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STUDY ON SIGNAL TRANSDUCTION OF CHICKEN PROTO-ONCOGENE C-*ROS*

BY

QINGHUA XIONG

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, the City University of New York

1995

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This manuscript has been read and accepted by the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

STUDY ON SIGNAL TRANSDUCTION OF CHICKEN PROTO-ONCOGENE C-*ROS*

by

Qinghua Xiong

Advisor: Lu-Hai Wang, Ph.D., Professor

Two chimeric receptors, ER1 and ER2, were constructed. ER1 has the extracellular (EC) and transmembrane (TM) domains from EGFR and the cytoplasmic domain from c-*ros*, whereas ER2 is identical to ER1 except for its TM domain derived form c-*ros*. Both chimeras promote colony formation with almost equal efficiency. Surprisingly, ER1 inhibits cell growth, while ER2 stimulates it. Flow cytometry analysis reveals that all phases of the cell cycle in ER1 cells are elongated in response to EGF treatment whereas the G1 phase is greatly shortened in ER2 cells.

Comparison of the signaling pathways triggered by the two chimeras reveals several differences: Several early signaling proteins are activated or phosphorylated to higher extent in ER1 cells in response to EGF; ER1 is less efficiently internalized and remains tyrosine phosphorylated for a longer time than ER2; However, phosphorylation of the 66 kD Shc, activation of MAPK and induction of c-*fos* and c-*jun* are either to a less extent or for a shorter time in ER1 cells; Cellular protein phosphorylation patterns are also different in ER1 and ER2 cells in response to EGF.

These observations suggest that over-stimulation of ER1, due to its higher EGF binding and

retarded internalization, apparently triggers a inhibitory response which counteracts the over-stimulated receptor resulting in growth inhibition.

I also tried to express chicken c-*ros*, its variants, and v-*ros* in different cells. The stable lines expressing c-*ros* and its kinase inactive mutant can not be established in any cell lines. The c-*ros* variant, ppros, that has a large EC domain deletion can be expressed in MDCK and CV-1 cells. The ppros and ppros-slt which has a three amino acids insertion in the TM domain of ppros can be expressed in CEF at low levels but not transforming. The v-*ros* can be expressed in NIH3T3 cells and is weakly transforming.

As an independent study, I and members of this lab cloned the chicken PTPase γ cDNA, which is 86.7% identical to human PTPase γ at the amino acid level. PTPase γ could be expressed as an enzymatically active form, but no detectable effects on cell growth and transformation were observed.

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ABSTRACT

In order to study the signaling function of c-ros, two chimeric EGFR-ros receptors, ER1 and ER2, which differ in their TM domains have been constructed. ER1 has its extracellular and TM domains from EGFR and cytoplasmic domain from c-ros, ER2 is identical to ER1 except for its TM domain that is derived from c-*ros*. ER1 and ER2 chimeras expressed in NIH3T3 cells are glycosylated and transported to the cells surface equivalently but bind EGF with different affinities according to their TM domains.

Both chimeras promote colony formation with almost equal efficiency in an EGF dependent manner, although ER2 has a high basal level activity. Surprisingly, ER1 inhibits cell growth, whereas ER2 stimulates it. The inhibitory effect of ER1 can not be overcome by the increase of serum concentration or the addition of IGF-1. Flow cytometry analyses indicate that the all phases of the cell cycle of ER1 cells are elongated upon EGF stimulation, however, the G1 phase of ER2 cells is remarkably shortened.

Comparison of the signaling pathways triggered by the two chimeras has revealed several differences: Several early signaling proteins are activated of phosphorylated to a higher extent in ER1 cells in response to EGF; ER1 is less efficiently internalized and remains tyrosine phosphorylated for a longer time than ER2; However, phosphorylation of

the 66 kD Shc, activation of MAPK and induction of c-*jun* and c-*fos* are either to a less extent or for a shorter time in ER1 cells; Cellular protein phosphorylation patterns are also different in ER1 and ER2 cells in response to EGF.

These observations suggest that over-stimulation of ER1, due to its higher EGF binding and retarded internalization, apparently triggers an inhibitory response which counteracts the over-stimulated receptor resulting in growth inhibition. Since the two chimeras expressing in the same cell type at similar levels have opposite effects on cell growth. Thus they provide a model system to further dissect the mechanisms by which a RPTK stimulates or inhibits cell growth.

INTRODUCTION

FUNCTION OF RECEPTOR PROTEIN TYROSINE KINASES (RPTKS)

Cell growth, differentiation and maintenance of homeostasis in multicellular organisms are coordinated events. This involves intercellular communication to respond to developmental and environmental signals. Receptor protein tyrosine kinases (RPTKs) play a key role in transmitting such signals (Yarden and Schlessinger, 1988). Currently more then 40 distinct RPTKs are known (Hunter et al, 1992). On the basis of sequence similarity and structural characteristics, these RPTKs can be divided into several families. They are epidermal growth factor receptor (EGFR) family which also include *erb*B2/Neu, *erb*B3, *erb*B4; Platelet-derived growth factor receptor (PDGFR) family which also include colony stimulating factor receptor (CSFR), c-*kit*, FLK 1, 2, 3, 4; Fibroblast growth factor receptor (FGFR) family which include FGFR 1, 2, 3, 4; Nerve growth factor receptor (NGFR) family which include Trk A, B, C; EPH family which include EPH, ELK, ECK, cek 4, 5; Insulin receptor (IR) family which include IR, insulin-like growth factor receptor (IGFR), IR-related receptor (IRR); *ret* family; And *ros* family. *ros* may also be considered as a member of the IR family since it shares great sequence homology with members of the family.

All of the RPTKs share a common structure that is an EC domain that binds a ligand, a TM domain, and a cytoplasmic domain harboring a protein tyrosine kinase (PTK) domain and substrate interaction regions. Binding of a ligand to its receptor leads to

dimerization and activation of the RPTK, resulting in interaction with and phosphorylation of cellular proteins that are involved in transmitting signals. These proteins are often called signaling proteins or transducers. These signaling proteins then elicit a chain of reaction called signal transduction or signaling pathway by interaction with and regulation of each other in a sequential order to generate diverse biological effects (Ullrich and Schlessinger, 1990, Liu and Wang, 1994).

Overwhelming evidence demonstrates that the majority of RPTKs are involved in cell growth control. The addition of growth factors such as EGF, PDGF, IGF, and insulin to tissue culture usually leads to increased DNA synthesis through activation of their receptors. Applying growth factors to the wound accelerates the healing, providing in vivo evidence for their growth control activities. The importance of RPTKs in cell growth control is also demonstrated by genetic manipulation of these genes encoding growth factors and their receptors. Mice carrying null mutation of the genes encoding IGF-1 or IGF-2 exhibit a growth deficiency, severe organ hypoplasia, and developmental delay (DeChiara et al, 1990, Liu et al 1993). Mice carrying the null mutation of IGFR gene exhibit more severe growth deficiency than those carrying null mutation of IGF-1 or IGF-2 genes and invariably die at birth of respiratory failure (Liu et al, 1993).

The importance of the cell growth regulation by RPTKs is also exemplified by the finding that more than a dozen of oncogenes have been documented to be altered RPTKs (Liu and Wang, 1994). Structural and functional alterations of RPTKs invariably lead to subversion of the normal regulation of cell growth. Truncation, internal deletion, point mutation, and gene amplification have been demonstrated to be common mechanisms for

constitutive activation of RPTKs, which consequently lead to uncontrolled cell growth. For example, *erb*B-2 amplification is found in some breast cancer cells and correlates with poor prognosis (Slaman et al. 1987). Besides amplification, a point mutation was found to be responsible for oncogenic activation of *neu*, a carcinogen-induced oncogene found in rat neurofibroblastoma (Drebin et al. 1984). The oncogenic Trk is originally isolated from a human colon carcinoma (Martin-Zanca et al 1986). This oncogene is generated by somatic rearrangement that results in fusion of the cytoplasmic and TM domains of Trk with a truncated non-muscle tropomyosin molecule (Martin-Zanca et al. 1986).

The mitogenic role of RPTKs may represent only a limited aspect of their normal spectrum of physiological activities such as development, cell differentiation, and morphogenesis. Evidence in support of this notion is mainly from genetic studies. Studies of Caenorhabditis elegans vulval development have shown that the *let*-23 (EGFR homolog) and *let*-60 (*ras* homolog) are essential for normal vulval formation (Aroian et al. 1990, Han et al 1990). The Drosophila EGFR homolog (DER) is also essential for embryonic development as a homozygous DER mutation is lethal (Price et al, Scheter et al, 1989). The *sevenless* (*sev*) gene product of Drosophila, which has no growth stimulatory effect at all, is required for the neuronal differentiation of one particular photoreceptor, the R7 cell, of the compound eyes. Further genetic studies have demonstrated that several gene products, including downstream receptor kinase (DRK), son of sev (*sos*), *ras*, and *rolled* (MAPK homolog) are also required for normal R7 cell differentiation and function downstream of *sev* (Bonfini et al 1992, Fortini et al 1992, Simon et al 1993, Brunner et al 1994). The Trk family RPTKs are the functional receptors for NGF family of neurotrophins,

targeted disruptions of Trk A, B, C in mice result in missing of certain population of neurons, respectively (Smeyne et al 1994, Klein et al 1993). C-kit is important in normal development of mice since mutation causes an impairment of the proliferation and/or migration of primordial germ cells and melanoblast, and deficiency of hematopoietic cells for multiple lineages (Chabot et al 1989, Flanagan et al 1990). vEGF and its receptor, FLK1, have been shown to be the major regulators of angiogenesis (Millauer et al 1993).

In vitro studies of PC12 cells suggest that NGF is not required for survival in the presence of serum but for differentiation since it can induce neurite outgrowth. The erbB2/Neu receptor can induce phenotypic differentiation of cultured human breast cancer cells, including altered morphology, synthesis of milk components, and growth inhibition upon stimulation with its ligand, Neu differentiation factor (NDF) (Peles et al 1992). This differentiation effect may be cell type and context dependent.

SIGNAL TRANSDUCTION

LIGAND INDUCED DIMERIZATION AND INTERNALIZATION OF RPTKS

The EC domain of a RPTK typically contains one or several copies of immunoglobulin-like, fibronectin type III-like, EGFR-like, cysteine-rich, or other domains. These domains are known to function in protein-protein interactions. For single chain RPTKs, it has been well demonstrated that, upon ligand binding, receptors undergo dimerization. Dimerization of a RPTK is followed by receptor autophosphorylation which mainly occurs by one receptor molecule phosphorylating the other in the dimer. IR family that normally exist as a heterotetramer containing two α and two β subunits don't form dimer. Binding of ligands to these receptors is thought to trigger a conformational change resulting in kinase activation.

Different strategies are utilized to achieve dimerization of a RPTK (Heldin et al 1995). Several of the ligands for RPTKs are themselves dimeric peptides, which thus contain two identical receptor-binding epitopes. Examples include PDGF and CSF-1, which are disulfide-bonded dimers (Li et al 1991), and stem cell factor (SCF) which is a non-covalent linked dimer. These ligands bind to two receptors simultaneously. RPTKs whose ligands are monomeric apply a different mechanism for dimerization. It is believed that, as exemplified by EGFR, ligand binding provides an allosteric regulatory signal that results in a conformational change and co-incidentally couples with kinase activation (Yarden and Schlessinger, 1987, Cochet et al 1988, Canals et al 1992). Interestingly, EGF which has an apparent monomeric configuration, however, has been shown to be able to bind

simultaneously to two receptor molecules (Lemmon and Schlessinger, 1994). RPTKs whose ligands are membrane proteins also undergo dimerization. NDF, the ligand of erbB2/Neu, is a transmembrane protein which can still induce dimerization of erbB2/Neu (Peles et al 1992).

Ligands for EPH family have a unique property. These ligands are unable to stimulate their receptors as conventional soluble factors. However, they do function when presented in membrane-bound form. Membrane attachment may serve to facilitate ligand dimerization or aggregation, thus leading to dimerization of their receptors. In support of this hypothesis, the addition of the antibody that aggregates the ligand activates the receptor (Davis et al 1992).

Another variation on the theme is FGFR. It is known that heparin is required for acidic FGF (aFGF) stimulation of biological responses, yet it doesn't bind to FGFR. However, aFGF forms 1:1 complex with the soluble EC domain of FGFR. It was recently observed that heparin could bind to many molecules of aFGF (Spivak-Kroizman et al 1994). The resulting aFGF-heparin complex would bind to multiple receptor molecules, leading to receptor aggregation and kinase activation.

Heterodimerization between different RPTKs has been observed. The emerging evidence suggests that this heterodimerization may have biological significance. In the case of the PDGFR family, the different isoforms of PDGF induce different dimeric forms of the receptors. PDGF has two different subunits A and B. Combination of the two subunits give rise to three dimeric isoforms, AA, AB, BB, which have different binding affinity for PDGFR subunit α and β. All three different isoforms of dimers among those

subunits have been detected. they are αα, αβ, ββ dimers (Heldin et al 1989, Kanaskaraj et al 1991, Seifert et al 1989). Differences in the signals transduced via these dimers have been observed.

Heterodimerization between EGFR family represents another example. Numerous observations have demonstrated that a critical level of over-expression of either EGFR or erbB2/Neu is necessary to achieve a significant growth advantage and transformation in tissue culture (King et al 1988, Kokai et al 1988). NIH3T3 cells that express Neu alone at a moderately high level (10^5/cell) are not transformed unless EGFR is co-expressed at an equivalent level (Hung et al 1988, Kokai et al 1989). Down-regulation of EGFR or Neu from the cell surface by anti-receptor antibody treatment reverses the transformed phenotype (Wada et al 1990). These results suggest that the two RPTKs can synergistically interact with each other leading to cellular transformation. This synergistic effect could be explained by the finding that the two receptors form heterodimer upon EGF treatment (Wada et al 1990). The heterodimeric complexes between erbB2 and erbB3, or erbB4 have also been detected (Peles et al 1993, Plowman et al 1993, Sliwkowski et al 1994). Moreover, the presence of erbB3 or erbB4 is necessary for high affinity binding and signal transduction through erbB2 to occur.

Heterodimerization in the PDGFR and EGFR family suggests that different types of dimeric complexes induced after ligand binding may provide a new array of responses different from the hemodimers.

The EC domain may be sufficient for dimerization. The soluble, EC domain of EGFR can form dimer in the presence of EGF (Lax et al 1991). The cytoplasmic domain deletion

mutants of either EGFR or erbB2 are still able to form dimer with full-length erbB2 or EGFR (Spivak-kroizman et al 1992, Qian et al 1994). However, the association of EGFR with mutant erbB2 lacking EC domain is undetectable.

Dimerization has been suggested as a general mechanism for RPTK activation. Some oncogenic activation of RPTKs correlates with constitutive dimer formation. Oncogenic erbB2/Neu has a single amino acid (aa) mutation in its TM domain. It is believed that this mutation changes the conformation of the TM domain and facilitates dimerization in the absence of its ligand (Brandi-Rauf et al 1990). Oncogenic Trk has been found to fuse to tropomyosin which normally exists as a dimer (Martin-Zanca et al 1986).

Soon after the binding of a ligand to its receptor on the cell surface, the ligand-receptor complex is thought to redistribute to form clusters and is internalized via receptor-mediated endocytosis, a pathway which involves specialized plasma membrane structures referred to as clathrin-coated pits (Schmid 1992). Although the same endocytosis pathway is involved in internalization of many RPTKs, the different RPTKs have specific structures and sequence motifs to determine the path of internalization and the fate of a ligand-RPTK complex. EGFR has its internalization motifs located at C-terminal region distal to the tyrosine kinase domain (Chen et al 1989, Chang et al 1992). Deletional analyses of EGFR have identified that several sequence motifs: FYRAL (residues 973-977), QQGFF (residues 996-1000) are essential for internalization. In addition, these motifs can induce internalization when inserted into other molecules (Chang et al 1993). Tyrosine kinase activity is required for internalization since kinase inactive mutant (Lys721) impaired internalization (Wiley et al 1991). In contradiction, another group showed that the kinase

inactive mutant EGFR was internalized at a similar rate as EGFR but was more efficiently recycled to the cell surface (Felder et al 1994). Ser/Thr phosphorylation by protein kinase C (PKC), which is considered as a mechanism to attenuate EGFR activity (Davis et al 1988), influences internalization. Single point mutation at Thr 654 which eliminates the PKC phosphorylation site reduces the internalization rate (Lund et al 1990). Internalization sequence motifs of PDGFR may lie in both C-terminal and juxtamembrane regions. Mutational studies revealed that C-terminal region truncation (Mori et al 1991)as well as point mutation of Tyr 579 to either Phe or Asp in the juxtamembrane segment (Mori et al 1994) impaired receptor internalization. However, unlike EGFR, kinase activity is not important for internalization of PDGFR (Escobedo et al 1988). IR family have their internalization motifs located in the juxtamembrane region. Several sequence motifs: GDLY (residues 950-953), NPEY (residues 957-960), KKITLL (residues 982-987) of IR and residues 940-957 of IGFR are important in for receptor internalization (Rajagopalan et al 1991, Haff et al 1994, Kaburagi et al 1993, prager et al 1994). The two Tyrosine based motifs (GDLY and NPEY) are thought to form type 1 turns that are important for interaction of the receptors' cytoplasmic tails with clathrin-associated adaptins (Pears et al 1990).

TM domain may also be involved in the regulation of RPTK internalization. The TM domain is generally thought to form an α-helix. However, the TM domain of IR has a gly^{933}-pro^{934} sequence which is predicted to interrupt the helical structure. To address the possible effect imposed by such interruption, gly^{933} and pro^{934} were mutated to ala. The resulting mutant was found to be indistinguishable from the wild-type IR with respect to insulin binding ability and PTK activity. However, it displayed an enhanced internalization

rate about 2-fold that of the wild-type IR (Goncalves et al 1993).

Following internalization, the ligand-receptor complex, as exemplified by EGF-EGFR, is delivered to early endosomal compartments and then to late endosomes and finally targeted to mature lysosomes (Beguinot et al 1984, Haigler, 1979, Miller et al 1986, Lai et al 1989). Several lines of evidence suggest that internalized ligand remains bound to its receptor until it reaches late endosomes and lysosomes. Electron microscopic studies of the localization of EGF after endocytosis in A431 cells revealed that EGF remained physically associated with the endosomal membrane (Carpenter et al 1987). Additionally, the finding that similar efficiency of chemical cross-linking of labeled EGF to the internalized receptors compared to the surface receptors indicated that internalized EGF was bound to its receptor (Sorkin et al 1991). Moreover, it was demonstrated that internalized EGFR remained dimerized to the same extent as the surface receptors after at least 10-15 min of internalization (Sorkin et al 1991). These observations indicate that the internalized ligand-receptor remains active, thus suggesting that signaling events, including interaction with signaling proteins in different compartments, may occur during intracellular trafficking of ligand bound RPTKs.

On the way to lysosomes where the ligand and the receptor are degraded, some of the receptors are recycled to the cell surface. The recycling rate varies greatly among the different RPTKs. EGFR undergoes rapid degradation, the half-life of EGFR decreases from 10 hrs in the absence of EGF to about 1 hr upon EGF treatment (Stoscheck et al 1984). IR, however, is more efficiently recycled to the cell surface (Carpenter et al 1985). Depletion of surface receptors caused by internalization and degradation has been

proposed to attenuate mitogenic signaling. One piece of evidence for this concept is from the study of internalization defective EGFR mutant. Cells that express the C-terminal truncated, internalization-defective EGFR exhibit a more potent mitogenic response at a lower ligand concentration than cells expressing the wild-type EGFR do (Wells et al 1990). In this study, however, one can not rule out the possibility that the signaling pathway is altered by the C-terminal truncation.

Both the soluble ligand and the transmembrane protein can induce receptor Internalization. The ligand of Sev is bride of Sev (boss) which is a transmembrane protein with 7 TM domains and is internalized along with Sev following the binding (Cogan et al 1992), suggesting that internalization is not only a general mechanism for receptor stimulation, but also represents a novel receptor-mediated protein transfer between cells.

RECRUITMENT AND ACTIVATION OF CYTOPLASMIC SIGNALING PROTEINS

RPTKs transmit signals to cytoplasmic signaling proteins or transducers through interaction and phosphorylation. The specific protein-protein interaction determines the path and specificity of the signal transduction pathway. The mechanism for the specific interaction has been illuminated by the identification of several protein-protein interaction domains. Studies on Src family PTKs and other PTKs have revealed , in addition to the conserved kinase domain, two other conserved non-catalytic regions designated as SH2 and SH3 domains (Mayer et al 1988). SH2 domain consists of about 100 aa which has been found mainly in non-receptor PTKs and many other signaling proteins with diverse

enzymatic activities or without any known catalytic functions (Pawson and Gerald 1992). Evidence has accumulated to clearly show that SH2 domain binds to protein phosphotyrosine motifs and mediates the interaction between enzyme molecules and their substrates with high affinity and specificity (Mayer 1990, Fanti 1992). Mutagenesis studies suggest that the highly conserved FLVRES sequence of SH2 domains and at lest three residues immediately carboxyl to the phosphotyrosine are required to maintain the specific interaction (Overduin et al 1992, Waksman 1992). Based on these observations, a general mechanism has been proposed that RPTK autophosphorylation creates phosphotyrosine residues which serve as molecule anchors to interact with cytoplasmic SH2 and SH3 containing substrates (Anderson et al 1990, Cantley et al 1991). These substrates, activated by a RPTK, will further interact with their downstream substrates, thus initiating a chain reaction to transmit the signal. A number of SH2 and SH3 containing proteins have been cloned or identified. Some of them, such as Grb2, Shc, Nck, Crk, don't have any enzymatic function are considered as adapters to couple downstream substrates to activated RPTKs.

Proteins with SH2 domains frequently possess a distinct sequence of about 50 aa, SH3 domain, which is also involved in protein-protein interaction during signal transduction (Mayer et al 1988, Stahl 1988). The binding motifs for SH3 domain have been characterized as proline-rich motifs which are present in proteins with a variety of functions (Ren et al 1993, Cohen et al 1995). The function of SH3 domain is well elucidated by studying these adapters. Grb2 interacts with RPTKs through its SH2 domain while its SH3 domains bind to Sos, thus bring Sos to the proximity of RPTKs. The fact that SH3 domain

is frequently found in many proteins associated with the cytoskeleton and membrane and that many SH3 binding proteins have GAP-like activities indicate that SH3 domain has other functions (Koch et al 1991).

Another domain of signaling proteins has been described. That is PH domain which is a region of approximately 100 aa found in a wide variety of signaling and cytoskeletal proteins (Cohen et al 1995). The pleckstrin protein, the major substrate of PKC in platelets, has two such domains and was the first protein in which the domain was recognized (Haslam et al 1993, Mayer et al 1993). Although the function of PH domain is not known yet, it was suggested that PH domain might not serve as mediators of protein-protein interactions, but rather as mediators of protein-lipid interactions (Gibson et al 1994).

Adding to the variety of protein domains is the recognition of a novel phosphotyrosine interaction domain (PID) (Bork and Margolis 1995). It was reported that a N-terminal region of Shc distinct from the SH2 domain could also bind to tyrosine phosphorylated proteins. This region appears structurally unrelated to SH2 domain and may impart on Shc its unique ability to bind to the Asn-Pro-X-Tyr motif found in many tyrosine phosphorylated proteins, including RPTKs. This domain was reported to be sufficient for association of Shc with a 145 kD cellular protein (Kavanaugh and Williams 1994). Based on sequence conservation, a new protein with a PID domain, named Sck, has been cloned (Kavanaugh and Williams 1994). The function of PID needs to be further analyzed.

MULTIPLE SIGNAL TRANSDUCTION PATHWAYS

Several signaling proteins have been shown to be physically associated with the activated RPTKs through either their SH2 domains or adapters. Thus they are considered as immediate substrates of RPTKs. These proteins include: phospholipase (PL) C-γ, phosphatidylinositol-3 kinase (PI3K), ras-GTPase-activating protein (GAP), Shc, Syp, Grb2 and Sos, insulin receptor substrate-1 (IRS1), STAT. Upon interaction with the RPTKs, these proteins are activated by either tyrosine phosphorylation, translocation, or other unknown mechanisms usually resulting in enhancement in their enzymatic activities. Then they interact with their own downstream signaling proteins and thus branch signals into different pathways. These signaling pathways act in concert to elicit diverse biological effects.

Ras is a membrane associated and farnesylated GTPase, participating in signaling between cytosol and membrane receptor in response to a variety of signals. In quiescent cells, Ras is predominantly bound to GDP. To convert to the active GTP bound state, Ras protein must first release bound GDP. this rate-limiting step is catalyzed by Sos (Wolfman et al 1990, Shou et al 1992). Sos forms complex with Grb2 and is activated upon translocation to the proximity of RPTKs (Chardin et al 1993, Rozakis-Adcock et al 1993). Sos is not tyrosine phosphorylated, but may be ser/thr phosphorylated. The mechanism by which Sos is activated upon translocation remained unclear. To examine the role of membrane translocation, Sos derivatives containing either a myristoylation or farnesylation signal, were expressed as a membrane associated protein (Aronheim et al 1994). Cells expressing these derivatives exhibit a constitutive activation of the Ras signaling pathway, suggesting that targeting of Sos to the plasma membrane in the vicinity of Ras appears to

be the primary mechanism leading to activation of the Ras pathway.

Three lines of evidence suggest that C-*raf* which is a ser/thr protein kinase functions downstream of Ras. First, viral *raf* oncogene does not require cellular Ras function to cause cell transformation (Feig et al 1988). Second, expression of oncogenic *ras* can induce phosphorylation of Raf and activation of its kinase activity (Morrison 1988). Third, transformation of NIH3T3 cells by *ras* can be blocked by dominant negative *raf* mutant (Kolch et la 1991). Direct interaction of Ras-GTP and Raf has also been observed (Vojtek et al 1993, Moodie et al 1993). It is thought that activated Ras binds to Raf, leading to its activation through translocation to the plasma membrane. Evidence in support of this is that requirement for Ras in Raf activation can be overcome by targeting Raf to the plasma membrane (Leevers et al 1994). However, one could criticize that overexpression may contribute to some extent to activation of the membrane associated Raf, but in physiological condition the mechanism other than translocation may play a role as well. Besides Ras dependent activation pathway, activation of Raf in response to a PKC activator 12-O-tetradecanoyl-phobol-13-acetate (TPA) was observed (Kolch et al 1993). TPA was found to induce Raf phosphorylation at Ser 499, a site not required for Ras or Src induced phosphorylation, suggesting that different mechanisms are employed to mediate Raf activation. To complicate matters further, 14-3-3, a family of highly conserved proteins with multiple functions were reported to be able to interact with Raf constitutively (Fuetal et al 1994, Fanti et al 1994, Irie, et al 1994), regardless of the activation state of Raf or whether it is bound to Ras. Although one of 14-3-3 isoforms could activate Raf kinase activity by direct interaction (Fanti et al 1994), it isn't clear what role 14-3-3 plays in

regulating Raf kinase activity.

Numerous observations have suggested that Raf is a good candidate to mediate activation of MEK (or MAPK kinase). MEK is constitutively active in v-*raf* transformed cells (Kyriakis et al 1992). and Raf protein purified after mitogen stimulation can reactivate the phosphatase 2A-inactivated MEK. Complexes of Ras-GTP with Raf and MEK were observed by using different approaches (Moodie et al 1993). MEK is a dual functional kinase that catalyzes both Ser/Thr and tyr phosphorylation. Consistently, MAPK (ERK), the substrate of MEK, requires both Ser/Thr and Tyr phosphorylation for its activation. Removal of phosphate from either Thr or Tyr results in inactivation (Anderson et al 1990). MAPK, which has two species, p44 (ERK1) and p42 (ERK2), phosphorylates several substrates including p90 and p70 RSKs. RSKs in turn phosphorylate ribosomal S6 and other unidentified substrates, resulting in activation of protein synthesis. MAPK has been reported to be able to translocate to nucleus (Chen et al 1992) where it possibly phosphorylates a variety of substrates, including transcription factors such as the ternary complex factor ($p62^{TCF}$) (Gille et al 1992). The phosphorylation enables $p62^{TCF}$ form ternary complex with a serum responsive factor ($p67^{SRF}$) and serum responsive element (SRE) in the promotor region of c-*fos* (Herrera et al 1989), resulting in increased transcription of c-*fos*, a member of AP-1 family. Transcription factors of AP-1 are also modified post-translationally. A novel Ser/Thr kinase, termed JNK (Derijard et al 1994), which binds to C-Jun and phosphorylates its N-terminal sites in response to UV irradiation and *ras* expression, was recently identified. Most cells express two isoforms of JNK, JNK1(46KD) and JNK2(55KD). JNK is highly similar to MAPK in terms of sequence and mechanism of

activation and thus is considered as members of MAPK family. N-terminal phosphorylation of C-Jun stimulates its transcriptional activity, while C-terminal phosphorylation inhibits it.

PI3K consists of a 110 kD catalytic subunit and a 85 kD regulatory subunit which acts as an adaptor coupling the catalytic subunit to an activated RPTK (Escobedo et al 1991, Osu et al 1991, Skolnik et al 1991). Alternatively, PI3K can be activated through interaction with IRS1 (Ruderman et al 1990, Sun et al 1992, Rodriguez-Viciana et al 1994) which is able to interact with IR family RPTKs. Recently it has been shown that PI3K can also be activated by Ras, bypassing the RPTKs. The activation may be mediated by interaction of the catalytic subunit of PI3K with the effector domain of Ras directly. The Ras mutant N^{17}-*ras* abolishes interaction and PI3K activation. In addition, N^{17}-*ras* leads to a 5-fold decrease in the levels of PI3K catalyzed products induced by EGF, suggesting that in intact cells Ras is a major regulator of PI3K. However, previous study has indicated that the RPTK may be the major activator of PI3K. Mutation of PDGFR that abolished PI3K binding site while leaving the Ras signaling pathway unaffected was no longer able to activate PI3K and transmit mitogenic signal (Valius 1993). To complicate this matter further, it has been shown that PI3K may act upstream of Ras since Ras is activated in the cells expressing constitutively active PI3K and dominant negative *ras* mutant could block the PI3K-mediated biological response (Hu et al 1995). It is known that PI3K phosphorylates inositol lipid at D3 position to generate several interconvertable products: phosphatidylinositol-3-monophosphate (PI-3-P), PI-3.4-P_2, PI-3.4.5-P_3. The physiological importance of these products remain elusive. Besides its lipid kinase activity, PI3K can also phosphorylate proteins at ser/thr position. P85 subunit (Carpenter et al 1993) and IRS1

(Lam et al 1994) were reported to be phosphorylated by PI3K, but the physiological significance was not defined. A significant progress in understanding the role of PI3K is the realization that activation of p70 RSK is mediated by PI3K (Cheatham et al 1994, Chung et al 1994). By using the specific PI3K inhibitors, wortmannin and ly294002, it was shown that activation of p70 RSK required functional PI3K.

PLCγ breaks down the phosphatidylinositol-4, 5-biosphsphate (PIP_2) to diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP3) (Berridge et al 1984). DAG stimulates PKC, while IP3 mobilizes intracellular calcium by binding to its receptor on endoplasmic reticulum (ER) membrane (Berridge et al 1989). Tyrosine phosphorylation of PLCγ is important for its activation and association with activated RPTKs. Although PLCγ is an important downstream component for a variety of RPTKs, it may not be essential for the mitogenic activity. Mutants of PDGFR, FGFR, IR, or IGFR that are unable to associate with or activate PLCγ can still cause increased DNA synthesis in response to ligand stimulation similar to the wild-type receptors (Nishibeet et al 1990, Mohammadi et al 1992).

IRS1 was originally identified as a signal transducer for IR and IGFR, but it also participates in signal transduction mediated by other receptors (Wang et al 1993). Once IRS1 is tyrosine phosphorylated it associates with RPTKs as well as a number of proteins, including PI3K, Grb2, Syp, and Nck (Mayer et al 1994). Thus IRS1 is an adaptor without SH2 and SH3 domains. Because IRS-1 can interact with so many signaling proteins and is involved in many signal transduction pathways, it is thought to be functional equivalent as C-terminal tail of PDGFR in the IR family. The mechanism by which the IRS-1 binds to RPTKs has not been defined. Acting as a downstream signaling protein for the IR family,

IRS1 is not only important for activation of PI3K, but is also involved in Ras activation since it can interact with Grb2. Recently it was shown that IRS1 could interact with integrin (Vuori et al 1994), a cell surface receptor for the matrix protein, and the interaction could lead to increased DNA synthesis, suggesting the diverse functions of IRS1. IRS1 may represent a member of a family proteins. In a myloid progenitor 32D cell line that lacks IRS1, 4PS, a IRS1-related protein, is believed to transmit the signaling events as IRS-1 does (Wang et al 1993). The observed reasonable level of PI3K activity in the tissues extracted from mice bearing null mutation in IRS1 gene after insulin or IGF-1 treatment may be attributed to the closely related protein 4ps/IRS2(Araki et al 1994).

The signaling pathway distinct from that described above has been demonstrated. In contrast to the signal transduction cascade, a novel family of transcription factors are tyrosine phosphorylated in cytoplasm by activated RPTKs or receptor associated PTKs and subsequently translocate to nucleus directly. The first evidence is from the studies of interferon α/β receptor mediated signaling. Interferon α/β receptor has no kinase activity, but has an associated PTK, named TYK2 (Velazquez et al 1992). Upon interferon stimulation, a transcription factor p91 (also known as ISGF3α, a component of interferon stimulated gene factor-3), is tyrosine phosphorylated immediately and migrates to nucleus where it forms complexes with a 48 kD transcription factor (ISGF3γ) and interferon stimulating responsive element (ISRE), resulting in interferon responsive gene transcription (Fu 1992, Schindler et al 1992). p91, also named as stat (Signal Transducer and Activator of Transcription) 1 or stat 91, contains SH2 and SH3 domains that enable its ability to interact with RPTKs or cytoplasmic PTKs, resulting in tyrosine phosphorylation.

Further studies have demonstrated that the same pathway is shared by many receptors, including receptors for different cytokines, EGFR, PDGFR (Fu et al, Larner et al, Ruff-Jamisen et al, Silvennoinen et al, Sakowski et al, 1993, Zhong et al 1994). Several new members of stat family have been identified or cloned, including stat2, stat3, stat4.

TRANSFORMATION AND PROLIFERATION

Transformation can be divided into morphogenic and mitogenic. Morphogenic transformation is characterized by the rearrangement of cytoskeleton and altered interaction with matrix and change of matrix components, lose of anchorage dependent growth. Mitogenic transformation results from increased proliferation rate, immortalization, growth in low serum and lose of contact growth inhibition. Transformation and proliferation are frequently linked events as tumors usually grow faster than the normal tissue, but substantial evidence suggests that it is not necessary. In some cases these two aspects of cell transformation even can be dissociated.

EGF is known to stimulate proliferation of a number of cell types, including endothelial cells, BALB/C3T3 cells (Gospodarowicz et al 1978). However, EGF treatment of A431 cells which express very high level of EGFR results in growth inhibition (Gill and Lazar 1981). Similar growth inhibition has been reported for the breast cancer cell line MDA-468 and squamous cell carcinoma lines which also express high level of EGFR upon EGF treatment (Filmus et al 1987, Hirai et al 1988). Based on these observations, one might think alternatively that amplification of EGFR or other proto-oncogenes may not be

involved in oncogenicity, instead, is a cellular defensive response to curb fast growing tumor cells.

Studies of the variants of squamous cell carcinoma lines which are resistant to EGF-mediated inhibition of cell growth have revealed two independent mechanisms by which the variants escape EGF-mediated growth inhibition (Hirai et al 1988). One mechanism involves the lose of the amplified EGFR gene, resulting in decreased expression level, and the other involves more efficient down-regulation of the cell surface receptor by internalization and degradation. In all those variants, however, EGF induces morphological changes, including a rapid shape change with formation of ruffles, exhibition of rounding and extension of long processes, and aggregation of cells into dense clusters, are all identical to those observed in the parental cells that are sensitive to EGF mediated growth inhibition (Lifshits et al 1983).

Other examples of growth inhibition associated with the over-expression of proto-oncogenes or oncogenes have described. For example, high concentrations of *mos* has been shown to coincide with cell mortality in moloney murine sarcoma virus-infected NIH3T3 cells (Papkoff et al 1982). Overexpression of Ha-*ras* results in transformation of a variety of established cell lines (Goldfarb et al 1982), but causes growth arrest of REF52 cells (Franza et al 1986), a established rodent cell line. C-*abl* is a nuclear PTK which negatively regulates cell growth (Sawyer et al 1994), but v-*abl* can inhibit or stimulate cell growth. It has been reported that v-abl expressing NIH3T3 cells exhibit two opposite phenotypes (Renshow et al 1992). In the majority of v-*abl* expressing NIH3T3 cells, v-*abl* causes a growth arrest at the G1 phase of the cell cycle; while in a minority of the cells, v-

abl abrogates the requirement for growth factors. V-*abl* expressing cells exhibiting either stimulatory or inhibitory growth effects undergo similar morphological alteration typical of transformed fibroblasts. It is believed that v-*abl* mediated opposite effects may be cell context dependent. In searching for the molecular basis underlining the opposite biological effects, it was observed that serum-responsive genes, including c-*fos*, c-*jun*, and c-*myc* are induced by temperature sensitive v-*abl* only in those "positive" cells.

Cell growth inhibition has also been observed in chicken embryo neroretina (CNR) cells expressing a combination of oncogenic *myb* and *myc* upon stimulation with basic FGF (bFGF) (Garrido et al 1993), although the cells are transformed and tumorigenic. The CNR cells expressing *myb* alone are not transformed and can be growth stimulated by bFGF. In this study, c-*fos* was found to correlate with the inhibitory effect since induction of c-*fos* by TPA or bFGF and overexpression of v-*fos* resulted in cell growth inhibition in these cells. Interestingly, v-*fos* also transforms the *myb* expressing CNR cells.

Studies on the tumor suppressor gene p53 also indicated that transformation and proliferation can be dissociated (Crook et al 1994). P53 is a transcription factor which shows growth and transformation suppression functions. Using a large series of p53 mutants, it is clearly demonstrated that transcription activation of p53 correlates with its growth suppression when such mutants are transfected into p53 null human cells. But not all transcription active mutants retain the ability to suppress transformation in primary rodent cells. Moreover, two tumor-derived mutants display both transforming and transactivating activities.

IGFR is required for optimal cell growth since IGFR-null mouse embryo fibroblasts

(MEF) can't grow in medium supplemented with less then 5% serum or serum-free medium supplemented with a combination of growth factors. The growth defective IGFR null-MEF, however, can still be weakly transformed by *ras*, although fully transformation can be achieved only after IGFR gene is introduced back into the MEF (Sell et al 1994).

C-*ROS* AND V-*ROS*

Avian sarcoma virus UR2 is a replication-defective virus that can induce sarcomas in chicken and transform chicken embryo fibroblast (CEF) in culture into a characteristic elongated form (Balduzzi et al 1981). The genome of UR2 contains a 1.2 Kb transformation-specific sequence, viral *ros* (v-*ros*) (Nechameyer 1984, 1985), which has a homologous counterpart, c-*ros*, in chicken cellular DNA (Shibuya et al 1982). UR2 was presumably generated by recombination between the UR2-associated virus UR2AV and c-*ros* at the expense of part of the *gag* and all of the *pol* and most of the *env* genes of UR2AV. As a result, *ros* is fused to the 5' region of the UR2AV sequence coding for part of the viral structural protein *gag* (Wang et al 1982). The fused *gag* and *ros* sequence codes for a transmembrane protein of 68 kD, called p68 which has PTK activity (Feldman et al 1982).

cDNAs for the c-*ros* were recently cloned from different species including human, rat, mouse, and chicken (Birchmeier et al 1990, Matsuchme et al 1990, Chen et al 1991, Riethmacher et al 1994). The chicken full-length cDNA is 8.3 Kb, which codes for a receptor-like PTK of 2311 aa sharing the greatest homology with *sev* and significantly homology with IR family RPTKs (Chen et al 1991). A characteristic 6 aa insertion in the PTK domain is shared with *sev*, but not with other *ros*-related RPTKs such as IR and IGFR. The large EC domain contains multiple cysteine residues and potential N-linked glycosylation sites. Eight fibronectin type-III repeats, a motif implicated in binding to cell surface proteins such as integrin and heparin, are identified in chicken c-*ros* and conserved

among *sev*, rat and human c-*ros*. Little is known about the biochemical and biological properties of c-*ros* since c-*ros* can not be stably expressed in several cell lines being tried and its putative ligand has not been identified. Epithelial restricted expression of c-*ros* suggests that c-*ros* may play some role in epithelia differentiation during embryogenesis and in their mature physiological function (Chen et al 1994, Sonnenberg et al 1991, Tessarrollo et al 1992). Besides the spontaneous transduction and activation of the tumorigenic potential of c-*ros* in ASV UR2, c-*ros* was implicated in the development of human glioblastomas as elevated level of c-*ros* expression and rearranged c-*ros* products were found in glioblastoma cell lines (Birchmeier et al 1987).

Comparison of the nucleotide sequences between c-*ros* and v-*ros* revealed 3 differences: First, v-*ros* is truncated at 7 aa upstream from TM domain and fused to *gag*; Second, v-*ros* contains a 9-nucleotide insertion within the TM domain. The insertion sequence codes for three non-polar aa Ser, Leu, and Thr; Third, v-*ros* has an internal 36 nucleotides deletion between nucleotide 6914 and 6953, and 3' terminal 27 nucleotides truncation and fusion to the *env* sequence. It has been demonstrated that modification of the regions upstream of the cytoplasmic domain is sufficient for activation of the transforming ability of c-*ros* (Zong et al 1993). Moreover, deletion of the 3-aa insertion in the TM domain of v-*ros* greatly reduces its transforming and mitogenic potency, as well as alters post-transnational modifications and substrates interaction despite of identical cytoplasmic domain and PTK activity, suggesting that TM domain has a profound effect on the signaling capacity of v-*ros*.

CHIMERIC RECEPTOR

In many examples, protein can be considered as a combination of multiple domains. Each domain is a relatively independent unit both structurally and functionally, providing the basis for construction of chimeric proteins which have been demonstrated to be valuable tools to dissect function of proteins.

Chimeric receptors have been successfully used as model systems to study the receptors or receptor-like proteins without available ligands. Using such model systems, several functional chimeras between different RPTKs have been created, including EGFR-NGFR, EGFR-erbB2, EGFR-Neu, EGFR-kit, EGFR-ret, EGFR-ELK, EGFR-IR, IR-EGFR, NGFR-ros (Yan et al 1991, Kraus et al 1993, Lehvaslaiho et al 1989, Pandiella et al 1989, DiFiore et al 1990, Riedel et al 1986, 1989, Lhotak et al 1993, Riethmacher et al 1994). In the study of EGFR-NGFR chimera (Yan et al 1991), It has been demonstrated that the $p75^{NGFR}$ with its EC domain replaced by the analogous region of EGFR can induce EGF-dependent neurite outgrowth when such chimera is expressed in PC12 cells, suggesting that $p75^{NGFR}$ has the potential to participate in signal transduction although it has a low affinity for NGF. Interestingly, the chimeric $p75^{NGFR}$ with its TM and EC domains derived from EGFR can't induce neurite outgrowth, indicating both TM and cytoplasmic domain are important for signaling capacity.

Chimeric receptors have also been used extensively to dissect the functions of domains or subdomains of a given receptor. Studies on the EGFR-IR and IR-EGFR chimeric receptors (Riedel et al 1989) have suggested that the cytoplasmic domain determines the

signaling specificity, including kinase specificity, mitogenic and transforming potential, and receptor routing, whereas the EC domain defines the ligand binding specificity. Function of the TM domain has also been dissected by using chimeric receptors. As mentioned above, two EGFR-NGFR chimeras differing in the origin of their TM domains have different effects on PC12 cell differentiation. Replacement of the TM domain of PDGFR with those of other receptors abolishes ligand-induced activation of tyrosine kinase activity (Escobedo et al 1988). These observations suggest that the TM domain actively participates in signal transduction other than passively serves as a membrane anchor. Aside from studying signal transduction, chimeric receptors have also been used to dissect the ligand binding domains. Chimeric IR receptors in which a portion of its α subunit is exchanged with the corresponding region of IGFR α subunit have allowed one to map the major insulin binding determinants within the amino acids 315 to 324 region of the IR α subunit (Schumacher et al 1993).

Signal transduction of cytokine receptors which do not have kinase activity, however, may have a different situation in that the specificity may lie in the EC domain. Chimeric receptors consisting of the EC domain of the erythropoietin receptor and the cytoplasmic domains of IL-2, or IL-3 receptors induce an erythropoietin-dependent tyrosine phosphorylation; However, chimeric receptors composed of the EC domain of IL-2 receptor and the cytoplasmic domains of the erythropoietin or IL-3 receptors transmit an IL-2-dependent signal (Chiba et al 1993).

The lack of a putative ligand and permanent expression lines have greatly hampered the study of c-*ros* function. In order to circumvent these problems, I have constructed two

chimeric receptors composed of the EC domain of the EGFR and cytoplasmic domain of c-*ros* with TM domain either from EGFR or c-*ros*. Such chimeras should mimic c-ros-mediated signaling under the control of EGF. Study on these chimeras have revealed that the two chimeras have opposite effects on cell growth despite of their similar colony forming abilities in soft agar. Comparison of the signaling pathways triggered by the two chimeras showed several differences that may account for their opposite effects on cell growth. the cytoplasmic signaling components that might be involved in c-*ros*-mediated signaling and the effects of different domains on this process.

MATERIALS AND METHODS

CELLS: NIH3T3 is maintained in DMEM with 5% calf serum.

REAGENTS: γ-^{32}P-ATP, α-^{32}P-dATP, ^{35}S-Met labelling mix, ^{3}H-Thymidine, and ^{125}I-EGF were purchased from Du pont-NEN. ^{125}I-Protein A was from ICN. NHS-LC-Biotin and BS^{3} were purchased from PIRECE. Human recombinant EGF (rHu EGF) and IGF were from Intergen. Immobilized rProtein A™ was from Repligen. Commonly used chemicals and enzymes are not listed here.

ANTIBODIES: Anti-Ros antibody (Ab) 219 and anti-IRS1 Ab that were made in this lab have been described before (Jong et al 1987, Jiang and Wang). Anti-phosphotyrosine Ab 4G10, anti-PLCγ, anti-Shc, and anti-EGFR (clone LA 22) Abs were from Upstate Biotechnology Inc. Anti-ERK-1 (C-16) was from Santa Cruz Biotech.. Anti-MAKP Ab TR-10 was a gift from Dr. Michael Weber. RC20, a recombinant anti-p-Tyr IgG conjugated with alkaline phosphatase (AP) was purchased from Transduction Laboratories. Goat-anti-rabbit IgG and rabbit-anti-mouse IgG conjugated with AP were purchased from Boehringer Mannheim.

CONSTRUCTION OF EXPRESSION PLASMIDS: Two epidermal growth factor receptor (EGFR)-*ros* chimeric receptors, named ER1 and ER2, were constructed. ER1 has the cytoplasmic domain from *c-ros* and the EC and TM domains from human EGFR. To

construct the expression plasmid, the plasmid pZIPER containing the full-length coding region of human EGFR (gift from Dr. Shibuya) were digested first with Sac1 and then partially with Nar1 to release the Sac1-Nar1 partial fragment (aa -24 to 646). The cytoplasmic domain of *c-ros* was generated by PCR from pECEROS plasmid (J. Chen and L-H. Wang) using the following pair of primers. Primer1, 5'*CGAAGGCGCCA*AAAGATGGAAATCCAGA, contains the sequence of EGFR (italic) and the Nar1 recognition site (underlined) at its 5' end and *c-ros* sequence corresponding to the region immediately carboxyl to its TM domain. Primer2, 5'CCGGATCCTCTAGACTCTCTTCTGTCCTCAAACAG, corresponds to the 3' noncoding region of *c-ros* and contains Xba1 site (underlined). The PCR product was first digested with Nar1 and Xba1 and then ligated to the pbluescript vector at the Sac1 and Xba1 sites together with the Sac1-Nar1 fragment of EGFR. the resulting plasmid containing the EC and TM domain of EGFR and cytoplasmic domain of *c-ros* was confirmed by sequence the whole PCR region. The chimeric cDNA was then released from pbluescript and ligated to a mammalian expression vector pECE which contains a SV40 promoter (Ellis et al 1986). The ER2 which has the EC domain of the *c-ros* replaced by corresponding region of EGFR was constructed as follows (Fig. 1): A Sac1-Bstx1 fragment(aa -24 to 625) coding for the entire EGFR extracellular domain was released from PZIPER. The fusion junction was generated by PCR using the following pair of primers; primer3, 5'*CATCGCCACTGGGATGG*ATATCACTACTGCTATTGTTGCT, contains sequence of EGFR (italic) including Bstx1 recognition site (underlined) and *c-ros* sequence corresponding to the TM domain; primer4, 5'CTCTGCTTGAGAAGGAAGAGTGCT,

corresponds to *c-ros* (aa 1941 to 1949) including the Ear1 recognition site (underlined). The PCR fragment was digested with Bstx1 and Ear1. A *c-ros* cDNA Ear1-Not1 fragment, together with the PCR fragment and the EGFR Sac1-Bstx1 fragment were ligated to Pbluescript (SK+) vector at Sac1-Not1 sites. The resulting plasmid containing the EGFR-*ros* fragment was confirmed by restriction map and sequencing at the junction regions. The EGFR-*ros* fragment was then transferred to pECE vector at Sac1-Xba1 sites.

TRANSFECTION AND SELECTION OF CELL LINE: Both electroporation and Calcium phosphate methods were used. For electroporation, DNA was mixed with $2x10^7$ cells which were suspended in 0.8 ml of phosphate-buffered saline (PBS). The mixture was placed on ice for 5 min and then subjected to electroporation at 300 V and 125 capacitance in a gene pulser apparatus (Bio-Rad). Cells were then placed on ice for another 5 min before plating on culture dish. For Calcium phosphate transfection, DNA was mixed with $CaCl_2$ and 2xHEPES buffer and then the mixture was added to the subconfluent cells. 40ug DNA was used and chloroquine (30 ng/ml) was added for transient transfection. For selection, 20 ug DNA plus 1 ug pSV2-neo plasmid were used. Stably transfected cell lines were selected with medium supplemented with 400 ug/ml of geneticin (G418, GIBCO Laboratories) per ml starting 2 days post-transfection. Geneticin resistant clones were then maintained in media supplemented with 100 ug geneticin per ml periodically.

COLONY FORMATION ASSAY: This was done according to published method (Hanafusa 1969). Briefly, cells (usually 10^5) suspended in 3 ml soft agar medium were seeded in a 60

mm plate containing 5 ml bottom agar medium. If EGF (usually 10 ng/ml) was included in the medium, the EGF containing medium (0.5% serum) were overlaid every 5 days and the control plates were overlaid with the soft agar medium supplemented with 0.5% serum only.

IMMUNOPRECIPITATION: Cell culture dishes were placed on ice, washed with PBS, and cells were lysed with RIPA buffer containing 50 mM Tris-Cl(pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% trasylol, 1 mM phenylmethylsulfonylfluoride(PMSF), 1 uM leupeptin, and 1 uM antipain. For PI3 Kinase assay, cells were lysed with NP-40 buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, and protein inhibitors as described above. For detection of protein association, cells were lysed with 0.1% NP-40 in the NP-40 buffer. Cell lysate were collected from plates, centrifuged at 12,000 RPM for 10 min at 4°C. The supernatant was then used for immunoprecipitation with indicated Ab for minimal of 1 hr followed by protein A beads binding for 1 hr. If monoclonal Ab was used, rabbit anti-mouse IgG was added together with protein A beads.

KINASE ASSAY: The immunoprecipitates were washed twice with high-salt RIPA buffer (same as RIPA except for 300 mM NaCl, 1% SDS, 10% glycerol), and twice with kinase buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MnCl_2$. The immunoprecipitates were finally resuspended in 50 ul kinase buffer. The kinase reaction was carried out at room temperature for 10 min after the addition of 1 ul (10 uCi) γ-^{32}P-ATP (6000 uCi/mM). The

reaction was terminated with the addition of excessive high-salt RIPA and washed with high-salt RIPA twice and once with low-salt RIPA (same as high-salt RIPA except for 10 mM NaCl). The samples were then resuspended in 20 ul 2x protein loading buffer and boiled for 3 min before loading onto a 7.5% SDS-gel. After electrophoresis, gel was fixed and dried. The kinase activity was detected by autoradiography.

WESTERN BLOT: Proteins in the immunoprecipitates were resolved by a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBS-T) containing 20 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% Tween-20, for 1 hr. Then membrane was incubated with indicated primary antibody for 1 hr followed by washing with TBS-T for three times. For detection of immunoreactive proteins, membrane was incubated with anti-rabbit IgG conjugated with AP for 30 min followed by color reaction according to the supplier's protocol. If monoclonal Ab was used for IP, rabbit anti-mouse IgG conjugated with AP was used to visualize the protein. For direct Western blot, cells were lysed with RIPA or RIPA containing 0.1% SDS if nuclear protein was desired and then mix with SDS-PAGE sample buffer and loaded onto the SDS-gel after boiling for 5 min.

PULSE-CHASE LABELLING OF CELLS: Cells were washed with PBS and incubated with methionine (Met)-free DMEM supplemented with 0.5% dialyzed calf serum, 2 mM L-glutamine for 2 hrs. Then cells were pulsed with 100 uCi of ^{35}S-methionine in 1 ml media for 20 min followed by chasing for indicated times. Cell lysis, IP, SDS-PAGE, and

autoradiography were performed as described above. In some experiments, tunicamycin was added to the cell culture at 10 μg/ml during starvation and labelling.

CELL SURFACE LABELLING: Cells were incubated in PBS containing 1 mg/ml NHS-LC biotin(Sulfosuccinimidyl 6-(biotinamido) Hexanoate) at $4C^0$ for 1 hr with occasional swirling. The reaction was stopped with the addition of 50 ml PBS containing 20 mM glycine. The cells were pelleted and washed two more times with PBS. Cell lysis, IP, and SDS-PAGE were done as above. Biotin labeled proteins were visualized with avidin conjugated with AP and color reaction.

EGF BINDING: For scatchard plot, cells were plated in 24-well plates at a density of 10^5 per well. The following day, plates were cooled on ice and then washed twice with ice-cold PBS-BSA (1 mg/ml BSA). Cells then were incubated with ^{125}I-EGF at the following concentrations: 0.2ng/ml, 1ng/ml, 5 ng/ml, 25 ng/ml, and 125 ng/ml in PBS-BSA at 4^0C for 2 hrs in the presence or absence of 100-fold cold EGF, followed by washing with PBS-BSA for 5 times. Cells were lysed directly by 0.2% SDS-0.2 N NaOH and the radioactivities were counted by using γ-counter. The cell associated counts (CPM)= CPM in the absence of cold EGF - CPM in the presence of cold EGF. For EGF binding, 5 ng/ml of ^{125}I-EGF was used.

DIMERIZATION: Cells were serum starved for at least 3 hrs and then incubated with EGF (100 ng/ml) in PBS-BSA (1 mg/ml) at 4^0C for 1 hr, followed by cross-linking with BS^3(1 mM in PBS-BSA) for 15 min at 4^0C. The cross-linking was stopped by the addition of Glycine

to the final concentration of 20 mM. Cells were then washed with PBS three times and lysed with RIPA. Cell lysates were immunoprecipitated with anti-Ros Ab, followed by in vitro kinase assay as described.

^{3}H-THYMIDINE INCORPORATION ASSAY: Cells were seeded at $5x10^4$ per well on 24-well plates. After overnight serum starvation, cells were stimulated with EGF at different concentrations for 16 hrs before the addition of ^{3}H-thymidine at 2 µci/ml. Cells then were incubated for another 4 hrs and lysed with lysis buffer containing 0.2% SDS and 0.2 N NaOH. A portion of the lysates were spotted on the DE-81 membrane (Whatman) and washed twice with 2X SSC and once with 70% ethanol. Radioactivities were counted using the scintination counter.

PI3 KINASE ASSAY: The PI3 Kinase assay was done essentially as described previously (Fukui and Hanafusa 1989). In brief, the cell lysates were incubated with either anti-Ros or anti-IRS1 Ab for 1 hr at 4^0C before the addition of 20 µl of Protein A beads. The immunoprecipitates were washed as described previously (Fukui and Hanafusa 1989), and the washed beads were resuspended in 25 µl of TGN buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 10 µg of the substrate phosphoinositol (PI, 20 µg/µl in dimethyl sulfoxide, Avanti Polar Lipids, Inc.) and mixed to make micelles of PI. The mixture was incubated at room temperature for 10 min before the pre-mixed γ-^{32}P-ATP (10 µci/assay) and $MgCl_2$ (final concentration of 20 mM) was added, and the mixture was incubated for another 10 min at room temperature. Phosphorylated PI was extracted and

analyzed on a thin layer chromatography silica gel 60 plate.

MAP KINASE ACTIVITY ASSAY: Cell lysates were immunoprecipitated with the TR-10 Ab and protein A beads and washed with RIPA buffer and then the MAP kinase assay buffer (10 mM Tris-HCl pH 7.5, 10 mM Mg acetate). Immunoprecipitates were then resuspended in 20 µl of MAP Kinase assay buffer and mixed with 20 µl MBP (myelin basic protein, 2 mg/ml) and 20 µl 3x hot mix (5 µCi/reaction of γ-^{32}P-ATP, 150 µM ATP, 30 mM Mg acetate, 30 mM hepes, pH 7.5) and incubated in 30^{0}C for 30 min. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiled for 5 min. The sample was centrifugated at 12,000 rpm briefly and the supernatant was loaded onto 15% SDS-gel followed by electrophoresis and autoradiography.

FLOW CYTOMETRY ANALYSIS: Cells were trypsinized, resuspended in PBS and fixed with ethanol at a final concentration of 70% by adding ethanol dropwise while vertexing. Cells were then kept at 4^{0}C. Before flow cytometry analysis, cells were pelleted by centrifugation at 1,600 rpm for 10 min and digested in 250 ul of PBS containing 2 mg/ml RNase A for at lest 30 min at 37^{0}C and then stained with Propidium Iodide (PI)staining solution (10x stocking solution containing PI 0.5 mg/ml, sodium citrate 10 mg/ml, 1% triton X-100) for 30 min at room temperature.

CELL STAINING: For sample preparation, cells were fixed with 4% parafomaldehyde in PBS for 10 min at room temperature and washed with PBS for three times. Cells were then

permealized with cold methanol and incubated at -20°C for 10 min, followed by washing with PBS three times. Staining was done using Histostain™ SP Kit (Zymed Laboratories Inc.) according to the supplied protocol. Briefly, after cells were blocked with blocking solution containing serum for 10 min followed by washing with PBS three times. cells were incubated with primary Ab (either anti-Ros or anti-MAPK monoclonal Ab B9) for 45 min followed by washing with PBS three times. Biotinylated secondary Ab (goat-anti-rabbit or anti-mouse) was added to the cells and incubated for 10 min followed by washing with PBS three times. Cells were then incubated with peroxidase conjugated streptavidin followed by color reaction with substrate-chromogen mixture. Then cells were mounted with mounting solution and covered with coverslip.

RNA SLOT-BLOT ANALYSIS: Total RNA was extracted from cells by using RNAzoL™B reagent according to manifacture's protocol (TEL-TEST, Inc.). RNA slot-blot was prepared by using Minifold II apparatus according to manufacture's instruction (Schleicher & Schuell). Pre- and hybridization were carried out in a solution containing 50% formamide, 6X SSC, 5X Danharts, 0.5% SDS, 100 µg/ml salmon sperm DNA at 42°C for 2 hrs and overnight, respectively. Filters were washed in 0.1X SSC, 0.2% SDS at 62°C for 30 min. cDNA probes were labeled with random priming kit (Biolab). Human c-jun cDNA and rat c-fos cDNA were gifts from Drs. Irwin Gelman and Selina Chen-Kiang.

RESULTS

CONSTRUCTION AND EXPRESSION OF CHIMERIC EGFR-ROS RECEPTORS

Two EGFR-ROS chimeric receptors which differ only in the origin of their respective TM domains were constructed (Fig. 1). One of them, named ER1, has the entire EC and TM domains of EGFR, including 2 aa residues carboxyl to the TM domain (aa residues -24 to 646 of EGFR) fused to c-*ros* at the cytoplasmic border of its TM domain (aa residues 1891 to 2311). The other, named ER2, has the entire EC domain (aa residues -24 to 625) of EGFR joined precisely at the EC border of the TM domain of c-*ros* (aa residues 1872 to 2311). The chimeras are placed under the control of the SV40 promoter in a mammalian expression vector pECE. To establish stable expression cell lines, ER1, ER2, or pECE vector, together with the pSV2-Neo plasmid containing geneticin resistant gene, was transfected into NIH3T3 cells. After selection with geneticin, the resistant colonies were picked up and analyzed for ER1 and ER2 expression by Western blot with anti-Ros Ab. A broad band could be visualized with apparent molecular mass of 160 KD in some of the resistant clones (data not shown).

The subcellular localization of chimeras was examined by cell surface labelling with NHS-LC biotin, followed by IP with anti-Ros Ab and Western blot with avidin conjugated with AP. A 160 kD protein could be detected in biotin treated cells (Fig. 2), suggesting that the chimeras were properly processed and transported to the cell surface. The apparent sizes of chimeras expressed in NIH3T3 cells are 160 kD, but the calculated molecular

mass of chimeras are about 120 kD, suggesting that they might have undergone extensive post-transnational modification. The EC domain of the chimeras is expected to be glycosylated. This was confirmed by the effect of tunicamycin treatment as it converted the apparent molecular mass of chimeras from 160 kD to 120 kD (Fig. 3). Much less proteins were detected in the presence of tunicamycin treatment, presumably due to increased degradation of the unglycosylated proteins.

The half-lives of the chimeras were examined by pulse-chase labelling with ^{35}S-met in the presence or absence of EGF. In the absence of EGF, both chimeras have half-lives of more than 4 hrs (Fig. 4 B), and this value doesn't decrease significantly in the presence of EGF, indicating that the effect of EGF on the half-lives of chimeras is negligible (Fig. 4 A). In contrast, EGFR has a half-life about 10 hrs in the absence of EGF in the human fibroblasts, but in the presence of EGF the half-life decreases dramatically to about 1 hr since EGFR undergoes rapid ligand-dependent down regulation (Stoscheck et al 1984).

EGF BINDING AND KINASE ACTIVATION OF CHIMERAS

The binding of EGF to ER1 and ER2 was detected by using ^{125}I labeled EGF and compared with that of control, A431 cells. For scatchard plot, cells were incubated with increased amount of ^{125}I-EGF with or without 100-fold cold EGF. The result is expressed as the amount of cell-bound EGF plotted against the ratio of bound/free. As expected, A431 cells which express a much higher level of EGFR have the highest EGF binding capacity (Fig. 5A, B). Two chimeras differing in their TM domains have different EGF

binding capacity, although their expression levels are similar (Fig. 5A, Insert). ER1 that has TM domain derived from EGFR has much higher EGF binding capacity (Fig. 5A, B), presumably due to a more conserved EC domain for EGF binding.

Ligand-induced dimerization of the chimeras and native EGFR were examined by chemical cross-linking. Cells expressing chimeras or EGFR were incubated with EGF at 4°C and then treated with BS^3, a membrane nonpermeable chemical cross-linker, followed by in vitro kinase assay (Fig. 6). As expected, dimeric form of EGFR was detected by using cross-linker. Similarly, dimeric forms of ER1 and ER2 about 320 kD were also detected in their expressing cells. ER1 and ER2 appeared to be dimerized as efficiently as the native EGFR. It should be pointed out that a band about 320 kD present in ER2 cells in the absence of BS^3 was not reproducible.

Generally, receptor activation upon ligand stimulation is fast and dosage dependent. This is also true for the two chimeras as they could be activated as rapidly as 30 seconds and activity increased proportionally as the dosage of EGF as shown by in vitro kinase assay and intracellular tyrosine phosphorylation (Fig. 7). As maximal stimulation of the kinase activity of the chimeras was reached with 50 ng/ml of EGF, this could be the saturation concentration for the ER1 and ER2 cells. After stimulation of EGF at saturation concentration for more than 10 min, in vitro kinase activities of both chimeras started to decrease, presumably the major autophosphorylation sites had already been phosphorylated and as a consequence couldn't be further phosphorylated in vitro.

BOTH CHIMERAS INDUCE CELL TRANSFORMATION, BUT ER1 INHIBITS CELL

GROWTH, WHEREAS ER2 STIMULATES IT.

The biological effects of chimeras on their expressing cells were examined. The anchorage-independent growth, an important characteristic of transformed phenotype, was analyzed by colony formation assay in soft agar. In one of the experiments, two individual clones each from ER1 and ER2 cells were analyzed. The result showed that both chimeras induced colony formation with about equal efficiency in an EGF dependent manner, although ER2 had a higher basal level activity (Table 1 and Fig. 8). No significant difference in the size and time of appearance of colonies were observed between ER1 and ER2 clones.

Morphology of the chimera expressing cells changed dramatically in response to EGF treatment. Both ER1 and ER2 cells transformed from flattened morphology to elongated, spindle-shape and piled up in groups and oriented in a crisscross manner (Fig. 9). However, the size of ER1 cells became bigger, indicating that these cells were not actively dividing.

Growth rate of the chimera expressing cells were determined in the presence or absence of EGF. Surprisingly, the two chimeras have opposite effects on cell growth (Fig. 10). ER1 inhibits whereas ER2 stimulates cell growth. The opposite effects were not due to clonal variation as all 3 independent ER1 clones were growth inhibited upon EGF treatment whereas all 3 ER2 clones were simulated. The opposite effects on cell growth could be detected when cells were treated with EGF at a concentration from as little as 0.25 to as high as 50 ng/ml and the effects were dosage dependent (Fig. 11). Neither

increase of serum concentration (Fig. 11) or the addition of IGF-1 (data not shown) could overcome the inhibitory effect. The medium harvested from ER2 culture after 2 days incubation with EGF could not rescue the EGF-mediated inhibition of ER1 cells. Conversely, such conditional medium from ER1 culture had no effect on ER2 cells (data not shown).

The opposite cell growth effects were further confirmed by ^{3}H-thymidine incorporation. As shown in Fig. 12, thymidine incorporation increased in ER2 cells as the dosage of EGF increased, whereas the opposite was true for ER1 expressing cells.

Cell detachment of chimera expressing lines in the presence or absence of EGF was monitored by counting cells in the medium. No obvious difference was noticed between the two chimera expressing lines before and after EGF treatment (data not shown), indicating that the inhibition of cell growth is not due to increased detachment of ER1 cells in the presence of EGF. DNA fragmentation, a hallmark of apoptosis, was analyzed by gel electrophoresis of genomic DNA extracted from the chimera expressing cells treated with EGF for 2 days. No DNA fragmentation was observed in the chimera expressing cells (Data not shown), indicating that apoptosis is not responsible for the growth inhibition of ER1 cells.

ALL CELL PHASES ARE ELONGATED IN ER1 CELLS WHEREAS G1 PHASE IS SHORTENED IN ER2 CELLS AFTER EGF TREATMENT

To further explore the opposite cell growth effects caused by the two chimeras, I

analyzed by flow cytometry the cell cycle distribution of chimera expressing cells in response to EGF. In this experiment, triplicate dishes of ER1 and ER2 cells were either treated with EGF for 2 days or without treatment. Cells were harvested before and after treatment, and cell numbers were counted to determine the doubling times before cell cycle analysis. The result showed (Table 2), unexpectedly, in response to EGF ER1 cells had slightly reduced proportion of cells in G1, and increased percentage in S and G2/M phases. However, cells in G1 phase decreased dramatically and cells in S and G2/M phases increased significantly in ER2 cells. The doubling time of ER1 cells were determined as 60 hrs when cells were grown in 0.5% serum without EGF whereas this value increased to 91 hrs after EGF treatment. By contrast, EGF treatment of ER2 cells shortened their doubling time from 77 to 38 hrs. From the cell cycle data in table 2 and the doubling times, the duration of each phase of the cell cycle was calculated (Table 3). For ER1 cells, all phases of cell cycle were elongated after EGF treatment, especially S and G2/M phases which were approximately doubled. For ER2 cells, however, the duration of G1 phase was reduced by 3 folds whereas that of S and G2/M phases remained essentially the same after EGF stimulation.

SIGNAL TRANSDUCTION BY THE CHIMERIC RECEPTORS

To understand the molecular mechanism underlining the biological effects of the chimeras, signaling pathways mediated by the chimeras were studied.

IRS1 is an important signaling component of IR family RPTKs. Upon

phosphorylation, IRS1 functions as an adaptor for the SH2 domain containing proteins, including PI3K and Grb2. My result showed that IRS1 was phosphorylated in both chimera expressing cells upon EGF treatment (Fig. 13 A).

PLCγ breaks down lipid to generate DAG and IP3 that are involved in PKC activation and calcium mobilization, respectively. PLCγ was also phosphorylated in chimera expressing cells in response to EGF treatment (Fig. 13 B). Both IRS1 and PLCγ were phosphorylated to higher extent in ER1 cells. This correlated with the higher EGF binding of ER1 than that of ER2.

PI3K is involved in mitogenic signaling for certain RPTKs. It can be activated through different mechanisms, including interaction with activated RPTKs through its p85 subunit or the adaptor IRS1. To examine whether PI3K was activated, the chimera- and IRS1-associated PI3 Kinase activity was analyzed. As shown in Fig. 14, EGF promoted association of PI3 Kinase with chimeras and IRS1. The fact that much higher IRS1- than chimera-associated PI3 Kinase activity suggests that PI3K interacts with the chimeras mainly through IRS1 adaptor. Similarly, both chimeras- and IRS1-associated PI3 kinase activities are higher in ER1 than in ER2 cells.

MAPK is a major signal transducer of the Ras signaling pathway. It has multiple substrates and thus can regulates a very wide range of events such as proliferation and differentiation. MAPK activation is well controlled events. The duration of MAPK activation and nuclear translocation may have a profound consequence on MAPK-mediated biological effects. MAPK activation was compared between the two chimera expressing cells. In both chimera expressing cells, MAPK was phosphorylated as evidenced by

mobility up shift in SDS-PAGE apparently due to hyperphosphorylation (Fig. 15 B), and its activity increased dramatically in response to EGF treatment (Fig. 15 A). MAPK constitutes p42 and p44 species, both of them were found to be retarded in SDS-PAGE in response to EGF treatment. The duration of MAPK activation was compared between the two chimera expressing cells(Fig. 15 C). The majority of MAPK was up shifted after EGF stimulation for 10 min followed by withdrawal for 10 min and then down shifted thereafter. In ER1 cells, about 50% of MAPK was shifted down after EGF withdrawal for 1 hr whereas it took 2 hrs in ER2 cells. I also examined nuclear translocation of MAPK in response to EGF treatment by cell staining. The result showed that MAPK was translocated to nucleus in both chimera expressing cells after EGF stimulation for as soon as 5 min and remained in nucleus for at lest 1 hr (Fig. 16).

ER1 IS LESS EFFICIENTLY INTERNALIZED AND REMAINS PHOSPHORYLATED FOR A LONGER TIME.

Following ligand binding, RPTK-ligand complex clusters in coated pits and is internalized via clathrin-coated vesicles. The ligand-RPTK complex is then delivered to lysosomes through endosomal compartments. Some of the RPTK are recycled to the cell surface while the others undergo degradation, although the efficiency of recycling varies greatly among different RPTKs. Both the cytoplasmic and the TM domains have been demonstrated to influence the rate of receptor internalization and degradation.

The two chimera expressing lines were analyzed for internalization of chimeras after

EGF treatment for various times. The result showed that ER1 was much less efficiently internalized than ER2 (Fig. 17). After 2 hrs of treatment with EGF, the majority of ER1 still remained on the cell surface as detected by cell surface labeling, while most of ER2 was internalized at this point. Both chimeras, however, were not degradated upon EGF treatment as detected by Western blot (Fig. 17 B) and pulse-chase metabolic labelling as mentioned before (Fig. 4).

The phosphorylation state of chimeras after EGF stimulation for 10 min followed by withdrawal for different times were analyzed. The result showed that ER1 remained tyrosine phosphorylated for a longer time than ER2 after EGF withdrawal (Fig. 18). Half of ER1 remained phosphorylated after EGF withdrawal for 2 hrs whereas half of ER2 were dephoaphorylated within 30 min, suggesting that dephosphorylation of chimeras may occur mainly in the cytoplasm after they were internalized. Although ER1 remained phosphorylated on cell surface for a longer time, it didn't support a sustained activation of MAPK (Fig. 15). However, the sustained activation of ER1 did have an effect on its immediate substrates such as IRS1, PLCγ, and PI3K, which were activated or phosphorylated to higher extent in ER1 cells.

CELLULAR PROTEIN PHOSPHORYLATION PATTERN IS DIFFERENT BETWEEN THE TWO CHIMERA EXPRESSING CLONES UPON EGF TREATMENT.

Upon ligand binding the RPTK become activated resulting in phosphorylation of itself and of cellular substrates. Different RPTKs phosphorylate distinct patterns of cellular

substrates, although some known cellular substrates have been found to be phosphorylated by a variety of RPTKs. The different tyrosine phosphorylation patterns of cellular proteins may reflect the different signaling specificity of the RPTKs. The potential cellular substrates of ER1 and ER2 were analyzed by Western blot with RC20. The result showed that the overall tyrosine phosphorylation patterns were similar in both chimera expressing cells in response to EGF treatment (Fig. 19). However, several proteins were differentially phosphorylated between the two chimera expressing cells. For example, a protein at about 200 kD appeared to be phosphorylated in ER2 cells, whereas two proteins lass than 43 kD were phosphorylated in ER1 cells. I do not know the significance of these differences. Nevertheless, they may suggest that different signaling pathways are triggered by the two chimeras despite of their identical cytoplasmic domains. To detect whether cellular proteins were recruited to the chimeras, cells were treated with EGF and lysed with mild detergent and immunoprecipitated with anti-Ros Ab, followed by Western blot with RC20. The result showed that several tyrosine phosphorylated proteins were associated with chimeras upon EGF stimulation, but no apparent difference in associated proteins was detected (Fig. 20 A). Alternatively, cells were labeled with ^{35}S-Met and lysed with mild detergent. The potential chimera associated proteins were detected by co-immunoprecipitation with anti-Ros Ab, followed by SDS-PAGE. The result showed that several proteins could be brought down by anti-Ros Ab, but none of them responded to EGF stimulation and no difference between the two chimeras were detected (Fig. 20 B).

THE 66 KD SHC PROTEIN IS DIFFERENTIALLY PHOSPHORYLATED IN ER2 CELLS

Shc consists of three species with apparent molecular mass of 46, 52, and 66 kD. Shc is an important cell growth regulator since overexpression of Shc in fibroblasts results in transformation. Shc can be tyrosine phosphorylated by and recruited to a number of RPTKs, but the specific function of each species of Shc is not known. I noticed that the 66 kD of Shc was phosphorylated to higher extent in ER2 cells, whereas 46 and 52 kD Shc proteins were equally phosphorylated in both chimera expressing cells in response to EGF (Fig. 21). Interestingly, a 190 kD protein could be detected in the anti-Shc immunocomplex after EGF treatment in ER2 but not in ER1 cells. Further experiments suggested that this 190 kD protein was not the chimera itself or IRS1 (data not shown). Differential phosphorylation of 66 kD Shc and the 190 kD protein provided another piece of evidence that the two chimeras have different signaling capacity.

INDUCTION OF C-JUN AND C-FOS BY THE CHIMERAS

c-*jun* and c-*fos* are among the genes that are rapidly induced in response to a variety of agents such as growth factors. The induction of c-*jun* and c-*fos* were analyzed in chimera expressing cells after EGF treatment for different times (Fig. 22). Both c-*jun* and c-*fos* were induced in ER1 and ER2 cells. However, the induction of c-*fos* was much less prominent in ER1 cells than that in ER2 cells whereas the induction of c-*jun* in ER2 cells lasted longer than that in ER1 cells. Interestingly, the kinetics of c-*jun* induction correlates with the duration of MAPK activation shown above, namely it is longer in ER2 cells. No kinetic difference of c-*fos* was observed between the two chimera expressing cells. This

might be that the interval between the two time points undertaken in this experiment is too long to reveal the difference since c-*fos* has a very short half-life.

Fig. 1 Construction of EGFR-ROS chimeras. Structures of the c-*ros*, EGFR and chimeras, ER1 and ER2, are shown. ER1 has the EC and the TM domains of EGFR (aa -24 to 646) fused to the cytoplasmic domain of c-*ros* (aa 1891-2311) whereas ER2 has the EC domain of c-*ros* replaced by the analogous region of EGFR (aa -24 to 625). Restriction sites used for cloning are shown. Primers, P1 and P2, were used to amplify the cytoplasmic domain of c-*ros* for construction of ER1, while P3 and P4 primers were used to amplify the TM and cytoplasmic region of c-*ros* for construction of ER2.

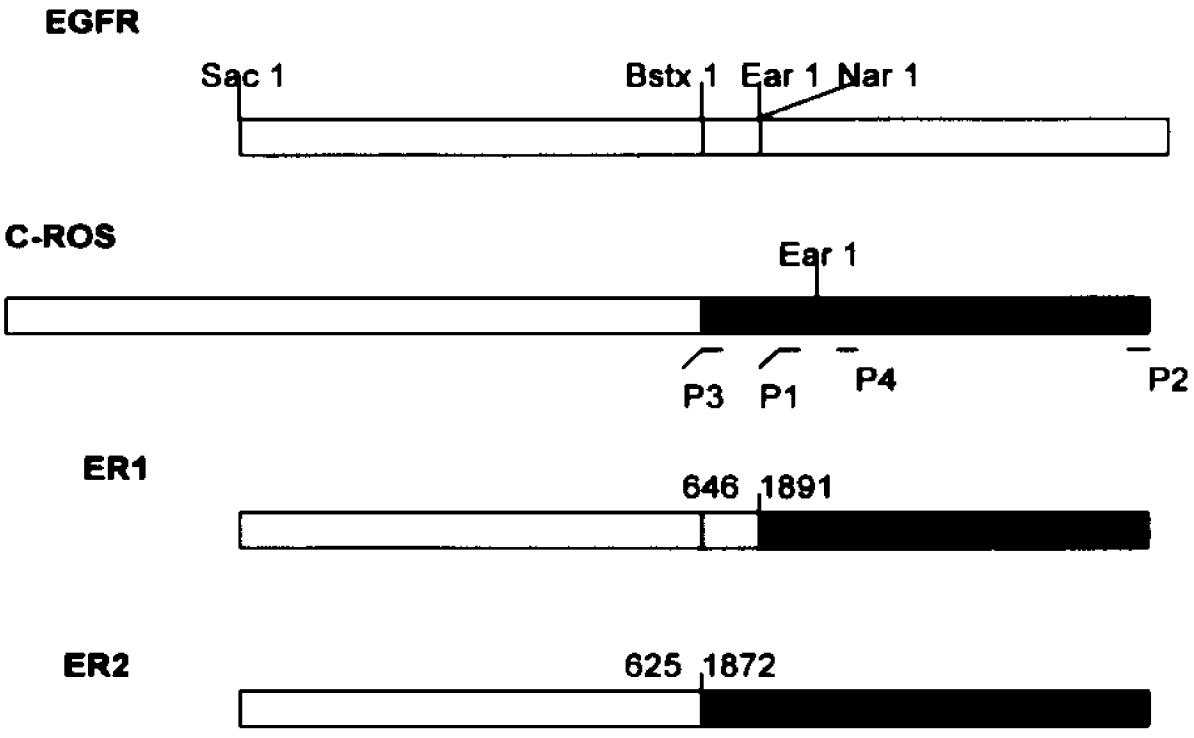


Fig. 2 Cell surface labelling with biotin. ER1 and ER2 cells were either treated with NHS-LC biotin (1 mg/ml) at 4°C for 1 hr or left without treatment. The reaction was stopped with the addition of 20 mM glycine. Cells were then washed with PBS, lysed, immunoprecipitated with anti-Ros Ab. The immunoprecipitates were resolved on 7.5% SDS-PAGE and Western blotted with avidin conjugated with AP.

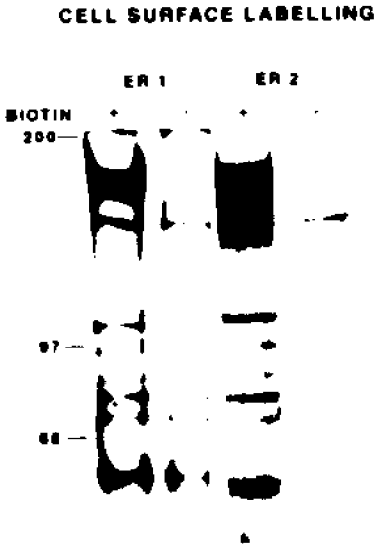


Fig. 3 Glycosylation of the chimeras. Cells were starved with methionine (Met)-free medium or Met-free medium containing 10 μg/ml of tunicamycin for 1 hr and then labeled with ^{35}S-Met for 4 hrs in the presence or absence of tunicamycin (10 μg/ml) as indicated. Cells were then washed with PBS, lysed, immunoprecipitated with anti-Ros Ab, followed by 7.5% SDS-PAGE and autoradiography. Note that tunicamycin treatment converted the apparent molecular mass of both chimeras from 160 kD to 120 kD. T: tunicamycin.

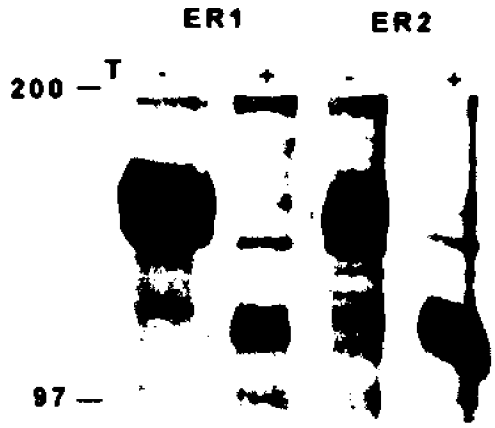


Fig. 4 Half-lives of chimeras. Cells were serum starved overnight and Met starved for 1 hr and labeled with ^{35}S-Met in the presence or absence of 100 ng/ml EGF for 20 min. Cells were then washed with PBS and incubated with serum-free medium supplemented with or without EGF (100 ng/ml) for indicated times before cells were lysed. IP and SDS-PAGE were done as described above.

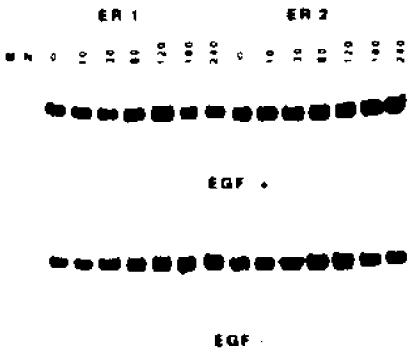


Fig. 5 A. EGF binding. Duplicate dishes of A431, ER1, ER2, and control NIH3T3 cells were incubated with 5 ng/ ml ^{125}I-EGF in the presence or absence of 100-fold cold EGF. Results shown here are cell associated radioactivity determined in the absence of cold EGF minus that in the presence of cold EGF. The insert shows the expression levels of EGFR in A431 cells, ER1 and ER2. Lysates from same number of cells for each cell lines were used. B. Scatchard plot of EGF binding to EGFR (A431), ER1, and ER2. Experiments were described above except that cells were incubated with ^{125}I-EGF at concentrations of 0.2 ng/ml, 1 ng/ml, 5 ng/ml, 25 ng/ml, and 125 ng/ml.

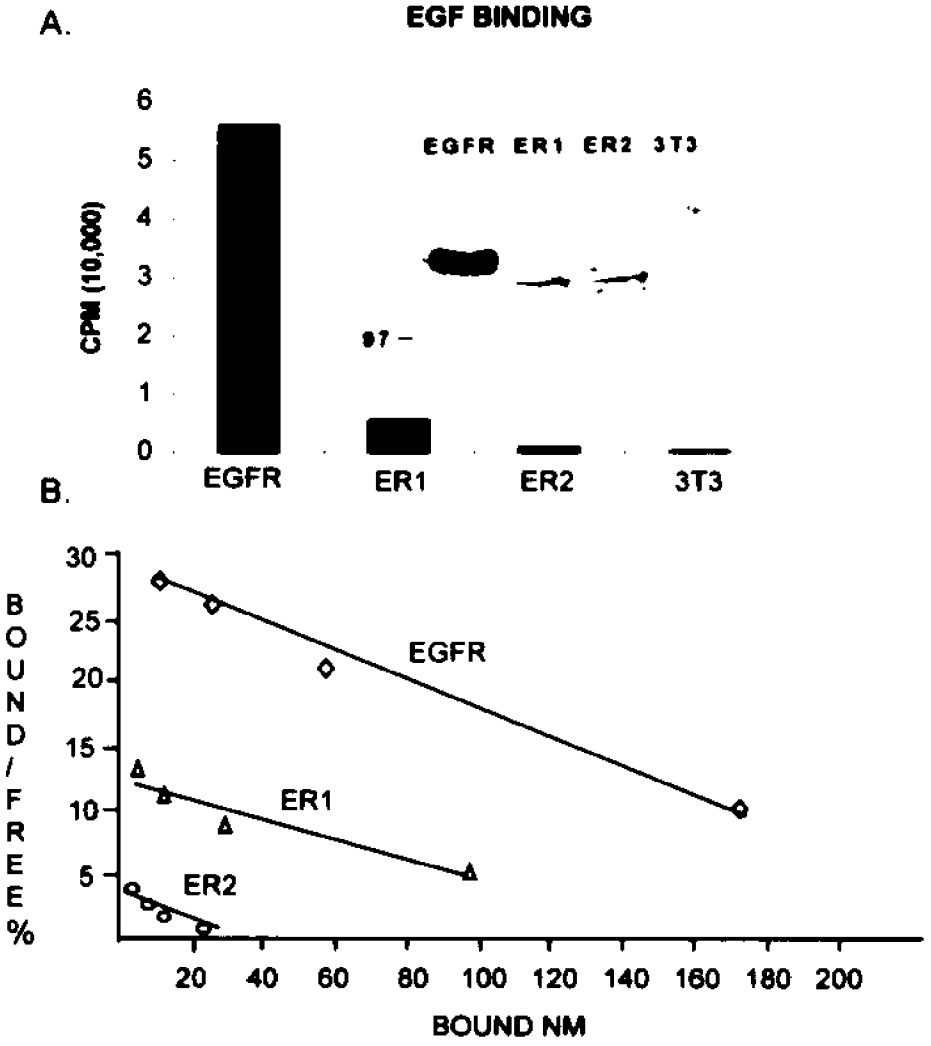


Fig. 6 Dimerization of the chimeras and native EGFR. A431, ER1, ER2 and control NIH3T3 cells were treated with 100 ng/ml of EGF at 4°C for 2 hrs, followed by cross-linking with BS^3 (1 mM) for 15 min or left without treatment. Cells were then lysed and the lysates were subjected to in vitro kinase assay as described in materials and methods.

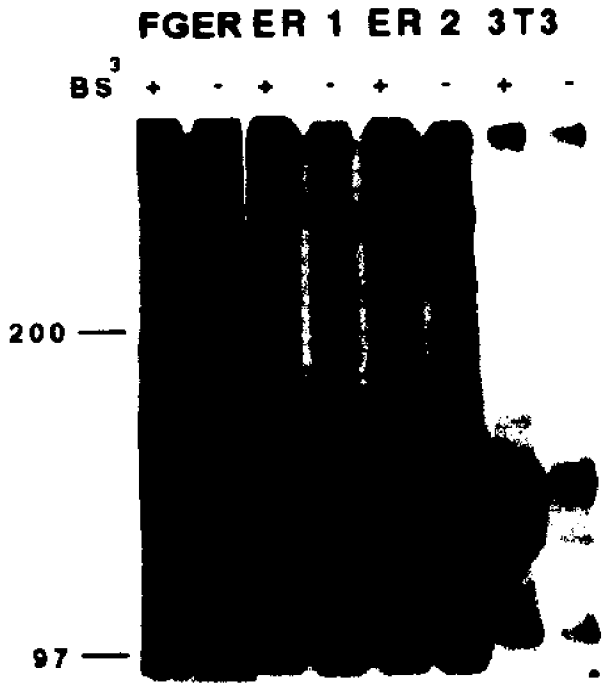


Fig. 7 Kinetics of chimera activation. ER1 and ER2 cells were serum starved overnight and treated with either different amount of EGF as indicated for 10 min or for different times with 100 ng/ml of EGF. Cells were then lysed and equal amounts of protein were used for immunoprecipitation with anti-Ros Ab. In vitro kinase assay was done as described. Intracellular tyrosine phosphorylation was detected by blotting with RC-20. Receptor amount used for kinase assay and intracellular tyrosine phosphorylation was measured by Western blot with anti-Ros Ab.

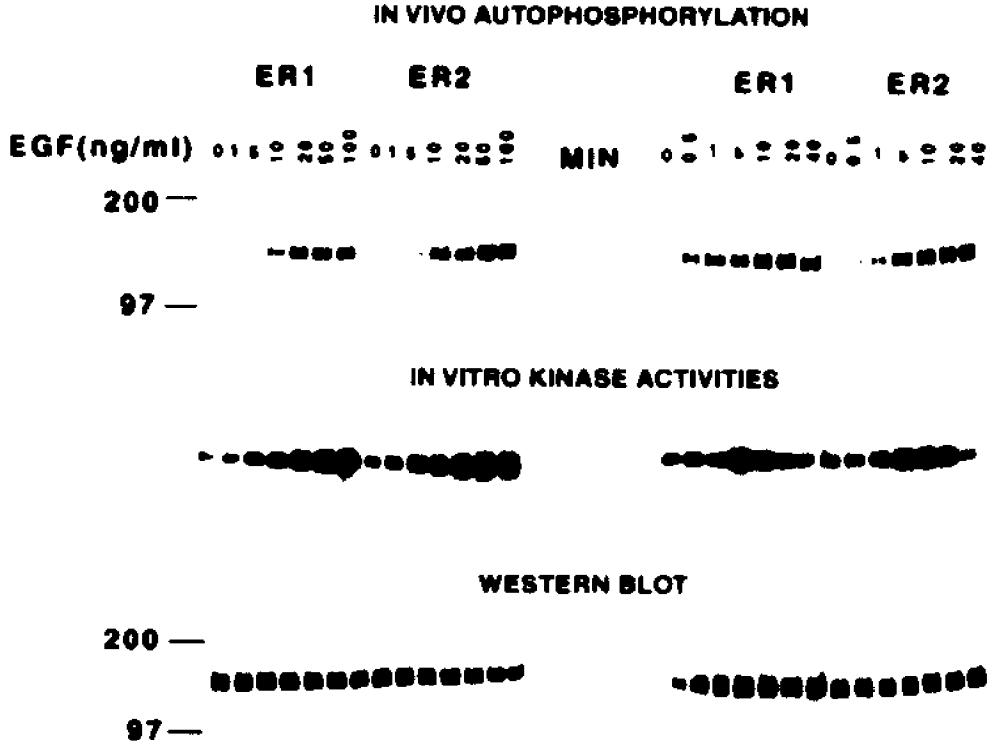


Fig. 8 Colony formation assay. Two individual clones each of ER1 and ER2 cells and control NIH3T3 cells were seeded in soft agar at 10^5 cells per 60 mm dish in the presence or absence of 10 ng/ml EGF. Every 5 days the cells were over-laid with soft agar medium containing either 0.5% serum or 0.5% serum plus 10 ng/ml EGF. Colonies were counted and pictures were taken after 20 days of incubation. Clones ER1-2, ER2-69 and 3T3-1 are shown.

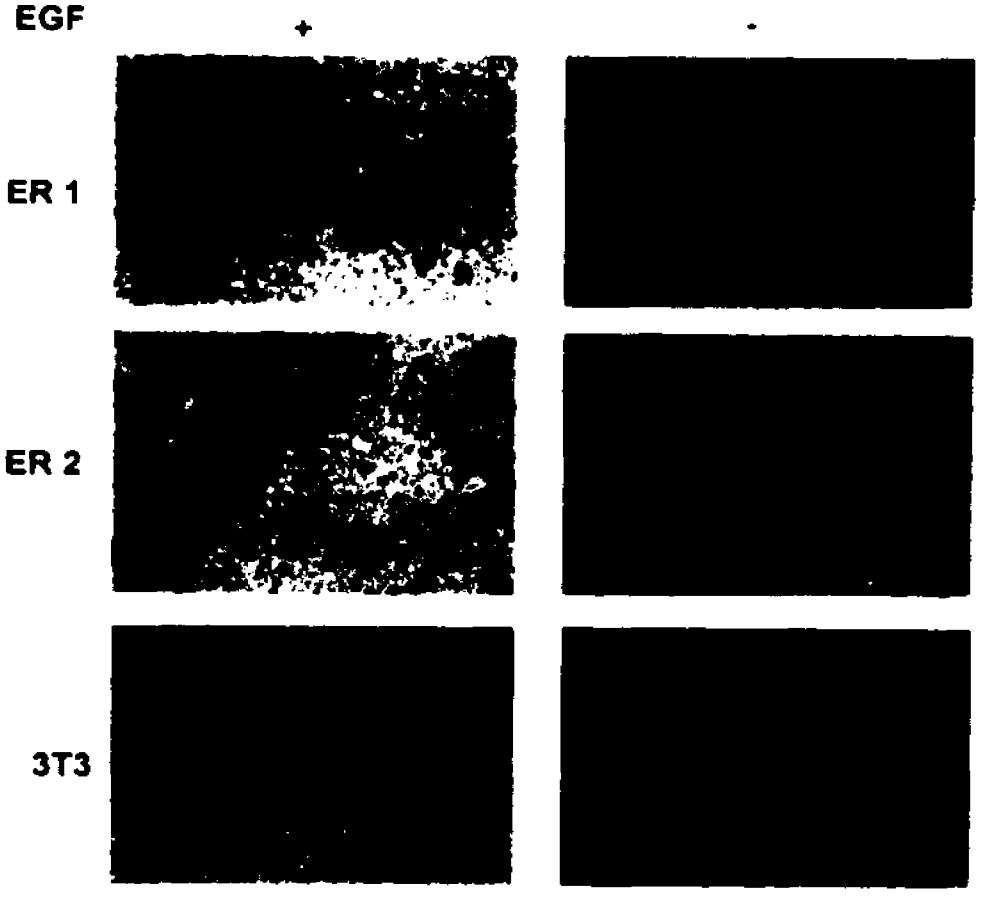


Table 1 Colony formation assay

Clones	EGF	
	+	-
ER1-2	1396	8
ER1-8	1316	8
ER2-51	1964	208
ER2-69	1872	480
3T3-1	82	10
3T3-2	72	8

Experiment was described in Fig. 3

Fig. 9 Morphological change of chimera expressing cells after EGF treatment. Cells were seeded at 10^5 cells per 60 mm plate. Following overnight serum starvation, cells were incubated with medium containing 0.5% calf serum or 0.5% serum plus 10 ng/ml EGF. Pictures were taken after EGF treatment for 3 days.

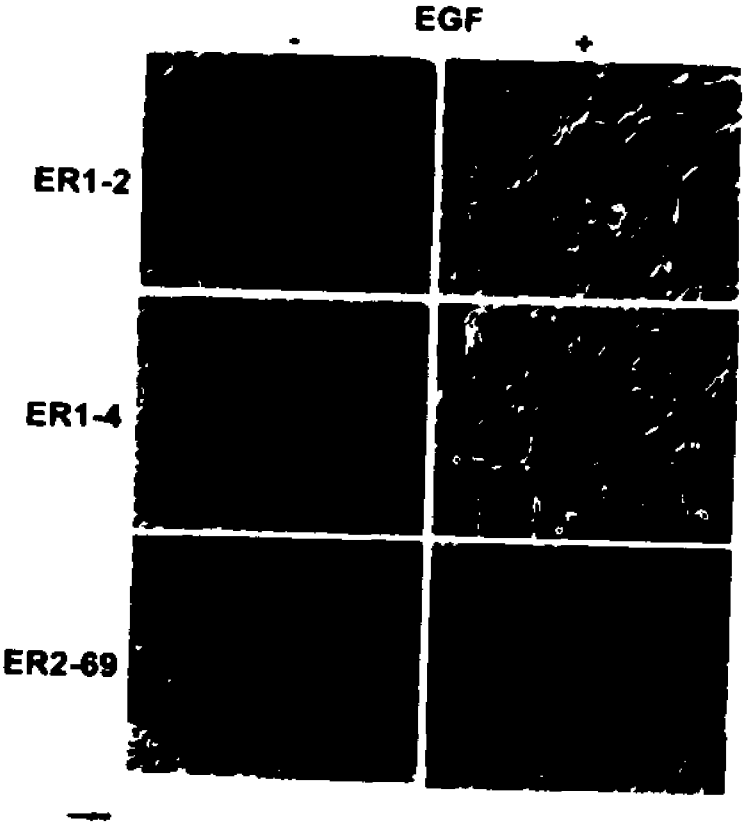


Fig. 10 Growth rate of chimera expressing cells. Experiment were the same as described in Fig. 9. Cell numbers were counted every other day. Three individual clones each of ER1 and ER2 cells were analyzed. They are ER1 clones 2, 4, and 8 and ER2 clones 51, 55, and 69. In addition, a control geneticin resistant clone was compared in parallel. day 0 is defined when EGF treatment started. Mean values of duplicate experiments are shown.

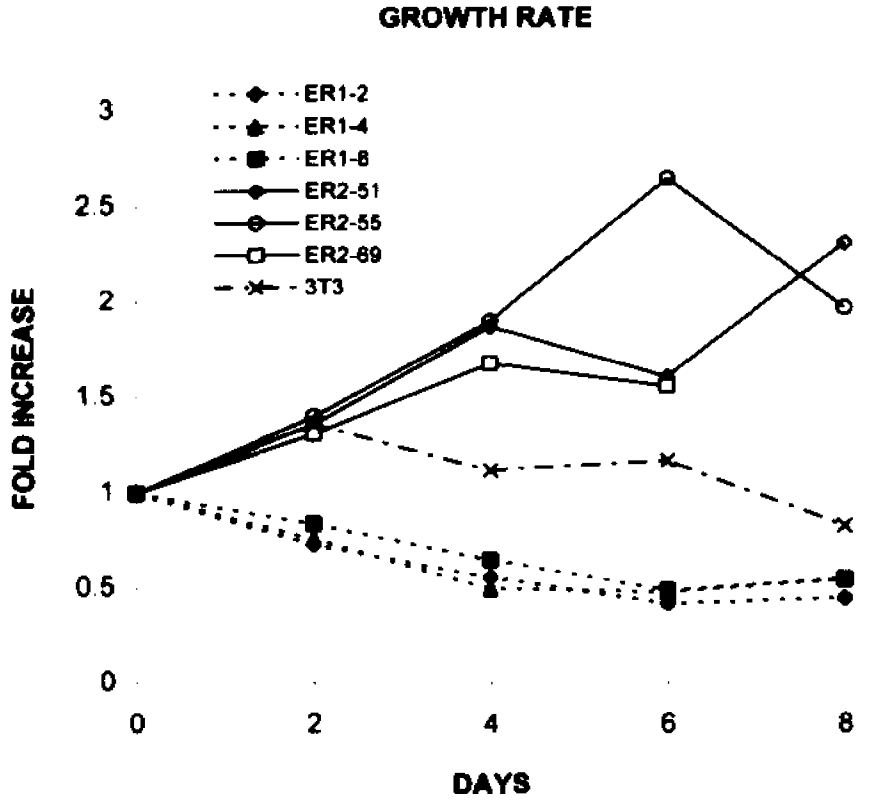
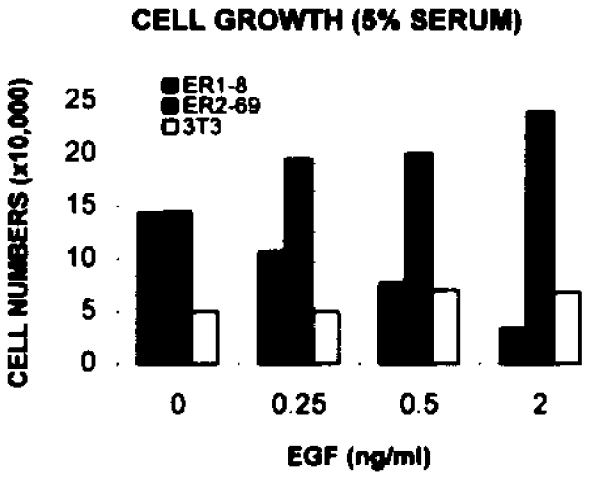
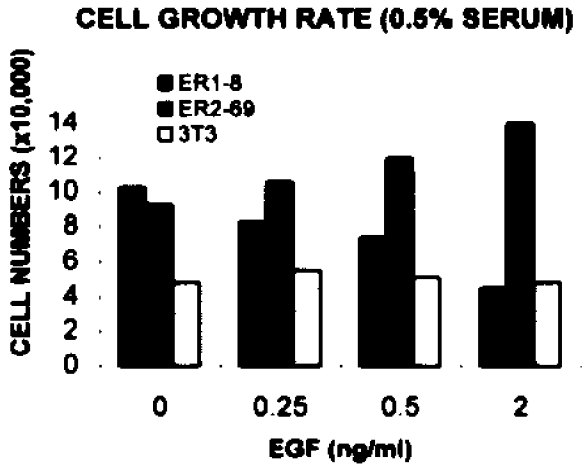


Fig. 11 The effects of the dosage of EGF and concentration of serum on ER1 and ER2 expressing cells. Cells were seeded at 1.5 X 10^4 cells per wells in 24-well plates. Note that less 3T3 control cells were seeded at the beginning. Following overnight serum starvation, cells were incubated in DMEM supplemented with either 0.5% calf serum (B) and 5% calf serum (A) plus different amount of EGF as indicated. Cell numbers in day 3 are shown. C. Experiment were described in Fig. 9 except that cells were treated with different amount of EGF as indicated. ER1 clone 2 and ER2 clone 69 are shown.

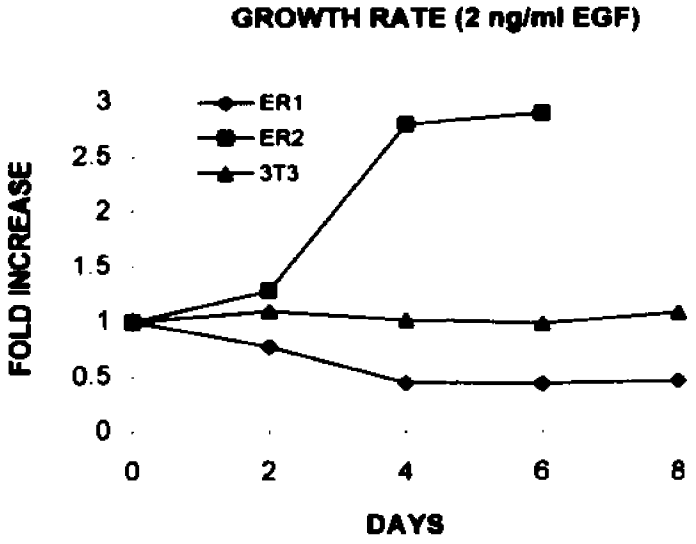
A.



B.



C.


GROWTH RATE (2 ng/ml EGF)
ER1
ER2
3T3
FOLD INCREASE
3
2.5
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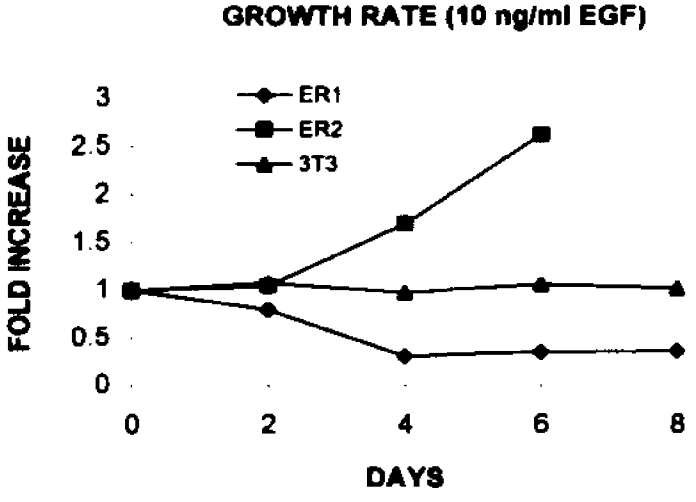
DAYS


GROWTH RATE (10 ng/ml EGF)
ER1
ER2
3T3
FOLD INCREASE
3
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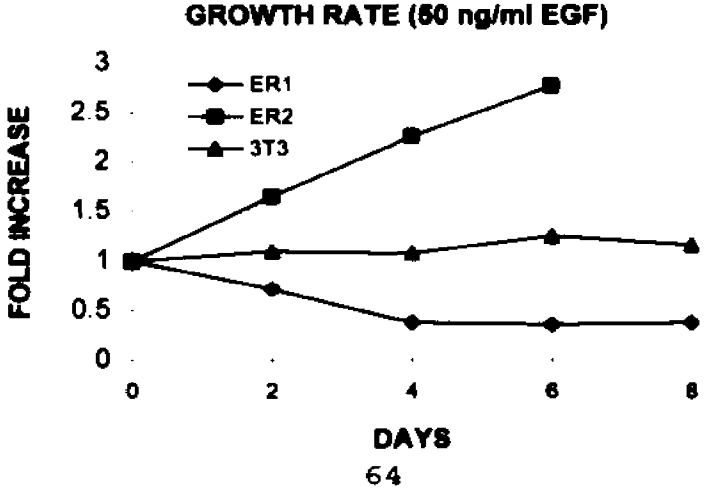
GROWTH RATE (50 ng/ml EGF)
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ER2
3T3
FOLD INCREASE
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
DAYS

Fig. 12 ^{3}H-thymidine incorporation. ER1, ER2, and NIH3T3 cells were seeded at 5x10^{5} cells per well in 24-well plates. following 48 hrs starvation with medium supplemented with 0.5% calf serum, cells were treated with EGF at different concentration as indicated for 16 hrs or left without treatment and then ^{3}H-thymidine was added and incubated for another 4 hrs. Detection of 3H-thymidine incorporation was described in materials and methods. Mean value of duplicate experiments are expressed as a ratio over basal value at no EGF treatment.

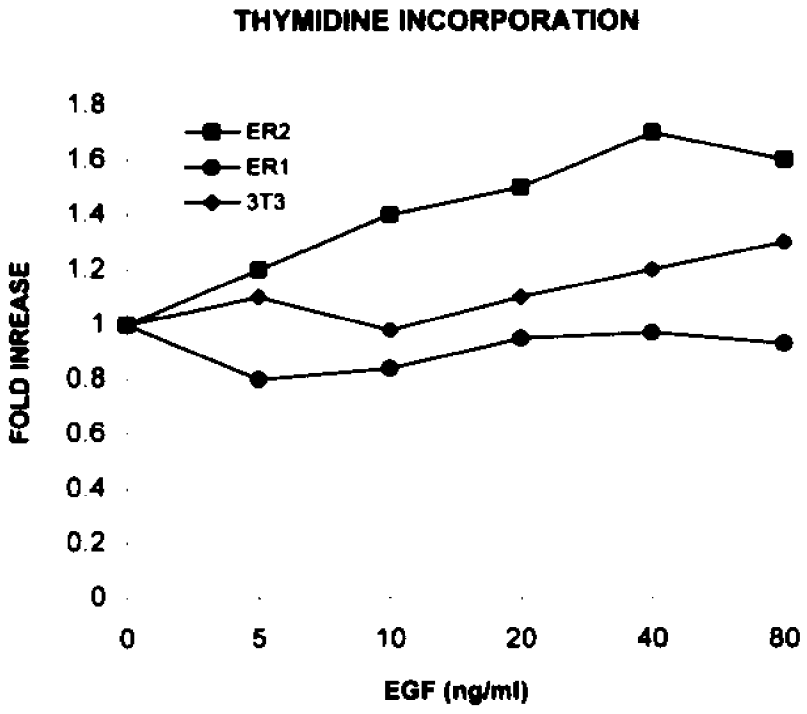


Table 2A Cell cycle distribution

Cells		% of cells in:		
		G1	S	G2/M
ER1	E-	66.6	19	14.2
	E+	56.1	21.3	22.3
ER2	E-	81.7	12	6.6
	E+	56.9	26	17.3

ER1 and ER2 cells were seeded at 200,000 cells per 6 cm dish. Cells were incubated in medium containing 0.5% serum for 2 days and then treated with EGF at 50 ng/ml for 2 days or left untreated. Cell number was counted and flow cytometry was analyzed just before or after EGF treatment for 2 days. Experiments were done in triplicate.

Table 2B Duration of each cell phase (hr)

Cells		Td	G1	S	G2/M
ER1	E-	60	40.1	8.5	11.4
	E+	91	51.0	20.3	19.4
ER2	E-	77	62.9	5.1	9.2
	E+	38	21.6	6.6	9.9

Duration of each cell phase was calculated from cell cycle distribution (Table 2a)and doubling times (Td)

Fig. 13 Tyrosine phosphorylation of IRS1 (A) and PLCγ (B). Cells were serum starved overnight and stimulated with 100 ng/ml of EGF for 10 min or without treatment. Lysates were immunoprecipitated with either anti-IRS1 (A) or anti-PLCγ (B)Ab, followed by 7.5% SDS-PAGE and Western blotted with RC 20 (A and B, upper panels), anti-IRS1 (A, lower panel), or anti-PLCγ (B, lower panel)Ab.

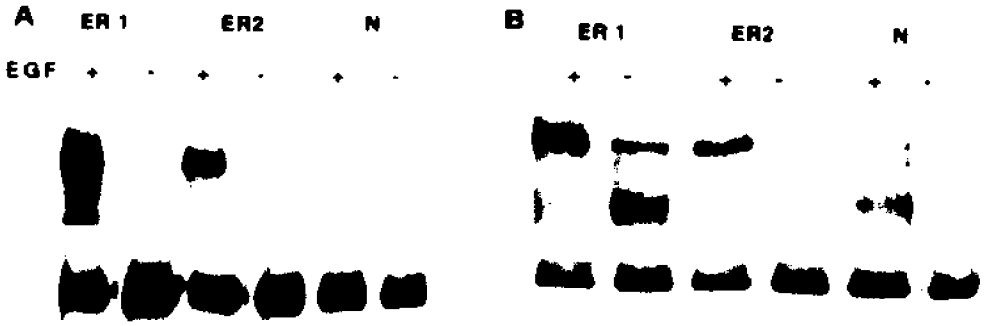


Fig. 14 Activation of PI3 Kinase by the chimeras. Serum starved cells were treated with 100 ng/ml of EGF for 10 min and lysed with 1% NP-40 buffer and immunoprecipitated with either anti-IRS1 or ant-Ros Ab. The IRS1- (A) or chimera- (B) associated PI3 Kinase activity was done as described in materials and methods.

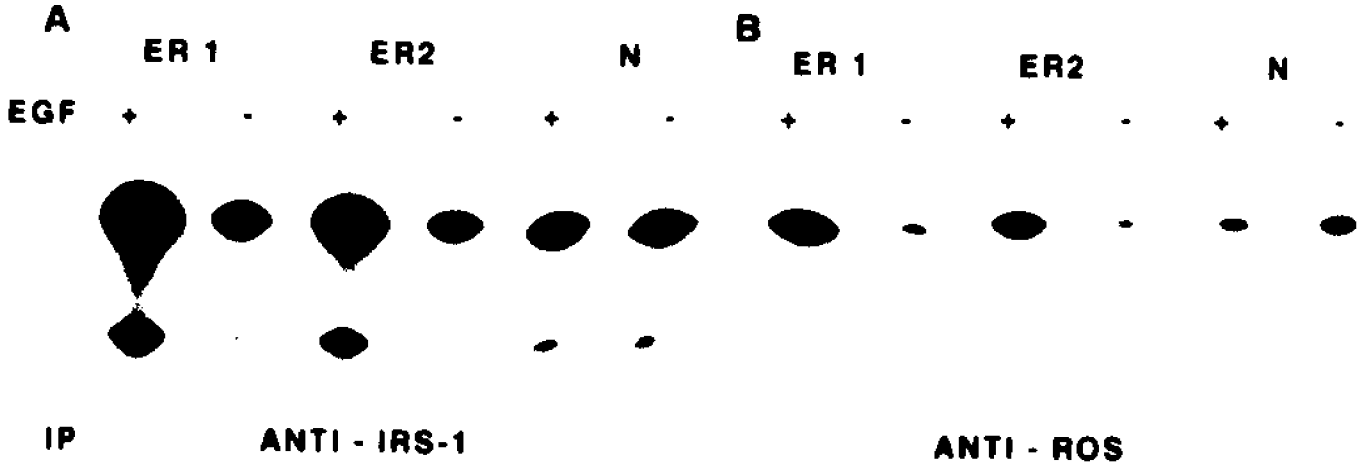


Fig. 15 Effects of EGF on the chimera-mediated MAPK activation. Serum starved cells were treated with 100 ng/ml EGF for 10 min before cells were lysed with RIPA containing 0.1% SDS. A. Activation of MAPK by the chimeras. For MAP Kinase assay, lysates were immunoprecipitated with TR-10 Ab. Labeled MBP were visualized by 15% SDS-PAGE, followed by autoradiography. B. Mobility shift of MAPK. 20 ug total cell lysates were loaded onto a 10% SDS-PAGE (Bisacrylamide:acrylamide equals 1:77) and Western blotted with anti-ERK-1 Ab. C. Duration of MAPK phosphorylation. Cells were stimulated with 100 ng/ml EGF for 10 min and then washed with serum free medium to remove EGF. Cells were then incubated in serum free medium for different times before lysis for Western blot.

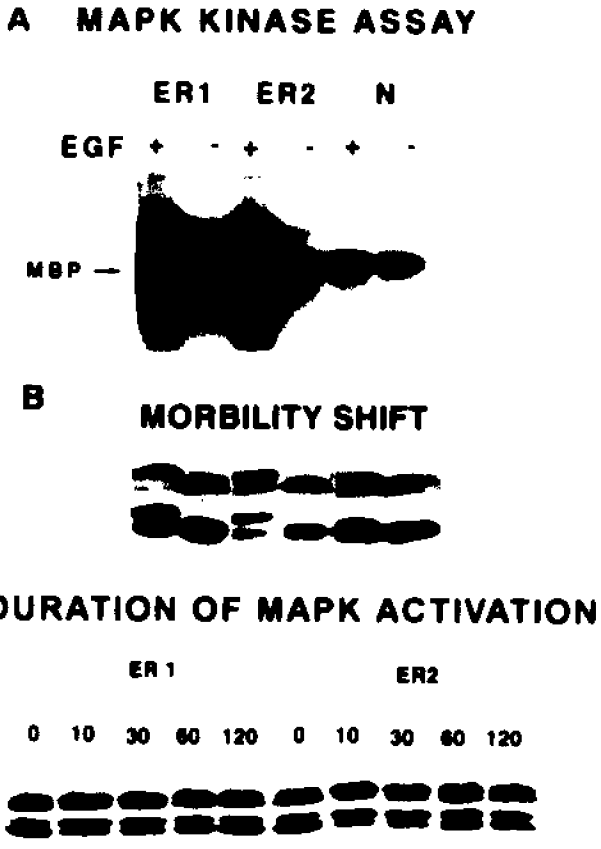


Fig. 16 Nuclear translocation of MAPK after EGF stimulation. Cells were seeded in 35 mm dishes at approximately 20% confluence. After overnight serum starvation, cells were treated with 100 ng/ml of EGF for the indicated times and then cells were prepared for staining as described in materials and methods. Pictures were taken at 40X magnification.

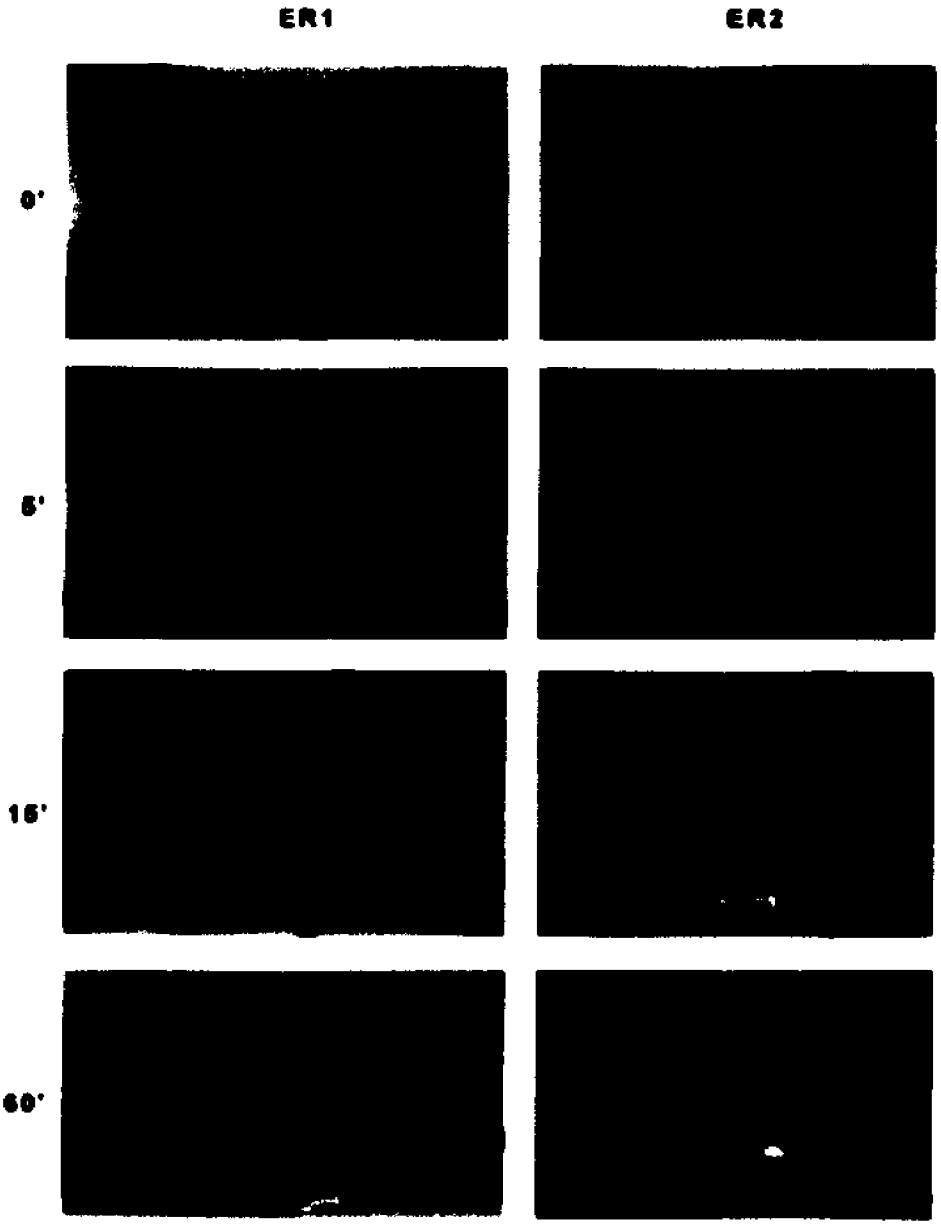


Fig. 17 ER1 is less efficiently internalized than ER2. ER1 and ER2 cells were serum starved overnight and treated with EGF for the indicated times. Cells were cooled on ice, washed with PBS and surface labeled with Biotin. Cells were then lysed, immunoprecipitated with anti-Ros Ab and Western blotting with avidin conjugated with AP (A) or anti-Ros Ab (B).

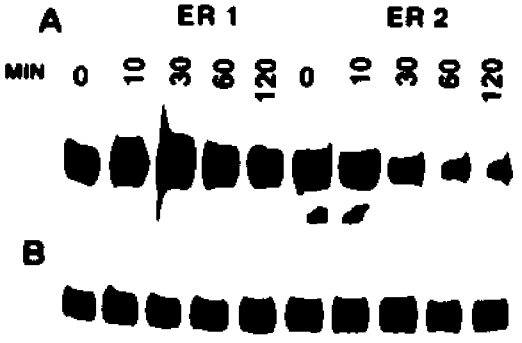


Fig. 18 ER1 remains tyrosine phosphorylated for a longer time after EGF withdrawal. Serum starved ER1 and ER2 cells were stimulated with 100 ng/ml of EGF for 10 min. Then EGF was washed away and cells were incubated with serum free medium for the indicated times before they were lysed. In vivo phosphorylation of chimeras and Western blot were done as described in Materials and Methods.

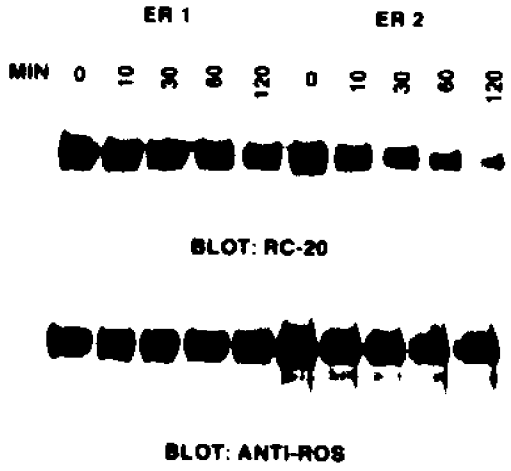


Fig. 19 Cellular protein tyrosine phosphorylation. Cells were serum starved overnight and treated with 100 ng/ml of EGF for 10 min. Cells were lysed and equal amount of protein were used for immunoprecipitation with anti-phosphotyrosine monoclonal Ab 4G10, followed by SDS-PAGE and blot with RC 20.

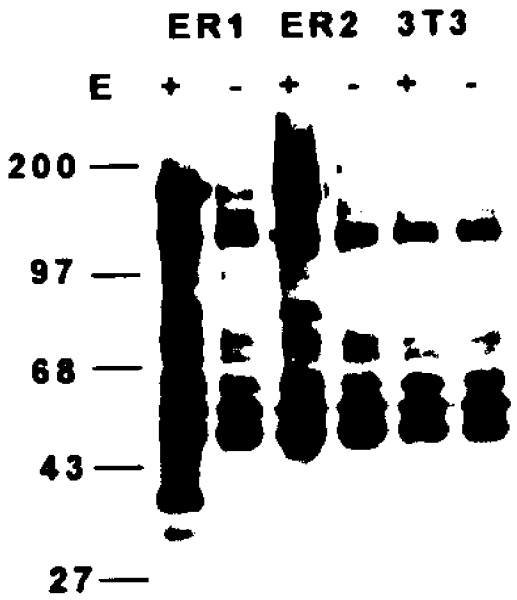


Fig. 20 Detection of chimera-associated proteins. A. Serum starved cells were treated with 100 ng/ml of EGF for 10 min and then lysed with mild detergent (0.1% NP-40). Cell lysates were immunoprecipitated with anti-Ros. The immunoprecipitates were resolved by 10% SDS-PAGE and blotted with RC 20. B. Cells were starved with Met-free medium for 1 hr and labeled with ^{35}S-Met overnight and then stimulated with 100 ng/ml of EGF for 10 min. Lysis, IP, and SDS-PAGE were done as above, and finally proteins were visualized by autoradiography.

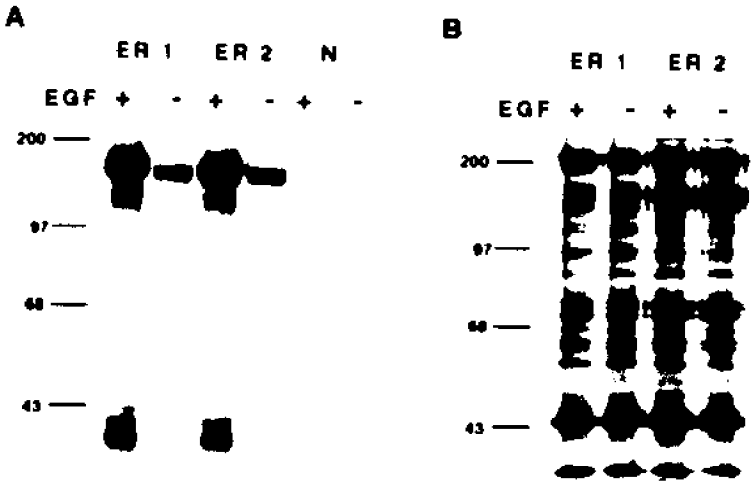


Fig. 21 Shc phosphorylation. Serum starved ER1 and ER2 cells and NIH3T3 cells were treated with 100 ng/ml of EGF for 10 min before cells were lysed. Cell lysates were immunoprecipitated with anti-Shc Ab followed by SDS-PAGE and Western blotting with either RC-20 (A) or anti-Shc (B).

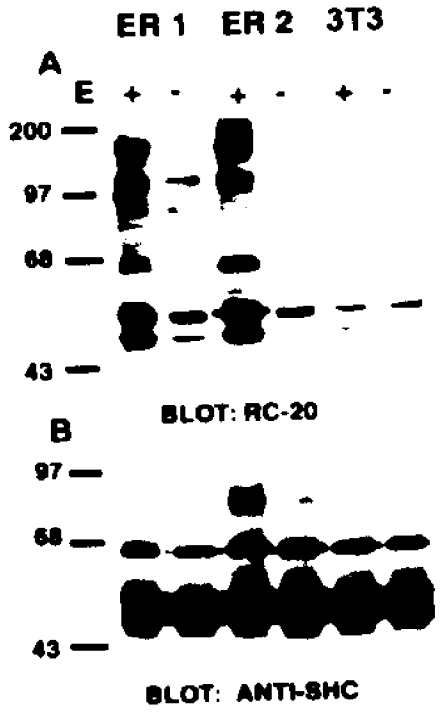
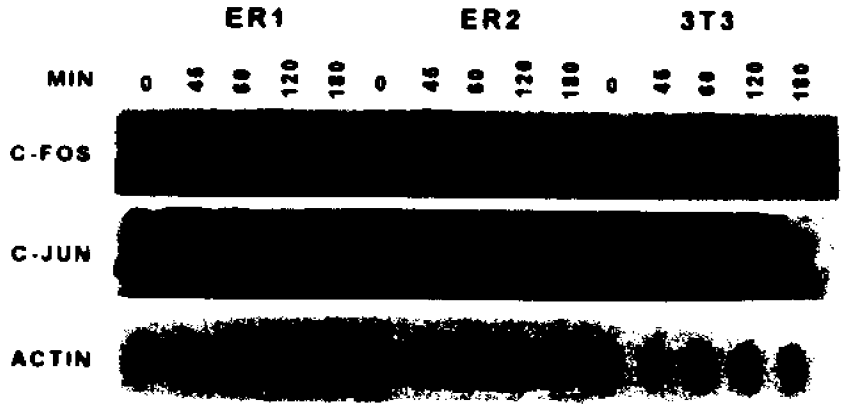


Fig. 22 Induction of c-*fos* and c-*jun* after EGF treatment. ER1, ER2, and 3T3 control cells were serum starved overnight and stimulated with 50 ng/ml EGF for the indicated times. Then total RNA was extracted and analyzed for c-*fos* and c-*jun* by slot-blot hybridization with rat c-*fos*, human c-*jun* and mouse actin cDNA probes, respectively. See details in Materials and Methods. Exposure times: c-*fos*: 2 days, c-*jun*: 1 day, and actin: 1 day.



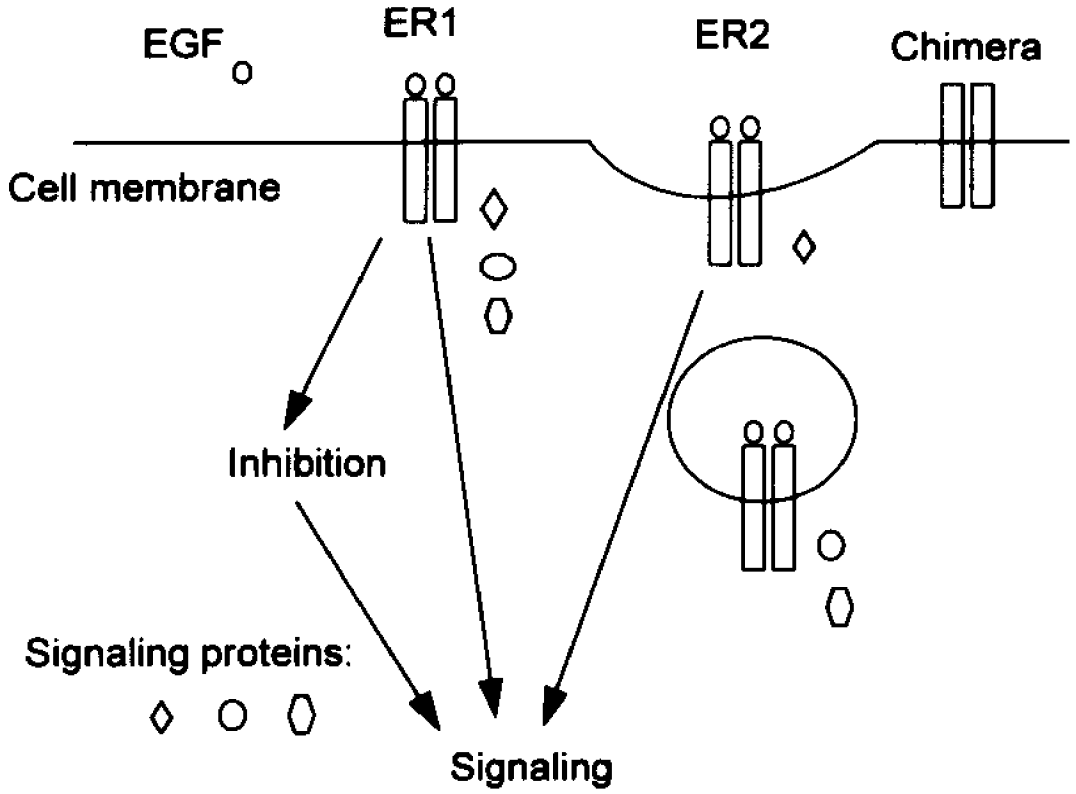


Fig. 23 Schematic diagram of signaling pathways mediated by ER1 and ER2. After EGF binding, ER2 which is internalized efficiently sends positive signals for cell growth. ER1 which is less efficiently internalized triggers an inhibitory signal that counteracts the positive signals.

DISCUSSION

The present study showed that the two EGFR-ros chimeras differing in their TM domains have opposite effects on cell growth despite of their similar transforming ability. Similar growth inhibitory effect has been observed. It is known that activation of EGFR promotes cell growth in a number of cell types (Gospodarowicz et al 1978). However. the addition of EGF to A431 and other tumor cells over-expressing EGFR, in contrary to what one might expect, results in remarkable growth inhibition without any effect on their transformed morphology (Gill and Lazar 1981, Lifshits et al 1983, Filmus et al 1987, Hirai et al 1988). Hepatocyte carcinoma cells are also growth inhibited upon treatment with hepatocyte growth factor (Tajima, et al 1991, Shiota, et al 1992).

Multiple reasons may account for the opposite growth effects of ER1 and ER2. It could be that the genetic differences between ER1 and ER2 cells render the ER1 cells susceptible to EGF-mediated inhibition. However, similar behavior of independent clones argues strongly against this possibility (Fig. 10). Although some early signaling proteins are activated to higher extent in ER1 cells, it is still possible that ER1 fails to activate a critical signaling component(s) required for cell growth such as the 66 kD Shc. In addition, activation of MAPK and Induction c-*jun* and c-*fos* are either to lass extent or for shorter time in ER1 cells. However, the failure to activate a signaling component may only renders a cell unresponsive to EGF but not growth inhibition. Furthermore, the fact that the increase of serum concentration and the addition of IGF-1 fail to rescue the inhibitory effect suggests that an inhibitory signal is triggered by ER1 that counteract the positive effect.

Previous studies have demonstrated that variants isolated from EGFR over-expressing squamous cell carcinoma lines that are resistant to EGF-mediated cell growth inhibition either loss their amplified EGFR gene or have the cell-surface receptor more efficiently internalized (Hirai et al 1988). Those observations, together with my data, suggest that over stimulation of a RPTK may be responsible for the inhibitory effect. Consistent with this model, the ER1 cells that is growth inhibited by EGF exhibit higher EGF binding and less efficient receptor internalization. In addition, several early signaling proteins of RPTKs, including IRS1, PLCγ, PI3K, are activated to higher extent in ER1 cells. Therefore ER1 is over-stimulated. With wealth of information about growth stimulatory function of RPTKs, it is unlikely that over-stimulation of a RPTK per se is directly responsible for the growth inhibitory effect. It is possible that high binding capacity of ER1 to EGF leads to over-stimulation which requires rapid down regulation as a physiological response to a RPTK. The retarded internalization of ER1 disrupts this normal physiological response and somehow triggeres an inhibitory signal(s) to counteract the over-stimulated receptor. When reaching a threshold, the inhibitory effect eventually overrides the positive signals imposed by ER1 activation and results in growth inhibition. The inhibition apparently does not act on the proximal signaling events since several immediate substrates are highly activated or phosphorylated in ER1 cells.

The current model of cell cycle control holds that transitions between different cell cycle states are regulated at checkpoints (Hunter and Pines 1995, Nurse 1995, Shear 1995). One of the most important checkpoints is START (also known as the restriction point in mammalian cells) in late G1. Cells are sensitive to a variety of external signals

including growth factors until they reach the restriction point late in G1, after which they can complete division cycle even only supplied with factors supporting their viability (Pardee et al 1989). Consistent with this notion, EGF treatment of ER2 expressing cells greatly shortens the G1 phase leaving the S and G2/M phases essentially unchanged. However, EGF-mediated growth inhibition of ER1 cells affects all phases of cell cycle. This suggests that the inhibition acts on multiple checkpoints in each phase that is involved in cell cycle progression. Interestingly, it was reported that all phases of cell cycle were elongated in mouse embryo fibroblasts (MEF) carrying null mutation of IGFR in comparison with normal MEF (Sell et al 1994).

^{3}H-thymidine incorporation showed that DNA synthesis in ER2 increased in response to EGF in a dosage dependent manner. In ER1 expressing cells, although thymidine incorporation decreased in response to EGF, the inhibitory effect was not as obvious as revealed in growth rate. The reason may be the following: First, The cells were growing in medium supplemented with 0.5% serum, at which condition cell growth was slow. Therefore, the negative effect on cell growth wouldn't be easily demonstrated. The obvious growth inhibition was observed after EGF treatment for at lest 2 days (Fig. 10 and 11), whereas the ^{3}H-thymidine incorporation was measured at 16 hrs; Second, Flow cytometry analysis revealed that the percentage of ER1 cells in S phase actually increased in response to EGF treatment which should lead to a increased thymidine incorporation. However, S phase was lengthened which would result in a decreased rate of DNA synthesis. Thus this two opposite effects accounted for a slightly decreased thymidine incorporation observed in ER1 cells.

Internalization of receptors has long been considered as a mechanism to attenuate signaling. One key evidence in support of this notion is that cells expressing the C-terminal truncated, internalization retarded EGFR mutant displayed a stronger response to EGF than those expressing wild-type EGFR (Wells et al 1990). However, one can not rule out the effects other than retarded internalization caused by the C-terminal truncation of EGFR. Internalization of a ligand bound RPTK is mediated through clathrin-coated vesicles. Following internalization, the vesicles deliver the ligand and receptor, as exemplified by EGFR and its ligand EGF, to the early endosomal compartment, the tubular-vesicular membrane structures located at the cell periphery, then to the late endosomal compartments located close to perinuclear, and finally to lysosomes. Numerous observations have demonstrated that ligand remains bound to its receptor and internalized receptor is still dimerized after 10-15 min (Sorkin et al 1991). This implies that internalized receptors remain activated, thus opening the possibilities that an activated receptor encounters cytoplasmic signaling proteins in different compartments during intracellular trafficking. If a receptor remains on the cell surface, it is less accessible to cytoplasmic signaling proteins except those exist in the proximity of cell surface or are membrane associated. As a consequence, signaling kinetics and specificity may change according to the efficiency of receptor internalization (Fig. 23).

Comparison of signaling pathways mediated by the two chimeras revealed several differences. They are: 1. several early signaling proteins of RPTKs are activated to greater extent in ER1 cells; 2. distinct cellular protein tyrosine phosphorylation patterns were observed in the two chimera expressing cells; 3. the phosphorylation of the 66 kD Shc,

activation of MAPK, and induction of c-*jun* and c-*fos* are either to less extent or for shorter time. These observations suggest that the two chimeras have different signaling specificities despite of their identical cytoplasmic domains. The mechanism is not clear, but it must lie in the TM and subtransmembrane regions. ER1 which has its TM and EC domains from EGFR has a higher EGF binding presumably due to a more conserved native conformation for EGF binding. However, the internalization signal of ER1 may be disrupted by fusion of the TM domain of EGFR with the cytoplasmic domain of c-*ros*. Since ER1 is less efficiently internalized, together with its higher EGF binding , it remains on the cell surface as an activated form for a longer time and thus has a longer time lapse to interact with its immediate substrates which presumably exist near the cell surface. This could explain why IRS1, PI3K, PLCγ and some unidentified proteins are activated or phosphorylated to higher extent in ER1 cells. However, different efficiency of internalization and subsequently intracellular trafficking of the two chimeras may resulting in their encountering different signaling proteins. This could explain that several cellular proteins including the 66 kD Shc are differentially phosphorylated in the two chimera expressing lines. Alternatively, substitution of the TM domain of c-*ros* with that of EGFR disrupts the structural integrity. As a consequence, the conformation of the cytoplasmic domain of ER1 is different from that of ER2 despite of their identical sequences. The changes of the conformation may alter the signaling specificities of the two chimeras. At this moment, I can not distinguish whether the different signaling specificity of the two chimeras is caused by the different levels of stimulation, different rate of internalization, changes of the cytoplasmic conformation, or a combined effects of these potential factors.

The TM domain may influence the signaling of a RPTK in different ways. Replacement of the TM domain of a RPTK with the analogous sequence from other receptors usually doesn't change the signaling capacity, but it isn't true for PDGFR (Escobedo et al 1988). Substitution of its TM domain with those from c-Neu, oncogenic Neu, or LDL receptor inactivates its kinase activity, indicating that the TM domain can contribute to the signaling function other than merely serving as a membrane anchor. Study of the two chimeric EGFR-p75^{NGFR} receptors showed that the one with its TM domain derived from p75^{NGFR} was able to induce differentiation of PC12 cells expressing such chimera, whereas the other with the TM domain of EGFR could not do so (Yan et al 1991). Previous studies have also demonstrated that a single charged aa substitution in the TM domain of Neu activates its oncogenic potential (Drebin et al 1984). Further experiments showed that the very same single mutation caused the dimerization of Neu in the absence of added ligand (Brandi-Rauf et al 1990). Studies on v-*ros* and its variants revealed that the three aa insertion in the TM domain of v-*ros* has a profound effect not only on its transforming activity but also on its posttranslational modification and substrate recognition (Zong et al 1994).

The present study shows that two chimeras differing in their TM domains have such diverse effects, suggesting the important roles of the TM domain. Since native EGFR and ER2 chimera internalized efficiently, the retarded internalization of ER1 is most likely due to the combined effects of EGFR TM domain and the c-*ros* subtransmembrane sequence. Studies of EGFR revealed that the C-terminal region as well as ser/thr phosphorylation by PKC are important for internalization (Chen et al 1989, Lund et al 1990, Chang et al 1992).

However, the function of its TM domain has not been addressed. Different receptors may employ different strategies to regulate internalization. Both the subtransmembrane and the C-terminal regions of IR and IGFR contain internalization sequence recognition motifs required for internalization (Rajagopalan et al 1991, Kaburgi et al 1993, Haff et al 1994, Prager et al 1994). TM domain of IR also affects its internalization as mutant IR carrying gly^{933}-pro^{934} in the TM domain substituted by ala-ala displayed enhanced internalization in comparison with the wild-type IR. The internalization sequence motifs of c-*ros* haven't been studied since the c-*ros* ligand has not yet been identified. If one assumes that the TM-subTM sequence of c-ros provides a sequence determinant for internalization, it would explain the efficient internalization of ER2, but not for ER1. Although ER1 is less efficiently internalized than ER2, the two chimeras have similar half-lives in the presence or absence of EGF. In contrast, EGFR rapidly undergoes EGF dependent degradation (Stoschck et al 1984). The distinct effects of EGF on its cognate receptor EGFR and the chimeras suggest that the cytoplasmic domain determines the rate of ligand-induced receptor degradation.

Substantial evidence suggests that the Ras signaling pathway is indispensable for cell growth. Constitutive activation of Ras or any known downstream components, including Raf, MEK, MAPK, leads to cell transformation in fibroblasts. PI3K is also involved in mitogenic signaling. The present study has shown that both MAPK and PI3K were activated in ER1 cells that were growth inhibited, suggesting that some inhibitory signal(s) has overridden those signaling pathways. However, whatever the presumed inhibitory signal is, it appears not affect the transforming abilities of the two chimeras. Shc is an

important cell growth regulator (Pelicci et al 1992). It is thought that Shc, together with Grb2, is involved in the activation of Ras (Rozakis-Adcock et al 1992). I noticed the differential phosphorylation of 66 kD Shc and its co-immunoprecipitated protein in the two chimera expressing cells, but its significance is not clear. I do not know if the reduced phosphorylation of the 66 kD Shc plays any role in growth inhibition of the ER1 cells.

EGFR-ros chimeras bearing different TM domains, when expressed in NIH3T3 cells, are glycosylated and transported to the cell surface equivalently but bind EGF with different affinities. Furthermore, EGF induces dimerization of both chimeras, resulting in stimulation of their kinase activities, and phosphorylation of themselves and of cellular proteins. These observations indicate that the cytoplasmic domain of Ros is capable of functioning as an inducible tyrosine kinase in NIH3T3 cells and leads to activation of an array of signaling components including the Ras pathway, common among several RPTKs. Thus c-*ros* is likely to be activated in vivo by binding of an appropriate ligand to its EC domain. However, the present study can't prove that c-ros undergoes ligand-dependent dimerization since the soluble EC domain of EGFR is sufficient for dimerization and may have coffered such property to the chimeras (Lax et al 1991).

Although both chimeras induce colony formation with almost equal efficiency, the picture is complicated by the finding that the chimeras have opposite effects on cell growth in monolayer culture. For reasons discussed above, I think that the growth inhibitory effect is caused by the over-stimulation of ER1 as seen in those cells over-expressing EGFR rather than the intrinsic function of c-*ros* since ER2 promotes cell growth. Thus c-*ros* has the potential to transmit mitogenic signals. The cell growth promoting activity of c-*ros* was

reported from other group by using a different approach in which the EC domain of murine c-*ros* is replaced by that from NGFR (Riethmacher et al 1994). However, these studies might not necessarily reflect the in vivo function of c-*ros* since it is specifically expressed in epithelial cells rather than fibroblasts. A precedent example is that c-Neu transmits mitogenic signals when expressed as EGFR-Neu chimera in NIH3T3 cells (Lehvaslaiho et al 1989), whereas it induces differentiation and growth inhibition of breast cancer cells that express c-Neu upon stimulation of NDF (Peles et al 1992). In view of the proposed role of c-ros in the formation of kidney collecting tubules and differentiation of intestinal villi epithelial cells, the potential mitogenic and differentiation function of c-ros may not be necessarily contradictory, rather it may be cell type dependent.

EXPRESSION AND CHARACTERIZATION OF CHICKEN C-ROS, ITS VARIANTS, AND V-ROS IN DIFFERENT CELLS

ABSTRACT

As a continuous effort to study the biochemical and biological properties of c-*ros*, its native form, variants, and v-*ros* were expressed in several cell lines. In no cases the stable lines expressing c-*ros* and its kinase inactive mutant can be established. The ppros that has a large extracellular domain deletion of c-*ros* can be expressed stably in CV-1 and MDCK cells but not in NIH3T3 cells, suggesting that cell type may affect the expression of c-*ros* variant. The ppros and ppros-slt which has a three aa insertion in the TM domain of the ppros can be expressed in CFE at low levels but neither of them are transforming. The ASV UR2 v-*ros* can be expressed in NIH3T3 cells and is weakly transforming.

INTRODUCTION

The retrovirus UR2 contains a transduced gene v-*ros* fused to the viral gag gene. The fusion sequence codes for a transmembrane gag-ros fusion protein with the *gag* portion protruding extracellularly (Wang et al 1982). UR2 v-*ros* is a potent transforming gene when expressed in CEF (Balduzzi et al 1981). CEF transformed by v-*ros* is characterized by fast growth rate, anchorage-independent growth in soft agar, elongated, fusiform shape, and increased light refractility. UR2 efficiently induces tumors when injected into chickens (Balduzzi et al 1981). The 5' truncation of c-*ros* and fusion to *gag* plus a 3-aa insertion in the TM domain account for the activation of the full oncogenic potential of c-*ros* (Zong et al 1993). Signaling proteins including PI3K, IRS1, PLCγ, and Shc are activated by v-*ros* (Zong et al 1993, 1994). In addition, tyrosine phosphorylation of a 88 kD protein complex is impaired in a v-*ros* variant that has greatly reduced transforming activity. Expression of v-*ros* in NIH3T3 cells showed that v-*ros* expression level was low and only weakly transforming as judged by the number and size of colonies formed by the expressing cells in soft agar (Jong and Wang).

C-*ros* is expressed mainly in epithelial cells, although truncation and amplification of c-*ros* gene has been detected in human glioblastoma cell lines (Birchmeier et al 1986). Little was known about the biochemical property of c-*ros*. It has been difficulty to stably express c-*ros* in CEF and several mammalian cell lines including Cos-7, NIH3T3, Rat1, Rat2, glioblastoma, and renal tumor cell lines, regardless of the promoters used (Chen and Wang, unpublished data). However, c-*ros* could be transiently expressed in most of the cell

lines tested. Studies of c-*ros* transiently expressed in Cos-7 cells revealed that c-*ros* protein is located on the cell surface and has very low kinase activity (Chen and Wang, unpublished data). The difficulty in establishing stable c-*ros* expressing lines had led to the suspicion that c-*ros* might function in promoting cell differentiation rather than proliferation.

As a continuous effort to study the function of c-*ros* in cultured cells. I have tried to express c-*ros*, its variants, and v-*ros* in different types of cells.

MATERIALS AND METHODS

CELLS: Chicken embryo fibroblasts (CEF) were maintained in F10 medium supplemented with 5% calf serum and 1% chicken serum. MDCK, MDBK, CV-1, Cos-7 cells were maintained in DMEM medium supplemented with 5% calf serum.

PLASMIDS: pECEROS which contains the full length coding sequence of c-*ros*, pECEROS PF3 which is kinase inactive mutant of c-*ros*, pCMVppros which is the c-*ros* variant with the large extracellular domain deletion of c-*ros* (aa 185 to 1846), and LTRppros were constructed by a previous student in our lab, Dr. J. Chen. pECEv-ros were constructed by a previous postdoc fellow in our lab, Dr. S. Jong. The help viral DNA pUR2AV and pUR2 was described before (Neskameyer and Wang 1984). pCMV vector has the CMV promoter and G418 resistant gene (Invitrogen). LTR vector contains two LTRs of UR2AV. pECEppros-slt which has a Ser-Leu-Thr (slt) residues insertion in the TM domain of ppros was constructed by using site directed mutagenesis kit (promega). Briefly, ppros fragment was released from pCMVppros by digestion with Not1 and ligated to pbluescript at the same site to give rise to pblueppros. A Sac1 and Xba1 fragment freed from pblueppros was ligated into pALT-1 mutagenesis vector provided in the kit. The insertion mutation was introduced by in vitro synthesis using single stranded DNA and the primer, 5'AAACAGTATGATTATTGTTAGTGAAGTCAAGCCCAGTAC3' (Insertion mutation is underlined). The resulting product was rescued in a plasmid and then the ppros-slt fragment was released by Not1 digestion and cloned back to the pCMV vector at the same

site. The final plasmid was named pCMVppros-slt. For expression of ppros-slt in CEF, ppros-slt fragment was released from pCMVppros-slt by digestion with Not1 and ligated to pbluesript and then the ppros-slt was released by digestion with Sac1 and BamH1 and inserted into LTRppros which was digested with the same enzymes. The resulting construct was named LTRppros-slt.

Transfection, protein analysis, colony formation assay, and immuno-staining were done as described in previous chapter.

RESULTS AND DISCUSSION

CONSTRUCTION AND EXPRESSION OF C-ROS VARIANTS

A c-*ros* variant, called ppros, containing 1661 aa deletion (aa 185-1846) of the EC domain is non-transforming when expressed in CEF. Its kinase activity is remarkably low compared with that of v-*ros*, but is significantly higher than that of c-*ros* (Chen and Wang. Unpublished observation). Considering the dramatic effect of the three aa insertion in the TM domain of v-*ros* on its transforming function, I have introduced the three aa into the TM domain of ppros by site directed mutagenesis. The new construct was named ppros-slt. ppros and ppros-slt were expressed in CEF under the control of UR2AV LTR. Western blot showed that the expression levels of both ppros and ppros-slt were extremely low in comparison with that of v-*ros* (Fig. 2-1 A). However, several experiments showed that expression level of ppros-slt was reproducibly higher than that of ppros. Previous study has shown that the v-*ros* variant with the three aa deletion in the TM domain is glycosylated whereas v-*ros* is not. Consistent with that observation I noticed that ppros-slt migrated faster than ppros on SDS-PAGE despite of a three aa insertion (Fig. 2-1 A), suggesting that ppros-slt which has the three aa insertion is not glycosylated. In vitro kinase assay showed that kinase activity of ppros-slt was very low and that of ppros was undetectable in comparison with v-*ros* (Fig. 2-1 B). Neither ppros nor ppros-slt expressing CEF was able to form colonies (data not shown). Attempts to establish permanent expressing lines of ppros and ppros-slt in NIH3T3 cells failed. However, transient expression showed that the

expression levels and kinase activities of ppros, ppros-slt, and v-*ros* were similar (Fig. 2-2 A, B). In contrast to what was observed in CEF, the mobility of ppros and ppros-slt was similar, suggesting that the effect of the three aa insertion on the protein modification is cell type dependent.

EXPRESSION OF C-ROS AND ITS VARIANTS IN MAMMALIAN CELLS

Since c-*ros* is mainly expressed in epithelial cells, one might have a better chance to express c-*ros* and its variants in epithelial cell lines. I tried to express c-*ros* and its kinase inactive mutant PFC in several kidney epithelial cell lines including CV-1, MDCK, MDBK, but did not succeed (data not shown). Failure to express even the kinase inactive mutant in any of those cell lines suggests that the kinase activity of c-*ros* is not responsible for the phenomenon.

I also tried to express ppros in MDCK and CV-1 cells and succeeded. In vitro kinase assay showed that 2 out of 15 G418-resistant MDCK clones and 1 out of 12 CV-1 clones expressed a kinase active protein (Fig. 2-3 and data not shown). Interestingly, ppros might also have undergone cell-type dependent modification since a very broad band was detected in the MDCK clones whereas a single narrow band appeared in the CV-1 clone. The morphology of these ppros expressing cells is not changed (data not shown). Since parental CV-1 and MDCK cells form colonies efficiently, the ability of ppros to promote colony formation in those cells can not be examined. The difference between the ppros and c-*ros* is that a large portion of the EC domain is deleted in the former, implying that

failure to obtain a stable c-*ros* expressing line is more likely due to the large EC domain rather than its kinase activity. As mentioned before, I failed to establish the stable expressing lines of ppros in NIH3T3 cells, although it could be expressed transiently. This suggests that cell type may indeed affect the expression of c-*ros* variants. This hypothesis can be further tested by expressing v-*ros* in the kidney epithelial and NIH3T3 cells. One would expect higher expression level of v-*ros* in kidney cells including CV-1 and MDCK cells than that in NIH3T3 cells.

EXPRESSION OF V-ROS IN NIH3T3 CELLS

Previous studies in expressing v-*ros* in NIH3T3 cells showed that a few clones expressed detectable levels of v-*ros* protein and acquired weak transformed phenotype. To study v-*ros* function in details in NIH3T3 cells, several more v-*ros* stable expressing clones were established (Fig. 2-4, A). The expression level of v-*ros* was comparable with that in previously established clone (clone SM15, Fig 2-5). Consistent with the low expression level, the kinase activity was also low (Fig. 2-4, B). The v-*ros* expression in these independent clones were further studied by immuno-staining with anti-Ros Ab. The result showed that cells were weakly but uniformly stained with anti-Ros Ab (Fig. 2-6, B).

Colony formation assay showed that v-*ros* expressing cells formed less and much smaller colonies than the EGFR-ROS chimera expressing cells (Fig. 2-7). In an attempt to increase v-*ros* expression level, those small colonies were picked up and pooled after incubation in soft agar for 3 weeks. However, Western blot analysis showed that the

expression level of v-*ros* in one of those clones recovered from the colonies (UR42R) was similar as that in the parental v-*ros* expressing clone UR42 (Fig. 2-5).

Weak transforming ability of v-*ros* in NIH3T3 cells in comparison with that in CEF and that of EGFR-ROS chimeras in NIH3T3 cells may partially correlated with the low expression level and much less in vivo phosphorylation of v-*ros* in NIH3T3 cells (Fig. 2-8). However, the species-specific difference may play a role as well. It is possible that the cellular factors involved in v-*ros*-mediated transformation are missing in NIH3T3. Previous studies have demonstrated that chicken v-*src* can transform both chicken and rodent cells, but some of the chicken v-*src* variants display a species-dependent transforming activities (DeClue and Martin, 1989, Verderame et al 1989, Hirai and Varmus 1990), namely they can transform chicken but not rodent cells.

Fig. 2-1. Expression of UR2 v-ros, ppros and ppros-slt in CEF. CEF was transfected with pUR2, LTRppros and LTRppros-slt together with pUR2AV by calcium-phosphate method. Two days late transfected cells were overlaid with the medium containing 0.375% agar for 4 days. Cell lysis, IP, western blot (A) and in vitro kinase assay (B) were done as described in materials and methods. 0.5 mg protein from v-ros transfected cells and 1 mg protein from ppros and ppros-slt transfected cells were used for IP. 1/3 of the immunocompolex was used for kinase assay and remaining for Western blot. Note that ppros-slt migrates faster than ppros despite of the three aa insertion. Line and arrows indicate the position of v-ros, ppros and ppros-slt, respectively.

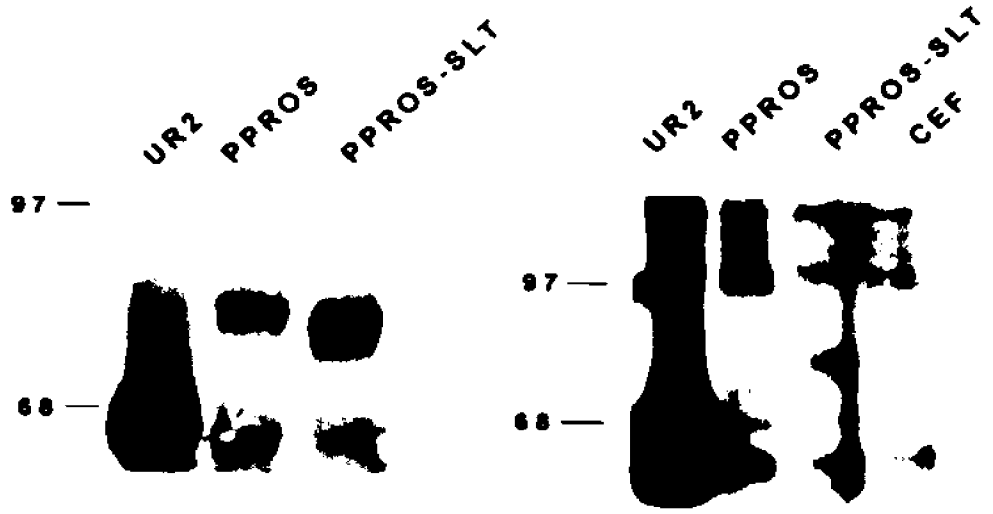


Fig. 2-2. Transient expression of UR2 v-ros, ppros and ppros-slt in NIH3T3 cells. NIH3T3 cells were transfected with pECEros, pCMVppros and pCMVppros-slt, respectively. 48 hrs after transfection, cells were lysed. Western blot (A) and in vitro kinase assay (B) were done as described. 0.8 mg and 0.5 mg protein was used for western blot and kinase assay, respectively.

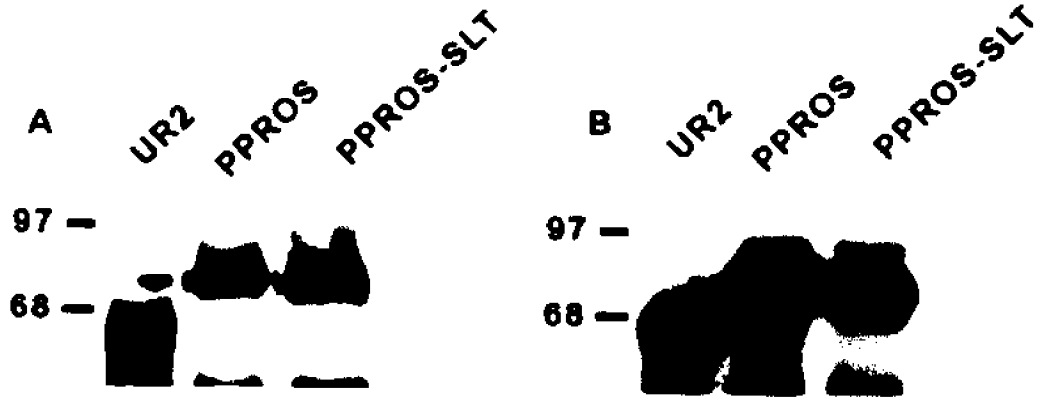


Fig. 2-3. Expression of ppros in MDCK and CV-1 cells. MDCK and CV-1 cells were transfected with pCMVppros. After G418 selection for 3 weeks, the resistant clones were analyzed for ppros expression by kinase assay. Only representative clones are shown. The numbers represent independent G418 resistant clones.

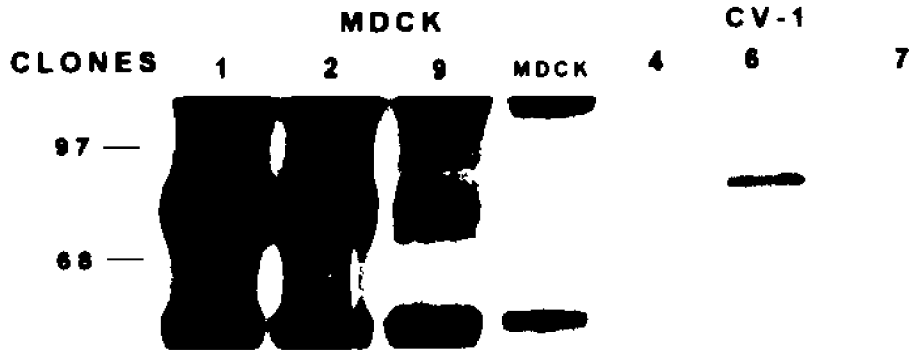


Fig. 2-4. Expression of v-ros in NIH3T3 cells. NIH3T3 cells were co-transfected with pECEros and pSV2-Neo plasmids. After 3 weeks of G418 selection, independent clones were analyzed for v-*ros* expression by Western blot (A) and in vitro kinase assay (B). Numbers represent independent clones. Cell lysates from 6 cm dishes were immunoprecipitated with anti-Ros Ab. 1/3 of the lysates was used for kinase assay and 2/3 for Western blot. Note that not identical clones are shown in (A) and (B). Arrow indicate the position of v-ros in (A).

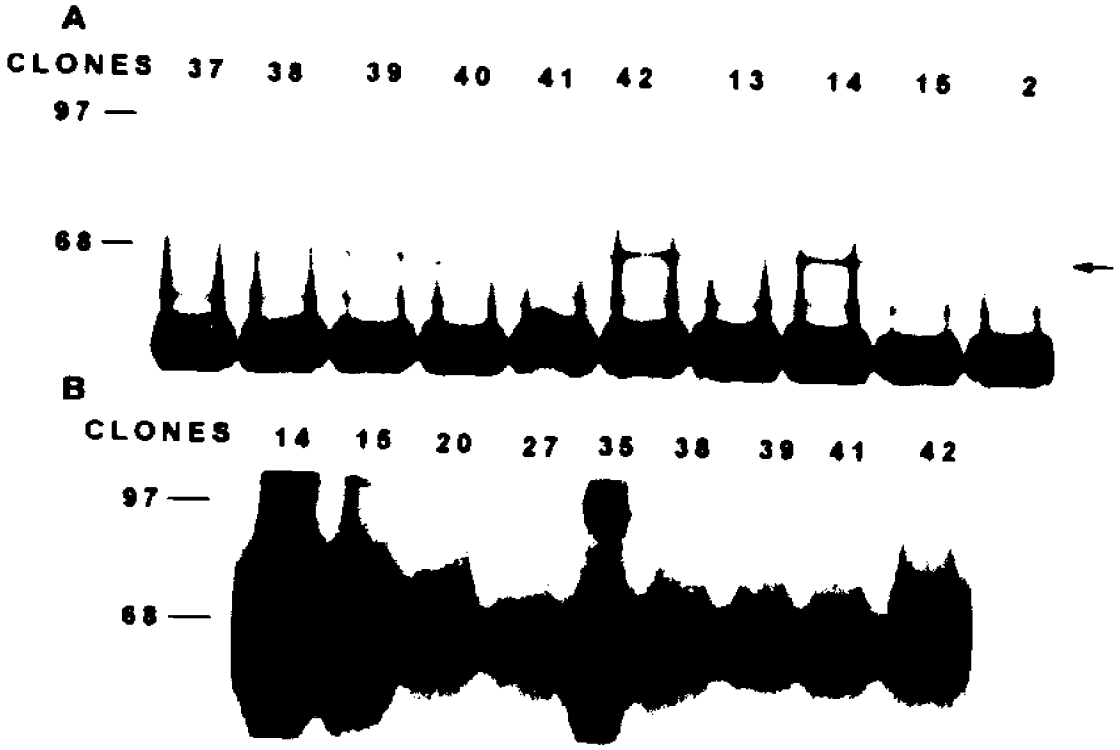


Fig. 2-5. Comparison of the expression levels of several v-*ros* expressing clones. 0.8 mg protein was used for Western blot in each sample. SM15 is a previously established clone. UR42R were established from combined colonies of UR42 NIH3T3 clone grown in soft agar for 3 weeks.

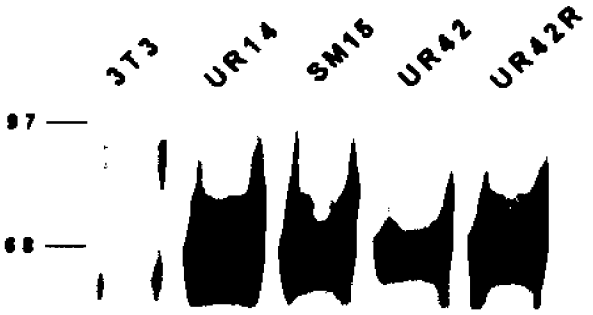


Fig. 2-6. Cell staining for v-ros expression. Staining procedure was described in materials and methods.

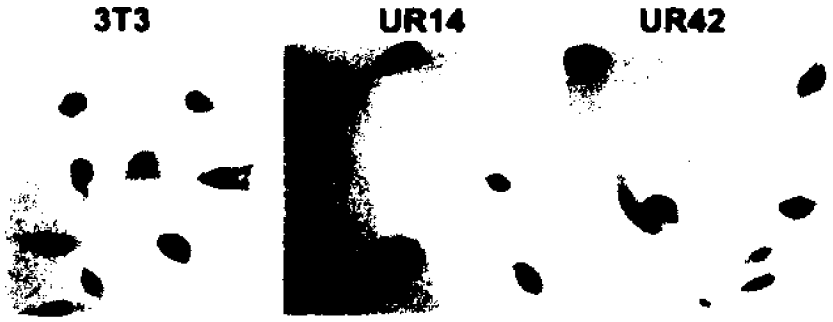


Fig. 2-7. Colony formation assay. Different UR2 expressing clones, EGFR-ROS chimera expressing clone (ER2-69) stimulated with 10 ng/ml of EGF (E+) or without stimulation (E-), and G418 resistant control NIH3T3 clone are shown. Pictures were taken after 20 days incubation in soft agar.

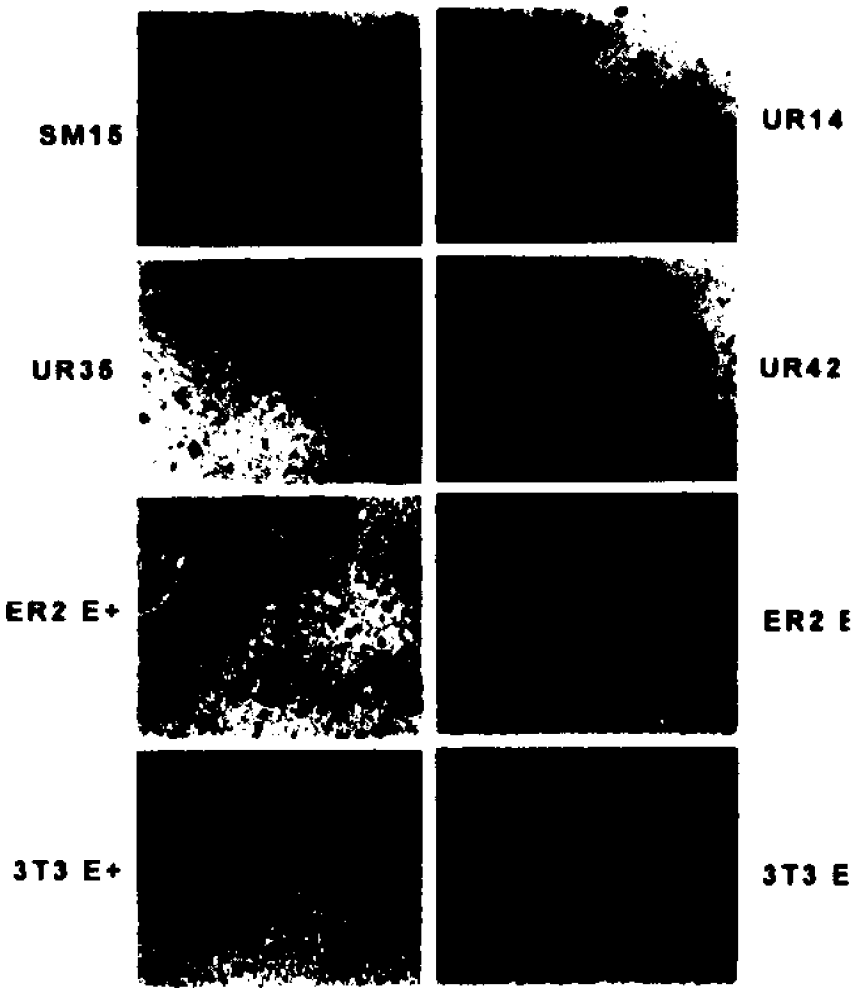
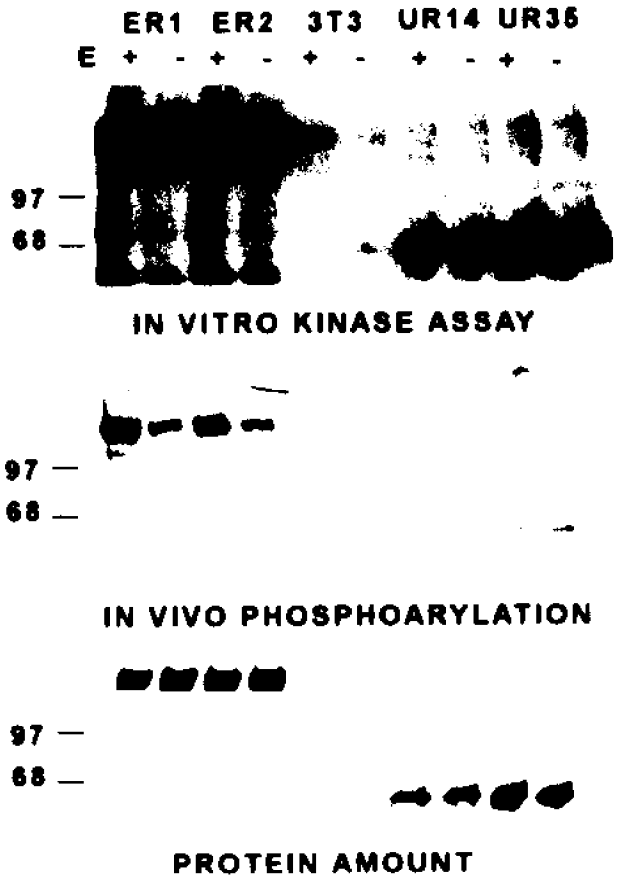


Fig. 2-8. Kinase activity (A) and in vivo phosphorylation (B) of EGFR-ROS chimeras (ER1 and ER2) and v-*ros* expressed in NIH3T3 cells. C. Protein amount control. 0.5 mg protein was used for each sample. E: EGF 10 ng/ml.



CLONING AND EXPRESSION OF CHICKEN PROTEIN TYROSINE PHOSPHATASE (PTPase)γ

ABSTRACT

Overlapping cDNAs of 5403 bp encoding the entire amino acid sequence of chicken PTPase γ were isolated and sequenced. Chicken PTPase γ, a receptor type PTPase, consists of 1422 amino acids which is 86.7% identical to human homolog. The homology extends throughout entire sequence including the carbonic anhydrase and fibronectin type III homologous regions in the extracellular domain, as well as the tandem linked catalytic sequence in the cytoplasmic domain. However, the chicken PTPase γ lacks 29 amino acids immediate downstream of the putative transmembrane domain in comparison with the human PTPase γ. Northern analysis revealed the presence of two transcripts of 6.3 kb and 9.5 kb in various tissues. The cytoplasmic domain of the PTPase γ could be expressed as a enzymatically active form in insect SF9 cells, although the expression level was very low. It could also be expressed in normal and *src* transformed NIH3T3 and Rat 1 cells as a gag-PTPase fusion protein, but no detectable biological effects on growth and formation of colonies in soft agar were observed in the expressing cells.

INTRODUCTION

Tyrosine phosphorylation is one of the most important protein modification involved in modulating a number of physiological and pathological processes, including cell growth, differentiation, and neoplastic transformation. The state of such phosphorylation depends upon the relative activities of two families of enzymes with opposing activities, namely PTK and Protein tyrosine phosphatase (PTP).

The first PTP to be extensively studied, PTP1B, was purified from human placenta (Tonks et al 1988). Enzymatic study and sequence analysis have clearly demonstrated that PTP 1B is distinct from protein-serine phosphatase, thus defining a new family of enzymes (Tonks and Charbonneau, Charbonneau et al, 1989). Since then a large number of PTPs have been identified and evidence suggests that PTPs, like PTKs, regulate diverse aspect of cellular functions.

PTPs can be divided into membrane-spanning receptor-like proteins (RPTP) and intracellular PTPs which can be further grouped based on the presence of SH2 domain (Hunter et al 1992, Feng and Pawson 1994). RPTP includes RPTP α, β, γ, δ, ε, λ, μ, CD45, LAR, DEP-1. All of these RPTPs have a EC domain, a TM domain and a cytoplasmic domain featured by two tandem catalytic sequences. Studies on the two tandem catalytic domains suggest that N-terminal catalytic domain is more active than the C-terminal one, which may have a regulatory function. Study of substrate specificity of PTPs has suggested that the chemical features in the primary sequence surrounding the

dephosphorylation site was found to contribute to the substrate specificity (Zhang et al 1994). However, no physiological substrates have been identified for a given PTP with certainty except for MAPK that is found to be a physiological substrate of MKP-1, a dual specificity phosphatase (Sun, et al, 1993). Localization of a PTP, however, does affect its substrate pattern. When different PTPs were co-expressed with a panel of RPTKs, it demonstrated clear differences in susceptibility of different RPTKs to the action of PTP 1B, T-cell phosphatase (TC-PTP), and CD45 (Lammers et al 1994). This suggests that cellular compartmentalization of a PTP is important factor affecting its substrate interaction and overall function.

The diverse nature of EC domains may indicate that RPTPs have very different aspect of functions (Mourey and Dixon 1994). The EC domains of RPTP γ and β contain a stretch of aa with striking homology to the Zinc-containing enzyme carbonic anhydrase (CAH) (Barnea 1993). Despite high similarity to the CAH domain, only one of the three His residues that are absolutely required for CAH catalytic activity is conserved in these RPTPs, suggesting that the CAH-like domain in RPTPs may have different function other than catalysis of hydration of metabolic CO_2. Interestingly, murine L-cells have lost one *ptprg* allele and sustained an intragenic deletion within the CAH domain in other (Wary et al 1993). Evidence also suggested that RPTP may be involved in cell-cell interaction. RPTP μ that has a EC domain containing a MAM (Meprin, A5 protein and PTP mu)domain, an Ig domain and four fibronectin type III repeats can binds homophilically (Brady-Kalnay et al 1994), i.e, RPTPμ on the surface of one cell binds to RPTPμ on a neighboring cell, and that the EC domain alone is sufficient for homophilic binding. Besides CAH-like

domain, the EC domain of RPTP β exhibits sequence similarity with a soluble, rat brain chondroitin sulfate proteoglycan (Shitara et al 1994). Moreover, RPTP β has a variant form composed of only EC domain possibly generated by alternative RNA splicing (Maurel et al 1994). Further experiments indicated that RPTP β is indeed a chondroitin sulfate proteoglycan and binds specifically to tenascin (Barnea 1994), an extracellular matrix protein that binds to neurons. The EC domain of RPTP κ contains an Ig-like domain, four fibronectin type III-like repeats, and a region of about 150 aa with similarity to the Xenopus A5 antigen, a putative neuronal recognition molecule. RPTP κ was shown to be able to mediate homophilic binding (Sap et al 1994).

Biological function of RPTPs are largely unknown. It was thought the PTPs may negatively regulate cell growth simply because that most RPTKs promote cell growth, emerging evidence, however, suggests that the effect of a PTP on cell growth can be negative or positive. Based on the observation that RPTP γ maps to a region of human chromosome 3, 3p21, that is frequently deleted in renal and lung carcinoma, RPTP γ has been considered as a candidate of tumor suppressor gene (LaForgia et al 1991). Another RPTP that is implicated to have negative effect on cell growth is DEP-1 (Ostmanetul et al 1994). DEP-1 is a unique RPTP since it has only one intracellular catalytic domain. Expression level of DEP-1 increases in dense cultures relative to sparse cultures. Also, DEP-1 activity increases in dense cultures. The increased expression level of DEP-1 occurs gradually with increasing cell contact and is initiated before saturation cell density is reached. These observations suggest that DEP-1 may contribute to the mechanism of contact inhibition of cell growth.

RPTP α may positively regulate cell growth. Evidence in support this includes that overexpression of RPTP α in rat embryo fibroblasts leads to cell transformation (Zheng et al 1992) probably through activation of signaling proteins, including Src, MAPK, and Jun (Zheng et al 1994). The kinase activity of Src is regulated by reciprocal tyrosine phosphorylation. Phosphorylation of Tyr 416 activates Src, whereas phosphorylation of Tyr 527 inactivates it. In RPTP α transfected cells, Tyr 527 is preferentially dephosphorylated. MAPK and Jun can also be activated and translocated to nucleus in RPTP α transfected cells, suggesting that RPTP α engages in signal transduction pathway, leading to cell growth. Further experiments suggest that Grb2 can bind to RPTP α and the binding site has been mapped to Tyr 789 both in vitro and in vivo by several groups (Su et al 1994, Den-Hertog et al 1994).

Much progresses have been made in understanding the function of cytoplasmic PTPs since the discovery of a subfamily of cytoplasmic PTPs containing SH2 domains. A PTP with various names, PTP1C, SH-PTP, HCP, or SHP, was first identified to be SH2 containing PTP. PTP1C is preferentially expressed in hematopoietic cells, although its expression has also been detected in epithelial cells, including breast cancer cell lines. Shortly thereafter, A second wildly expressed SH2 containing PTP was identified, and called Syp, PTP1D, SH-PTP2, SH-PTP3 or PTP2C. Syp is most closely related to the product of Drosophila corkscrew (CSW) gene. Function of CSW was well elucidated by genetic mutagenesis of CSW in Drosophila (Perkins et al 1992). It was established that the DER (Drosophila EGFR, or torso), and D-raf are involved in a signal transduction pathway, leading to terminal development of embryo. Mutation of CSW or double mutation of CSW

and D-*raf* resulted in loss of termini, providing evidence that CSW acts downstream of *torso* and in concert with D-*raf* to positively transduce the *torso* signal that is required for termini development. Mammalian homolog Syp was also found to positively participate in signal transduction pathways. Syp has been shown to be able to interact with a number of activated RPTKs through its SH2 domains. These RPTKs include PDGFR, EGFR, Kit, erbB2/Neu, and IR (Staubs et al , Tauchi et al, Bennett et al, 1994). The binding site of PDGFR was mapped to Tyr 1009, the site was known for long time to interact with a 64 kD substrate that turned out to be Syp (Kazlauskas et al 1993). Further observation suggested that Syp is required for Ras activation in response to a variety of growth factors whereas enzymatically inactive Syp blocks the Ras signaling pathway (Noguchi et al, Milarski et al, Bennet et al 1994). Syp was also shown to be tyrosine phosphorylated and this phosphorylation correlated with an enhancement of its catalytic activity (Vogel et al 1993), Thus, PTK and PTP, although having opposite enzymatic activities, do not simply oppose each others' action; rather they may work in concert to maintain a fine balance of effector activation needed for the regulation of cell growth and differentiation.

Conversely, PTP1C appears to be predominantly a negative regulator of growth factor signaling. Several lines of evidence suggest that PTP1C regulates multiple hematopoietic growth factor signaling pathways. PTP1C is tyrosine phosphorylated upon stimulation of macrophage with colony-stimulation factor 1 and upon T cell receptor stimulation (Yeung et al 1992, Lorenz et al 1994). PTP1C has also been reported to associate with the IL-3 receptor β chain and c-Kit (Yi et al, Ihle et al 1993), but the specific binding site in these receptors has not been identified. Most importantly, mutations in the

PTP1C gene are the cause of the motheaten phenotype in mice (Shultz et al 1993, Tsui et al 1993). Mice harboring a PTP1C null mutation (motheaten) or a deletion in the phosphatase domain of PTP1C (motheaten viable) display a panoply of hematopoietic abnormalities, indicating a central role for PTP1C in the regulation of hematopoiesis. In the erythroid lineage, absence or reduction of enzymatic activity of PTP1C results in hypersensitivity to erythropoietin (EPO) stimulation Shultz and Sidman 1987, Van Zant and Shultz 1989). PTP1C was shown to bind to EPO receptor through its SH2 domains and in vitro studies suggested that Tyr 429 in the EPO cytoplasmic domain is the binding site. Mutant EPO receptor lacking Tyr 429 is unable to bind PTP1C, and cells expressing such mutant are hypersensitive to EPO and display prolonged EPO-induced autophosphorylation of JAK2. These results suggest that activation of PTP1C by binding to the EPO receptor plays a major role in terminating proliferative signals, presumably through dephosphorylation of JAK2 (klinmuller et al 1995).

In this paper, we present the cloning of chicken PTPase γ and study of its biochemical and biological properties.

MATERIALS AND METHODS

CELLS: NIH3T3 and Rat1 cell lines trasformed by *src* were isolated by infection with subgroup A Rouse Sarcoma Virus (L-H. Wang unpublished).

LIBRARY SCREENING: The original chicken PTPase γ 4.1 clone was isolated by low stringency screening of a λgt10 chicken kidney cDNA library (Chen et al 1992) with a probe of full length human CD45 cDNA (obtained from H. Saito of Dana Farber Cancer Institute). Subsequently, the 5' fragment released from the 4.1 clone by digestion with EcoR1 (the 5' cloning site) and Bsa1 was used as a probe to screen a λgt10 chicken brain cDNA library (Levy et al 1987) at high stringency condition. Several upstream cDNA clones were isolated (Fig. 3-1). Then the further 5' fragment was used as a probe to screen the library again. After several rounds of screening, a set of 5' cDNA clones were isolated which together with the 4.1 cDNA clone allowed us to assemble into a total composite of 5403 bp cDNA. Hybridization was carried out at 42°C overnight in a solution of 5X SSC, 50% formamide, 20 mM Tris-HCl (pH7.4), 0.5% SDS, 1 mM EDTA, 4 mg/ml salmon sperm DNA and 10^7 CPM/ml of probe. For low stringency condition, filters were washed in 37°C for 30 min in 2X SSC, 0.2% SDS and then processed for autoradiography. For high stringency condition, filters were washed in 0.1X SSC, 0.2% SDS at 65°C for 30 min. The 4.1 clone was initially isolated and sequenced by X. Guo, a previous member of our lab.

RNA ANALYSIS: Total RNAs from various chicken tissues were isolated by guanidinium

thiocyanate extraction followed by centrifugation on a Cesium Chloride cushion according to the published methods (Glisin et al, 1974). Polyadenylated RNAs were purified as described previously (Wang et al, 1981). For Northern blot, RNA electrophoresis and transfer to nylon membrane were done as described (Sambrooke et al 1981). Riboprobe was prepared from the clone 4.1 subcloned into the pbluescript vector (Strategen) using T7 RNA polymerase. Hybridization was carried out in a solution containing 5X SSC, 1% SDS, 50 mM Na_3PO_4 (pH 6.5), 8X Denhart's solution, 0.5 mg/ml yeast RNA, 5 µg/ml poly(C) at 72°C overnight. Nylon membranes were washed three times in 0.1x SSC, 0.2% SDS at 78°C. Northern blot of brain, heart, and CEF was done by Yixing Jiang of our lab.

GENERATION OF ANTIBODY (by X. Guo): The full length clone 4.1 was inserted into the expression vector pSEM3 at Sma1 and EcoR1 sites to generate a lacZ-PTPase fusion cDNA. The 160 Kd fusion protein was expressed in bacteria strain W3110 and extracted from the insoluble fraction and purified by eluting from the SDS-PAGE. The immunization of rabbit and collection of serum were done following standard procedure.

EXPRESSION OF THE CYTOPLASMIC DOMAIN OF PTPASE γ IN SF9 CELLS USING BACULOVIRUS VECTOR: To generate the initiation codon for the PTP cDNA, the authentic initiation codon of polyhedrin that had been mutated to ATT in the PVL1393 vector (Webb and Summers 1990) was mutated back to ATG by PCR using the following primers: 5' primer, 5'ATTATAGTTGCTGATATCAT (the EcoR5 site used for cloning is underlined), and 3' primer, 5'

CCCGGGATCCGACGCCCCGATGGTGGGACGGTATGAATAATCCGGCATATT (the BamH1 cloning site is underlined). The 3' primer corresponds to a sequence from -3 to +46 of PVL1393 vector, but has an A insertion at position +36 and T to G change at position +3 to generate an initiation codon and reading frame-shift (Fig. 3-5 A). The PCR product was digested with EcoR5 and BamH1 and ligated to PVL1393 which was cut by the same enzymes. The resulting plasmid which has an authentic polyhedrin initiation codon and a changed reading frame starting at codon 12 was named PVL1394. The cytoplasmic cDNA fragment corresponding to aa residues 756 to 1422 of PTPase γ was released from the clone 4.1 in the pbluescript vector by digestion with BstU1 and EcoR1 and inserted into the PVL1394 vector at Sma1 and EcoR1 sites. As the result of construction, 12 aa from the baculoviral polyhedrin and 3 aa from the linker sequence were fused to the cytoplasmic portion of PTPase γ. The expressing plasmid was co-transfected into SF9 cells with the linearized viral helper DNA (Pharmingen). This helper DNA has a lethal deletion so that only the recombinant virus can survive. Recombinant virus selection, infection and maintenance of SF9 culture were carried out according to the suggested procedures (Summers and Smith 1987).

CONSTRUCTION OF GAG-PTPASE FUSION PROTEIN EXPRESSION PLASMID (by Cong S. Zong): An Xba1-EcoR1 cDNA fragment of PTPase γ including the TM and entire cytoplasmic domain was ligated to the pbluescript vector. Then a Xba1 fragment released from that vector including the entire PTPase sequence and the linker region was transferred to the pECE vector to give rise to pECE-PTPase plasmid. A Sac1-EcoR5 cDNA

fragment of UR2 including the 5' noncoding and first 150 aa of gag p19 was ligated to the pECE-PTPase plasmid at the Sac1 site and Xba1 site which was generated by partial digestion and then blunted.

ASSAY OF PTPASE ACTIVITY: The peptide substrate Raytide (Oncogene Science) was tyrosine phosphorylated with $pp60^{src}$ immunoprecipitated from extract of Rous sarcoma virus infected CEF. The kinase reaction was carried out according to the instruction (Oncogene Science). After removing the protein-A bound Src immunocomplex, 50 ul of supernatant was precipitated with the addition of 0.5 ml of 20% (W/V) Trichloroacetic acid (TCA) in 20 mM NaH_2PO_4 and 0.1 ml 5 mg/ml BSA and incubated on ice for 10 min. After brief spin at 12,000 RPM, the precipitates were washed with 20% TCA in 20 mM NaH_2PO_4 and then dissolved in 0.2 M Tris-HCl (pH 8.0). Phosphatase assay was carried out by adding the labeled Raytide ($1\text{-}5x10^5$ CPM) to PTPase immunoprecipitates resuspended in a total of 50 ul reaction solution containing 25 mM HEPES (pH 7.3), 5 mM EDTA, 10 mM DTT for 15 min at 37°C. The reaction was terminated by the addition of 20% TCA to a final concentration of 5%. After removing the PTPase immunoprecipitate, the supernatant that contains free phosphate (Pi) and Raytide were spotted on a sheet of Whatman paper and developed decendingly with 5% TCA. The amount of Pi released was counted by scintination counter.

RESULTS AND DISCUSSION

ISOLATION AND SEQUENCING OF CHICKEN PTPASE γ cDNAs

Taking advantage of the conservation of catalytic domain among different PTPases, a chicken kidney cDNA library was screened with the full-length CD45 cDNA as a probe under the low stringency condition. A 4.1 Kb cDNA clone was initially isolated. Subsequently a chicken brain cDNA library was screened using a 5' cDNA probe of the original cDNA clone under high stringency. After several rounds of screening, a set of cDNA clones with overlapping sequences were isolated and subloned into pbluescript vector and sequenced on both strands. Those overlapping sequences were assembled into a total cDNA length of 5403 bp(Fig. 3-1). This 5.4 kb cDNA contains a open reading frame of 1422 aa which has two hydrophobic amino acid stretches (Fig. 3-2). One is at the very amino-terminal end which is a putative signal peptide, the other is at aa 743 to 768 which is a putative TM domain. Sequence comparison between human PTPase γ and our cDNA reveals that they are 86.7% identity at amino acid level and 82% identity at nucleotide level. Thus we think this cDNA represents the chicken PTPase γ. Sequence analysis of human and mouse PTPase γ showed that the EC domain of PTPase γ contains a region of 266 aa residues with striking sequence similarity to the enzyme carbonic anhydrase and a sequence resembling fibronectin type III repeat, and the cytoplasmic domain contains two tandem stretches of typical PTPase catalytic sequences (Barnea et al 1993). The second domain has a Asp residue at position 1351 instead of a conserved

Cys residue thought to be essential for the catalytic activity. All those domains and structural features are well conserved in the chicken homolog. However, the region encompassing about 100 aa residues immediate upstream of the TM domain is relatively less conserved among the three species. Interestingly, a 29 aa stretch immediate carboxyl to the TM domain is deleted in the chicken homolog in comparison with human and mouse PTPase γ. I have isolated several independent cDNA clones spanning this region. They all have this 29 aa deletion, suggesting that this is a genuine difference between chicken and mammalian species. Chicken PTPase γ has nine potential N-glycosylation sites. Eight of them are conserved in human whereas six of them are conserved in murine homolog.

EXPRESSION OF CHICKEN PTPASE γ mRNA IN DIFFERENT TISSUES

Two transcripts of 6.3 kb and 9.5 kb were detected at varying ratio in different tissues by Northern blot analysis (Fig. 3-4). They are bigger than the murine counterparts that were reported to be 5.5 Kb and 8.5 Kb (Barnea et al 1993). Only the 6.3 kb transcript was detected in CEF. RNA slot-blot hybridization revealed that PTPase γ mRNA were detected in various tissues including brain, heart, intestine, lung, kidney, ovary, spleen, and stomach, but not in bursa, liver, muscle, and thymus (data not shown).

Northern result indicates that the 5.4 Kb cDNA composite does not represent the full length copy of the mRNA, although sequence comparison with human and murine PTPase γ suggests strongly that the entire coding region of chicken PTPase γ has been identified. However, I have no independent evidence to confirm that the initiation codon in

the cDNA is an authentic one.

EXPRESSION OF THE CYTOPLASMIC DOMAIN OF CHICKEN PTPase γ IN SF9 CELLS

To study biochemical properties of the chicken PTPase γ, its cytoplasmic domain was engineered into a modified PVL1393 vector that contains a baculovirus polyhedrin promoter and an initiation codon (Fig. 3-5 A). The expression plasmid was co-transfected into SF9 cells with the linearized helper viral genomic DNA containing a lethal deletion so that only the recombinant virus generated by recombination between the helper and the expression plasmid can survive. After transfection, the recombinant virus was harvested and the titer was determined as 10^6 PFU/ml. The PTPase γ protein expression was detected by metabolic labelling of the recombinant virus infected cells, followed by cell lysis, immnuoprecipitation with anti-PTPase γ Ab and SDS-PAGE. The result showed that a protein of expected size was detected in the recombinant virus infected cells at a very low level, but not in the wild-type virus infected cells (Fig. 3-5 B). Phosphatase assay showed that the cytoplasmic domain was enzymatically active (Fig. 3-6, Lanes 5 and 6). The protein was initially detected at 24 hrs after infection and reached the highest level at 48 hrs and then decreased thereafter (Fig. 3-5 B).

More than 100 recombinant proteins have been expressed using recombinant baculovirus system at levels ranging from 1-500 mg/L (Webb and Summers 1990). The factors that influence the level of expression in baculovirus infected insect cells are not completely understood. The 3' noncoding sequence, the context of the initiation codon, and

nature of the foreign gene all affect the expression level of a recombinant protein. I have constructed the PTPase γ expression plasmid using the authentic polyhedrin initiation codon, and fusing the cDNA to the polyhedrin sequence several amino acid downstream of its initiation codon. It has been reported that fusion proteins containing short polyhedrin sequence at the amino-terminal end are expressed at higher levels than nonfused proteins (Webb and Summers 1990). Therefore, the low level of expression of PTPase γ is likely due to its intrinsic nature rather than inappropriate initiation sequence. It could be that this protein is not compatible to the SF9 cells, or its phosphatase activity somehow interferes with the expression. Cell death apparently was not the reason for the low expression since that no obvious difference in the cytopathic effect of the recombinant- and wild-type virus-infected cells was observed.

EXPRESSION OF GAG-PTPASE γ FUSION PROTEIN IN NORMAL AND SRC TRANSFORMED NIH3T3 AND RAT1 CELLS

Fusion to the *gag* sequence is commonly seen in retrovirus transduced oncoproteins, including oncogenes *ros, fps, yes, myc, erbA, abl, fms, kit, and raf* (Weiss et al 1982). The function of the *gag* sequence is thought to provide an initiation codon and help to stabilize and form active oncoproteins (Prywes et al 1985, Jong et al 1990). This lab has shown that IR and IGFR genes could be truncated and fused to *gag* to form active oncogenes (Liu et al 1993). Therefore, I thought there was a good chance to stably express PTPase γ as a gag-PTPase fusion protein in mammalian cells. The PTPase γ

cDNA fragment including the TM and cytoplasmic domains (aa 679 to 1422) was linked to the gag sequence of UR2 to form a gag-PTPase coding sequence in the mammalian expression vector pECE (Fig. 3-7 A). The plasmid was co-transfected with the pSV2-Neo into NIH3T3 cells and a number of stable expressing clones were isolated. However, the PTPase γ expression levels in those clones were very low (Fig. 3-7 B). Since PTPase γ was suggested to be a candidate of tumor suppressor based on its chromosomal localization (LaForgia et al 1991) and because of its presumed activity against PTKs, *src* transformed NIH3T3 and Rat1 cell lines were used to establish stable gag-PTPase γ expressing lines and to observe the effects of PTPase γ. Several clones from both *src*/NIH3T3 and *src*/Rat1 cells were established and shown to express a protein at the anticipated position as detected by metabolic labelling (Fig. 3-7 B). Phosphatase assay confirmed that this fusion protein was able to dephosphorylate the Raytide, a peptide substrate (Fig. 3-6, Lanes 1, 2, 3, and 4). The low expression level of PTPase γ in mammalian cells is consistent with that in insect cells and suggests that its low level of expression is likely due to its intrinsic property.

To detect whether gag-PTPase γ can reverse the transformed phenotypes of *src*/NIH3T3 and *src*/Rat1 cells, colony formation assay was performed. The result showed that gag-PTPase expressing cells gave rise to similar numbers of colonies as the parental cells, although the colony numbers varied among individual clones (Table 5-1). To determine cell growth rate, parental *src*/NIH3T3 and *src*/Rat1 clones and gag-PTPase γ expressing clones were seeded in 24-well plates. After incubation in 5% serum for 24 hr, cells were grown in 0.5% serum and cell numbers were counted after 1 and 3 days

incubation. No difference was observed between gag-PTPase expressing cells and parental cells either (data not shown).

PTPs can regulate cell growth positively and negatively. For examples, the cytoplasmic PTPase 1B can suppress transformation by *neu* oncogene in PTPase 1B expressing NIH3T3 cells (Brown-Shimer et al 1992), and microinjection of PTPase 1B into Xenopus oocytes demonstrated that it could block the maturation induced by insulin (Tonks et al, Cicirelli et al, 1990). The SH2 domain containing PTPase, PTP1C, inhibits cytokine induced mitogenic response in hematopoietic cells (Klinmuller et al 1995) whereas another SH2 containing PTPase Syp is required for signal transduction mediated by a number of RPTKs and thus positively regulates cell growth (Feng and Pawson 1994). Expression of receptor-type PTPase CD45 in C127 murine cells has been shown to inhibit ligand-dependent phosphorylation of IGFR and PDGFR and their mitogenic response to IGF-1 and PDGF, respectively (Mooney et al 1992). Conversely, expression of PTPase α in rat fibroblast results in transformation (Zheng et al 1992). Since *src* transformation requires its continuous PTK activity, our result indicates that expression of PTPase γ is unable to inactivate the $pp60^{src}$ or to block its essential substrate(s) for cell transformation. This could be duo to low level expression of PTPase which is insufficient to overcome the activity of Src. Alternatively, the specificity of PTPase γ or its compartmentalization preclude its interaction with Src or its substrates.

Fig. 3-1. Isolation of cDNAs coding for chicken PTPase γ. The open box depicts the coding region. The filled box represent the TM domain. The dotted lines represent the non-coding regions. The solid lines represent individual cDNA clones. One representative clone from each round of screening is shown. The thickened lines represent the DNA fragments used as probes for library screening. The restriction enzyme sites used for construction of various PTPase γ expression plasmids and for generation of probes are shown.

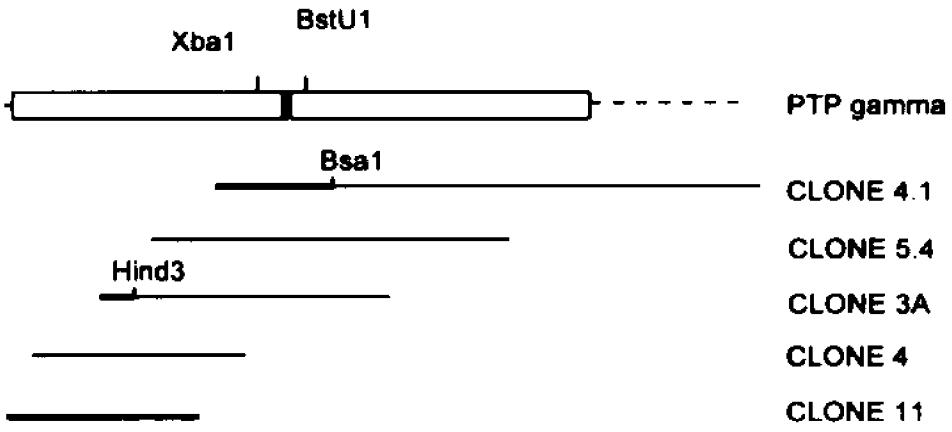


Fig. 3-2. Nucleotide and derived amino acid sequences of chicken PTPase γ cDNA. Hydrophobic amino acid stretches are underlined.

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                                                                TTTTAGCCCCTCTCCCCTCTTTCGATGTGCGGCTTTGGAC      40
ATGCGGAGGCTACTGCAACCGTGTTGGTGGATCTTTTTCTTGAAAATCACCAGCTCCGTGCTCCATGATGTGGTGTGCTTCCCCGCTCTG     130
MetArgArgLeuLeuGlnProCysTrpTrpIlePhePheLeuLysIleThrSerSerValLeuHisAspValValCysPheProAlaLeu      30

ACTGAAGGTTATGTTGGCTCTCTGCATGAAAGCAGACATGGAAGTTCTGTGCAGATACGGAGGCGAAAGGCTTCGGGGGATCCTTACTGG     220
ThrGluGlyTyrValGlySerLeuHisGluSerArgHisGlySerSerValGlnIleArgArgArgLysAlaSerGlyAspProTyrTrp      60

GGATACTCGGGTACCTATGGTCCCGAGCACTGGGTTACTTCCAGCGAGAAGTGCGGGGGGTCCCACCAGTCTCCCATAGACATCGTGGAC     310
GlyTyrSerGlyThrTyrGlyProGluHisTrpValThrSerSerGluLysCysGlyGlySerHisGlnSerProIleAspIleValAsp      90

CATCAGGCTCATGTTTTATATGAGTATCAAGAACTGCAGCTCGATGGTTTTGACAATGAATCTTCTAACAAGACTTGGATGAAAAACACA     400
HisGlnAlaHisValLeuTyrGluTyrGlnGluLeuGlnLeuAspGlyPheAspAsnGluSerSerAsnLysThrTrpMetLysAsnThr     120

GGAAAAACGGTTGCTATCCTGCTCAAGGACGATTACTTTGTCAGTGGCGCCGGGTTGCCAGGCAGATTCAAGGCAGAGAAGGTGGAGTTC     490
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CACTGGGGTCAGAGCAATGGATCAGCAGGCTCTGAGCACAGCATCAATGGCAAGAGGTTCCCCGTTGAGATGCAGATTTACTTCTACAAT     580
HisTrpGlyGlnSerAsnGlySerAlaGlySerGluHisSerIleAsnGlyLysArgPheProValGluMetGlnIleTyrPheTyrAsn     180

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ProAspAspPheAspSerPheGlyThrAlaValLeuGluAsnArgGluValGlyAlaMetAlaValPhePheGlnValSerGlnArgAsp     210

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LeuLeuProThrSerLeuGlySerTyrTyrArgTyrThrGlySerLeuThrThrProProCysSerGluIleValGluTrpIleIlePhe     270

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ArgLysProValProIleSerTyrHisGlnLeuGluAlaPheTyrSerIlePheThrThrGluGlnGlnAspHisValLysSerValGlu     300

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TyrLeuArgAsnAsnPheArgProGlnGlnArgLeuAsnAsnArgLysValSerLysSerAlaValLysAspAlaTrpSerGlnAspMet     330

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ValThrSerThrLeuLeuAlaGlyLeuGlyPheSerGlySerSerIleSerSerProSerSerValTrpProThrArgLeuProThr     540

GCAGCTGCCCCCACCAAGCAGGCCGGGCGGCCTGTGGTAGCCACCACTGAGCCCGCTGCTGCCTCCCCAGGGCCAGAGCGGGACTCGGCA    1750
AlaAlaAlaProThrLysGlnAlaGlyArgProValValAlaThrThrGluProAlaAlaAlaSerProGlyProGluArgAspSerAla     570

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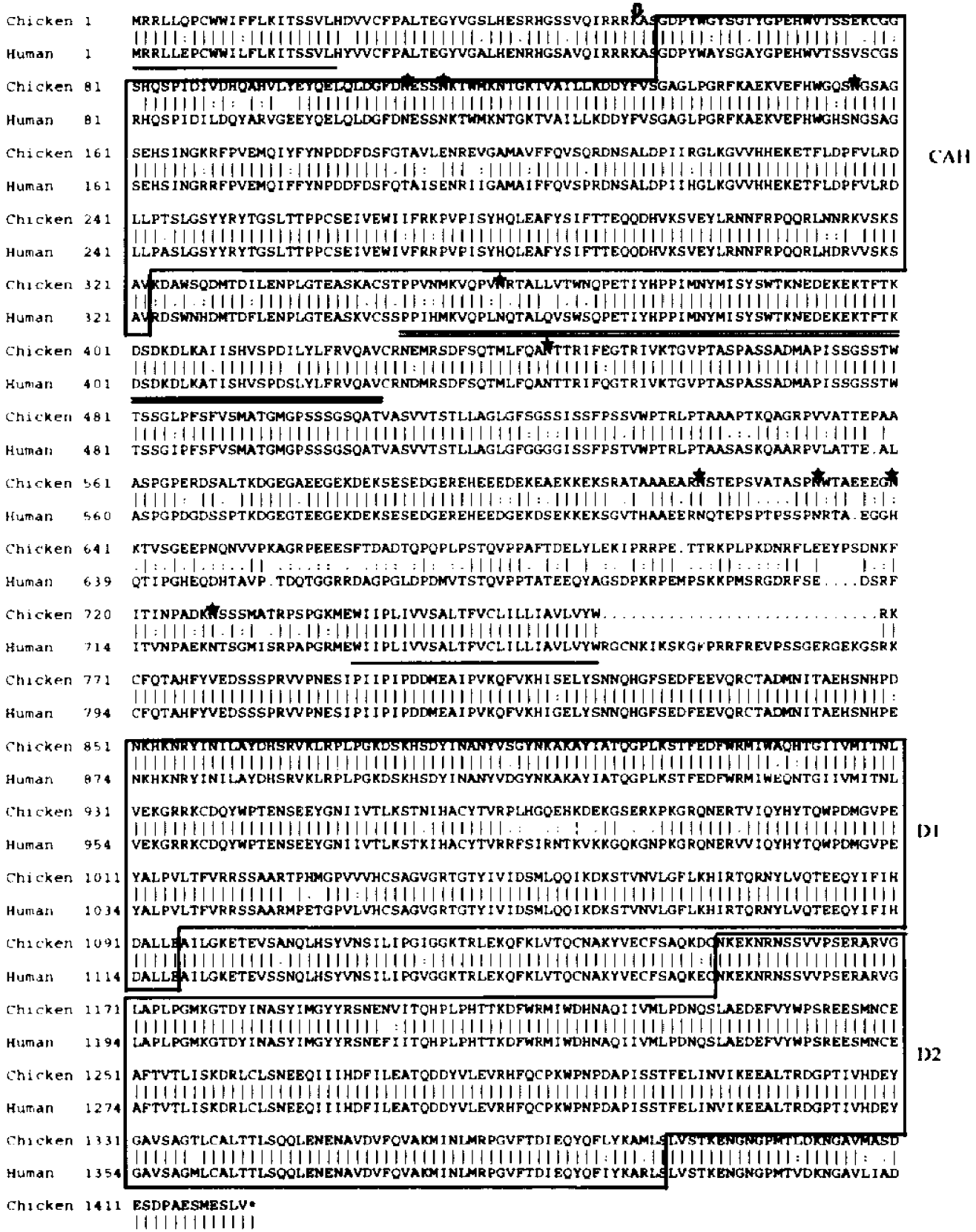
Fig. 3-3. Comparison of predicted amino acid sequence of chicken PTPase γ with that of the human homolog. Symbols: |, identical amino acid; :, and ., similar amino acid. Carbonic anhydrase (CAH)- like domain and phosphatase domains 1 (D1) and 2 (D2) are boxed. The hydrophobic sequences are underlined. Fibronectin III repeat is double-underlined. Asterisk and arrow represent glycosylation and cleavage sites, respectively.


Chicken 1 MRRLLQPCWWIFFLKITSSVLHDVVCFPALTEGYVGSLHESRHGSSVQIRRRKASGDPYWGYSGTYGPEHWVTSSEKCGG
Human 1 MRRLLEPCWWILFLKITSSVLHYVVCFPALTEGYVGALHENRHGSAVQIRRRKASGDPYWAYSGAYGPEHWVTSSVSCGS
Chicken 81 SHQSPIDIVDHQAHVLYEYQELQLDGFDNESSNKTWMKNTGKTVAILLKDDYFVSGAGLPGRFKAEKVEFHWGQSNGSAG
Human 81 RHQSPIDILDQYARVGEEYQELQLDGFDNESSNKTWMKNTGKTVAILLKDDYFVSGAGLPGRFKAEKVEFHWGHSNGSAG
Chicken 161 SEHSINGKRFPVEMQIYFYNPDDFDSFGTAVLENREVGAMAVFFQVSQRDNSALDPIIRGLKGVVHHEKETFLDPFVLRD
Human 161 SEHSINGRRFPVEMQIFFYNPDDFDSFQTAISENRIIGAMAIFFQVSPRDNSALDPIIHGLKGVVHHEKETFLDPFVLRD
Chicken 241 LLPTSLGSYYRYTGSLTTPPCSEIVEWIIFRKPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQRLNNRKVSKS
Human 241 LLPASLGSYYRYTGSLTTPPCSEIVEWIVFRRPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQRLHDRVVSKS
Chicken 321 AVKDAWSQDMTDILENPLGTEASKACSTPPVNMKVQPVNRTALLVTWNQPETIYHPPIMNYMISYSWTKNEDEKEKTFTK
Human 321 AVRDSWNHDMTDFLENPLGTEASKVCSSPPIHMKVQPLNQTALQVSWSQPETIYHPPIMNYMISYSWTKNEDEKEKTFTK
Chicken 401 DSDKDLKAIISHVSPDILYLFRVQAVCRNEMRSDFSQTMLFQANTTRIFEGTRIVKTGVPTASPASSADMAPISSGSSTW
Human 401 DSDKDLKATISHVSPDSLYLFRVQAVCRNDMRSDFSQTMLFQANTTRIFQGTRIVKTGVPTASPASSADMAPISSGSSTW
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Chicken 561 ASPGPERDSALTKDGEGAEEGEKDEKSESEDGEREHEEEDEKEAEKKEKSRATAAAEARNSTEPSVATASPNWTAEEEGN
Human 560 ASPGPDGDSSPTKDGEGTEEGEKDEKSESEDGEREHEEDGEKDSEKKEKSGVTHAAEERNQTEPSPTPSSPNRTA.EGGH
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Human 639 QTIPGHEQDHTAVP.TDQTGGRRDAGPGLDPDMVTSTQVPPTATEEQYAGSDPKRPEMPSKKPMSRGDRFSE....DSRF
Chicken 720 ITINPADKNSSSMATRPSPGKMEWIIPLIVVSALTFVCLILLIAVLVYW.....RK
Human 714 ITVNPAEKNTSGMISRPAPGRMEWIIPLIVVSALTFVCLILLIAVLVYWRGCNKIKSKGFPRRFREVPSSGERGEKGSRK
Chicken 771 CFQTAHFYVEDSSSPRVVPNESIPIIPIPDDMEAIPVKQFVKHISELYSNNQHGFSEDFEEVQRCTADMNITAEHSNHPD
Human 794 CFQTAHFYVEDSSSPRVVPNESIPIIPIPDDMEAIPVKQFVKHIGELYSNNQHGFSEDFEEVQRCTADMNITAEHSNHPE
Chicken 851 NKHKNRYINILAYDHSRVKLRPLPGKDSKHSDYINANYVSGYNKAKAYIATQGPLKSTFEDFWRMIWAQHTGIIVMITNL
Human 874 NKHKNRYINILAYDHSRVKLRPLPGKDSKHSDYINANYVDGYNKAKAYIATQGPLKSTFEDFWRMIWEQNTGIIVMITNL
Chicken 931 VEKGRRKCDQYWPTENSEEYGNIIVTLKSTNIHACYTVRPLHGQEHKDEKGSERKPKGRQNERTVIQYHYTQWPDMGVPE
Human 954 VEKGRRKCDQYWPTENSEEYGNIIVTLKSTKIHACYTVRRFSIRNTKVKKGQKGNPKGRQNERVVIQYHYTQWPDMGVPE
Chicken 1011 YALPVLTFVRRSSAARTPHMGPVVVHCSAGVGRTGTYIVIDSMLQQIKDKSTVNVLGFLKHIRTQRNYLVQTEEQYIFIH
Human 1034 YALPVLTFVRRSSAARMPETGPVLVHCSAGVGRTGTYIVIDSMLQQIKDKSTVNVLGFLKHIRTQRNYLVQTEEQYIFIH
Chicken 1091 DALLEAILGKETEVSANQLHSYVNSILIPGIGGKTRLEKQFKLVTQCNAKYVECFSAQKDQNKEKNRNSSVVPSERARVG
Human 1114 DALLEAILGKETEVSSNQLHSYVNSILIPGVGGKTRLEKQFKLVTQCNAKYVECFSAQKEQNKEKNRNSSVVPSERARVG
Chicken 1171 LAPLPGMKGTDYINASYIMGYYRSNENVITQHPLPHTTKDFWRMIWDHNAQIIVMLPDNQSLAEDEFVYWPSREESMNCE
Human 1194 LAPLPGMKGTDYINASYIMGYYRSNEFIITQHPLPHTTKDFWRMIWDHNAQIIVMLPDNQSLAEDEFVYWPSREESMNCE
Chicken 1251 AFTVTLISKDRLCLSNEEQIIIHDFILEATQDDYVLEVRHFQCPKWPNPDAPISSTFELINVIKEEALTRDGPTIVHDEY
Human 1274 AFTVTLISKDRLCLSNEEQIIIHDFILEATQDDYVLEVRHFQCPKWPNPDAPISSTFELINVIKEEALTRDGPTIVHDEY
Chicken 1331 GAVSAGTLCALTTLSQQLENENAVDVFQVAKMINLMRPGVFTDIEQYQFLYKAMLSLVSTKENGNGPMTLDKNGAVMASD
Human 1354 GAVSAGMLCALTTLSQQLENENAVDVFQVAKMINLMRPGVFTDIEQYQFIYKARLSLVSTKENGNGPMTVDKNGAVLIAD
Chicken 1411 ESDPAESMESLV*
Human 1434 ESDPAESMESLV*
CAH
D1
D2

Fig. 3-4 Expression of chicken PTPase mRNA in different tissues. Northern analysis. Two independent northern analyses are shown. 10 μg of poly(A$^+$) RNA was used for each sample. Riboprobe transcribed off clone 4.1 (Fig. 3-1) in pbluescript vector was used for hybridization. Molecular weight markers are in kb. Several RNA samples were separated in agarose gel, stained with ethedium bromide and photographed as shown under the Northern blot.

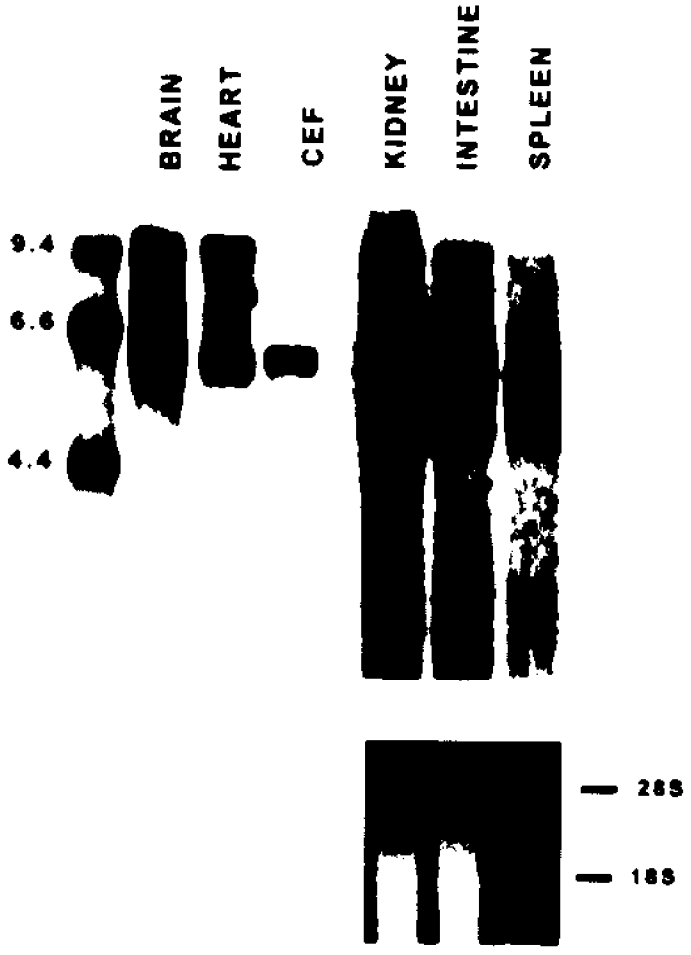
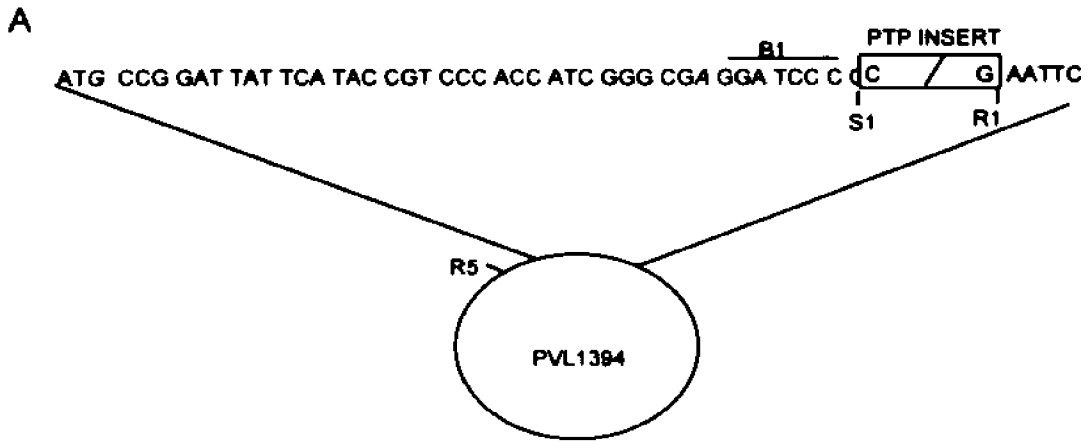


Fig. 3-5 Expression of the cytoplasmic domain of PTPase γ in SF9 cells. A. Schematic representation of the expression plasmid. The pVL1394 vector is derived from pVL1393 with substitution of T by G at position +3 and an A insertion at position +36 indicated as italic. E5: EcoR5, B1: BamH1, S1: Sma1, and R1: EcoR1. E5 and B1 were used for construction of PVL1394 and S1 and R1 were used for insertion of PTPase cDNA fragment. The open bar represents PTPase γ sequence. B. Sf9 cells were metabolically labeled with ^{35}S-Met after infection with the PTPase γ recombinant virus for indicated times. Cell lysis, IP, and autoradiography were done as described in previous chapter. 1 mg protein (from 10 cm tissue culture dish) for each sample was used for the assay.



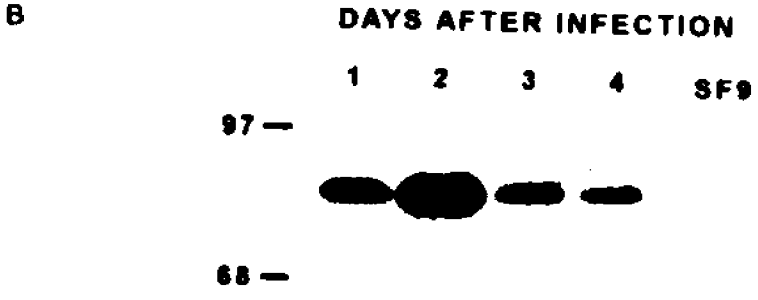


Fig. 3-6 Phosphatase assay. 1 mg protein from Cell lysates were immunoprecipitated with anti-PTPase γ Ab. 1/10 of the immune complexes were presented to the ^{32}P labeled Raytide peptide for 15 min at 37°C. The free phosphate (Pi) were separated by paper chromatography and counted with scintination machine. Pi release(%)=CPM(free Pi)/CPM(Raytide + free Pi). 1. *src*/NIH3T3 cells, 2. gag-PTPase γ expressing *src*/NIH3T3 cells, 3. *src*/Rat1 cells, 4. gag-PTPase γ expressing *src*/Rat1 cells. 5. SF9 cells 6. SF9 cells infected with recombinant virus for 2 days.

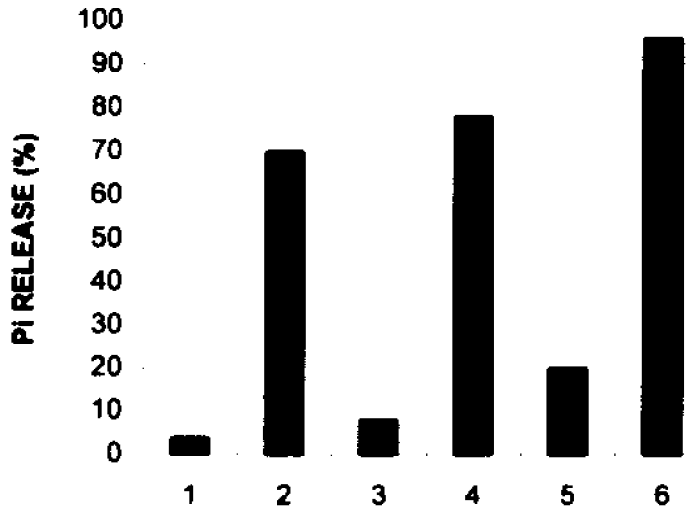
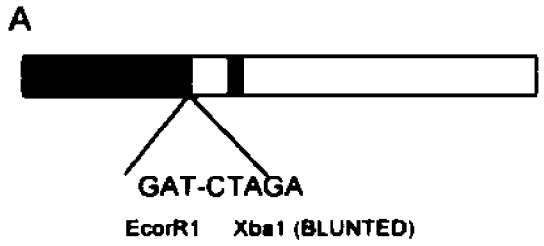


Fig. 3-7 Gag-PTPase γ expression in normal and *src* transformed NIH3T3 and Rat1 cells. A. Structure of gag-PTPase γ fusion cDNA. Hatched bar represents gag sequence of UR2. Open bar represents PTPase γ sequence and filled bar represents the TM domain. The restriction sites used for cloning and the fusion junction are shown. B. NIH3T3, *src*/NIH3T3 and *src*/Rat1 cells were transfected with gag-PTPase γ expression vector and pSV2-Neo plasmid. After drug selection, resistant clones were analyzed for gag-PTPase γ expression by metabolic labelling with ^{35}S-Met. Representative clones and pSV2-Neo transfected cells from each cell type are shown.



B

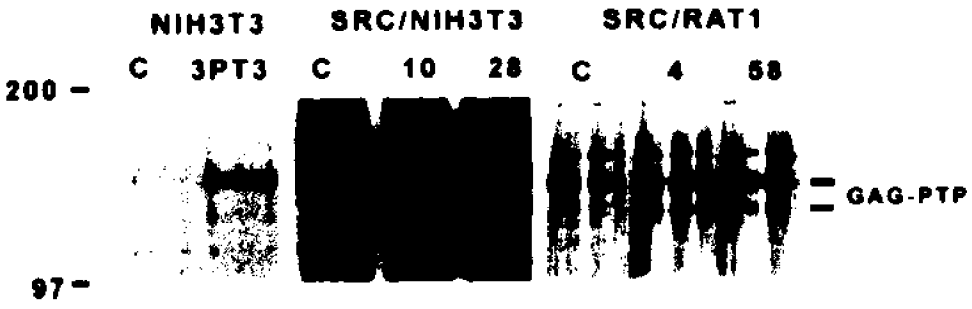


Table 3-1. Colony formation of GAG-PTP expressing cells

Clones	Colony No.	Clones	Colony No.
src/3T3-1	230	PTP/src/3T3-1	274
src/3T3-2	523	PTP/src/3T3-2	415
src/Rat-1	150	PTP/src/Rat-1	130
src/Rat-2	218	PTP/src/Rat-2	212

Parental *src* transformed NIH3T3 (src/3T3) and Rat1 (src/Rat) clones and the GAG-PTP expressing clones were seeded at 2000 cells per 6 cm dish. Colonies were counted after 3 weeks incubation.

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