecular levels with modern molecular and cellular biology methodologies. Biochemical, physical or electric changes in inner or outer environment are constantly sensed and consequently relevant molecular activities are induced or

inhibited in the cells. Although genes on chromosomes are the scripts for cell functions, the effects of genes on cell functions can only be implemented at the molecular interaction level such as protein-protein and protein-DNA interactions. The molecular interactions transducing signals and regulating the cell functions are compositions of the chains of biochemical reactions, which form signal transduction pathways. Accumulating experimental evidence supports the notion that signal transduction pathways can be linear or non-linear (divergent or converged). In fact, the pathways from one upstream molecule to a downstream molecule are often woven into a nonlinear subnet, which is a part of a larger network. Two other types of biological networks have been intensely studied. One is a metabolic network that is collections of metabolic pathways. The other is a genetic network, which deals with gene regulations. Regardless of the categorization, all three types of networks are common in that all can be detailed at the biochemical level. An important task for biological network analyses is the assessment of the output (change in other molecules or cellular status) after modification of the input (perturbation in one molecule). The quantitative analysis, with differentiation equations, of the effects of the perturbation in an upstream molecule on a downstream molecule is the most precise approach. However, this approach needs a complete set of the kinetic parameters (such as binding constants and rate constants) which are usually lacking at this stage, especially for signal transduction pathways. At the same time, several qualitative or semi-quantitative modeling approaches for analyses of signalling networks, metabolic networks or genetic networks have been proposed. Specifically, the concepts and techniques of Bayesian network (Sachs et al., 2002), Boolean network (Tatsuya et al., 2000), Probabilistic Boolean networks (Shmulevich et al., 2002), artificial neural network

(Vohradsky, 2001) and Petrinets (Hofestadt and Thelen, 1998) have been used. All of these approaches were attempted to reveal the causal relationship of the molecules. Meanwhile, another line of the studies on the biological networks has been focused on topological structures and their biological consequences. Interesting results from this line of research include scale-free (Jeong et al., 2000) and small-world (Fell and Wagner, 2000) properties of large-scale metabolic networks. One recent study revealed the biological importance of the scale-free property of protein networks based on the correlation between links proteins have, and their importance for cell survival (Jeong et al., 2001). However, this study failed to take into account the pathways in which the proteins were involved. Another study showed that biochemical networking contributed more to genetic buffering in metabolic pathways than did gene duplication, based on evolution of genes with or without redundant networks (Kitami and Nadeau, 2002). Although pathways were considered in that study by checking the number of the pathways in which a particular protein was involved, the result was not derived on the basis of network topology. In fact, no pathway and topology-based parameters have ever been proposed and evaluated for robustness of a network.

For dissecting the complex networks, we have previously proposed the concepts of pathnet and pathwome (Gong and Zhao, 2002). Both concepts cope with direct and/or indirect connections between a pair of molecules. A pathnet is defined as a subnet between a pair of molecules in the complete networks, including all the pathways/reaction steps used for transducing signals or materials from one molecule to another. Pathnet has its own topological structure, i.e., how the involved components are connected with each other. To capture the features of its structure, it is further dissected

into a set of different alternative pathways, and the collection of them is called the pathwome of the pathnet. For the length of each pathway and the size of the pathwome (number of pathways in a pathwome) are ready to be determined for a given pathnet, it is feasible to design an approach to quantitatively assess the topological structure for each pathnet.

The objective of the present study was to determine whether it was possible to infer the genetic buffering (robustness) of a causal relationship, i.e., the effect of perturbation of an upstream molecule on a downstream molecule in signal transduction pathnet, based on the network topological structure. Our results show that the pathnet score (PNS, see below for detail) can be used to reveal the resistance of signal transduction between molecules to random gene mutations, i.e., its robustness. Furthermore, this PNS-based assessment is in accordance with and more accurate than, the mean connectivity the nodes possess, a quantity having been used as a measurement of complex, large-scale networks.

METHODS AND RESULTS

Pathnet Space

Random artificial and abstract networks were used to illustrate our concepts and test our hypothesis. The pathnets composed of six proteins were considered. They are the beginning of the signaling cascade (assigned A), the end of the cascade (assigned Y), and four intermediate proteins (B, C, D and E) which transduce the signal from A to Y. The pathnet from A to Y could have different possible topological structures, depending on how these proteins are connected or interacting with each other. Figure 4.1 shows two

examples of the structures with different complexity. The arrows represent the directional interactions, which can be best interpreted as the flow of signal. The topological space, or pathnet space, for these assumed components of the network were constructed to include all possible structures, by randomly changing the arrow status (existence and direction) between any two nodes. For simplicity, all pathnets in the space satisfy the following conditions. 1) All interactions are directed, from one protein (output) to another (input). 2) There is output only, and at least one output from A. 3) There is input only, and at least one input to Y. 4) Each of B, C, D and E is involved in at least one pathway. 5) There is no loop (self-interaction) and cycle (pathway returning to a node itself) in the pathnet. A computer program for graph generation, written with PHP (codes are available on request), resulted in a big topological space (over 43000 graphs in the space). However, there was a considerable amount of redundancy in the topological structures (the graph isomorphism, see Figure 4.2 for examples). The isomorphic graphs possess the identical structural features, thus they were avoided in the pathnet space for this study. This was fulfilled with a program implementing a recursive split algorithm described elsewhere (http://links.math.rpi.edu/devmodules/graph_isomorphism/), with a minor modification. The modification was that during splitting of the graphs, both input and output for each protein were considered. For graphs a and b in Figure 4.2, the starting status was: A:01, B:12, C:11, D:22, E:11, Y:20; and A:01, B:11, C:12, D:11, E:22, Y:20, respectively. The first digit following each node represents the input number, the second one the output number. The final split string for both graphs a and b is 000000010000(1) 000000000110(1) 000000100001(1) 100001000001(1) 001000000010(1) 000110100100(1) (number of the nodes possessing the same split value is in the

parenthesis following each value). All the pathnets with the identical final split string are considered isomorphic, thus only one of them (no matter which one) appears in the final pathnet space. Implementation of the algorithm resulted in a much smaller final pathnet space containing over 2000 pathnets instead of over 43000 with isomorphism.

Pathwome: Dissecting the Pathnet

Pathwome (collection of pathways in a pathnet) for each pathnet in the space was searched. For these directed graphs, a pathway was defined as a linear sequence of the nodes, starting from A and ending at Y, connected by directed arrows with all the arrows pointing to the end of the pathway (For an example, see Figure 4.1a). Several algorithms had previously been implemented to search pathways (Jeong, 2000; Kanehisa, 2000; Fukuda and Takagi, 2001; Takai-Igarashi and Kaminuma, 1999; Helden et al., 2000). All of them are based on width-first algorithm that has been adopted for this study. Generally, starting with a binary interactions in which node A has an output as an input to another node (level 1), the binary interactions for 1 level further were searched and recorded, and each new pathway (when an input to Y is encountered) was added to the pathwome table. The procedures were repeated until no more pathways were found. For the pathnets in the established space, the number of pathways in a pathnet ranged from 1 to 16. For each pathwome, the number of pathways from A to Y, called the pathwome size, was counted. If more than one pathway exist in the pathwome, the pathnet is redundant. The distribution of the pathnets with different sizes was shown in Figure 4.3.

Pathway Distance (PWD), Pathway Score (PWS) and Pathnet Score (PNS): Quantifying the Pathnet Structural Features

While the pathnets had been dissected into pathways and each pathnet has its own pathwome, it is possible to use the pathways in the pathwome for extracting topological features for the pathnet quantitatively. One feature of the pathway that can be described quantitatively based solely on its structure is its length. Therefore pathway distance (PWD) is defined as the number of steps between the head (A) and tail (Y) molecules of a pathway. Pathway score (PWS) is simply calculated as 1/PWD. The longer (more steps between the head and tail molecules) a pathway, the less its PWS is (see Figure 4.4). Suppose the probability for each gene to mutate, and thus be inactivated, is r ($0<r<1$) and the number of the intermediate proteins (genes) between A and Y is n (Note, PWD=n+1), then the probability for the pathway to be blocked is that at least one of the intermediate gene is mutated, $1-(1-r)^n$, because the mutation in any of these proteins blocks the pathway (Note, here the mutation of A or Y themselves are not considered). Thus, the less the PWS, the more sensitive the pathway is to random gene mutation.

To capture the feature of pathnet quantitatively from its pathwome, a direct way is to add up the PWSs of all its pathways and assign this value for the pathnet as pathnet score (PNS). In another word, PNS is calculated as ΣPWS for all pathways in the pathwome. An intuitive is, like PWS, the larger the PNS, the more resistant the pathnet is to random gene mutation. This is illustrated in Figure 4.5, in which all the pathways have the equal PWS (0.5), and the larger the pathnet size is, the larger its PNS. For the first pathnet in Figure 4.5, inactivation of B results in blocking of signalling from A to Y. For the last pathnet, the probability for the pathnet to be blocked is the product of all the

probabilities for B, C, D and E to be mutated. Generally, the blocking probability for this type of simple pathnet is r^n, where r and n are probabilities of mutation for each gene and the intermediate gene number in the pathnet, respectively. It needs to be emphasized that different pathnet wiring needs different calculation of the probability for pathnet blockage.

The above shown relationship between PNS and the pathnet robustness also holds for more complex pathnets that contain the same set of molecules but have different topological structures. As illustrated in Figure 4.6, the three pathnets possess the same set of molecules and same pathwome size (2), but different structures, and they could have the equal PNS (for the first and second pathnet, PNS=0.85), or different PNS (for the third pathnet, PNS=1). As described in the legend, their different resistance to gene mutation also relates to their topological structures and PNS.

The Relationship between PNS and Pathwome Size

The regression of PNS to pathwome size for all the pathnets in the pathnet space revealed the linear correlation between them (Figure 4.7), implying that both of them could be of the similar value as measurement of the robustness of the pathnet.

PNS and Connection Number of the Proteins

The correlation between the connections of the proteins and the PNS was further examined. Each intermediate protein could have input and output connections from and to other protein(s), and the numbers of them were counted separately. The average numbers of input and output connections for a pathnet were then calculated. As shown in

Figure 4.8, both pathwome size and PNS increased with the average number of input connections of the proteins in the pathnet. Similar relationships between pathwome size and PNS, and the average number of output connections of the proteins were also established (data not shown). In fact, this phenomenon could be explained as, the more connections a node has, the more pathways it is involved in (or, the more new pathways it creates), and the more it contributes to PNS. Interestingly, when input connection was plotted to pathnet size, a three-stage curve was obtained (Figure 4.9), where the pathnets with equal average input/output connections could possess different pathwome size.

DISCUSSION

To study the network as a whole could provide some important characteristics of the network such as scale-free organization and help understand the wiring of the networks. However, the ultimate goal for biological network analyses should be the inference of the consequences of molecular perturbation at the level of molecules, or more importantly at the level of cells and tissues. As experimental results accumulate for the biological networks, it is necessary to dissect the whole network into smaller and more manipulatable subnets. This is not only for easiness of analyses, but also for better understanding the designing principle of the networks. In fact, the decomposition of the networks is drawing attention from more and more researchers recently. There are two main streams concerning the decomposition methodology. One is motif/module-based. For example, Lee et al. (2002) showed the identification of several network motifs from the yeast network of regulator-gene interactions. In addition, Ravasz et al. (2002) demonstrated that the metabolic networks of 43 distinct organisms were organized into

many modules that combine in a hierarchical manner into larger, less cohesive units. However, it is worth to note that till now, the identified motifs/modules are in the sense of abstract structures or graph topology only, but not in the sense of reusability. Another line of the decomposition methodology is pathway/flux-based, or network-based pathway definitions, as described by Palsson et al. (2003). Two main concepts in this category are elementary flux mode and extreme pathway. A review and detailed comparison of the two concepts can be found elsewhere (Klamt and Stelling, 2003). The latter decomposition approach is intended for the causal relationships of the molecules; thus it is more potential to reveal the correlation between network structures and functions, for the cell functions are actually the status of the molecule profiles which depends on the dynamics of the pathways.

In this study, we proposed the concepts of pathnet and pathwome, which are decompositions of the network at different levels. The concept of pathwome is similar to previously proposed network-based pathway definitions. In fact, the pathways in the pathwome are similar to the element flux modes or extreme pathways. Although the element flux modes and extreme pathways are also specified for pairs of molecules, they are less organized in the sense that they are from the whole network directly and different element flux modes/extreme pathways for the same pair of substrate/product are not grouped as an entity. Nor the features of the pathways or groups of pathways as a whole are characterized. In contrast, the concept of pathnet proposed in this study is aimed to hold the subnet between a pair of molecules, which is decomposed from the whole network; and the concept pathwome is aimed to hold the pathways decomposed from a pathnet. Thus both pathnet and pathwome together provide a bridge between the whole

network and the single individual pathways and individual molecular interactions as well. This provides potential means for constructing subnets and networks based on a huge number of individual interactions between bio-molecules held in large-scale interaction databases, such as BIND (Bader et al., 2003). Further, our decomposition leads to the quantitative characterization of their structural features, making it feasible to assess the pathnet robustness quantitatively, as illustrated in this study.

In general, network robustness reflects the networks' resistance to perturbations, including changes in the environments, in kinetic parameters and in topological structures/connectivity resulted from node dropping from the network (when a gene is mutated for biological networks). The assessment of the robustness/reliability for the communication networks has been called the all-terminal network reliability problem (or two-terminal reliability when the connection of two particular nodes is considered). This problem has been under extensive studies for a long time, because of its applications in the design of communication networks (see Karger, 1999 and the references therein). For this problem was identified as NP-hard (at least as hard as any NP-problem, where NP-problem is verifiable in polynomial time by a nondeterministic Turing machine) (Provan and Ball, 1983), a great deal of research has been devoted to estimate the failure probability. Although in the situation of the biological networks, the assessment of the reliability seems similar to the above problem, they are different in at least three ways. First, in the communication networks, any pair of nodes could be assigned an edge, thus the network could be a complete graph. Therefore, the all-terminal network reliability problem has been considered based on this assumption. However, this assumption is not true for biological networks, in which the connections between nodes are very specific,

with one node connects to a specific set of neighbor node(s). Second, in all-terminal network reliability problem, usually the failures in edges are considered. However, the result of gene mutation is the failure of a node in the bio-networks. This could cause the failure of several edges. While a randomized fully polynomial time approximation scheme was proposed for the communication networks (Karger, 1999), the author was aware of that "no results on the structure of minimum vertex cuts that could lead to the same results as we have derived here for edge cuts". Third, a communication network is undirected, communication on each edge needs to be two-ways. The above author also realized that "the same obstacle arises in directed graphs" (as in the vertex failures). In contrast, directed edges exist in biological networks. The directions represent the signal or material flow. Furthermore, the biological networks are usually in much smaller-scale (hundreds or thousands of nodes, or approximately tens of thousands at the upper bound if all genes in a big genome, such as human genome, are taken into account) than the communication networks (millions or more of nodes). Thus it is necessary and also possible to propose a novel protocol for robustness assessment of the biological networks. The new protocol, such as that proposed in this study, should be more precise and take more topological details into consideration.

The problem of topology-robustness relationship has recently been tackled in the field of military and civilian communications (Dekker and Colbert, 2004). With the graph-theoretic concepts of connectivity, it was revealed that node connectivity (the smallest number of nodes whose removal results in a disconnected or single-node graph) is the most useful measure of the robustness of network topologies (Dekker and Colbert, 2004). As the node connectivity used in our study is the number of links the nodes

possess, the result is not comparable to that by Dekker and Colbert (2004). Again, our approach is focused on the detailed topological structures of the network, in which the networks are analytically dissected and PNS is then calculated from the dissected pathwome. This makes PNS significantly different from the connectivity, which can be easily derived computationally from the connection matrix directly. We argue here that our topology and pathway-based measurement is more solid and informative. However, a space of small pathnets has been used in this study, and validation of our results with larger pathnets of different sizes is also NP-hard (roughly, the number of the pathnet structures to be checked, given n intermediate nodes, is $2^n \times 3^{n(n-1)/2} \times 2^n$). Similar algorithms of estimation used for communication networks should be devised for larger-scale pathnets in the future, for validating our results and for further application in real biological networks which are expected to appear in an accelerated speed.

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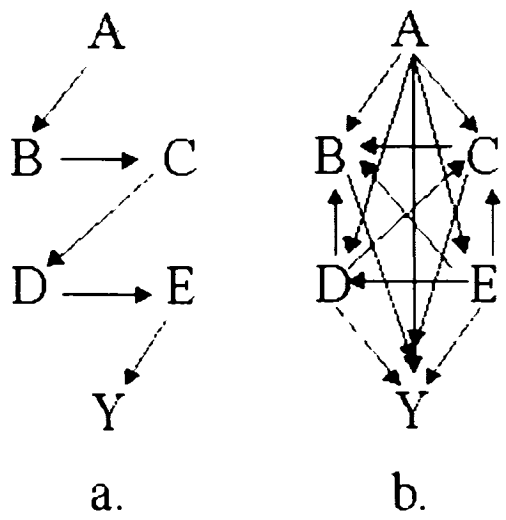

Figure 4.1. Different topological structures of the pathnets for same amount of molecules. Panel a shows the simplest structure in which only one pathway exists between A and Y. Panel b shows a much more complex structure in which 16 pathways compose the pathwome (the shortest pathway is A→Y, and the longest is A→D(or E)→C→B→Y).

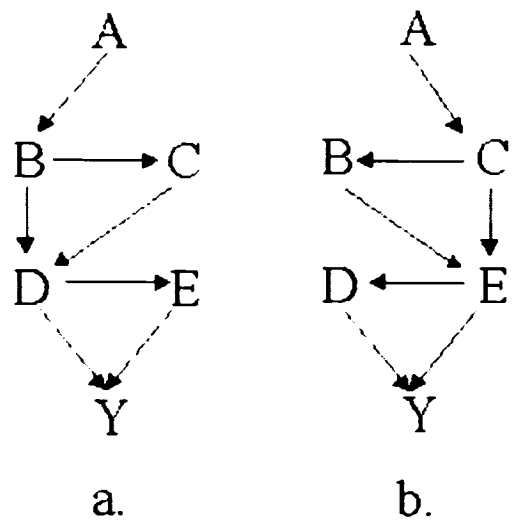

Figure 4.2. Two isomorphic graphs (pathnet). They are identical graphically. By exchanging B and C, and D and E in a, another graph which is identical to b can be obtained.

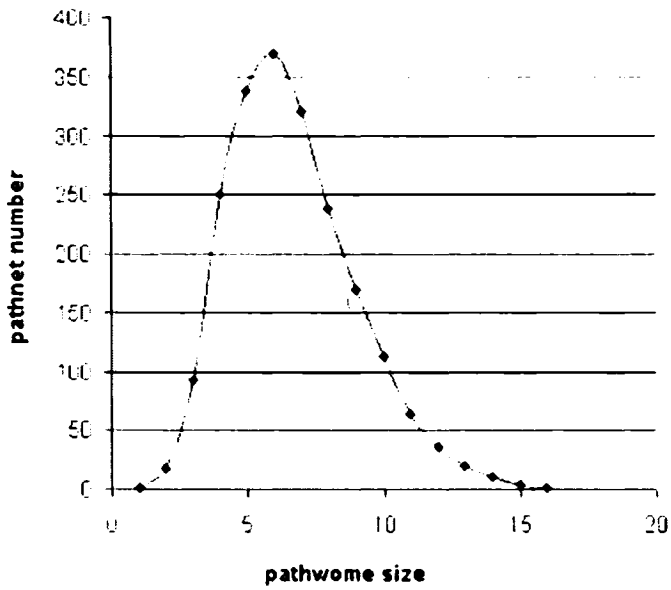

Figure 4.3. Distribution of pathnets with different sizes of pathwome. The random artificial pathnet space was constructed and graphical isomorphism was eliminated as described in the text.

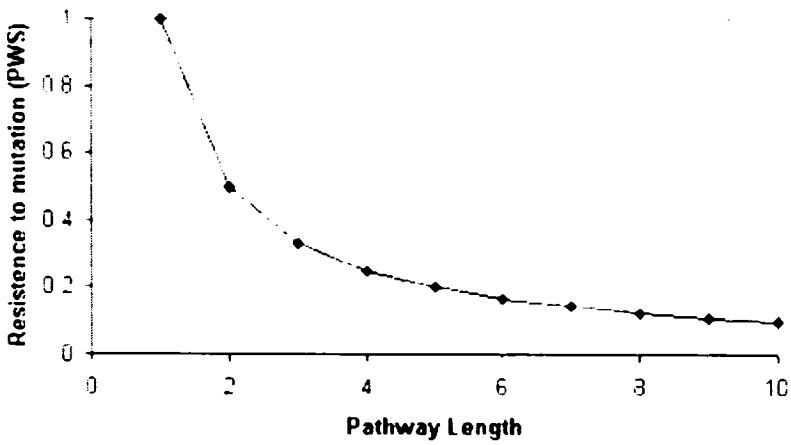

Figure 4.4. For the pathways with different length, PWS is decreased with the function PWS=1/PWD.

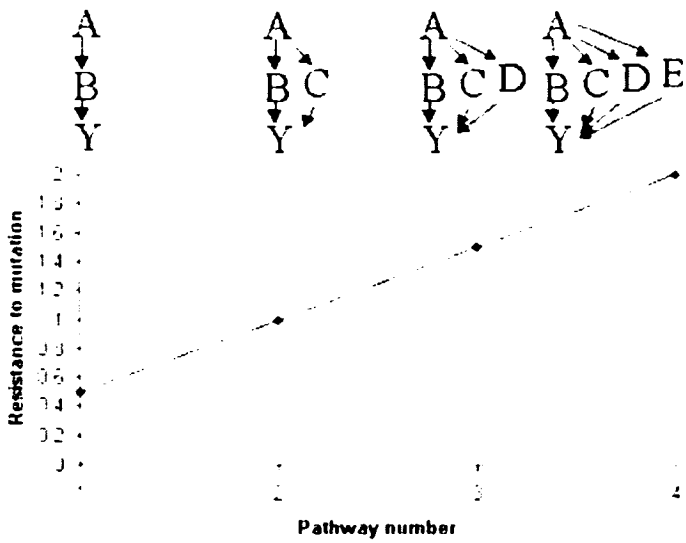

Figure 4.5. For the pathnet with different sizes and all the pathways have the same length (2), PNS increases with the function PNS=0.5*pathnet size. Note that the larger the PNS is, the more genes needed to be mutated to block the signaling from A to Y.

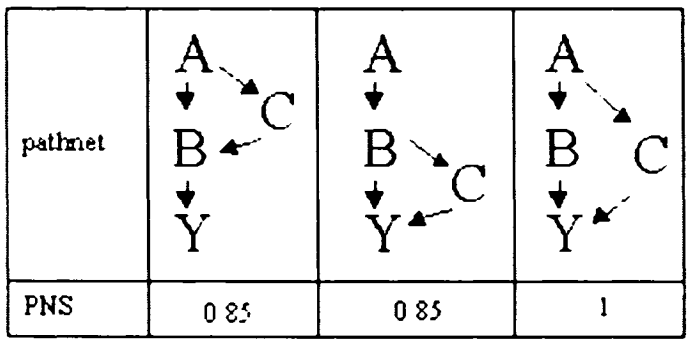

Figure 4.6. For pathnets with same number (2) of intermediate molecules (B and C) and same number of pathways (2) but different topological structure, PNS could be same or different. Note that in the pathnet with lower PNS (0.85), mutation of only one gene (B) is enough to block the signal transduction from A to Y; in the third pathnet with PNS value 1, single gene mutation (B or C) is not enough to block the signaling.

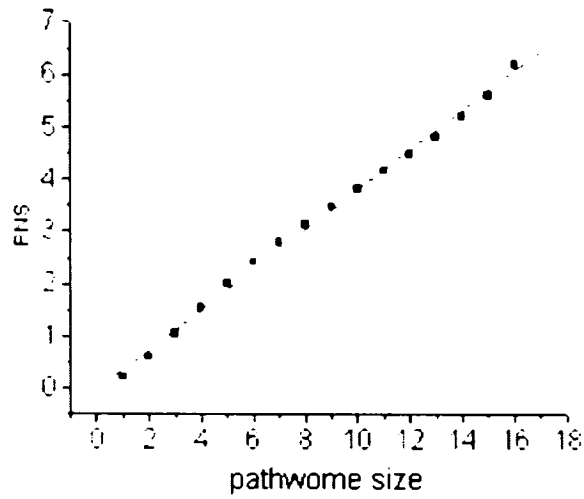

Figure 4.7. PNS values increase with pathwome size. Regression revealed a linear correlation following the equation: PNS= -0.032 + 0.383*pathwome size.

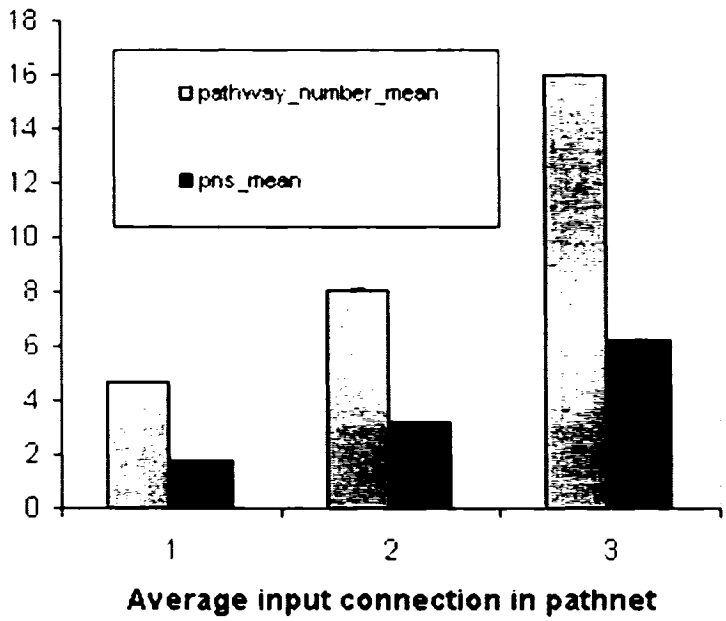

Figure 4.8. Increases in pathwome size and PNS value with the mean input connectivity of the proteins in the pathnet.

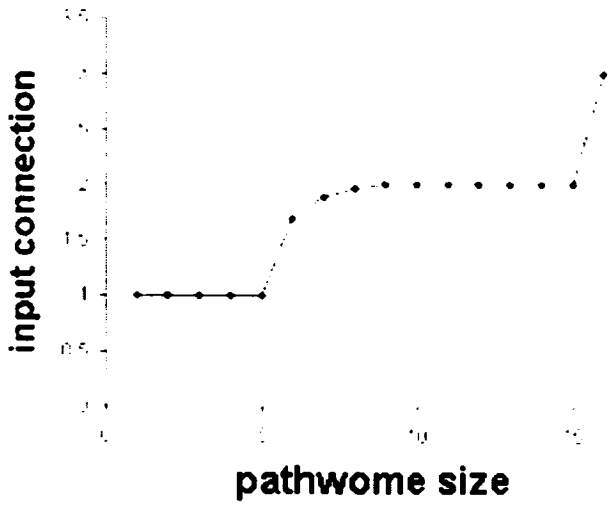

Figure 4.9. Correlation between pathwome size and node connectivity.

CHAPTER V. CONCLUSIONS AND GENERAL DISCUSSION

Epithelial cells need ECM to attach on, survive and subsequently proliferate as the consequence of growth factor stimulation. In this study, degradation of an ECM component, fibronectin by tPA was shown to induce apoptosis of bovine mammary gland epithelial cells using the cell lines transformed with active tPA genes. The direct degradation of fibronectin by tPA was supported by the fact that the culture medium of the cell lines used in this study lacked the plasminogen/plasmin and uPA activities. The pro-apoptotic effect of this degradation could involve two mechanisms, cell detachment-induced cell death (anoikis), and fibronectin fragment-induced cell death. The in vivo situations were mimicked by supplement of plsminogen to the cell cultures, which induced a large-scale cell detachment. Thus, the in vivo tPA could induce cell loss in one more way: induction of plasmin activity via activation of plasminogen. As reviewed previously, apoptosis could be induced by detachment via two pathways, increase of death ligands or inhibition of anti-apoptotic proteins. The detailed pathways in MAC-T cells used in this study need to be examined in future research. The exact mechanism for fibronectin fratments-induced apoptosis remains unclear. Furthermore, because of the multi-substrates of plasmin, more pathways are involved in apoptosis induction in vivo. While some of these have been partially identified (such as TNF-alpha releasing), others are still unknown. Much more research is still needed to delineate all the pathways.

Cell detachment by degradation of ECM components can not only induce apoptosis, but also deprive the cells of proliferation stimulated by growth factors.

Although no model for integration of ECM receptor signaling and growth factor receptor signaling is available, a relatively mature and quantitative model of EGFR stimulation of MAPK, an important mediator for cell proliferation, had been proposed. In this model, MAPK activation by EGFR was through two pathways, the Shc-dependent and the Shc-independent. The relative contributions of these two pathways were assessed in this study. A protocol for such an assessment has been proposed. It is based on the reaction specific quantity, the flux and accomplished by the concentration and parameter scanning approaches as provided by the biochemical simulator, GEPASI. It was revealed that the Shc-dependent pathway was both redundant and dominant. The redundancy of the Shc-dependent pathway is not due to the cross talk with the Shc-independent pathway, rather due to the flow of the majority of the common precursor of the two pathways to this pathway. The key steps for the dominance have been identified. Our conclusion is that cells may prefer a longer pathway to a shorter alternative one during signal transduction. Considering that Shc is a common mediator for multiple receptor-stimulated signaling cascades, it was hypothesized that the dominance of the Shc-dependent pathway was important for EGFR to compete with other receptors. Validation of this hypothesis can only be possible when a larger-scale model, which is composed of signal transduction pathways induced by different cell surface receptors, is available.

The possibility that the Shc-dependent and -independent pathways co-exist in order to provide robustness for EGFR signaling cascade was ruled out based on its topological structure. However, as the topologies of signaling cascades could be different from one to another, it is possible that in some redundant cascades the alternative pathways are expected to increase the robustness of the cell signaling. Thus it is still

important to make a quantitative assessment of their robustness based on the topologies. To this end, two concepts were proposed to decompose the signaling networks, the pathnet and the pathwome. These concepts serve as the bridge between network itself and its underlying individual pathways. The merit of this decomposition becomes clear when the features of the pathnet are quantitatively characterized based on the pathway length and pathwome size. Using several abstract cascades with simple topologies, it has been demonstrated that the pathnet score (PNS) is correlated with the robustness of the pathnet. Using a simulated pathnet space, it has been shown that PNS is actually linearly correlated with pathwome size. Thus these two quantities are of similar value as the index for pathnet robustness. The correlation between these quantities and the molecule connections, once used to characterize the network structure, was also investigated. It is concluded that pathwome size, and the PNS as well, are more accurate assessment of the robustness. With the expanding of the researches on bio-molecular networks, the broader usage of the concepts proposed in this study can be expected, such as assessment of the evolution of signal transduction cascades, or mechanisms behind the difference between different types of cells to resist malicious environments.

With rapid accumulation of the experimental data, the management and analysis of the cell signaling cascades are getting beyond the capability of the human brains. The future tasks for us are to establish the database systems for information storage (Bader et al., 2003) and the algorithms for data mining.

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