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Liu, Delong, Ph.D.

City University of New York, 1993

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Transforming Potential and Signal Transduction of the Human Insulin-like Growth Factor I Receptor

by

Delong Liu

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

1993

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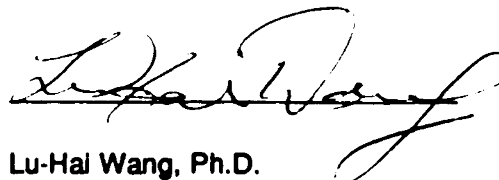

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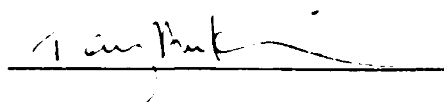

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Abstract

Transforming Potential and Signal Transduction of The Human Insulin-like Growth Factor I Receptor

by

Delong Liu

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

Human insulin-like growth factor I receptor (hIGFR) is a transmembrane (TM) protein tyrosine kinase (PTK). The IGFR PTK domain shares high sequence homology with those of the insulin receptor and the *ros* oncoprotein of avian sarcoma virus UR2. To explore the potential of IGFR to induce cellular transformation, 10 mutants were constructed from human IGFR cDNA by mutating its extracelluar (EC) or carboxy terminal sequences. Those mutants were fused in frame to the 5' sequence of UR2 *gag* p19 and expressed in chicken embryo fibroblasts (CEF). The full length hIGFR was able to induce CEF transformation. A gag-IGFR fusion protein retaining 36 amino acids of the IGFR EC domain and the entire TM and cytoplasmic domains had an enhanced transforming potential, which is correlated with its increased tyrosine kinase activity. The EC 36 aa sequence of IGFR in the *gag*-IGFR exerts modulatory effects on the protein's transforming and tumorigenic potential. The 20 aa residues immediately upstream of the TM domain have an inhibitory effect on the tumorigenic potential of *gag*-IGFR. The elevated association of phosphatidylinositol (PI) 3-kinase activity with IGFR proteins seem to be correlated with the transforming potency of the IGFR mutant proteins. The most C-terminal

27 amino acids of IGFR were dispensable, further deletion of 20 or 60 amino acids drastically affected the PTK activity and transforming ability of the *gag*-IGFR fusion protein. Surprisingly, deletion of C-terminal 67 aa appears to have little effect. Association of PI 3-kinase with IGFR requires its kinase activity and correlates with its tyrosine phosphorylation. In summary, this thesis contains the following findings: 1. The EC sequence has negative effects on PTK activity, transforming and tumorigenic potential of the hIGFR; 2. The C-terminal sequence from 1250 to 1310 is critical for the above biological functions and biochemical properties of IGFR; 3. PI 3-kinase is involved in IGFR signal transduction. Association of PI 3-kinase with IGFR relies on kinase activity and/or tyrosine phosphorylation of IGFR.

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Format of Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter III contains results published as Liu et al, Journal of Virology (1992) 66:374-385. Chapter IV contains results published as Liu et al, Journal of Virology (1993) 67:9-18. Chapter III, IV, V has specific introduction and discussion for each section, with a general introduction and a general discussion at the beginning and end of the thesis. To avoid redundancy, "Materials and Methods" and "References" have been consolidated. Tables and figures for Chapter I, III, IV, and V are placed at the end of each chapter.

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Chapter I Introduction

I. Receptor-type protein tyrosine kinases and cell growth control

II. Insulin receptor, insulin receptor-related receptor, and insulin-like growth Factor I receptor

III.Retrovirus UR2 and oncogene ros

IV. Signal transduction by protein tyrosine kinases

I. Receptor-type protein tyrosine kinases and cell growth control

Cellular proliferation and differentiation are normally regulated by growth factors through transmembrane receptors with intrinsic protein tyrosine kinase (PTK) activity. These receptor-type PTKs (RPTKs) have a common structural configuration. They all consist of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular domain where lies the tyrosine kinase catalytic sequence (Yarden and Ullrich, 1987, 1988; Ullrich and Schlessinger, 1990). The mitogenic signals of growth factors are transmitted into cells through their cognate receptors by activating their intrinsic PTK activity. The PTK activity is absolutely required for the signal transduction. Autophosphorylation of the activated receptors is usually the earliest response upon ligand stimulation. Why this is so has remained a puzzle until recently. Studies in the past several years on proteins containing src homology domain 2 and 3 (SH2 and SH3) have revealed that the PTK autophosphorylation creates sites (phosphotyrosine motifs) for SH2 binding and thus initiates a chain of protein-protein interaction, fulfilling the signal transmission from extracellular growth factor to nucleus resulting in gene activation, DNA synthesis and cell proliferation (Cantley et al, 1991; Koch, et al, 1991).

The importance of cell growth control by growth factors and the RPTKs is best exemplified by the findings that more than 10 oncogenes have been documented to be

altered cellular RPTKs (Table 1-1). Structural and functional alteration of RPTKS invariably lead to subversion of normal regulation of cell growth (Aaronson, 1991; Heldin, et al, 1987). Truncation, internal deletion, point mutation and amplification of RPTK genes have been demonstrated to be common mechanisms for constitutive activation of RPTKs, which consequently cause cell transformation in vitro and oncogenicity of host cells in vivo (Westermark and Heldin, 1988).

The first example is the oncogenic counterpart of EGF (epidermal growth factor) receptor, v-erbB, which was found to arise from c-erbB by almost complete truncation of the extracellular sequence and deletion of the C-terminal 34 amino acids (aa) including one major autophosphorylation site (Downward, et al, 1984). In addition, v-erbB harbors multiple point mutations in comparison with c-erbB. Those structural changes render v-erbB constitutively active in its kinase activity and oncogenicity (Shu et al, 1991; Massoglia et al, 1990; Velu et al, 1989).

A second example of a truncated RPTK gene is v-fms transduced by the Gardner-Arnstein strain of feline sarcoma virus (Coussens, et al, 1986). The oncogene codes for a mutant version of the CSF-1 (colony stimulating factor) receptor (Table 1-1). v-fms oncoprotein is synthesized as a precursor fusion protein, gag-fms, which is subsequently cleaved and processed into $gp140^{v\text{-}fms}$. In contrast to the v-erbB product, $gp140^{v\text{-}fms}$ appears to contain complete ligand binding domain of the CSF-1 receptor (Coussens, et al, 1986). The C-terminal 40 aa in the CSF-1 receptor have been replaced by 11 irrelevant residues in v-fms, including one tyrosine residue. Similar to v-erbB product, this carboxyl terminal alteration may contribute to the increased tyrosine kinase activity and oncogenicity (Sacca, et al, 1986).

Another example of transduced RPTK genes comes from the discovery of v-ros, an oncogene originally found in avian sarcoma virus UR2 (Balduzzi et al, 1981; Wang et al, 1982; Neckameyer et al, 1984). The proto-oncogene c-ros encodes a RPTK-like molecule (Neckameyer et al, 1986; Birchmeier et al, 1990; Chen et al, 1991; Matsushime

et al, 1986, 1990). Similar to the transduction process of v-erbB, v-ros has almost a complete truncation of c-ros extracellular domain (only 7 aa left) and alteration of its carboxyl terminal sequence (Neckameyer, et al, 1986). The resulting oncoprotein $p68^{gag\text{-}ros}$ has constitutive kinase activity and highly transforming and oncogenic (see below for more detail).

The *met* oncogene was found in carcinogen treated human osteosarcoma cells and seems to be activated by gene rearrangement such that a DNA segment from human chromosome 1 joints the 5' truncated c-*met* gene (Cooper et al,1984; Park et al,1986; Tempest et al, 1986). The fusion protein expressed from the *met* oncogene is a tyrosine phosphorylated doublet of 60/65 Kd as opposed to the 165 and 140Kd proteins encoded by the c-met proto-oncogene (Tempest et al, 1986). C-*met* proto-oncogene may be the proto-type of a new class of tyrosine kinase receptor with a heterodimeric subunit structure consisting of a 50Kd α subunit disulfide linked to a 145Kd ß subunit (Giordano et al,1988 ,1989a and 1989b). The c-*met* proto-oncogene has been found to be amplified and overexpressed both in GTL-16 gastric carcinoma cell line and in transfected NIH 3T3 cells(Park et al,1986). Evidence for transforming capability of the amplified c-*met* gene has been derived from transfection of NIH 3T3 cells with normal cellular DNA where cells from a high portion of spontaneously occurring foci was found to contain a 4-8 fold amplification of c-*met* and at least a 20-fold overexpression of a normal c-*met* transcript (Cooper et al,1986). A recent finding shows that improper processing of met precursor can cause oncogenic activation (Mondino et al, 1991). The met precursor, which is normally cleaved into two subunits, is not processed in a protease-deficient tumor cell line LoVo. As a result, PTK activity of the uncleaved met is constitutively activated. This may represent a new mechanism for constitutive activation of the RPTK. It is recently shown that the ligand for c-met turns out to be the hepatocyte growth factor (Bottaro et al,1991).

More extensive truncation and deletion are found in another retroviral oncogene, v-*kit*, which corresponds to c-*kit* transduced by Hardy-Zuckerman 4 strain of feline

sarcoma virus (Besmer et al, 1986). Compared with c-kit, v-kit has lost the entire extracellular and TM domains. The C-terminal 49 aa were replaced by 5 unrelated residues, again including the deletion of a potential autophosphorylation site (Yarden et al, 1987). Unlike the oncoprotein v-erbB, v-fms or v-ros, the v-kit gene product becomes associated with plasma membrane via the myristilated gag sequence fused at the N-terminus of v-kit (Besmer et al, 1986). c-kit has been demonstrated to be the receptor for mastocyte growth factor (Flanagan and Leder, 1990; Huang et al, 1990; Williams et al, 1990). The above examples suggest that truncation of the extracellular domains and/or deletion of the C-terminal tails remove negative regulatory elements, resulting in constitutive activation of erbB, fms, ros, and kit.

A point mutation, rather than deletions or truncations, is found to be responsible for oncogenic activation of neu, a carcinogen-induced oncogene found in rat neuroblastoma (King et al, 1985; Schechter et al, 1984). The single point mutation results in a change of valine to glutamic acid in the transmembrane sequence. This mutation results in constitutive dimerization of the neu receptor with higher affinity for ligand binding (Ben-Levy et al, 1992). There appears to exist several ligands for the neu receptor as demonstrated recently from cloned cDNAs coding for the putative ligands (Holmes et al, 1992; Peles et al, 1992; Wen et al,1992).

The oncogene *trk* was originally isolated from a human colon carcinoma cDNA library. This oncogene was generated by somatic rearrangement that resulted in the fusion between a protein tyrosine kinase with a transmembrane domain and a truncated non-muscle tropomyosin molecule(Martin-Zanca et al, 1986). Proto-oncogene trk encodes a receptor molecule with ligand binding domain which is replaced in the oncogenic trk by tropomyosin sequence (Martin-Zanca et al, 1989; Coulier et al, 1989). In this case, truncation and fusion to non-muscle tropomyosin activated the oncogenic potential of c-trk, although other mechanisms like point mutation, deletion and duplication of kinase domain could also cause oncogenic activation of the proto-oncogene (Coulier et al, 1990). It is

now known that the trk proto-oncogene product is a high affinity receptor for nerve growth factor (Klein et al, 1991a). Several different forms of trk genes have been cloned recently and they code for a family of receptors with different specificities for a class of neurotrophic factors (Klein et al, 1991b; Lamballe et al, 1991).

The *ret* oncogene was activated by recombination between two unlinked segments of human DNA which occurred most likely during the transfection of NIH 3T3 cells (Takahashi et al,1987). This oncogene encodes a fusion protein consisting of an amino terminal part from an unknown protein sequence and a carboxyl-terminal part of a tyrosine kinase with a transmembrane domain. As in the case of the *trk* oncogene, the regulatory extracellular domain of a growth factor receptor has been replaced by another sequence, presumably causing a constitutively activated growth factor receptor with tyrosine kinase activity.

The oncogenic RPTKs and their normal counterparts discussed above have provided informative clues to the understanding of the mechanisms for the activation of normal growth factor receptors and also invaluable tools to study their signal transduction in the process of cell transformation as well as physiological functions. A separate group of oncogenic PTKs, the non-receptor PTKs, such as src, fps/fes, abl, yes, and so on, have also contributed tremendously to our understanding of the mechanisms for cellular transformation and tumorigenesis (Jove and Hanafusa, 1987; Bishop, M.J., 1991). The accumulated evidence has clearly demonstrated that protein tyrosine phosphorylation plays a crucial role in cellular signal transduction, growth control and oncogenesis.

II. Insulin receptor (IR), insulin receptor-related receptor (IRR) and insulin-like growth factor I receptor (IGFR)

The polypeptide hormone, insulin, induces a wide spectrum of physiological responses on target cells. It stimulates uptake of glucose and amino acids, increases

synthesis of metabolic enzymes like glycogen synthetase and synthesis of lipid, protein, DNA and RNA (Jacobs and Cuatrecasas, 1981). This hormone is required for maintenance of glucose homeostasis and diabetes develops when insulin is deficient. The rapid metabolic effects and long-term mitogenic actions of insulin are initiated by its binding to specific cell surface receptors and activating the tyrosine kinase activity of the receptors, which were first isolated by Cuatrecasas (1972). The IR is composed of two α and two β subunits, forming a heterotetramer which is anchored on the plasma membrane. Cloned human placental IR cDNA encodes a 1370 or 1382 aa polypeptide (1370 aa reported by Ullrich et al, 1985; 1382 aa reported by Ebina et al, 1985), which is further cleaved and processed into α subunit of 135 Kd and β subunit of 95 Kd. The difference between the two cDNA clones were due to alternative splicing of a single gene at exon 11. As a result, 12 aa were inserted to the C-terminus of α subunit (Wilden et al, 1992). The cloned cDNA also shows sequence homology to EGFR, src oncogene family and ros (Ullrich et al, 1985; Ebina et al, 1985), but highest homology is found between IR and IGFR (Ullrich et al, 1986). With its heterotetramer structure, membrane localization, and intrinsic PTK activity, IR represents a prototype for a new class of RPTK family (Ullrich and Schlessinger, 1990). Although no oncogene has ever been found to arise from IR, it has been shown that IR harbors the potential to induce cell transformation and tumorigenesis (Poon et al, 1991; Wang et al, 1987). Overexpression of IR induces ligand-dependent transformation of fibroblast and ovarian cells (Giorgino et al, 1991). A more recent study demonstrated surprisingly that IR may have intrinsic serine kinase activity, although the significance of this newly described property remains unclear (Baltensperger et al, 1992).

A new member of IR family, IRR (insulin receptor-related receptor), was cloned from human genomic DNA library (Shier and Watt, 1989). IRR gene codes for a receptor highly homologous to both IR and IGFR. However, its C-terminal sequence is shorter than those of IR and IGFR. A study using chimeric receptors clearly indicated that IRR is a new

receptor for a distinctive ligand different from insulin or IGF-1 (Zhang and Roth, 1991).

The IGF-1 receptor, similar to the insulin receptor, is synthesized as a single glycosylated polypeptide precursor of 180 kilodalton(Kd). The precursor is further processed into two polypeptides, α subunit of 135Kd, and ß subunit of 95Kd(Jacobs et al. 1983a; Fig. 1-1). The α subunit is located extracellularly and possesses the IGF-1 binding domain and is disulfide-linked to the transmembrane ß subunit of which the cytoplasmic domain contains the catalytic region of protein tyrosine kinase (PTK) (Czech 1982, 1989). Cloned human placental IGFR cDNA encodes a 1337-aa polypeptide which includes 710-aa and 627-aa for α and ß subunits, respectively. The ß subunit consists of a 195-aa extracellular domain(EC), a 24-aa transmembrane domain(TM), a 408-aa intracellular domain including a PTK conserved region of 257-aa and a diverged carboxyl tail of 108-aa (Ullrich et al. 1986). The mature IGFR, like the insulin receptor, is a ß-α-α-ß heterotetrameric receptor complex (Steele-Perkins et al. 1988; Ullrich et al. 1986; Rechler and Nissley 1985). The receptor undergoes phosphorylation on its ß subunit upon binding of IGF-1 in both intact cells and cell lysate preparations (Jacobs et al. 1983b; Rubin et al. 1983; Steele-Perkins et al. 1988; Lammers et al. 1989). Phosphorylation of the IGF-1 receptor occurs on both tyrosine and serine residues in living cells (Roth et al, 1988; Steele-Perkins et al. 1988), whereas under in vitro conditions, the phosphorylation takes place exclusively on tyrosine residues (Rubin et al. 1983). The autophosphorylation of ß subunits appears to be catalyzed by the neighboring ß subunit kinase within the complex (Feltz et al. 1988; Boni-Schneitsler et al. 1988). The major tyrosine residues that are involved in IGFR autophosphorylation are likely the tyrosine 1131, 1135 and 1136 (Czech 1989; Chen et al. 1991), similar to those in IR (Tornqvist et al. 1987; Ellis et al. 1986; Debant et al. 1988).

Despite structural similarities between IGFR and IR which have 84% amino acid sequence identity in their PTK domains (Ullrich et al. 1986), their physiological functions are, however, distinctive. Insulin primarily regulates rapid anabolic metabolism, including

glucose uptake, lipid and glycogen synthesis, while IGF-I appears to be one of the primary regulators of cell growth (Rechler et al. 1987; Steele-Perkins et al. 1988). In one study using IR and IGFR chimeras, the cytoplasmic domain of IGFR was shown to be 10 times more active in stimulating DNA synthesis than that of IR (Lammers et al. 1989). It has been reported that the tyrosine phosphorylation and activation of IGFR may be at least in part involved in the altered growth regulation induced by $pp60^{v\text{-}src}$(Kozma et al. 1990a&b). Moreover, many tumors have been found to secrete IGF-I which has been suggested to be a significant growth promoter in human breast cancer (Cullen et al. 1990; Huff et al. 1986; Jaques et al. 1988; Minuto et al. 1986). IGFR expression has also been shown in benign and malignant breast tumors (Cullen et al. 1990; Pekonen et al. 1988; Peyrat et al. 1988a&b), and altered IGFR has been found in human leukemic HL-60 cells (Kellerer et al. 1990). Almost all of the human breast cancer cell lines and tumor specimens tested were positive in IGFR expression (Cullen et al. 1990; Peyrat et al. 1988). One of them, MCF-7, expresses surprisingly high level of IGFR proteins (Liu, D. and Wang, L.-H., unpublished data). Another study reported that IGFR level was higher in cancer tissues than in adjacent normal tissues (Pekonen et al. 1988). Overexpression of native human IGFR in NIH3T3 cells has been demonstrated to be able to induce transformation of these cells in the presence of human IGF-1 and the transformed NIH3T3 cells could form tumors in athymic mice(Kaleko et al, 1990). Antisense IGF-1 has recently been shown to be capable of inhibiting tumorigenicity of rat glioblastoma cells (Trojan et al, 1992). These studies suggest that IGFR may be involved in cell transformation and tumorigenesis.

III. Avian sarcoma virus UR2 and ros oncogene

Avian sarcoma virus (ASV) UR2 was originally recovered from a chicken pancreas myofibroma by R. E. Luginbuhl and later isolated by P. Balduzzi and his colleagues at the

University of Rochester, after which the UR2 was named (Balduzzi et al, 1981; Wang, L-H., 1988). UR2 is defective in replication and an associated helper virus UR2AV is needed for propagation of UR2. Molecular studies revealed that UR2 contains a unique oncogene, v-ros, which was fused to gag p19 (Wang et al, 1982; Neckameyer, 1984, 1985). The fusion protein, $p68^{gag\text{-}ros}$, is a transmembrane molecule with intrinsic PTK activity (Garber et al, 1985; Feldman et al, 1982; Jong and Wang, 1987; Neckameyer et al, 1985). Molecular cloning of the proto-oncogene, c-ros, shows that it may represent a new member of RPTK family (Birchmeier et al, 1990; Chen et al, 1991, Matsushime et al, 1986, 1990). Membrane association and gag sequences in the $p68^{gag\text{-}ros}$ were demonstrated to be essential for its transforming activity (Jong and Wang, 1990, 1991). Sequence comparison shows that ros is most homologous to the sevenless of *D. Melanogaster*, IR and IGFR in their PTK domains (Birchmeier et al, 1990; Chen et al, 1991; Matsushime, 1990; Wang, L.-H., 1988). However, the ros PTK domain can not substitute functionally for the IR PTK domain since the IR-ros hybrid, consisting of IR ligand-binding domain and ros PTK domain, failed to mediate physiological responses normally induced by insulin (Ellis et al, 1987). Studies on heterologous chimeras of EGFR/Neu, EGFR/PDGFR, EGFR/IR, and IR/IGFR invariably show that biological functions of those chimeric receptors are determined by the cytoplasmic PTK domain of the chimeras (Balloti et al, 1989; Riedel et al, 1989; Lammers et al, 1989, 1990; Lee et al, 1989; Lehvaslaiho et al, 1989; Segatto et al, 1992; Seedorf et al, 1992). These lines of evidence indicate that postreceptor signaling is specific for the individual PTKs and distinctive signaling pathways may exist for different RPTKs. Besides, it appears that the src-like kinases interact with significantly more cellular substrates than do RPTKs as suggested by the study using v-ros and v-src recombinants and comparing their cellular substrates (Jong et al, 1992). Recent studies showed that c-ros was highly expressed in kidney and intestines and suggested that it may be important in early organogenesis and later in physiological functions of the kidney and intestines (Chen,J. and Wang, L-H.,

unpublished data; Tessarollo et al, 1992). A putative ligand for this RPTK-like molecule remains to be defined.

IV. Signal transduction by protein tyrosine kinases

Although molecular cloning of genes encoding protein tyrosine kinases, including RPTKs and non-receptor PTKs (e.g. *src* family), has led to tremendous advances in understanding of the structural alterations involved in constitutive activation of tyrosine kinase activity and oncogenic potential (Hunter and Cooper, 1985; Jove and Hanafusa, 1987; Ullrich and Schlessinger,1990), not much is known about how the activated PTKs achieve their ultimate biological effects--causing cell transformation and oncogenicity. Since oncogenicity of the PTK oncogenes all depends on their increased tyrosine kinase activity, cellular targets of the activated PTKs are logically the molecules that relay the mitogenic signals and regulate the signaling pathways. The initial efforts have been directed to look for tyrosine phosphorylated cellular proteins by western blotting with anti-phosphotyrosine antibodies (Wang, 1985; Kamps and Sefton, 1988; Kozma et al, 1990; Hamaguchi et al, 1988; Roth et al, 1988; Kanner et al,1990; Jong et al, 1992). A wide spectrum of cellular tyrosine phosphoproteins have been described. However, their molecular identities are rarely known, let alone their functions.

A new era begins for the study of signal transduction when the non-catalytic regions of *src*-like kinases, src homology domains 2 and 3 (SH2 and SH3), were implicated in directing protein-protein interactions (Sardowski et al,1986; Stahl et al 1988; Mayer et al,1988; Katan and Parker, 1988). Since then, more than 20 proteins have been reported to contain SH2 and/or SH3 domains (Table 1-2). There is increasing evidence indicating that these non-catalytic domains are of primary importance in mediating cellular protein-protein interactions and participating in signal transmission (Koch et al,1991; Cantley et al, 1991; Hunter,1991; Pawson, 1988, 1992). Recent progresses in the study

of signal transduction by protein tyrosine kinases are discussed below and are sketched in Figure 1-2.

SH2 and SH3 domains:

Studies on cytoplasmic PTKs, such as src, fps/fes, abl, yes, fyn, lck, fgr, etc., have revealed highly conserved non-catalytic regions among the PTKs (Hanks et al, 1988; Sardowski et al, 1986; Mayer et al, 1988). One of the non-catalytic region lying upstream of the PTK domain in src and fps was designated SH2 domain, which might direct specific protein interactions (Sardowski et al, 1986). The importance of the SH2 domain was clearly recognized when H. Hanafusa and his colleagues discovered a unique retroviral oncogene, v-crk, which has no PTK catalytic sequences but only SH2 (B+C box) and SH3 (A box) domains (Mayer et al, 1988).

The SH2 domain consists of about 100 amino acids which has been found in non-receptor PTKs and in many other seemingly unrelated proteins (Table 2). Accumulated evidence clearly shows that the SH2 domain binds to protein phosphotyrosine motifs and directs interaction between enzyme molecules and their substrates with high affinity and specificity (Pawson,1988; Koch et al,1991; Cantley et al, 1991; Fantl et al, 1992; Hidaka et al, 1991; Kashishian et al, 1992). Mutations in the SH2 region have been shown to cause dramatic changes in biochemical properties and biological functions of those SH2-containing proteins (src,abl and crk) (Mayer et al,1992; Fukui et al,1991; Mayer and Hanafusa, 1990; Matsuda et al, 1992). There are several well conserved sequence motifs separated by more variable sequence elements inside the SH2 domain (Koch et al 1991). Crystal and solution structures of the SH2 domains have been recently reported, which are characterized by a large central antiparallel β-sheet flanked by two α-helices (Booker et al, 1992; Overduin et al, 1992; Waksman et al, 1992). The large central β-sheet is further divided into five anti-parallel small β-sheets. Binding of SH2 domain to phosphotyrosine peptide is suggested to be mediated by the N-terminal α helix , β sheet,

and intervening loops (Waksman et al, 1992; Overduin et al, 1992). The highly conserved, positively charged residues (for instance, Arg 155, Arg 175 and Lys 203 in v-src) form a small, shallow cleft and are closely involved in correct positioning and binding of phosphotyrosine motif (Waksman et al, 1992). Arg 155 is particularly important since it recognizes both the phosphate group and the aromatic ring of phosphotyrosine. Arg 175 in the sequence FLVRES is strictly conserved among SH2 domains and is involved in specific hydrogen-binding with two of the four phosphate oxygens of phosphotyrosine. Dual SH2 domain-containing proteins might be able to affix themselves to dimeric targets, such as activated receptor PTK complex (Oveduin et al, 1992).

Most SH2-containing proteins are accompanied by a separate sequence motif of about 45 aa known as SH3, which was first described in v-crk and PLC-148 (Mayer et al, 1988; Stahl et al, 1988). Subsequently, the SH3 domain has been identified in many proteins that associate with cytoskeleton and membrane (Table 2). Several gene products have more than one SH3 domain, such as p47, p67, Crk, Nck, Shc, GRB2/ASH and Vav (Table 2). Very little is known about the function of the SH3 domain. The association of SH3-containing proteins with cytoskeleton and membrane implies a potential role of the domain in subcellular localization and morphogenesis (Koch et al, 1991). Sequences of the partial cDNA clone of a SH3 binding protein, 3BP-1, show homology to GAP-related proteins (rho-GAP), implying a functional association of SH3 proteins with GAP-like proteins and Ras-GTP binding proteins (Cicchetti et al, 1992). Mutations in the SH3 domain of the v-src oncogene result in a different morphology of the mutant transformed cells, suggesting that the SH3 domain might be involved in morphological transformation by v-src (Wages et al, 1992). Crystal structure of the α-spectrin SH3 domain contains five anti-parallel β sheets and those most conserved residues are closely positioned. The β sheets form a smooth surface which is suggested to be putative ligand binding site (Musacchio et al, 1992).

Signaling proteins for RPTKs

Recently, several signaling proteins have been shown to be physically associated with activated receptor and non-receptor PTKs. They include phospholipase (PL) C-γ (Margolis et al, 1989; Meisenhelder et al, 1989; Wahl et al, 1989), Ras GTPase-activating protein (GAP) (Adari et al,1988; Anderson et al, 1990; Ellis et al,1990; Kaplan et al, 1990; kazlauskas et al, 1990b; Liu and Pawson, 1991), and phosphatidylinositol (PI) 3-kinase (Bjorge et al, 1990; Carpenter et al, 1990; Coughlin et al, 1989;Fukui and Hanafusa, 1989, 1991; Kazlauskas and Cooper, 1990; Kypta et al, 1990; Whitman et al, 1985). GAP, PLC-γ and PI 3-kinase all contain the SH2 domain (Koch et al, 1991; Cantley et al, 1991).

GAP is a 120kd protein that directly interacts with cellular ras protein and enhances GTPase activity of the Ras, thereby acting as a negative regulator by returning Ras from the active GTP-bound form to the inactive GDP-bound state (McCormick, F., 1989; Trahey and McCormick, 1987; Trahey et al, 1988; Vogel et al, 1988). Molecular cloning of GAP cDNAs shows that GAP contains two SH2 and one SH3 domains at its N-terminal part and a GTPase activating domain at its C-terminus (Trahey et al, 1988; Vogel et al, 1988). GAP becomes tyrosine phosphorylated by and physically associated with activated EGFR and PDGFR (Anderson et al, 1990; Kaplan et al, 1990; Kazlauskas et al, 1990). The SH2 domain of GAP has been shown to mediate binding of GAP to specific sequence motifs of the activated EGFR and PDGFR (Fantl et al, 1992). GAP protein is also found in a multiprotein complex including the PDGFR, raf-1, PLC-γ and PI 3-kinase (Kaplan et al, 1990). It is interesting to note that GAP fails to associate with activated PDGFR in ras-transformed 3T3 cells, even though the PDGFR has normal PTK activity and still associates with PLC-γ and PI 3-kinase (Kaplan et al, 1990). This result suggests that GAP is crucial in PDGFR signal transduction and in regulation of the Ras activity. However, it is still not clear how increased tyrosine phosphorylation of GAP affects its function. Tyrosine kinase also induces GAP to associate with two other tyrosine

phosphorylated proteins, p6? and p190 (Ellis et al, 1990; Moran et al, 1990). Surprisingly, p62 cDNA encodes a nucleic acid binding protein homologous to a putative hnRNP, GRP33 (Wong et al, 1992). GAP binds to tyrosine phosphorylated p62 through its SH2 domain. p190 is shown to be a multidomain protein, homologous to other GAP activity containing molecules, such as n-chimaerin, BCR (break point region) oncoprotein, and rhoGAP (Broach, 1991; Settleman et al, 1992). The p190 could be a potential link between signal transducer from membrane-associated tyrosine kinases to rasGAP and then to nucleus (Settleman et al, 1992; Hall, A., 1992). The role of GAP domains in signal transduction becomes more intriguing when two clinically important genes, NF1 (neurofibromatosis type 1 susceptibility gene) and bcr, are shown to have GAP activity (Xu et al, 1990a, 1990b; Maru and Witte, 1991). GAP domains are highly conserved in yeast (IRA1,IRA2,CDC42 in S. cerevisiae; gap1 in S. pombe) and in Drasophila (gap1) (Hall, A., 1992). It is worthwhile to mention that GAP has a function other than down-regulating Ras, namely, coupling K^+ channel to atrial muscarinic cholinergic receptor (Martin et al, 1992). Thus, GAP's role in signal transduction seems more complicated than simply down regulation of ras.

PLC-γ1 is one of several PLC isoforms which breaks down the phosphatidylinositol 4,5-bisphosphate (PIP2) to the diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) (Majerus et al,1986; Nishizuka, Y., 1984; Rhee et al, 1989). DAG stimulates protein kinase C activity while IP3 increases release of intracellular calcium by binding to IP3 receptor on the ER membrane (Beridge, MJ.,1984; Berridge and Irvine, 1985, 1989; Mignery and Sudlhof, 1990). The IP3 receptor has multiple transmembrane domains in its C-terminus and may regulate calcium channel opening (Mignery and Sudlhof, 1990). The enzyme activity of PLC-γ1 can be stimulated by PDGF, EGF, and FGF (Anderson et al, 1990; Hill et al, 1990; Nishibe et al, 1990; Kumjian et al, 1989; Mohammadi et al, 1991; Kim et al, 1991; Wahl et al, 1989; Williams, L.T., 1989). cDNA clones of PLC-γ contains SH2 and SH3 domains (Stahl et al, 1988; Suh et al,1988). PLC-γ1 protein physically associates with

activated PDGFR,EGFR and FGFR through its SH2 domain (Margolis et al, 1989, 1990a,1990b, 1990c). Tyrosine phosphorylation of PLC-γ1 is important for its activation and association with activated RPTKs (Meisenhelder et al, 1989; Morrison et al, 1990; Nishibe et al,1990; Kim et al, 1991). It seems that PLC-γ1 is an important component for signaling by the PDGFR, EGFR, and FGFR. However, the PLC-γ1 may not be essential for the mitogenic activity of FGFR or PDGFR since mutants of those RPTKs unable to associate with or activate PLC-γ still cause increased DNA synthesis in response to ligand stimulation (Peters et al,1992; Mohammadi et al, 1992; Hill et al, 1990). In additon, insulin and IGF-1 receptors can mediate mitogenic responses without phosphorylation of PLC-γ, implying that PLC-γ is neither essential for the mitogenicity of the IR or IGFR (Nishibe et al, 1990). The studies on FGFR association with PLC-γ also suggested that PI turnover and calcium influx is not required for mitogenesis.

Two types of PI kinases have been reported (Carpenter and Cantley, 1990). Type I, PI 3-kinase, phosphorylates the inositol ring of PI or PI derivatives (PI4P, PI 4,5-P2) in the 3-position (Whitman et al, 1987, 1988; Fukui et al, 1989a, 1989b). Type II, PI 4-kinase, phosphorylates the inositol ring at 4-position (Whitman et al, 1985, 1987; Stephens et al,1991). PI 4-kinase is known to be involved in the pathway of production of PI-4,5-P2 which is the precursor for the well-known second messengers DAG and IP3 as described previously. The physiological role of the PI 3-kinase product (PI-3-P, PI3,4-P_2, and PI-3,4,5-P_3) is not clear yet. The phospholipases that cleaves PI, PI-4-P, and PI-4,5-P_2 do not hydrolyze any of the D-3 phosphoinositides, suggesting that the PI-3-P pathway is independent of the well-characterized PI-4-P pathway (Carpenter and Cantley, 1990). Accumulated evidence indicates that PI 3-kinase are involved in mitogenic signaling of many oncogenes (Fukui et al, 1989a, 1989b; Whitman et al, 1985, 1987, 1988). Purified PI 3-kinase is a heterodimer that consists of 85kd (p85) and 110kd (p110) polypeptides (Carpenter et al, 1990; Morgan et al, 1991). Molecular cloning of cDNAs coding for PI 3-kinase shows that the p85 has no catalytic sequences, instead it contains SH2 and SH3 domains (Escobedo et al, 1991b; Otsu et al, 1991; Skolnik et al, 1991), whereas cDNA

coding for p110 has PI 3-kinase catalytic sequences and p85 is not required for the kinase activity of p110 (Hiles et al, 1992). Increased PI 3-kinase activity is associated with both activated RPTKs like EGFR,PDGFR,IR, Ros, CSF-1R, c-Kit, and non-receptor PTKs, such as src and Fyn (Cantley et al, 1991; Koch et al, 1991; Endemann et al, 1990; Ruderman et al; 1990; Fukui et al, 1989; Fukui and Hanafusa, 1989, 1991; Sugimoto and Erikson, 1985; Yamanashi et al, 1992). The association of PI 3-kinase with tyrosine phosphoproteins is through the SH2 domain (McGlade et al,1992a; Escobedo et al, 1991a). The structure of p85 SH2 domain is reported to be similar to those of v-src and c-abl (Booker et al, 1992; Overduin et al, 1992; Waksman et al, 1992). Mutations of specific tyrosine phosphorylation sites on PDGFR abolish its association with PI 3-kinase, preventing the growth factor induced mitogenesis (Fantl et al, 1992; Kashishian et al,1992). PI 3-kinase activity is also complexed with $p21^{ras}$ upon insulin or IGF-1 stimulation, suggesting a link between activated RPTKs (e.g. IR and IGFR) and $p21^{ras}$ pathway (Sjolander et al, 1991). PI 3-kinase is able to associate with almost every activated RPTKs and src-like PTKs examined so far, but GAP is not detected in CSF-1R or c-Kit complex, while PLC-γ fails to bind CSF-1R (Cantley et al, 1991; Ullrich and Schlessinger, 1990; Williams, L.T., 1990; Table 3). Whether IR and IGFR bind GAP or PLC-γ remains to be elucidated. These lines of evidence suggest that there exist specific signal transduction pathways activated by different RPTKs but there are overlaps in the signaling pathways.

src and the related non-receptor PTKs, such as fyn, yes, may directly participate in RPTK signal transduction, since src, fyn, yes are physically associated with, and are phosphorylated by, the ß-PDGFR (Kypta et al, 1990). PDGF stimulation results in an increase in src kinase activity (Gould and Hunter,1988). Src seems also to have an interplay with IGFR since in src transformed cells, tyrosine phosphorylation and kinase acitivity of IGFR are increased (Kozma et al, 1990a, 1990b). However, the biological significance of this interaction remains unclear.

Signaling proteins for non-receptor PTKs

Many proteins in Rous sarcoma virus- transformed cells become tyrosine phosphorylated and are potential substrates of $pp60^{src}$ (Reynolds et al, 1989; Koch et al, 1989; Hamaguchi et al, 1990; Kozma et al, 1990a). A glycoprotein, gp130, is a major substrate of src. Phosphorylation of the gp130 seems to correlate with cell transformation (Hamaguchi et al,1990). The molecular identity of the gp130 remains unknown. It could be identical to the p130 described by others (Reynolds et al, 1989; Koch et al, 1989). Another tyrosine phosphorylated protein, p110, is also tightly associated with src (Reynolds et al,1989). p130 binds to the SH2 domain of src, while p110 may associate with the SH3 (Koch et al, 1991). A 95 kd protein tyrosine phosphorylated by v-src turns out to be the IGFR ß subunit, suggesting a possible interplay between the signaling pathways of the two PTKs (Kozma et al, 1990a, 1990b). Using cellular extracts containing enriched tyrosine phosphorylated proteins from v-src transformed cells as immunogens, Parsons and his colleagues obtained a panel of monoclonal antibodies specific for proteins tyrosine phosphorylated by v-src (Kanner et al, 1990). Several cDNAs coding for the proteins have been molecularly cloned by using the antibodies as screening probes. Among them, an 85 kd polypeptide is a cytoskeletal protein containing SH3 domain and capable of binding to filamentous actin (Wu et al, 1991). A 125 kd protein is a new cytoplasmic PTK associated with the focal adhesions in src-transformed cells ($p125^{FAK}$, for focal adhesion kinase). The tyrosine phosphorylation of $p125^{FAK}$ appears to correlate with cell transformation by *src* and with the ability of anchorage-independent growth of the transformed cells (Guan and Shalloway, 1992). Two other proteins are also found in the src complex. One is the GAP protein, the other one being the p62 now known to be homologous to the hnRNP, GRP33, indicating that these two proteins are also involved in src signal transduction (Ellis et al, 1990; Moran et al, 1990; Wong et al, 1992).

c-crk, originally found as an oncogene (v-crk) in ASV CT10, codes for a 35kd

protein containing one SH2 and two SH3 domains without any recognizable catalytic sequence (Mayer et al, 1988; Reichman et al, 1992). Compared with c-crk, v-crk is fused to the viral gag sequence and has a large deletion of 100 aa in the corresponding C-terminus (Reichman et al, 1992), which contains another SH3 domain in the c-crk product. Tyrosine phosphorylation of cellular proteins of 62,70,120, and 135-155kd is markedly elevated in v-crk transformed cells. The three major species of 70, 120, 135-155 kd are specifically associated with the Crk in vitro (Mayer and Hanafusa, 1990). Some cellular tyrosine kinase activities are coimmunoprecipitated with v-crk. The $p47^{gag-crk}$ oncoprotein can also complex with $p60^{v-src}$ in vitro and tyrosine phosphorylation of the $p60^{v-src}$ is required for their association (Matsuda et al, 1990a, 1990b). It is recently shown that the SH2 domains of Crk and PLC-γ can protect tyrosine phosphorylated EGFR from dephosphorylation in vitro by tyrosine phosphatases (Birge et al, 1992; Riotin et al, 1992). It may explain at least to some extent why Crk can enhance tyrosine phosphorylation of cellular proteins.

It has been known that phosphorylation of Tyr-527 of src has inhibitory effect on its tyrosine kinase activity (Jove and Hanafusa, 1987). A distinct cellular protein, CSK (c-terminal src kinase), is molecularly identified as a cytoplasmic tyrosine kinase specifically phosphorylating the Tyr-527 (Okada and Nakagawa, 1989; Nada et al,1992; Sabe et al,1992a). The c-src kinase activity is decreased after its phosphorylation by the Csk which can also phosphorylate other src family members like $p56^{lyn}$ and $p59^{fyn}$ at tyrosine residues corresponding to the Tyr-527 of c-src (Okada et al, 1989). Coexpression of v-crk and c-src causes transformation of rat 3Y1 fibroblasts where c-src kinase activity was elevated. However, v-Crk and c-Src did not seem stably complexed (Sabe et al, 1992a, 1992b). Csk is shown to be capable of suppressing the transformation induced by coexpression of v-crk and c-src, but unable to reverse transformed phenotype induced by v-src or c-src527F (Tyr-527 changed to Phe). This study suggests that v-crk may activate c-src kinase in vivo by modulating the phosphorylating state of Tyr527. On the other

hand, the oncogenic potential of pp60$^{c\text{-}src}$ can be activated by overexpression of phosphatase α (PTPaseα) in rat embryo fibroblast, and the Tyr-527 of c-src is specifically dephosphorylated by the PTPase α (Zheng et al, 1992). It seems that v-crk, csk and probably certain tyrosine phosphatases are involved in regulation of src kinase activity and thus in src signal transduction.

PI 3-kinase appears also involved in src signal transduction since it has been found to associate with p60$^{v\text{-}src}$ (Sugimoto and Erikson, 1985; Fukui and Hanafusa, 1989, 1991). Their association is likely mediated by the SH2 domain of v-src and a tyrosine phosphorylated region of PI 3-kinase (Fukui and Hanafusa, 1989). Moreover, the SH3 domain of v-src seems also implicated in their association (Wages et al, 1992). The src-associated PI 3-kinase activity is correlated with cellular transformation induced by src. However, several nontransforming src or kinase inactive src mutants are also associated with PI 3-kinase activity. These results suggest that association of PI 3-kinase with src is not sufficient for transformation (Fukui and Hanafusa, 1989).

Ras, an integration point in signal transduction pathways of RPTKs and src-like PTKs

p21^{ras}, a well-characterized proto-oncogene with intrinsic GTPase activity, plays a critical role in the signal transduction by activated tyrosine kinases. Activation of the NGF, EGF, PDGF, insulin receptors and c-kit all increase the proportion of GTP-bound, activated forms of p21^{ras} (Gibbs et al, 1990; Medema et al, 1991; Qui and Green, 1991; Satoh et al, 1990; Duronio et al, 1992). Studies using a dominant inhibitory mutant of c-Ha-ras, Ha-ras(S17N), and microinjection studies using neutralizing anti-ras antibody have shown that the function of cellular ras protein is essential for NGF, FGF, CSF-1, and v-src signal transduction (Smith et al, 1986; Hagag et al, 1986; Feig and Cooper, 1988; Kremer et al, 1991; Wood et al, 1992; Thomas et al, 1992). Cross-linking of the T cell receptor and stimulation of mast cells (R6X and MC-9) with IL3, IL-5, and GM-CSF also caused

accumulation of GTP-bound $p21^{ras}$ (Downward et al, 1990; Duronio et al, 1992). Although T cell receptor and those interleukin receptors have no tyrosine kinase activity, src-like kinases (lck, fyn, lyn) and other tyrosine kinases like ZAP-70 have been shown to be involved in the signal transduction by those receptors (Hatakeyama et al, 1991; Cooke et al, 1991; Klausner and Samelson, 1991; Chan et al, 1992). Genetic studies on Drasophila eye development have shown that ras protein is a key signaling element for two tyrosine kinases, *sevenless* (ros homolog) and *Ellipse* (Simon et al, 1991). SOS (son of sevenless) is found to be a downstream signal transducer for *sevenless* but upstream of ras (Simon et al, 1991; Bowtell et al, 1992). Studies on the vulval development of *C. elegans* also demonstrate that ras (let-60) is required for EGFR (let-23) signal transduction (Horvitz and Sternberg, 1991). Ras function is required for transmitting signals from NGF receptors and phobol ester mitogens through raf-1, MAP kinase (mitogen activated protein kinase), and RSK (ribosomal S6 kinase) (see below for more detailed discussion). It is likely that ras protein represents a converging point standing in the middle of the signal transduction pathways of RPTKs, non-receptor PTKs, and probably phobol ester activated protein kinase C (PKC).

Downstream signaling serine/threonine kinases

Raf-1 is a 74kd protein serine/threonine kinase originally found as a retroviral oncoprotein (Rapp et al, 1988; Li et al, 1991). It is tyrosine phosphorylated by and physically associated with PDGFR tyrosine kinase (Morrison et al, 1989, 1990). Recent investigations, however, indicate that it can be activated through insulin receptor without being accompanied by tyrosine phosphorylation (Blackshear et al. 1990; Kovacina et al. 1990), suggesting that Raf-1 may not be a direct substrate of IR tyrosine kinase though it may be involved in the IR signal transduction pathway. The earliest evidence that suggests raf-1 as a downstream signal transducer for ras and src came from

microinjection of anti-Ras antibodies (Smith et al, 1986). Those neutralizing antibodies blocked the activity of both Ras and tyrosine kinases like $p60^{src}$ and v-fms, but not v-raf, implying that raf lies downstream of ras, src and fms. Phosphorylation and kinase activity of $pp74^{c\text{-}raf}$ are increased in cells transformed by src-like oncogenes (Morrison et al, 1989). However, c-raf lacks SH2 and SH3 domains, so its activation and phosphorylation by src-like PTKs do not seem to take place by direct physical association. Antisense RNA for c-raf-1 and expression of kinase -defective raf-1 inhibit serum-induced NIH 3T3 cell proliferation and v-raf transformation. ras oncogene-induced cell transformation was blocked by the above measures (Kolch et al, 1991). This study provided direct evidence that raf-1 is a signal transducer downstream of ras.

MAP kinases represent another family of serine/threonine kinases involved in signal transduction by tyrosine kinases and PKC (Thomas, 1992). Though commonly referred to as MAP kinase, the enzyme is also known as erk (extracellular signal regulated kinase), MPK (meiosis-promoting kinase) (Blenis, 1991). There exist several forms of MAP kinases , $p44^{mapk}$ (erk1) and $p42^{mapk}$ (erk2) (Boulton et al, 1991; Thomas, 1992). Growth factors like insulin, EGF, NGF and phobol esters activate MAP kinase by increasing its tyrosine and threonine phosphorylation. Activated MAP kinase then phosphorylate another serine/threonine kinase, RSK (Sturgill et al, 1988; Sturgill and Wu, 1991; Gomez and Cohen, 1991; Chen et al, 1992). A fraction of the activated MAP kinase and RSK enter the nucleus (Blenis, 1991; Chen et al, 1992). MAP kinase has been shown to phosphorylate c-jun and activate its transactivating activity (Pulverer et al, 1991).

MEK1 (MAP kinase or Erk Kinase), a protein tyrosine/threonine kinase having long been suspected to phosphorylate MAP kinase, is recently purified and molecularly cloned (Gomez and Cohen, 1991; Crews and Erikson, 1992; Crews et al, 1992; Alessandrini et al, 1992). MEK1 expressed in bacteria can phosphorylate Erk on tyrosine and threonine residues (Crews et al, 1992). Purified MEK from PC12 cells was activated by tyrosine/threonine phosphorylation after NGF stimulation (Gomez and Cohen, 1991). MEK

may be a substrate for raf-1 kinase since it has been shown that raf-1 can activate purified MAP kinase kinase in vitro. The MAP kinase (erk1 and erk2) and MAP kinase kinase are constitutively activated in v-raf- transformed fibroblasts (Kyriakis et al, 1992; Howe et al, 1992).

Signaling proteins with no catalytic domains

The p85 subunit of PI 3-kinase has only SH2 and SH3 domains, while the catalytic activity of PI 3-kinase resides in p110 (Hiles et al, 1992). Therefore, the p85 serves as a regulator of PI 3-kinase. It has been documented that many activated RPTKs and src-like kinases associate and activate the PI 3-kinase through binding to the SH2 domain of the p85 subunit (Koch et al, 1991; Pawson, T., 1988; Cantley et al, 1991;Kavanaugh et al, 1992; McGlade et al, 1992a). The p85 subunit appears to function as a "docking" protein to affix the catalytic p110 of PI 3-kinase to tyrosine phosphorylated RPTKs or to the SH2 domains of src-like PTKs.

The p85 of PI 3-kinase may be a prototype of a class of proteins which have only SH2 and/or SH3 domains while lacking catalytic domains. Crk may function in a similar manner since in v-crk transformed cells, tyrosine phosphorylation of a number of proteins are apparently elevated (Matsuda et al, 1990a; Mayer and Hanafusa, 1990a, 1990b). One mechanism may be that the Crk oncoprotein acts as an adaptor to bridge a PTK with certain substrates or that Crk may prevent PTPase from dephosphorylating PTKs, locking them in the active state.

In addition to crk gene product, a couple of SH2- and SH3- containing small proteins have been identified recently. The human nck cDNA codes for a cytoplasmic protein of 377 amino acids which contains one SH2 and three SH3 domains but has no catalytic sequences (Lehman et al, 1990). Overexpression of Nck caused transformation of 3Y1 rat fibroblasts and NIH 3T3 cells (Chou et al, 1992; Li et al, 1992). Nck is

physically associated with EGFR, PDGFR, pp60$^{v\text{-}src}$ and cytoplasmic serine/threonine kinases (Li et al, 1992; Chou et al, 1992). Phosphorylation of Nck on serine and tyrosine residues is increased in response to phobol ester, cAMP, and a variety of receptors including EGFR, PDGFR, NGFR, T cell receptor, B cell receptor and Fc receptor (Li et al, 1992; Park and Rhee, 1992; Meisenhelder and Hunter, 1992). However, total phosphotyrosine levels in the transformed 3Y1 fibroblasts were not elevated (Chou et al, 1992). These studies suggest that Nck plays an important role in cell growth control. Another putative adaptor cDNA, SHC, was isolated from cDNA library of Burkitt lymphoma cells (Pelicci et al, 1992). SHC cDNA encodes a SH2 containing protein with no identifiable catalytic sequence. The SHC protein also contains an α1 collagen-like domain. SHC is tyrosine phosphorylated by and associated with EGFR through its SH2 domain. SHC may also be a cellular substrate for v-src and v-fps (McGlade et al, 1992b). Overexpression of SHC in NIH 3T3 cells resulted in their transformation and the transformed cells can form tumors in nude mice. These data suggest that SHC may function in src, fps, EGFR and probably other RPTK signaling pathways. It may play an important role in mitogenic activities of those PTKs. Unlike v-crk, the SHC transformed cells do not demonstrate prominent increase in tyrosine phosphorylation of cellular proteins, even though SHC itself is heavily tyrosine phosphorylated by activated EGFR. This study suggests that SHC might associate with some signaling proteins different from those complexed with the crk protein. Interestingly, a new SH2 and SH3 containing cDNA, GRB2/ASH, was isolated recently which again has no catalytic sequences (Lowenstein et al,1992; Matuoka et al, 1992). GRB2 (growth factor binding protein 2) and ASH (abundant src-homology) cDNAs encode an identical protein of about 25 kd. Human and rat GRB2/ASH amino acid sequences are identical. This 25kd protein binds to activated EGFR, PDGFR, but not FGFR, through its SH2 domain. It appears to be a homolog of *C. elegans*'s sem-5 gene, which is involved in let-23 (EGFR-like) and let-60 (ras-like) signaling pathway crucial for the vulval development of *C. elegans* (Clark et al, 1992).

Indeed, GRB2/ASH protein seems involved in ras mitogenic signaling since co-injecting the GRB2 and normal Ras can induce DNA synthesis of quiescent REF-52 cells (Lowenstein et al, 1992). Consistent with this result, antisense ASH cDNA interferes with 3Y1 cell growth (Matuoka et al, 1992).

Vav proto-oncogene product (95 kd) perhaps represents a new class of signaling proteins that contain SH2 and SH3 domains. The vav oncogene was initially isolated from esophageal carcinoma genomic DNA fragments through gene transfer assay (Katzav et al, 1989; 1991; Coppola et al, 1992). Loss of the N-terminal sequence activates the oncogenicity of the vav proto-oncogene (Katzav et al, 1989, 1991). The vav proto-oncogene is exclusively expressed in hematopoietic cells. The vav protein becomes rapidly tyrosine phosphorylated when T or B cell antigen receptors are activated (Margolis et al, 1992b; Bustelo et al, 1992; Bustelo and Barbacid, 1992). Besides, activation of EGFR or PDGFR leads to marked increase in tyrosine phosphorylation of the $p95^{vav}$. Vav also directly associates with activated EGFR through its SH2 domain (Margolis et al, 1992b). These studies suggest that the Vav may serve as a direct messenger transducing signals from the cell membrane to the nucleus. A similar mechanism may be employed by interferon α (IFNα) for its signal transduction. A group of SH2-containing transcription factors, ISGF-3α (p113, p91/84), have been shown to be tyrosine phosphorylated in response to IFNα stimulation (Fu, X-Y., 1992; Fu et al, 1992; Schindler et al, 1992). The tyrosine phosphorylation is necessary for the formation of the transcription complex. A cytoplasmic tyrosine kinase, tyk2, might be the putative enzyme that is involved in IFNα/β signaling pathway (Velazquez et al, 1992). Another possible member of this family is HS1, a 75 kd protein strictly expressed in hematopoietic cells (Kitamura et al, 1989). The cDNA product of HS1 contains an SH3 domain at its C-terminus and a Helix-loop-helix domain at its N-terminus. The HS1 is found both in the cytosol and in the nucleus. Currently, little is known yet about the function of this protein. ISGF-3α, and HS1 may represent a new class of signal transducers that may transmit signals directly from the cell membrane to

the nucleus.

Even though the SH2- and SH3- containing proteins become increasingly popular in signal transduction and protein-protein interactions, a totally different signal transducer, IRS-1 (insulin receptor substrate 1), may be a prototype of yet another new family of signaling proteins. The IRS-1 cDNA was originally cloned from a rat cDNA library and codes for a component of 185 kd protein that has been found to be a major substrate for insulin receptor PTK (White et al, 1985; Sun et al, 1991; Nishiyama et al, 1992). The IRS-1 has neither SH2 or SH3 domains nor catalytic domains. However, it contains over 10 tyrosine phosphorylation sites, 8 of which are YMXM or YXXM motifs (Shoelson et al, 1992; Sun et al, 1991). The tyrosine phosphorylated YXXM and YMXM motifs have been shown to mediate binding of SH2- domains of GAP and PI 3-kinase to activated tyrosine kinase receptors (Fantl et al, 1992; Shoelson et al, 1992). The IRS-1 binds to IR and to PI 3-kinase in response to insulin stimulation (Backer et al, 1992; Shoelson et al, 1992). Thus, IRS-1 may serve as a multivalent bridging protein that connects signaling partners in a complex. It is conceivable that more IRS-1 like proteins will be identified in the near future.

Table 1-1. Receptor protein tyrosine kinases (RPTK)

RPTK	Ligand	Oncogene	Origin	Reference
EGFR	EGF	v-erbB	AEV-R(ES4)	Downward et al, 1984
			AEV-H	Yamamoto et al, 1983
Neu	NDF	neu	neuroblastoma	Schechter et al, 1985
HER3	?	erbB3		Kraus et al, 1989
CSF-1R	CSF-1	v-fms	FeSV	Coussens et al, 1986
Kit	MGF	v-kit	FeSV	Besmer et al, 1986
Ros	?	v-ros	ASV UR2	Neckameyer et al, 1984
Met	HGF	met	osteosarcoma	Cooper et al, 1984
NGFR	NGF	trk	colon carcinoma	Martin-Zanca et al,1986
	NT-3	trkB		Klein et al , 1991b
		trkC		Lamballe et al, 1991
FGFR	aFGF	flg		Ruta et al, 1989
	bFGF	bek		Pasquale et al, 1989
Ret	?	ret		Takahashi and Cooper, 1987
Sea	?	v-sea	ASV S13	Smith et al, 1989
PDGFR	PDGF			
IR	insulin		human placenta	Ebina , 1985; Ullrich, 1985
IGF-1R	IGF-1		human placenta	Ullrich et al, 1986
IRR	?			Shier and Watt, 1989
Xmrk	?			Wittbrodt et al, 1989
Flk-2	?		hematopoietic cell	Matthews et al, 1991
Ltk	?		lymphocyte, Neuron	Haase et al, 1991
Axl	?	axl	myeloid leukemia	O'Bryan et al, 1991

Abbreviations: AEV, avian erythroblastosis virus; ASV, avian sarcoma virus; CSF, colony stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; FeSV, feline sarcoma virus; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IRR, insulin receptor-related receptor; NDF, Neu differentiation factor; NGF, nerve growth factor; NT, neurotrophin; MGF, mastocyte growth factor; PDGF, platelet-derived growth factor.

Table 1-2. Proteins containing SH2 and/or SH3 domains

Protein	Feature	Size(kDa)	Structure	Reference
ABP1p	actin binding	65	---------------SH3-----	Drubin et al, 1990
CDC25	RAS pathway	?	-----------SH3---------	Broek et al, 1987
c-Crk	proto-oncogene	35	------SH2---SH3--SH3--	Reichman et al, 1992
Csk	src kinase	50	---SH3--SH2---PTK	Noda,1990; Sabe,1992a
FUS1	yeast fusion	?	----------------SH3------	Truehart et al, 1987
GAP	GTPase Activating	120	--SH2--SH3--SH2--GA	Trahey, 1988; Vogel, 1988
GRB2	PTK signaling	25	---SH3---SH2---SH3	Loweinstein , 1992
GRB7	EGFR signaling?	?	-----------GH---------SH2	Margolis et al, 1992a
HS1	?	75	HLH---------------SH3	Kitamura et al, 1989
ISGF3α	IFN signaling	84,91,113	-------------SH3--SH2----	Fu, X-Y, 1992;Shindler, 1992
Myo 1B	actin binding	?	--------------------SH3--	Jung et al, 1987
Myo 1L	actin binding	?	--------------------SH3--	Jung et al, 1989
Nck	PTK signaling	?	---SH3--SH3--SH3--SH2-	Lehman et al, 1990; Ref.A
PI3K p85	PI 3-kinase noncatalytic subunit	85	-----SH3---SH2--SH2--	Escobedo,1991b; Otsu,1991; Skolnik et al, 1991
PLC-γ	phospholipase	140	PLC--SH2-SH2--SH3--PLC	Stahl et al, 1988; Suh, 1988
PTP1C	phosphatase	67?	--SH2--SH2----PTPase--	Shen et al, 1991
Shc	PTK signaling	46,52,66	--------CH----------SH2--	Pelicci et al, 1992
Spectrin	membrane protein	?	----------------SH3-----	Wasenius et al, 1989
Tensin	actin binding	90	--------------SH2--------	Davis et al, 1991
Vav	PTK signaling	95	------SH3--SH2--SH3	Katzav et al, 1989
ZAP-70	TCR signaling	70	SH2---SH2------PTK	Chan et al, 1992
p55	membrane protein	55	---------------SH3------	Ruff et al, 1991
p47	NADPH oxidase	47	---------SH3-------SH3---	Volpp et al, 1989
p67	NADPH oxidase	67	---------SH3------SH3----	Leto et al, 1990
p85	actin binding	85	----------------------SH3---	Wu et al, 1991

Abbreviations: GA: ras GTPase activating domain; CH: collagen homology domain; HLH: helix-loop-helix domain; PTPase: protein tyrosine phosphatase domain; PTK: protein tyrosine kinase domain; PLC: phospholipase C. Myo: myosin. Src-like kinases (fps, yes, abl, etc.) are not listed. For their structures, see Hanks et al, 1988 and Russel et al, 1992. cDNA sequence of GRB2 rat homolog (ASH) was published (Matuoka et al, 1992). ISGF3α has three different components, p84/91 and p113. Shc protein has three different species as indicated. Ref. A: Chou et al, 1992; Li et al, 1992; Park and Rhee, 1992; Meisenhelder and Hunter, 1992. TCR: T cell receptor. Relative positions of SH2 and SH3 domains in proteins were indicated in "structure", but sizes of those proteins were not shown in scale.

Table 1- 3. Specificity of signal transduction by receptor protein tyrosine kinases

	PDGFR	EGFR	CSF-1R	c-Kit	IGFR	IR	Ros
PI 3-kinase	+	+	+	+	+	+	+
ras GAP	+	+	-	-	?	?	?
PLC-γ	+	+	-	+	?	?	?

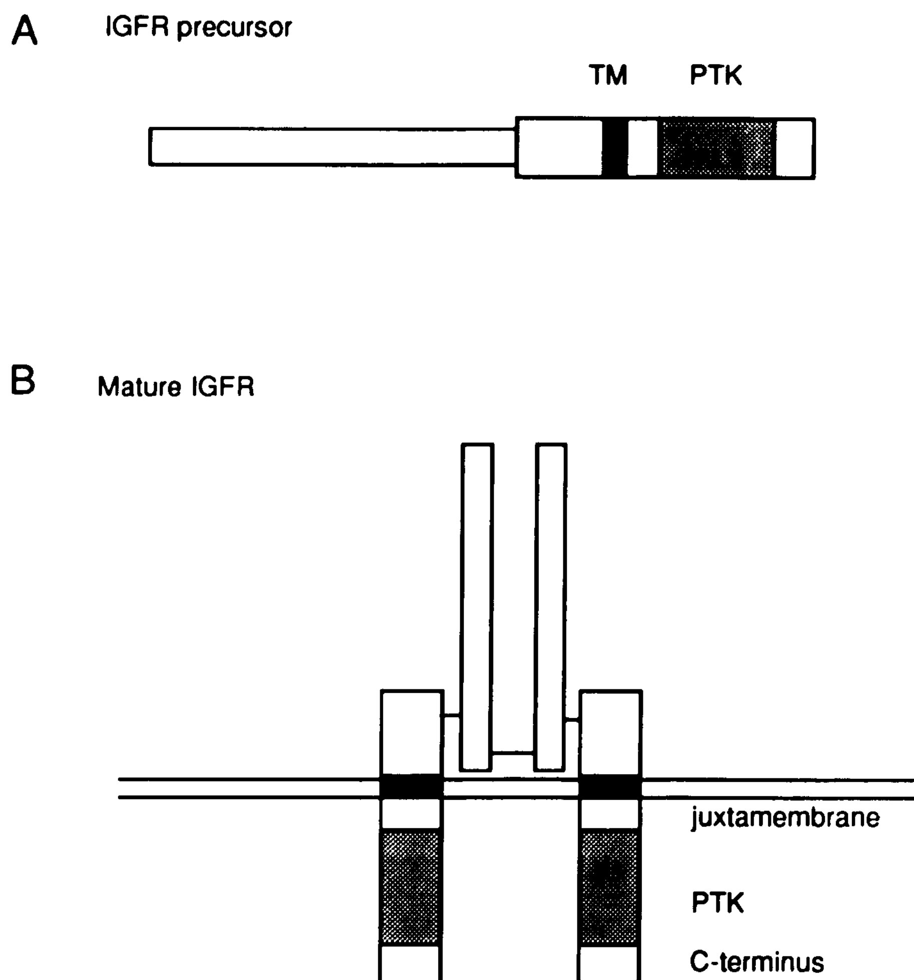

Fig. 1-1. Structure of Insulin-like growth factor I receptor (IGFR). (A). Structure of IGFR precursor. IGFR precursor consisits of (thin box) and ß (thick box) subunits. The ß subunit contains the protein tyrosine kinase domain (PTK) . (B). Structure of mature IGFR. Mature IGFR is a disulfide bond-linked hetreotetramer (-ß-ß-). Three regions of the cytoplasmic domain are indicated. TM, transmembrane domain.

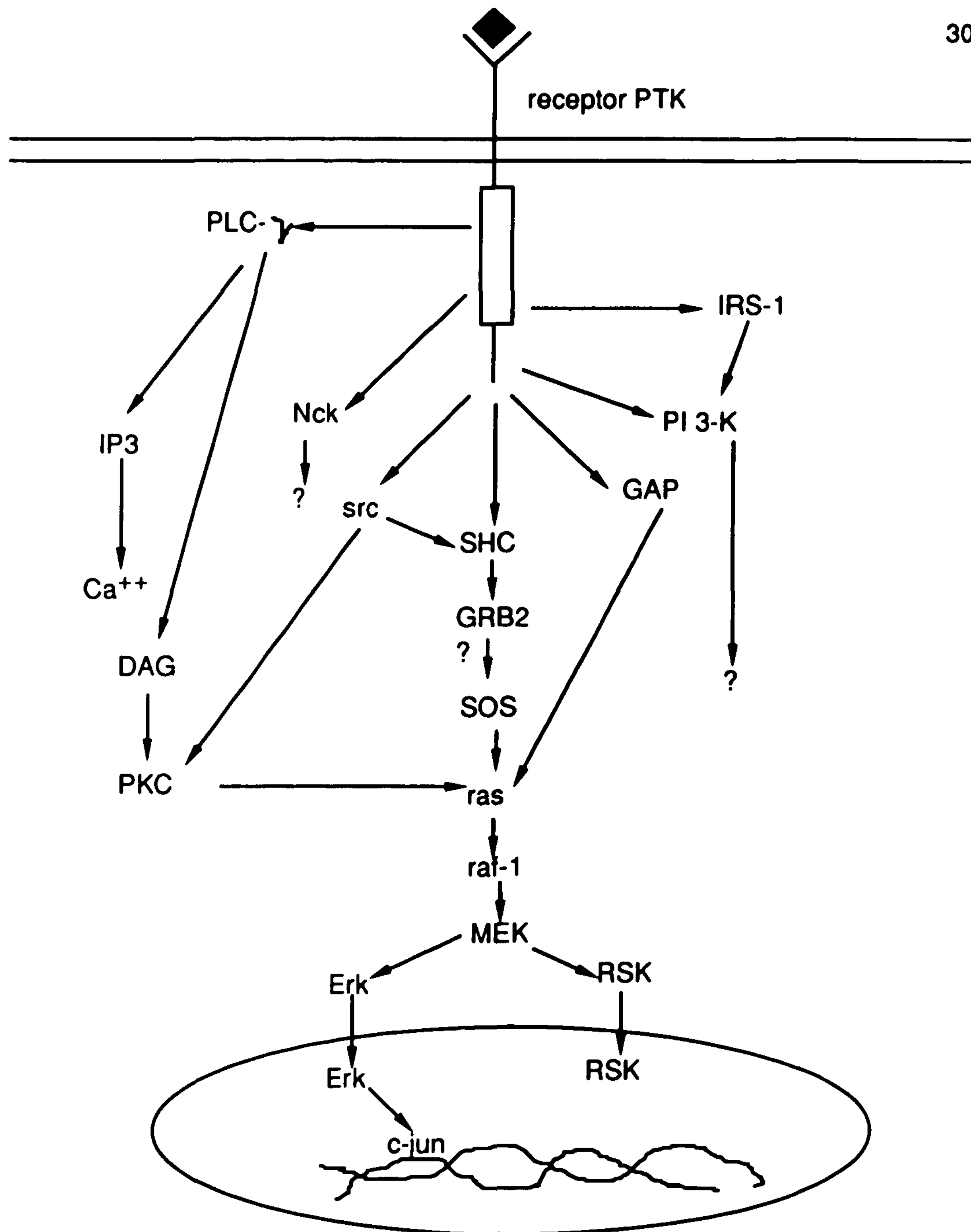

Fig. 1-2. Schematic representation of protein tyrosine kinase signaling pathways. The drawing represents a summary of what has been discussed in the text. The question marks indicate that the signaling orientation is not well documented yet or that nothing is known about the downstream signal flow. IP3, inositol-1,4,5-P3; DAG, diacylglycerol; PTK, protein tyrosine kinase. The blank box represents the PTK domain of the receptor PTK molecule. The black diamond represents the ligand.

Chapter 2

Materials and Methods

Cells and viruses. The preparation of CEF and colony formation assay of virus-infected CEF were done according to published procedures (Hanafusa, 1969). CEF were routinely maintained in F10 medium supplemented with 5% bovine calf serum and 1% chicken serum (GIBCO Laboratories) unless otherwise indicated. Avian sarcoma virus UR2 and its associated helper virus, UR2AV, have been described elsewhere (Balduzzi et al, 1982; Neckameyer et al, 1984, 1985, 1986; Wang et al, 1982). Virus stocks were obtained by collecting culture medium from the transfected cells around 12 days after the transfection. Recovery of viruses from tumors was done as described previously (Hanafusa et al,, 1977). Briefly, the portions of tumors without necrosis were dissected and minced into tiny pieces in a tissue culture plate on ice. The tissue was transferred to a 25 ml flask containing 10 ml of 1% trypsin-0.02% EDTA in Tris-Glu buffer (TGB) (25 mM Trizma base, 5.6 mM glucose, 150 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO4; the pH of this solution was adjusted to 7.4 with 1N HCl) and incubated at room temperature for 15 min with gentle stirring. The suspension containing tumor cells was collected and the remaining tissue was similarly trypsinized. The combined cell suspensions were centrifuged at 1,500 rpm for 4 min and the cell pellet was resuspended in culture medium. After being washed once, the cells were resuspended in 2 ml of culture medium and seeded onto 60 mm culture plates that had been preseeded with 10^5 primary CEF. Medium was changed after overnight incubation and 3 ml of fresh medium was added. Culture medium was recovered 5-10 hr later and used as the virus stock.

Recombinant plasmid construction.

Construction of plasmids pUIGFR, pUfIGFR, and pS-I. Plasmid pMXIGFR (Fig. 1A)

contains the 4.4Kb full-length cDNA of the IGFR gene, including about 500 bp of 5' untranslated region(Steele-Perkins et al, 1988). Restriction sites of the IGFR cDNA coding region were based upon a map derived from the published cDNA sequence of IGFR(Ullrich et al, 1986). A 1420 bp SmaI-BamHI fragment of the IGFR cDNA (from nucleotide positions 2736 to 4155, see ref. Ullrich et al, 1986), coding for 36 aa of the extracellular region as well as the entire transmembrane and cytoplasmic domains of the IGFR ß subunit, was freed from pMXIGFR and used for subcloning. pUR2H1 bearing the entire UR2 genome(Wang et al, 1987) was digested completely with SmaI and StuI to remove the v-*ros* coding sequence and the large vector fragment was isolated. The 1.4Kb SmaI-BamHI IGFR fragment was then blunt-end ligated in frame to the 5' *gag* of the pUR2H1 vector DNA. The resulting plasmid containing the 3.2Kb UR2-IGFR recombinant DNA was designated pUIGFR. The structure of pUIGFR was confirmed by restriction enzyme analysis and the resulting viral genome structure is shown in Fig.1B. The 4.4Kb full length IGFR cDNA was liberated from pMXIGFR by first cutting with EcoR1, filling the ends with Klenow enzyme, and digesting the plasmid with BamH1. This 4.4Kb cDNA was inserted into SmaI-BamH1 digested pUIGFR-ΔATG vector(see below). The resulting UR2 retroviral vector containing the full length hIGFR cDNA was named pUfIGFR. The plasmid clone was confirmed by restriction enzyme analysis. The recombinant UIGFR is expected to encode a 516-aa *gag*-IGFR fusion protein containing 49 aa of UR2 p19 followed by 468 aa from human IGFR ß subunit, whereas UfIGFR would encode the entire hIGFR precursor of 1337 aa.

To prevent translation initiation from the UR2 p19 ATG codon and thus allow the initiation at the native ATG site of hIGFR, the ATG of p19 was mutated into AGT by using synthetic oligonucleotides and polymerase chain reaction(PCR) (Saiki et al, 1988). Two pairs of oligonucleotide primers were used, DLNco (5'-GGGACCATGGTATGTATAGGC-3')/DLBgl3 (5'-TTCACTGCTAGATCTCCGGG-3') for generating DNA fragment 5' to the mutation site and DLBgl5 (5'-CCCGGAGATCTAGC<u>AGT</u>GAA-3')/DLSph3(5'-

CTCACGCATGCTTGCGGCCT-3') for producing DNA fragments containing the mutation site. Primer DLBgl5 contains a mutation of ATG to AGT. Plasmid pUIGFR was used as the template DNA. The PCR reaction mixture contained 200μM each of the four deoxynucleotide triphosphates, 100 ng of each primer pairs, 20 ng of DNA template, 2.5 u of Taq DNA polymerase and reaction buffer(Promega). PCR amplification was carried out for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min in a programmable thermocycler(Perkin Elmer Cetus). The reaction was finished with a final cycle at 72°C for 10 min. The PCR products were purified by passage through PrimerErase column(Stratagene) and digested with Nco I /Bgl II and Bgl II /Sph I, respectively. The two fragments were then ligated into Nco I /Sph I digested pUIGFR vector. The resulting plasmid was designated pUIGFR-ΔATG. The full length hIGFR cDNA was inserted into this vector as described above.

For preparation of polypeptide from bacteria, a 1710 bp ClaI-BamHI fragment(from nucleotide position 2446 to position 4155, Fig. 1B) of the IGFR ß subunit cDNA sequence was cloned into bacterial expression vector pSJH57 (Chen, 1985). Briefly, pSJH57 was digested with ClaI and BamHI, and then the ClaI-BamHI IGFR fragment was ligated in-frame into the vector fragment. The recombinant plasmid was confirmed by restriction enzyme analysis and designated pS-I, which is expected to encode a 64 KDa fusion polypeptide containing 12aa of *lambda* phage cII protein and the carboxyl 567 aa from the IGFR ß subunit.

Construction of plasmid pBUIGFR-II. The prototype vector, pBUIGFR-II, containing a nonpermuted viral genome was constructed as follows. pUIGFR was digested with NheI and EcoR1, and the fragment containing the viral DNA was isolated and ligated into SpeI-EcoRI- cut pBluescript SK(+)(Stratagene), the resulting plasmid pBUIGFR contains a permuted proviral genome with long terminal repeat located upstream of the *gag*-IGFR fusion gene. An NruI-KpnI fragment containing the same LTR DNA sequence was inserted downstream of the remaining *env* sequence at the 3' end to make a nonpermuted proviral

genome. This final construct, pBUIGFR-II, was used for the construction of plasmids pNM1, pNM2, pNM3 and pNM4.

Construction of plasmids pNM1, pNM2, pNM3, and pNM4. DNA fragments containing deletion or specific mutations were generated by PCR using synthesized oligonucleotide primers. The conditions were the same as described previously. In all cases, the template used for PCR was plasmid pUIGFR. The subsequent cloning procedure was done according to standard protocols (Sambrook et al, 1989). Oligonucleotide primers used in PCR had the following sequences (nucleotide and amino acid positions for UR2 and IGFR are numbered according to the published sequences [Neckameyer, 1986; Ullrich et al, 1986]; underlined sequences in the primers are added restriction sites):

ASN 5'AAC CCG GGG (UR2 526) / (IGFR 2743) **CAA** TAC ACA GCC CGA ATT CAG GCC ACA TCT CTC TCT GGG **CAA** GGG 3'.

Bam3 5'GC GGA TCC ATT CCC AGA GAG AGA (IGFR 2770) 3';

Bam5 5'CC GGA TCC (IGFR 2790) / (IGFR 2851) CTG ATC ATC GCT CTG CCC 3';

Bgl2 5'(UR2 366) CCCGG AGA TCT AGC ATG GAA 3';

DL5 5'GGC CCG GGA (UR2 526) / (IGFR 2851) CTG ATC ATC GCT CTG CCC GT 3';

Sma5 5'AAC CCG GGG (UR2 526) / (IGFR 2791) TGG ACA GAT CCT GTG TTC 3';

Sph3 5'CTC ACG CAT GCT TGC GGC CT (IGFR 3155) 3'.

An IGFR fragment with a deletion of sequence coding for the EC 36 aa residues (aa 870-905) was generated by PCR using primers DL5 and Sph3. The PCR product was digested with SmaI and SphI and then reinserted into the SmaI/SphI- digested pBUIGFR-II vector . This plasmid was named pNM1.

Sequence encoding aa 870-885 including the two N-linked glycosylation sites was

deleted in a DNA fragment generated by PCR with use of primers Sma5 and Sph3. The PCR product was digested with Sma I and Sph I and reinserted into the pBUIGFR-II vector. The resulting plasmid was designated pNM2.

Sequence encoding aa residues 886-905 was deleted in DNA fragments generated by PCR with use of two pairs of primers, Bgl2/Bam3 and Bam5/Sph3. The two fragments generated were digested with Bgl II/BamHI and BamHI/SphI, respectively, and ligated into Bgl II/SphI- digested pBUIGFR-II vector. This construct, named pNM3, contains artificial Bgl2 and BamHI restriction sites (underlined sequences in primers Bgl2 and Bam5, respectively) which facilitated subcloning and screening without changing encoded amino acid residues.

The two N-linked glycosylation sites within the 36 aa residues were mutated by converting asparagines (codon AAT) to glutamines (codon CAA, bold letters in primer ASN) by PCR using primers ASN and Sph3. The fragment was inserted into pBUIGFR-II exactly as described for pNM1. The mutant plasmid was named pNM4. An artificial EcoRI restriction site (underlined sequence in primer ASN) was engineered into the ASN primer without altering the authentic amino acid residues in order to facilitate screening of the mutants.

Construction of plasmids CM1, CM2, CM3, CM4, CM5, and CM6. By using PCR and synthetic oligonucleotide primers, five DNA fragments containing deletions within the C-terminal region (aa 1250-1337) were generated. The oligonucleotide primers had the following sequences (corresponding nucleotide positions in the original published nucleotide sequence of IGFR and UR2 are indicated; restriction sites for Stu I and Bam H I are underlined):

Stu5: 5' C (IGFR 3803) C AAG ATG AGG CCT TCC TTC 3'

CM1: 5' GC GGA TC CTA G(IGFR 4095)TT CAT GTG GGC GTA 3'

CM2: 5' GC GGA TC CTA G(IGFR 4065)AA GCT GGC GCG GAG 3'

CM3: 5' GC GGA TC CTA G(IGFR 4005)TG TCT GTC GGG CAG 3'

CM4: 5' GC GGA TC CTA C(IGFR 3945)AT GTT CTC TGG CTC 3'

CM5: 5' GC GGA TC CTA G(IGFR 3882)AA GGA GAC CTC CCG 3'

DNA fragments of CM1 to CM5 were made by PCR with use of Stu I (sense primer) and one of the CM primers (antisense primers) for each corresponding fragments. Fragments of CM1 to CM5 have gradually longer deletions (range of deletions are marked by the junctions in each CM primer). The DNA fragments were each digested with Stu I and Bam H I and inserted individually into pBUIGFR-II replacing the wild type sequence between Stu I and Bam H I (from 3805-4155 in IGFR cDNA). The final constructs were named pCM1, pCM2, pCM3, pCM4, and pCM5 after each corresponding CM primer.

pCM6 plasmid was generated by removing Stu I-Bam H I fragment in pBUIGFR-II with Stu I and Bam H I digestion, blunting the restriction ends with Klenow, and ligating the blunted fragments. Bam H I restriction site was regenerated after ligation and used for screening of the mutant.

The DNA sequence of the PCR fragments containing the mutations in the four NM and five CM plasmids were confirmed by dideoxy sequencing.

After CM4 was found to cause CEF transformation while CM3 and CM5 did not, unexpected mutation outside of the PCR- derived region in pCM4 was suspected. To exclude this possibility, the CM4 fragment derived from PCR was subcloned into pCM3 backbone, replacing the CM3 fragment. The PCR derived CM4 region subcloned into pCM3 vector was resequenced to ensure no further mutation within the fragment was present. The newly derived pCM4 in a pCM3 background was reassayed for its biological and PTK activities and has been used since then.

Antibodies. The fusion protein, S-I, encoded by pS-I was purified as described previously (Chen, 1985). Purified protein (250μg) was emulsified with equal volume of complete Freund's adjuvant and injected subcutaneously into four sites on the dorsal area of each New Zealand White Rabbit(2.5kg, female). The rabbits were boosted twice by the same route of injection with 150μg of purified protein preemulsified with an equal volume

of incomplete Freund's adjuvant. Bleeding was performed weekly starting 10 days after the second boost and the antiserum was named α-IB. The αIB antiserum was used at 1:1,000 dilution for both immunoprecipitation and immunoblotting. Preparation and characterization of anti-phosphotyrosine antiserum(α-pTyr) was described elsewhere (Jong and Wang, 1992). Monoclonal antiphosphotyrosine antibody PY20 was purchased from ICN (see ref. Glenney et al, 1988), 4G10 was purchased from Upstate Biotechnology, Inc.(UBI, IgG2bK). PY20 was used at a 1:2,000 dilution and 4G10 was used at a 1:3,000 dilution. Polyclonal antiserum against the rat p85 subunit of PI 3-kinase (anti-p85) was purchased from UBI.

DNA transfection. Plasmids pUfIGFR and pUIGFR contain permuted proviral inserts. Before transfection, they were digested with Hind III to free the proviral inserts. The insert fragments were gel purified and briefly ligated(1-2hr) at 20°C to form nonpermuted proviral DNA. pUR2AV containing the helper viral genome was digested with Sac I to liberate the proviral insert. The plasmids (10μg) containing nonpermuted proviral genome were used directly for transfection into CEF. Transfection of viral DNA into CEF was done by a published procedure(Kawai et al, 1984). Briefly, $7x10^5$ CEF cells were seeded in 60-mm dishes; 18h later the culture medium was removed, and 1 μg of ligated insert DNA from pUfIGFR or pUIGFR and 1 μg of Sac I digested pUR2AV were added into each dish in 1 ml of fresh medium, 30 μg of polybrene was then added (Aldrich Chemical Co., Milwaukee, Wis.). The culture was incubated for 6h at 37°C with occasional shaking. At the end of this incubation period, the cells were treated with 2ml medium containing 30% dimethyl sulfoxide at room temperature for 4 min, washed twice with fresh medium and incubated in 3 ml of fresh medium at 37°C. The cells were transferred to 100-mm dishes at confluence and overlaid with soft agar medium the next day to enhance the growth of transformed cells. The cell passage and soft agar overlay procedures were repeated until morphologically uniform transformation of CEF was achieved, which usually takes 2-3 rounds of soft agar overlay.

Biological assays for cell transformation and tumorigenicity. Cell

transformation was monitored by observing morphological changes and by examining anchorage-independent growth of the transfected CEF (Hanafusa, 1969). Relative virus titers were measured either by determination of colony forming units in soft agar medium or by slot blotting analysis of viral RNAs. Tumorigenicity of the recombinant retroviruses was assayed as described previously (Jong and Wang, 1990; Poon et al, 1991). Briefly, equivalent titers of various mutant viruses were injected subcutaneously into the wing-web region of each wing of one-day old chicks. The injected chicks were observed for two to three months.

Metabolic labeling. Two different labeling media were used, methionine-free minimum essential medium (MEM) for radiolabeling with ^{35}S-methionine, and phosphate-free medium 199 for labeling with ^{32}p-orthophosphate (GIBCO Laboratories). Both were supplemented with 5% dialyzed calf serum (GIBCO Laboratories). Cells were rinsed once with TGB and prestarved in the labeling medium for 2 hr. Cells were radiolabeled either with 200 μCi of ^{35}S-methionine (Trans^{35}Slabel, ICN) or with 1 mCi of ^{32}p-orthophosphate (ICN) in 3 ml of the labeling medium per 100 mm dish for 4 hr prior to harvesting (Feldman et al, 1982; Garber et al, 1985a; Jong and Wang, 1990). Cells were rinsed twice with TGB and lysed in RIPA buffer (50 mM Tris-HCl [pH7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1mM Na_3VO_4, 1mM phenylmethanesulfonyl fluoride, 20 μg aprotinin per ml, 20 μg leupeptin per ml). Cell lysates were immunoprecipitated as described below.

Immunoprecipitation. Cells were solubilized in either RIPA buffer or Nonidet P-40 (NP40) buffer (20 mM Tris-HCl [pH7.5], 5 mM EDTA, 150 mM NaCl, 1% NP40, 1mM Na_3VO_4, 1mM phenylmethanesulfonyl fluoride (PMSF), 100mM NaF, 50mM sodium pyrophosphate). The cell lysates were centrifuged at 12,000xg for 10 min at 4°C to remove protein aggregates and nuclei. Proteins in the lysates were quantitated with Bradford assay solution (Bio-Rad). To analyze IGFR proteins, αIB antiserum was added to the supernatant containing equal amounts of proteins and the mixture was incubated

for 2 hr at 4°C with rotating. 15 µl of protein A-agarose beads (Repligen) was added and the mixture was incubated for one hour at 4°C. The beads were spinned down and washed once with high-salt (0.3M NaCl) RIPA, once with low-salt (10mM NaCl) RIPA, and once with regular RIPA. The washed immunoprecipitates were ready for further analysis. To analyze proteins with monoclonal antibodies (MoAb), rabbit anti-mouse IgG (1:1,000 dilution, Sigma) was added to the mixture of MoAb and cell lysate after the initial 2 hr incubation, and this mixture was incubated for 1 hr at 4°C before 15 µl of protein A-agarose beads was added. The mixture was further incubated and washed as described above.

In vitro kinase assay. The immunoprecipitates obtained as described above were washed once more with 50 mM Tris-HCl (pH 8.0). The in vitro kinase reaction was initiated by adding 20 µl of kinase assay buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MnCl_2$) plus 10 µCi of γ-^{32}P-ATP (6,000 Ci per mmole, NEN) (Ellis et al, 1986; Jong and Wang, 1987). The reaction mixture was incubated for 10 min at room temperature. Kinase reaction was terminated by adding 1 ml of RIPA buffer and the labeled beads were recovered by centrifugation for 5 seconds in a microfuge. The beads were resuspended in 30 µl of Laemmli sample buffer and boiled for 4 min to elute the labeled proteins, which were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). To examine whether truncated IGFR fusion protein can form covalent dimers, 2-mercaptoethanol was ommitted in the sample buffer as described previously (Li and Schlessinger, 1991).

The procedure for treatment of UfIGFR virus transformed cells with trypsin and subsequent protein analysis has been described previously (Jong and Wang, 1987). Extraction and fractionation of membrane proteins were done as described (Addison, 1987; Garber et al, 1985b; Jong and Wang, 1990). For the glycosylation-inhibition experiment, cells were pretreated with 10 µg tunicamycin per ml and 20µM 2-deoxy-glucose(Sigma Chemical Co.) for 2h and then ^{35}S-methionine labeled in the presence of

the inhibitors for 4h before being subjected to protein extraction. The molecular weights of protein bands of interest were determined by semilogarithmic plotting of their mobility as determined in comparison with the mobility of molecular weight standards (Bethesda Research Laboratories, Inc.). Autoradiography was performed wiht Kodak X-omat AR film and DuPont intensifying screens at -70^{0}C.

Western Immunoblotting. Western analysis was done as described previously (Hamaguchi et al, 1988; Kamps and Sefton, 1988; Jong and Wang, 1990) with the following modifications. After separation of proteins by SDS-PAGE, the gel was immediately placed onto an electrotransferring apparatus (without being presoaked in transfer solution composed of 25 mM Trizma base, 192 mM glycine, and 20% methanol) and electrotransferred to a nitrocellulose filter for 3h at 70V or overnight at 35V at room temperature. The filter was blocked at room temperature for 2h with 5% nonfat dry milk in TTSS (10mM Tris-HCl[pH7.5], 1% Triton X-100, 0.9% NaCl, 0.02% sodium azide) and then subjected to binding with αIB in 0.5% milk-containing TTSS for 6 hours or longer at 4^{0}C. After binding the filter was rinsed once with TTSS and then twice for 20-min each time with TTSS at room temperature. The washed filter was blocked again as described above and then labeled with 1 μCi of ^{125}I-protein A(ICN) in 10 ml of TTSS containing 0.5% milk at 37^{0}C for one hour. The filter was then washed as described above, dried under an infrared light, and autoradiographed as described above. For immunoblotting with monoclonal antibodies, the procedure was similar except that after initial binding, the filter was rinsed twice with TTSS and hybridized with rabbit anti-mouse antibody(1:1000 dilution, Sigma) for two hours at 4^{0}C. The filter was then washed, reblocked, labeled and autoradiographed as described above. To reuse PY20 blotted filter membrane for blotting with αIB antibody, the ^{125}I-labeled filter was incubated in elution buffer containing 0.2M glycine-HCl (pH2.0) and 1mM EGTA (Sambrook et al, 1989) for 20min at room temperature. This procedure was repeated twice. It was noticed that further elution with the above elution buffer did not reduce the signal any further. The eluted filter was blocked

overnight at 4°C with rocking in 5% nonfat milk- containing TTSS. The radioactive signal was further decreased. The filter was then blotted with αIB and the subsequent procedure was exactly as described above.

Ligand binding studies. A total of 10^5 cells were plated into each well of 24-well culture plates. After overnight incubation, the medium was removed and the cells were washed twice with TGB and incubated with 18,000 cpm ^{125}I-IGF-I (Amersham) and various amounts of unlabeled competitors (hIGF-1 [Collaborative Res. Inc.], Insulin [Sigma], or calf serum [Hyclone]) for 2 h at 4°C in binding buffer (100 mM Hepes, pH 7.9, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM EDTA, 15 mM sodium acetate, 5 mg of bovine serum albumin per ml) in a total volume of 200 μl. The cells were washed twice with binding buffer, solubilized in 0.05% SDS and counted in a gamma counter (Steele-Perkins et al, 1988).

To determine whether tyrosine phosphorylation is increased after ligand stimulation, 10^6 CEF were plated into each 60-mm dish. The medium was then removed, the cells were washed twice Tris-Glu buffer and serum free F10 medium was added to the dishes; 15 h later, recombinant hIGF-1 (Collaborative Res. Inc.) was added in 1 ml of serum free F10 medium. Proteins from the ligand stimulated CEF were analyzed for tyrosine phosphorylation by immunoblotting as described above.

PI 3-kinase assay. The PI 3-kinase assay was done essentially as described previously (Fukui and Hanafusa, 1989;Fukui et al, 1989) with slight modification. Transformed CEF were lysed in NP40 buffer. Cell lysates were immunoprecipitated with αIB as described above. The immunoprecipitates were washed as described previously (Fukui and Hanafusa, 1989; Fukui et al, 1989), and the washed beads were resuspended in 25μl of TGN buffer (20mM Tris-HCl, pH7.5, 100mM NaCl, 0.5mM EGTA). 10μg of the substrate PI (20μg per μl in dimethyl sulfoxide, Avanti Polar Lipids, Inc.) was then added to the resuspended immunoprecipitates and mixed to make micelles of PI. The mixture was incubated at room temperature (23°C) for 10 min. Pre-mixed γ-^{32}P-ATP (10μCi per

assay, NEN) and $MgCl_2$ (final concentration 20 mM) were then added, and the mixture was incubated at room temperature for 10 min. PIP was extracted and analyzed on a thin layer chromatography silica gel 60 plate (Merck) exactly as described previously (Fukui and Hanafusa, 1989).

Sucrose gradient sedimentation. Cell lysates were prepared in NP40 buffer as described above, layered onto 5-20% continuous sucrose gradients, and centrifuged in a Beckman SW50.1 rotor at 46,000 rpm for 7.5hr as described previously (Fukui and Hanafusa, 1989). The gradients were divided into 10 fractions and each fraction was diluted with NP40 buffer (1:1) and immunoprecipitated with αIB as described above. After collection of the protein A -beads containing αIB immunoprecipitates, the supernatants were reimmunoprecipitated with antiserum against the p85 subunit of rat PI 3-kinase protein (UBI). ^{14}C-labeled high-range molecular weight markers (BRL) were also sedimented through parallel 5-20% sucrose gradients. Fractions were precipitated with 10% trichloroacetic acid for 30 min on ice, and the precipitated proteins were washed twice with acetone, boiled in the Laemmli sample buffer and analyzed on 8% SDS-PAGE gels.

Chapter 3

Enhancement of Transforming Potential of Human Insulin-Like Growth Factor I Receptor by N-terminal truncation and fusion to Avian Sarcoma Virus UR2 *gag* sequence.

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Abstract

To assess the transforming potential of hIGFR, we introduced the intact and altered hIGFR into chicken embryo fibroblasts (CEF). The full length hIGFR cDNA (flGFR) was cloned into a UR2 retroviral vector replacing the original oncogene v-*ros*. flGFR was able to promote the growth of CEF in soft agar and cause morphological alteration in the absence of added hIGF-I to the medium containing 11% calf and 1% chicken serum. The transforming ability of hIGFR was not further increased in the presence of 10 nM exogenous IGF-1. The 180KDa protein precursor of hIGFR was synthesized and processed into α and ß subunits. The overexpressed hIGFR in CEF responded to hIGF-1 stimulation with increased tyrosine autophosphorylation. The cDNA sequence coding for part of the ß subunit of IGFR, including 36 amino acids of the extracellular domain and the entire transmembrane and cytoplasmic domains, was fused to the 5' portion of the *gag* gene in the UR2 vector to form an avian retrovirus. The resulting virus, named UIGFR,

was able to induce morphological transformation and promote colony formation of CEF with a stronger potency than did fIGFR. The UIGFR genome encodes a membrane-associated, glycosylated *gag*-IGFR fusion protein. The specific autokinase activity of the mature form of the fusion protein, P75, is 6-fold higher *in vitro* and 3-fold higher *in vivo* than that of the native IGFR ß subunit, P95. In conclusion, overexpression of the native or an altered IGFR can induce transformation of CEF with the *gag*-IGFR fusion protein possessing enhanced transforming potential, which is consistent with its increased *in vitro* and *in vivo* tyrosine kinase activity.

INTRODUCTION

Human insulin and insulin-like growth factor I receptors belong to a distinct class of receptor-type protein tyrosine kinase (PTK) family (Ullrich and Schlessinger, 1988). The PTK domains of insulin receptor (IR) and insulin-like growth factor I receptor (IGFR) share a close homology with that of the v-ros oncogene of avian sarcoma virus UR2. The v-*ros* is a 5' truncated version of its cellular counterpart c-*ros* coding for a growth factor receptor-like molecule with PTK activity (Neckameyer et al, 1985, 1986; Matsushime et al, 1986; Wang, 1988). The PTK domain of *ros* is also highly homologous with that of Drosophila *sevenless* protein (Birchmeier et al, 1990; Matsushime and Shibuya, 1990; Chen et al, 1991). In exploring whether the IR has transforming potential, we have fused the 5' truncated human IR cDNA coding for portion of the ß subunit to the 5' *gag* sequence in UR2 genome and demonstrated that this *gag*-IR fusion protein was able to transform chicken embryo fibroblasts (CEF) (Wang et al, 1987). Moreover, further mutations of the *gag*-IR protein rendered it highly tumorigenic *in vivo* (Poon et al, 1991). Since IGFR is highly homologous to and presumably more potent in promoting cell growth than IR, it would be interesting to know whether IGFR has higher transforming and tumorigenic potential. Overexpression of the native human IGFR (hIGFR) in NIH3T3 cells

can induce transformation of these cells in the presence of human IGF-1 and the transformed NIH3T3 cells form tumors in athymic mice (Kaleko et al, 1990). However, nothing is known about the biochemical properties of the overexpressed hIGFR or about the events leading to the cell transformation.

The purpose of this study was to explore and compare the transforming potential of native and truncated versions of the IGFR gene, and to further characterize the biochemical natures of proteins encoded by them . The full length hIGFR cDNA and 5' truncated ß subunit sequence, encoding 36 amino acids (aa) of the extracellular domain and entire transmembrane and cytoplasmic domains, were inserted into the avian sarcoma virus UR2 vector replacing the original v-*ros* sequence. Their transforming potential was examined in CEF. Our results show that both the full length and truncated IGFR genes have transforming potential, and the latter has a significantly higher potency.

RESULTS

Transforming potential of the intact and 5'- truncated IGFR. The UflGFR and UIGFR recombinant DNA inserts were freed from pUflGFR and pUIGFR, respectively (Fig. 3-1). Equivalent amounts of DNA were transfected into CEF together with DNA of the helper virus UR2AV, which provided necessary replicative functions. The resulting retrovirus containing the full length hIGFR cDNA sequence was designated UflGFR, while the retrovirus containing the extracellular 36aa and the rest of the human IGFR ß subunit sequence was designated UIGFR. The transfected cells underwent morphological transformation about two weeks after initial addition of viral DNAs. The morphological change developed faster(one week) after infection of primary CEF with UIGFR virus stock obtained from the transfected cell culture, which most likely reflected the fact that viral infection is more efficient than DNA transfection. The UflGFR- and UIGFR- transformed CEF in monolayer culture assume an elongated shape (Fig. 3-2), similar to that of UR2-

and UIR- transformed CEF (Wang et al, 1987). No clear difference in morphology was observed between UfIGFR- and UIGFR transformed cells. No enhancement of morphological transformation of UfIGFR transformed CEF was observed in the presence of 10nM hIGF-I (Table 3-1). Cell transformation was also monitored by anchorage-independent growth (Hanafusa, 1969). Both UfIGFR- and UIGFR-transformed CEF formed colonies in soft agar medium (Fig. 3-2), with a morphology similar to that of UIR-induced colonies but distinctive from that of Rous sarcoma virus- and UR2- induced colonies (Wang et al, 1987). It is noteworthy that UIGFR is markedly stronger than UfIGFR in promoting anchorage independent growth of transformed CEF, reflected by the number and size of colonies formed in soft agar and by the time(latency) required to reach a given colony size (Table 3-1). The added recombinant hIGF-I at 10 nM to 100 nM, did not significantly enhance the ability of colony formation of the UfIGFR transformed CEF (Table 3-1 and other data not shown). We suspected that the IGF-1 present in the 5% calf and 1% chicken serum was probably sufficient to support the anchorage independent growth of the UfIGFR transformed CEF, we therefore lowered the serum concentration 5-fold and included 10 nM hIGF-1 in the colony formation assay. Unfortunately, no colonies from either UfIGFR- or UIGFR-transformed CEF developed under such conditions (data not shown), indicating that growth factors other than IGF-1 present in the serum are indispensable for cell growth in soft agar. We noticed that the initial plating density of CEF can greatly affect the number of colonies formed, which could explain the difference of colony forming efficiency shown in Table 3- 1. We conclude that both the full length and 5'-truncated *gag*- fusion protein of human IGFR are capable of transforming CEF, the *gag*-IGFR fusion protein appears to have a stronger potency. To determine whether the two viruses are tumorigenic, equivalent amount of the retroviruses were injected into wing webs of 1-day-old chicks. The injected chicks were observed for two to three months. No tumors were observed for UIGFR (three experiments) or UfIGFR (one experiment)(Table 3- 1). UR2 virus was used as a positive control in parallel which caused sarcomas in all

animals in 10 to 14 days after injection.

To further investigate whether the overexpressed hIGFRs in CEF retain ligand-binding capacities and whether the calf serum used contains IGF-1, the hIGFR-transformed CEF were incubated with labeled and unlabeled hIGF-1, insulin, or calf serum (Fig. 3-3). The transformed CEF were found to bind hIGF-1 ligand with high specificity and affinity, whereas insulin binding to hIGFR was over 50-fold weaker. Weak competition of hIGF-1 binding by insulin demonstrated that the binding was specific to hIGFR and not due to IGF-1 binding to other nonspecific proteins. Scatchard analysis of the binding of ^{125}I-IGF-1 to fIGFR- transfected CEF indicated that there are approximately 8.1 x 10^5 receptors per cell with an apparent *Kd* of 5.4 x 10^{-9} M for its ligand. It was also observed that 10% calf serum contains sufficient IGF-1 ligand to compete for the overexpressed hIGF-1 receptors as efficiently as 15 nM hIGF-1, which is consistent with the observation that 10 nM hIGF-1 did not significantly augment anchorage-independent growth in soft agar medium containing 11% calf serum and 1% chicken serum (Table 3- 1). Untransfected CEF expressed either only background levels of IGFR or very low affinity IGFR for human IGF-1. The exact number of IGFR molecules in CEF could not be determined because of such a low-level and seemingly nonspecific binding of hIGF-1 (Fig. 3-3). It has been shown previously that chicken IGF-1 has 50% cross-reactivity with hIGF-1 (Ballard et al, 1990), whereas bovine IGF-1 is identical to human IGF-1 in terms of amino acid sequence and binding to hIGFR (Ballard et al, 1990, Read et al, 1986).

Protein analysis The UfIGFR transformed CEF were examined for production of the intact hIGFR. The 180 kDa polypeptide precursor as well as 135 kDa α and 95 kDa(P95) ß subunits were detected by ^{35}S-Met labeling and immunoprecipitation with αIB, indicating that hIGFR was expressed and properly processed in CEF (Fig. 3-4). In Western blotting with αIB, the 135 kDa protein was not visible as expected since αIB was raised against the 3' portion of the ß subunit (data not shown). To assess the function of hIGFR in CEF, the transformed CEF were stimulated with human IGF-1. The hIGFR overexpressed in

CEF indeed responded to ligand stimulation in terms of autophosphorylation in a dosage-dependent manner (Fig. 3-5). The response reached maximum at about 30 min of stimulation with 10 nM hIGF-1. A higher concentration of hIGF-1 was required to achieve comparable stimulation when incubation was shortened to 10 min. It is clear that the increased autophosphorylation after ligand stimulation was not due to increased IGFR synthesis since the physical amount of IGFR proteins present remained constant during the stimulation period(Fig. 3-5B). The response to IGF-1 treatment indicates that the hIGFR expressed in CEF is transported to the plasma membrane and can be stimulated by the ligand.

The UIGFR transformed cells were analyzed for the production of *gag*-IGFR fusion protein. Four species of proteins with sizes of 57 (P57), 60 (P60), 64 (P64) and 75 (P75), were detected with antiserum αIB (Fig. 3-4). 57 KDa is the expected size of the fusion protein based on the construct. The three species of proteins with sizes larger than the expected 57 KDa could be due to posttranslational modifications, such as glycosylation and/or phosphorylation. Since the UIGFR construct retains two potential asparagine-linked glycosylation sites in the remaining extracellular sequence of the β subunit of IGFR(Ullrich et al, 1986), the possibility of glycosylation was examined. A single 57 KDa protein band was observed after cells had been incubated with tunicamycin and 2-deoxy-glucose and labelled with ^{35}S-methionine(Fig. 3-4). This result confirmed that P60, P64, and P75 were glycosylated *gag*-IGFR fusion proteins. This result is also consistent with the diffusive appearance of the most heavily glycosylated P75. The 78 KDa protein visible only after tunicamycin treatment(open triangle, Fig. 3-4) is very likely the stress-related cellular protein GRP78-BiP which remained tightly associated with the unglycosylated IGFR proteins probably because of its abnormal conformation(Ng et al, 1990). To further confirm that all four species were IGFR proteins, an excess amount of the S-I antigen purified from bacteria was added during immunoprecipitation of cell lysates. All the four species of proteins were abolished, indicating that they are indeed UIGFR proteins(data not shown

).

The *in vitro* autophosphorylation assay was performed to examine the PTK activity of the intact IGFR and *gag*-IGFR fusion protein. The P75 and P57-64 UIGFR proteins were all tyrosine phosphorylated *in vitro* (Fig. 3-6), although the three smaller species, P57, P60 and P64, were not separated well. P75 appeared to be preferentially phosphorylated over the other three species. All four species of presumed *gag*-IGFR proteins were abolished by the S-I IGFR polypeptide, confirming their identity. A 95 KDa protein appeared in the *in vitro* autokinase assay in both transformed and normal CEF lysates, but was not visible in ^{35}S-met metabolic labeling(Fig. 3-4). This minor 95 KDa band most likely represented the chicken IGFR ß subunit recognized by our antiserum since it was also abolished by the S-I polypeptide. The purified S-I polypeptide retained weak PTK activity in this autophosphorylation assay(Fig. 3-6). As for the kinase activity of intact IGFR, the 95 kDa ß subunit of the IGFR was autophosphorylated *in vitro* whereas the ligand binding α subunit was not (Fig. 3-6). The 180 kDa precursor was also phosphorylated since it also contains the ß subunit.

The phosphorylation profile of the IGFR proteins was also investigated in intact cells. Transformed CEF were metabolically labeled with ^{32}Pi and the cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody, alternatively, unlabeled protein extracts were separated and immunoblotted with anti-phosphotyrosine antibody. Although P64 was phosphorylated under *in vitro* conditions, P75 was the major species of tyrosine phosphorylated *in vivo* as detected by both methods (Fig. 3-7A and 7B). In Western blotting the P75 was sometimes resolved into a doublet which probably reflected heterogeneity of glycosylation or phosphorylation. Autophosphorylation of the 95 kDa IGFR ß subunit encoded by UfIGFR was also detected in the intact transformed CEF, but phosphorylation of the 180 kDa precursor was very weak and sometimes not detectable. Tyrosine phosphorylation of the α subunit was not detected in the living cells (Fig. 3-7C).

It has been shown that increased specific PTK activity of *gag*-IR proteins is

correlated with the stronger transforming and tumorigenic activity of the protein encoded by the UIR19t virus (Poon et al, 1991). From *in vitro* autokinase assay and *in vivo* autophosphorylation experiments, we noticed that P75 was always phosphorylated to a greater extent than the other three protein species (Fig. 3-6 and 7), although the physical amount of the P75 was much less than those of the other three species. We therefore compared the specific PTK activities of the proteins encoded by UfIGFR and UIGFR. Equal amount of protein extracts from uniformly transformed CEF cells were analyzed by immunoblotting with αIB and anti-phosphotyrosine antibodies in parallel for determining *in vivo* specific kinase activity. To determine *in vitro* specific kinase activity, protein extracts were immunoprecipitated with αIB, the immunoprecipitates were analyzed by *in vitro* kinase assay, and equal amounts of protein extracts were simultaneously analyzed by immunoblotting with αIB to reflect the physical amount of specific proteins present in the extracts used for the kinase assay. Results from two independent experiments are summarized in Table 3- 2. P75 was preferentially phosphorylated *in vitro* at about 6-fold higher efficiency than were the other two UIGFR proteins, and the specific kinase activity of P75 *in vivo* was about 2-fold higher than that of P60/64. The specific kinase activity of P75 was 4-6 fold higher than that of P95 *in vitro* and 2.5-3 fold greater than that of P95 *in vivo* as reflected in autophosphorylation, consistent with the higher transforming potency of the fusion protein. It appears that P57 was very poorly phosphorylated *in vitro* and *in vivo*. The putative 95kDa endogenous CEF IGFR ß subunit was efficiently phosphorylated *in vitro* even though it was hardly detectable by ^{35}S-Met labeling; however, the CEF 95 kDa protein was very poorly phosphorylated in intact CEF and virtually undetectable by both metabolic ^{32}Pi labeling and α-pTyr immunoblotting (Fig. 3-7).

Association of UIGFR proteins with the membrane fraction. As the UIGFR gene retains the transmembrane domain of the IGFR, it may be associated with the cellular membrane as is the case for P68 $^{gag\text{-}ros}$(Jong and Wang, 1987). The UIGFR-transformed CEF were homogenized and cellular extracts were subjected to membrane fractionation

by differential ultracentrifugation followed by *in vitro* autokinase analysis. The UIGFR encoded proteins were found in the membrane rich P100 fraction(Fig. 3-8A). To further differentiate whether the UIGFR proteins are associated with the cytoplasmic membrane, the P100 fraction of the membrane preparation was subjected to sucrose gradient ultracentrifugation to separate the light plasma from the heavy endoplasmic membranes (Garber et al, 1985; Jong and Wang, 1990). P75 was detected in both light (Fig. 3-8A, lanes 7 to 9) and heavy (Fig. 3-8A, lanes 2 to 4) membrane fractions, but P60 and P64 appeared to be only associated with the heavy membrane fraction(Fig. 3-8A, lanes 2 to 4). This finding suggests that P75, the most heavily glycosylated species of the UIGFR proteins, is the mature form that is transported to plasma membrane whereas the P60/P64 represent immature forms that need to be further glycosylated in heavy membrane fractions(endoplasmic reticulum and Golgi). P75 associated with the heavy membrane fraction may be the newly synthesized mature proteins that are being transported to plasma membrane.

It has been demonstrated that the $P68^{gag\text{-}ros}$ in UR2-transformed cells traverses the plasma membrane, with the p19 portion protruding extracellularly(Jong and Wang, 1987). This is a unique example among the *gag*-onc fusion proteins(Jong and Wang, 1990). Since UIGFR also encodes a fusion protein containing UR2 p19 N-terminal sequence, we examined the configuration of the *gag*-IGFR fusion protein on the plasma membrane. The UIGFR transformed CEF were first treated with neuraminidase and hyaluronidase and then subjected to trypsin digestion. Most of the P75 was diminished after trypsin digestion, but P60/P64 were unaffected(Fig. 3-7B), indicating that the P75 is indeed associated with and traverses the plasma membrane, whereas the P60/P64 are apparently located intracellularly, further supporting the result from sucrose gradient fractionation. Although there were no new distinct bands which would correspond to the digested P75, there was an increased intensity in the range of 45 to 50 KDa which could represent the digestion products(Fig. 3-7B). Judging from the sizes of the P75 digestion products, it is most likely

that the N-terminal sequence including 49 aa of UR2 p19 and 36aa of the IGFR ß subunit protrudes extracellularly. We conclude from these studies that UIGFR encoded P75 *gag*-IGFR protein is, like the intact IGFR, a transmembrane molecule.

DISCUSSION

We have shown that both native and 5' truncated IGFR genes have transforming potential which can be activated upon insertion into a retroviral genome. It appears that after fusion to *gag*, the 5' truncated IGFR has a stronger transforming potency than the native receptor in terms of the latency and the number and size of the transformed CEF colonies. This finding may indicate that removal of the α subunit releases the constraints of normal ligand regulation of the intact IGFR, as a result, the receptor protein tyrosine kinase is constitutively active, which brings about the enhanced growth property of the transfected CEF. Similar mechanisms have been suggested for the transforming activity of *ros* in UR2 (Neckameyer et al, 1985), truncated hIR in UIR (Wang et al, 1987), and erbB in avian erythroblastosis virus(Downward et al, 1984). Since the specific autokinase activities of P75 *gag*-IGFR is 4-6 fold higher *in vitro* and 2.5-3 fold higher in intact cells than those of P95 hIGFR-ß, it is possible that the *gag*-IGFR fusion protein more efficiently transmit the signal in promoting transformation. Overexpressed native IGFR in CEF behaves like a transforming protein in the absence of exogenously added ligand stimulation as evidenced by morphological change and anchorage-independent growth of the transfected cells. This finding differs from that of an earlier study in which the transforming ability of the overexpressed native hIGFR in NIH3T3 cells was ligand-dependent and required a higher serum concentration(Kaleko et al, 1990). It was found that 10% calf serum competed for the overexpressed hIGFR as efficiently as did 15 nM hIGF-1, which might imply that the 11% calf serum and 1% chicken serum contained in the soft agar medium may already have sufficient IGF-1 to activate the hIGFR overexpressed in CEF. Since chicken serum has been shown to contain about 5.5 nM IGF-1 (Daughaday et al, 1985) and chicken IGF-1 has 50% cross-reactivity with human IGF-1, we can not exclude the possibility that an autocrine and/or paracrine mechanism might also be responsible at least in part for the stimulation of hIGFR and the exogenous

ligand-independence. However, it is clear that the cell transformation is mediated through overexpressed hIGFR as normal CEF have no detectable transformed phenotypes even in the presence of hIGF-1 and express only background levels of IGFR in terms of IGF-1 ligand-binding capacities (Fig. 3-3). Another possible explanation for the discrepancy of ligand dependence between the earlier and our current study might reside in the number of receptors expressed in different cell systems, for instance, in our system more receptors might be expressed which might decrease the threshold requirement for ligand, although we do not have any evidence yet to document the receptor number expressed in CEF. A third explanation may be the fundamental difference between NIH 3T3 cells and CEF, such as growth requirements for factors other than IGF-1 contained in the different culture media.

The intact hIGFRs expressed in CEF are properly processed into α and ß subunits with the expected sizes and respond to the cognate ligand stimulation in a dose-dependent manner in terms of tyrosine autophosphorylation, which indicates that the receptor molecules are located on the plasma membrane with the proper configuration. In addition, the kinetics of the morphological transformation in UIGFR and UfIGFR transfected CEF are about the same. We therefore consider that the original fIGFR rather than its mutant(s) is responsible for the observed CEF transformation. Since both the native hIGFR and the *gag*-IGFR fusion protein induced transformation of CEF, the *gag* sequence may not be as critical as that of UR2 P68$^{gag\text{-}ros}$, in which the *gag* sequence has been shown to be essential for transforming activity(Jong and Wang, 1990). However, we can not disregard the possibility that the *gag* portion plays some role in the enhancement of transforming potency of the *gag*-IGFR fusion protein. Similar to the *gag*-IR encoding virus, UIR, the *gag*-IGFR encoding virus UIGFR, like UfIGFR, is transforming but not tumorigenic. Parallel studies carried out in this laboratory on UIR 19t and c-*ros* containing viruses suggested that sequences immediately upstream from the transmembrane domain of these receptor PTK oncogenes appeared to exert a negative effect on their

transforming and tumorigenic potentials (Poon et al, 1991; B. Poon, C. Zong, and L.-H. Wang, unpublished observation). It is thus possible that the extracellular 36 aa of IGFR in the UIGFR virus may have a similar inhibitory effect. It would be interesting to determine whether deletion of the 36aa can activate the tumorigenic potential.

Four IGFR proteins were detected in the UIGFR transformed cells. The 57 KDa protein is apparently the unmodified *gag*-IGFR fusion polypeptide, and the 60 KDa, 64 KDa and 75 KDa proteins are apparently the differentially phosphorylated and glycosylated forms. There are two potential N-linked glycosylation sites in the extracellular 36 aa and one in the cytoplasmic domain of the IGFR in the UIGFR construct. Experiments with tunicamycin confirmed the glycosylation of the P60, P64 and P75 fusion proteins. The significance of these differential post-translational modifications is unknown. Another unresolved question is the identity of the active protein component responsible for cell transformation by UIGFR. Studies on PTK oncogenes so far indicate that the active transforming PTK proteins are all capable of autophosphorylation as well as of phosphorylating cellular proteins *in vivo*. The P75 species is preferentially phosphorylated both *in vivo* and *in vitro*, and has the highest specific PTK activity. It appears to be the most stable species as indicated by our ^{35}S-Met pulse-chase experiment(unpublished data). P75, but not the P64/60 complex, is the major tyrosine phosphorylated protein species *in vivo*(Fig. 3-6). Accumulated evidence has also underscored the importance of membrane association of a tyrosine kinase for its transforming activity. The transforming activity of the cytoplasmic protein tyrosine kinase pp60$^{v\text{-}src}$ requires its association with the plasma membrane(Cross et al, 1984,1985; Krueger et al, 1982, 1983). Membrane localization has also been shown to be necessary for the transforming activity of the receptor-type tyrosine kinase oncoprotein v-*fms*(Roussel et al, 1984). Our recent study also demonstrated that membrane association is necessary for the transforming function of P68$^{gag\text{-}ros}$(Jong and Wang, 1991). Our data suggest that P75 is the only species that is transported to, and traverses, the plasma membrane (Fig. 3-7). Taken together, the results

suggest that it is most likely that P75 is the active transforming protein in UIGFR transformed CEF. However, we still can not completely rule out the possibility that P75 is merely a preferred substrate of another species of IGFR protein which is responsible for cell transformation. Site-specific mutations at the glycosylation sites and/or in the transmembrane region will be necessary to address this question.

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Table 3-1. Growth Characteristics of Transformed CEF Cells

A[d]		IGF-1 ng/ml (nM)	Morphology	Anchorage-independent Growth			[a]Tumorigenicity
				No. of Colonies(%)[b]	Colony size (mm)	Latency(days)[c]	
	CEF		spindle	0			0/10
	UIGFR		fusiform	10^4(2)	≥0.30	8-10	0/15
	UfIGFR		fusiform	10^3(0.2)	≥0.15	>14	0/8
	UR2		fusiform	ND	ND	ND	6/6
B[e]	CEF	0	spindle	0			
		77(10)	spindle	0			
	UfIGFR	0	fusiform	563(0.56)	≥0.15	>14	
		77(10)	fusiform	411(0.41)	≥0.15	>14	

[a] Viruses equivalent to about 5×10^4 colony forming units were injected into each wing-web of each 1-day-old chick. The chicks were observed for over two months. Each result was derived from three injections for control medium and UIGFR virus, two injections for UR2 virus, and one injection for UfIGFR virus.

[b] Only colonies 0.15 mm or greater were counted. Each value represents the number of colonies formed out of number of cells plated. ND, not determined.

[c] Time required for appearance of colonies over 0.15 mm in soft agar medium. The data were averaged from three independent experiments.

[d] For the assay of anchorage-independent growth, 5×10^5 of uniformly transformed cells were plated in soft agar F10 medium supplemented with 11% calf and 1% chicken serum in 100-mm dishes; colonies were counted 2-3 weeks after plating. The numbers of colonies shown were averaged from two independent experiments.

[e] hIGF-1 was added as indicated, and cells were bathed in hIGF-1 for more than one week, during which time the growth factor was supplied fresh daily. Cell morphology was monitored

during and after growth factor treatment. For the anchorage-independent growth assay, 10^5 uniformly transformed cells were plated in 60-mm dishes and hIGF-1 was added into the soft agar medium as indicated. Colonies were quantitated four weeks after plating of the cells. The data represent averages from duplicate dishes.

Table 3-2. [a]Specific tyrosine phosphorylation (STP) of intact and truncated IGFR proteins

	[b]*in vitro* STP(arbitrary unit)						[c]*in vivo* STP(arbitrary unit)					
	Western(αIB)		kinase		STP		Western(αIB)		Western(PY20)		STP	
	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2
UfIGFR P95	63.0	62.5	63.0	46.5	1.0	0.74	86.5	81.6	25.6	48.8	0.30	0.60
UIGFR P75	30.5	37.5	188	113	6.2	3.01	43.0	8.8	38.0	13.0	0.88	1.48
UIGFR P64	120.5	23.5	92.5	20.0	0.77	0.85	176	31.2	72.2	TWTD	0.41	
					Relative STP						Relative STP	
P75/P95					6.2	4.07					2.93	2.47
P75/P64					8.05	3.54					2.15	

a. Specific tyrosine phosphorylation (stp) IS expressed as units of tyrosine phosphorylation per unit amount of IGFR proteins. Units of immunoblotting and *in vitro* autophosphorylation are defined as units of densitometry per equivalent exposure time. STP is obtained from units of autophosphorylation divided by units of immunoblotting signal (in vitro STP) or units of PY20 immunoblotting divided by αIB immunoblotting signal (in vivo STP). TWTD, too weak to be determined.

b. Cell extracts were immunoprecipitated with αIB and then subjected to an in vitro autokinase assay. Equal ammounts of cell extracts were subjected to immunoblotting with the same antiserum (αIB). The exposure time of autoradiography was controlled in the linear

range to make sure that the intensities of bands were proportional to the actual signals. The intensities of the protein bands on the autoradiogram were determined by densitometric scanning.

c. Equal amount of cell extracts were analyzed by immunoblotting with αIB or antiphosphotyrosine PY20. The intensities of protein bands were determined as for the in vitro assay.

Fig. 3-1. Structures of plasmids of pUIGFR and pUfIGFR. (A). pMXIGFR. This plasmid contains the full length cDNA of human IGF-1R cloned into the pMX plasmid vector downstream of the Moloney murine leukemia virus long terminal repeat (LTR). TM, transmembrane domain; SV40, simian virus 40. (B). Construction of pUIGFR recombinant from UR2 and hIGFR. The cDNA sequence encoding part of the ß subunit, including 36 aa of extracellular domain, and the entire TM and cytoplasmic domains, was fused to 5' portion of the *gag* gene in the avian sarcoma virus UR2 replacing the original *ros* oncogene of the virus. (C). pUfIGFR. The full length hIGFR cDNA was cloned into pUIGFR replacing the IGFR-ß insert. The initiation codon ATG of *gag* p19 in the UR2 vector was mutated to AGT(ΔATG) (see "Materials and Methods"). utr, untranslated region; s, signal peptide sequence.

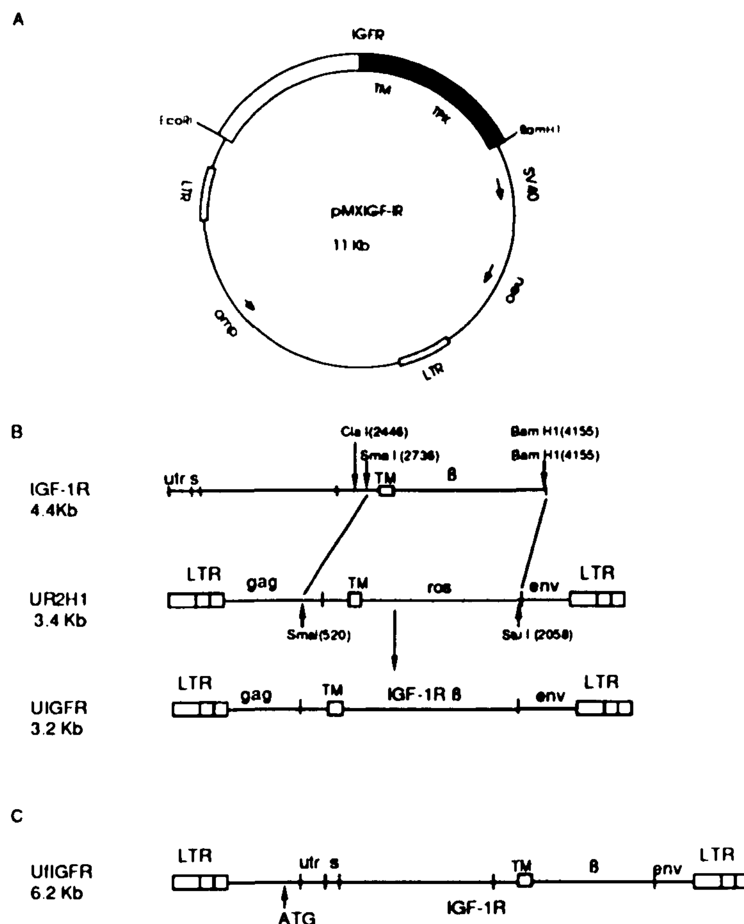


Fig. 3-2. CEF transformation by UfIGFR and UIGFR. (A). Morphology of normal and transformed CEF on monolayer culture. CEF were transfected with proviral DNA from pUfIGFR or pUIGFR together with pUR2AV and selected by overlaying the cell culture with soft agar medium(see "Materials and Methods"). Each photograph shows the transfected CEF after four passages following transfection. (B). Recombinant virus-induced CEF colonies. Equal numbers of uniformly transformed CEF were plated in soft agar medium for the anchorage independent growth of the cells. Shown are colonies 2 weeks after plating of the cells in soft agar medium.

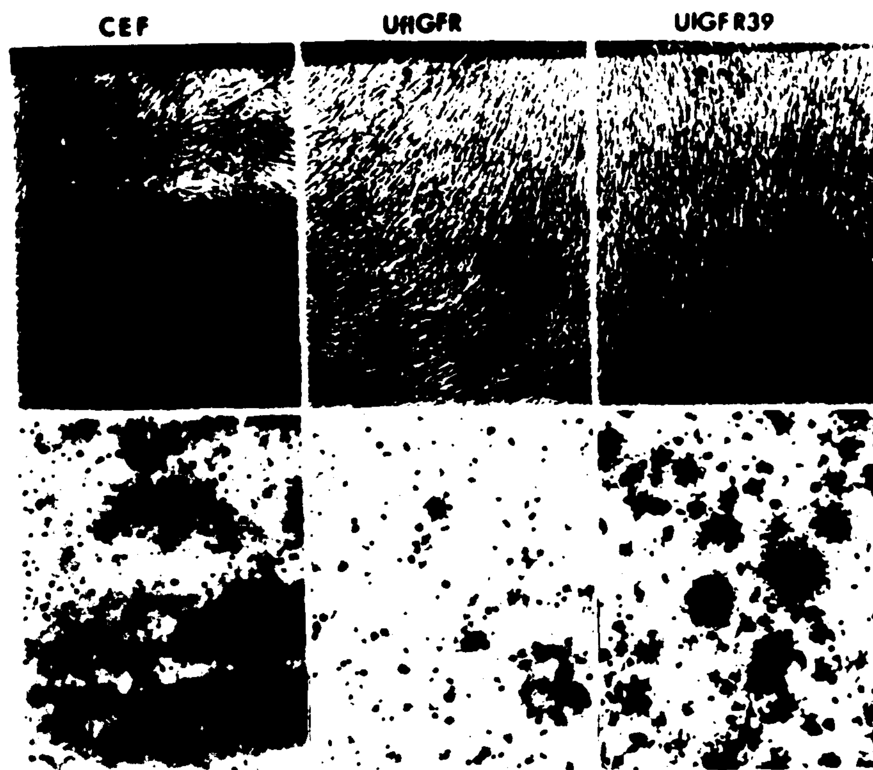


Fig. 3-3. Ligand binding to hIGFR expressed in CEF. (A) Duplicate wells of cells were incubated for 2 h at 4°C with ^{125}I-IGF-1 (18,000 cpm, 32 pM) and different amounts of unlabeled factors. Cells were washed, lysed, and counted as described in Materials and Methods. The averages of duplicate counts were used. Most of the averages were within a 5% difference. The competitor used fo untransformed CEF (+) was hIGF-1 (0, 1, 5, 10, 100, and 200 nM); Competitiors used for hIGFR- transformed CEF were hIGF-1 (concentration as stated above), insulin (0, 1, 5, 10, 1,000, and 5,000nM), and calf serum (1, 5, 10, 20, and 50%). (B) Scatchard plot of IGF-1 binding to hIGFR- transformed CEF. Linear regression analysis was used to determine the slope and intercept of the plot (straight line, r=0.93). The dashed line represents the curve before regression ploting.

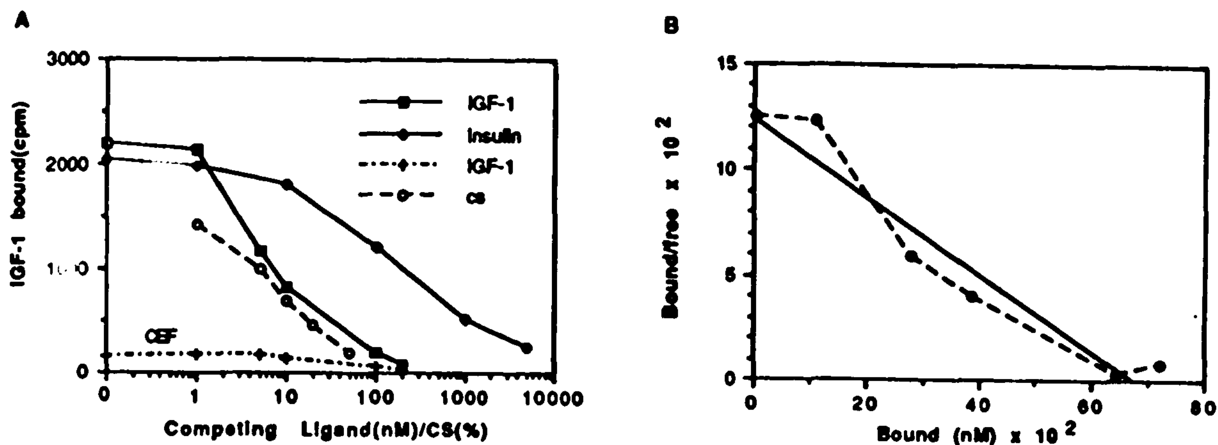


Fig. 3-4. Analysis of proteins from transformed CEF. (A) UfIGFR-transformed CEF were labeled with ^{35}S-methionine (see "Materials and Methods"). Labeled protein extracts were immunoprecipitated with rabbit antiserum α-IB raised against the IGFR ß subunit. The immunoprecipitates were dissolved and analyzed on an 8% SDS-polyacrylamide gel. (B) UIGFR-transformed CEF were treated with(+) or without(-) tunicamycin and 2-deoxy-glucose (T+G) and labeled with ^{35}S-methionine. Protein extracts were immunoprecipitated and analyzed as described above. Protein size markers are shown in kilodaltons. Arrows indicate IGFR proteins with molecular weights represented in kilodaltons. The open triangle indicates the possible 78 KDa "BiP" protein. 39 and 41 are parallel UIGFR clones.

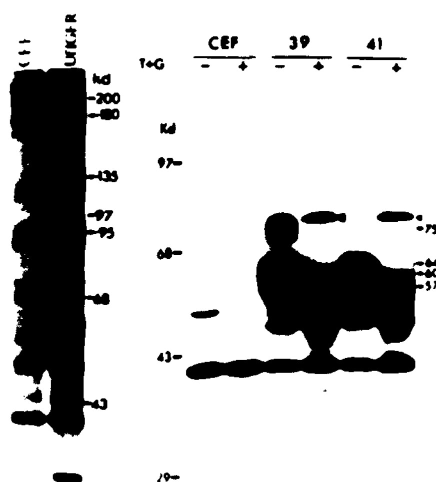


Fig. 3-5. Ligand stimulation of hIGFR expressed in UfIGFR transformed CEF. transformed CEF (10^6) were starved overnight in serum free F10 medium. The cells were then incubated in serum free F10 medium with 10 nM hIGF-1 for different length of time or with different amounts of IGF-1 for 10 minutes. The cell lysate was prepared at the end of IGF-1 treatment and immunoprecipitated with αIB. The immunoprecipitated proteins were then subjected to immunoblotting with the monoclonal anti-phosphotyrosine antibody, PY20(A). The radioactive label was stripped off("Materials and Methods") and the nitrocellulose filter was rehybridized with αIB(B). The degree of tyrosine phosphorylation and abundance of the hIGFR were quantitated by densitometric scanning of the 95KDa protein bands on the respective autoradiogram(C).

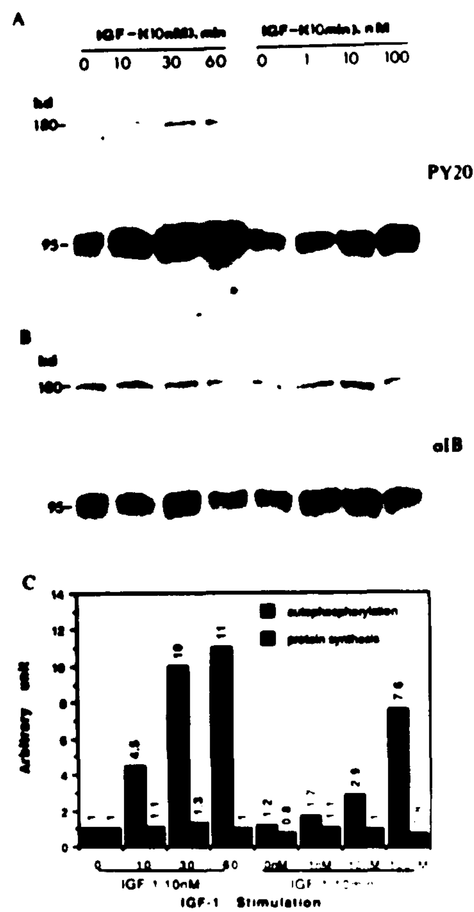


Fig. 3-6. Autophosphorylation of IGFR proteins *in vitro*. Total proteins were extracted from UfIGFR- (A) and UIGFR- (B) transformed CEF, and the extracts were immunoprecipitated with α-IB antiserum. The *in vitro* kinase reaction was carried out with resuspended immunoprecipitates and analyzed as described previously (Jong and Wang, 1991). For UIGFR transformed CEF, protein extracts were immunoprecipitated with αIB in the presence(+) or absence(-) of 10 μg of polypeptide antigen S-I purified from pS-I-transformed *Escherichia coli* (see "Materials and Methods").

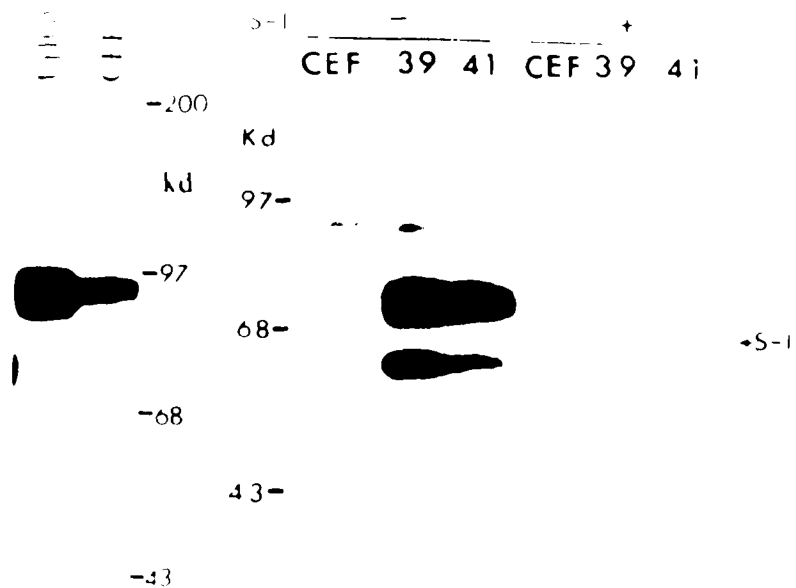


Fig. 3-7. Autophosphorylation of IGFR proteins in transformed CEF. (**A**) ^{32}P metabolic labeling. UIGFR transformed CEF were starved for 2 h in P_i-free medium and then labeled with ^{32}Pi for 4 h. The cellular lysate was divided into two equal aliquots and immunoprecipitated with α-IB or α-pTyr antiserum. Resuspended immunoprecipitates were analyzed by 8% SDS-PAGE and the gel was then treated with 1N KOH at 55°C for 1hr to remove serine and threonine phosphorylation as described previously (Jong and Wang, 1991). (**B**) Immunoblotting. Cell lysates of UIGFR transformed CEF were loaded duplicately onto an 8% SDS-PAGE. After separation, proteins were transferred to a nitrocellulose membrane. The membrane was cut into two parts, hybridized with α-IB or α-pTyr antiserum, respectively, and then labeled with ^{125}I-protein A. (**C**) Immunoblotting (imp). Protein extracts from UfIGFR transformed CEF were immunoprecipitated first with αIB; the immunoprecipitates were then separated by 8% SDS-PAGE and immunoblotted with PY20 as described in "Materials and Methods". The lower dense band is the IgG heavy chain. Sizes of protein markers are indicated in kilodaltons.

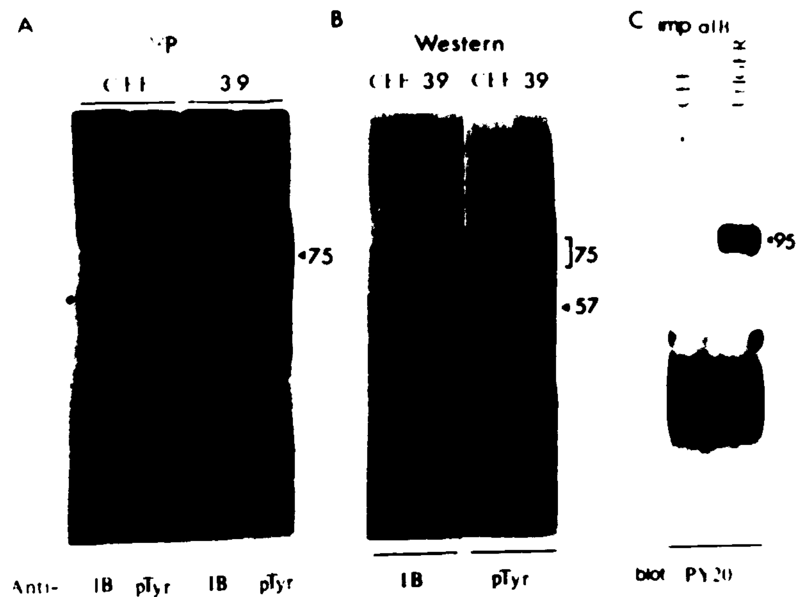
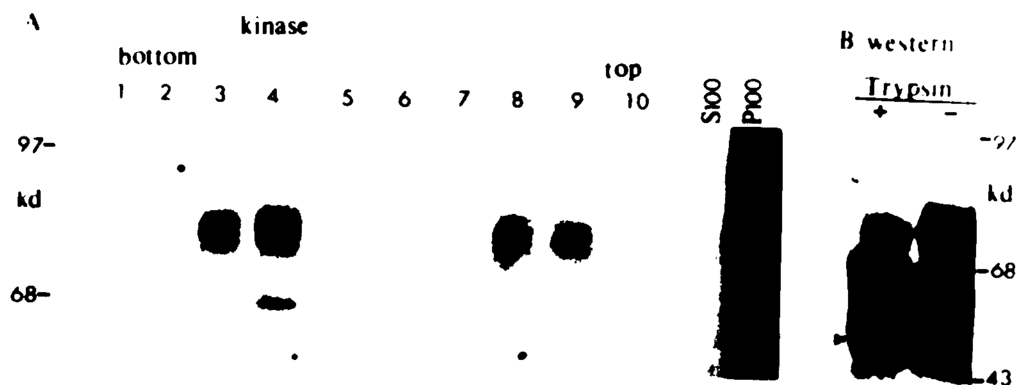


Fig. 3-8. Subcellular localization of *gag*-IGFR proteins. (**A**) UIGFR infected CEF were homogenized before being subjected to differential ultracentrifugation. S100 and P100 represent the supernatant and pellet, respectively, of the postnuclear fraction after centrifugation at 100,000xg. Proteins of the P100 fraction were further fractionated by sucrose gradient ultracentrifugation as described before (Jong and Wang, 1990). The sucrose solution was fractionated into 10 aliquots. Proteins from different fractions were immunoprecipitated with α-IB and the *in vitro* kinase reaction was performed with the immunoprecipitates as previously described. **B**. UIGFR transformed CEF were treated first with neuraminidase(1 U/ml, Sigma) and hyaluronidase(1500 U/ml, Sigma) for 15 min at 37°C and then digested with TPCK-trypsin(5 mg/ml, Sigma) for 30 min as described previously (Jong and Wang, 1991). Protein extracts from these cells were analyzed by immunoblotting with αIB. The arrow head indicates possible degraded products of *gag*-IGFR P75.



Chapter 4

Modulating Effects of the Extracellular Sequence of the Human Insulinlike Growth Factor I Receptor on Its Transforming and Tumorigenic Potential

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Abstract

An N-terminally truncated insulinlike growth factor I receptor (IGFR) fused to the avian sarcoma virus UR2 *gag* p19 (gag-IGFR) had a greater transforming potential than did the native IGFR, but it failed to cause tumors in vivo. To investigate whether the 36 amino acids (aa) of the IGFR extracellular (EC) sequence in the *gag*-IGFR fusion protein encoded by the retrovirus UIGFR have a modulatory effect on the biological and biochemical properties of the protein, four mutants, NM1, NM2, NM3 and NM4 of the EC sequence were constructed. NM1 lacks the entire 36 aa residues; NM2 lacks the N-terminal 16 aa residues (aa870-885) including two potential N-linked glycosylation sites of the EC sequence; NM3 contains a deletion of the C-terminal 20 aa residues (aa 886-905) of the EC sequence; and NM4 contains N to Q substitutions at both N-linked glycosylation sites. NM1 was the strongest of the four mutants in promoting anchorage independent growth of the transfected chicken embryo fibroblasts, while NM2 and NM4 had weaker transforming potential than did the original UIGFR. Only NM1 and NM3 were able to induce sarcomas in chickens. The four NM mutant transformed cells expressed

the expected proteins with comparable steady-state levels. The in vitro tyrosine kinase activity of $P53^{NM1}$ was about 4 fold higher than that of the parental $P57\text{-}75^{UIGFR}$; whereas NM2 and NM4 proteins exhibited at least 4-5 fold lower kinase activities. Despite lacking the IGFR EC sequence, $P53^{NM1}$ formed covalent dimers similar to those formed by the parental $P57\text{-}75^{UIGFR}$. Increased phosphatidylinositol (PI) 3-kinase activity was found to be associated with the mutant IGFR proteins. Among the mutants, relatively higher PI 3-kinase activity was associated with the NM1 and NM3 proteins than with the NM2 and NM4 proteins. Elevated tyrosine phosphorylation of cellular proteins of 35, 120, 140, 160, and 170 kDa was detected in all mutant IGFR transformed cells. We conclude that the EC 36 aa sequence of IGFR in the *gag*-IGFR fusion protein exerts intricate modulatory effects on the protein's transforming and tumorigenic potential. The 20 aa residues immediately upstream of the transmembrane domain have an inhibitory effect on the tumorigenic potential of *gag*-IGFR, whereas N-linked glycosylation within the EC sequence appears to have a positive effect on the transforming potential of UIGFR. Increased in vitro kinase activity and, to a lesser extent, in vivo tyrosine phosphorylation as well as the elevated association of PI 3-kinase activity with IGFR proteins seem to be correlated with the transforming potential of IGFR mutant proteins.

Introduction

Normal receptor protein tyrosine kinases (PTKs) are involved in control of cell growth and altered versions of these receptor-type PTKs are responsible for oncogenic transformation (Cantley et al, 1991; Hunter, 1991; Ullrich and Schlessinger, 1990). Several proteins, such as PI 3-kinase, GAP, and PLC-γ, are involved in signal transduction of these receptor PTKs. Insulin receptor (IR), a member of the receptor-type PTK family, was shown to harbor the potential for cell transformation and tumorigenesis (Giogiano et al, 1991; Poon et al, 1991; Wang et al, 1987). Insulin stimulation results in an increased IR associated PI 3-kinase activity, suggesting the involvement of PI 3-kinase in IR signal transduction (Endemann et al, 1990; Ruderman et al, 1990). Human insulinlike growth factor I receptor (IGFR) is a receptor PTK most closely related to the IR(Czech, 1982, 1989). The tyrosine kinase of IGFR is activated upon IGF-1 binding, resulting in rapid autophosphorylation on tyrosine residues and stimulation of cell growth (Czech, 1982, 1989). However, little is known about the intracellular events following receptor activation.

Given the sequence homology among IR, IGFR, and the receptor-like PTK encoded by the oncogene *ros* (Birchmeier et al, 1990; Matsushime and Shibuya, 1990; Chen et al, 1991), we examined whether IGFR exhibits transforming and tumorigenic activities and demonstrated that overexpressed IGFR was able to transform primary chicken embryo fibroblasts (CEF). Moreover, truncation of most of the extracellular (EC) sequence of IGFR and fusion of the remaining ß subunit to avian sarcoma virus UR2 *gag* p19 significantly enhanced the protein's transforming potential. However, the IGFR fusion protein encoding virus failed to induce tumors in vivo.

This study was intended to examine whether the remaining EC 36 amino acid (aa) residues of IGFR in gag-IGFR fusion protein have an effect on the protein's transforming and tumorigenic potential and to correlate the biochemical properties of the variant

receptor proteins with their biological functions.

Results

Construction of mutants. We have shown previously that UIGFR virus containing a *gag*-IGFR fusion gene is strongly transforming in vitro but can not induce tumors in vivo (see Chapter 3). A *gag*-IR containing virus, UIR, was found to become tumorigenic only after deletion of the EC 44 aa upstrem of the IR transmembrane (TM) domain (Poon et al, 1991). We examined whether the EC 36 aa remaining in the *gag*-IGFR fusion protein had a similar inhibitory effect on the protein's tumorigenic potential by constructing pNM1 lacking the 36 aa (Fig. 4-1). To characterize more precisely the sequence involved in the modulating effect, the 16 aa residues containing two potential N-linked glycosylation sites were removed in pNM2, whereas the 20 aa residues adjacent to the TM domain were deleted in pNM3. To examine whether N-linked glycosylation plays some modulating role, the two asparagine residues in the 36 aa sequence were mutated to glutamines in pNM4 (Fig. 4- 1, see "Materials and Methods" for details).

In previous studies (see Chapter 3; Poon et al, 1991; Wang et al, 1987), vectors containing permuted proviral DNA were used. To facilitate DNA transfection and increase the transfection efficiency, plasmid vector pBUIGFR-II containing a nonpermuted proviral genome was constructed (Fig. 4- 1A).

Biological properties of mutant UIGFR retroviruses. 10μg plasmids pBUIGFR-II, pNM1, pNM2, pNM3 or pNM4, was transfected into primary CEF together with 1μg of SacI-digested plasmid DNA of the helper virus UR2AV (Neckameyer and Wang, 1984). pBUIGFR-II and pNM1 transfected CEF were morphologically transformed about two weeks after transfection. The procedure included 3-4 passages and two soft agar overlays to promote growth of the transformed cells. The other three mutant- transfected CEF developed uniform morphological transformation about three weeks after transfection (data

not shown). All of the transformed CEF exhibited a remarkably elongated (fusiform) phenotype. The transforming potency of mutants was also evaluated by their ability to promote anchorage- independent growth (Hanafusa, 1969). All four mutants induced colony formation in soft agar medium (data not shown) with morphologies similar to those of UIGFR- and UIR-induced colonies (see Chapter 3;Wang et al, 1987). NM1 and UIGFR had similar colony forming potencies but were markedly stronger than NM2, NM3 and NM4 as judged by the number and sizes of colonies and latency required to reach a given colony size (Table 4-1). Our earlier study has shown that UIGFR can not induce tumors in vivo even though it causes obvious cell transformation in vitro (see Chapter 3). To determine whether any of the four NM mutants has tumorigenicity, equivalent titers of the NM viruses were injected into the wing webs of 2-day old chicks (Table 4-1). The NM1 and NM3 viruses were found to induce tumors about 3 weeks after virus injection (Table 4-1). No tumors developed in chicks injected with the NM2 or NM4 viruses even after 10 weeks. Viruses recovered from NM1- and NM3- induced tumor tissues induced a similar morphological transformation in CEF and caused sarcomas with similar latencies when reinjected into newborn chicks (Table 4-1). These viruses, NM1t and NM3t, encoded *gag*-IGFR fusion proteins which were indistinguishable from those encoded by the NM1 and NM3 viruses (data not shown). These results strongly suggest that tumors were induced by the NM1 and NM3 viruses *per se* and that no further mutations were needed for their tumorigenicity. These data suggest that the remaining short EC sequence of IGFR modulates the transforming and tumorigenic potential of the gag-IGFR fusion proteins. The 20 aa residues immediately upstream of the TM domain exert an inhibitory effect on the tumorigenic potential of the fusion receptor.

Tyrosine kinase activity of the mutant *gag*-IGFR proteins. We examined the expression and kinase activities of the NM proteins. The four mutant viruses encoded fusion proteins of the predicted sizes (Fig. 4- 2A). Several larger-sized protein species were also detected in both in vivo ^{35}S- and in vitro ^{32}P- labeled immunoprecipitates by αIB.

These species may represent posttranslationally modified *gag*-IGFR products, as glycosylation and phosphorylation are responsible for the appearance of larger species proteins of 60, 64 and 75KDa in addition to the expected P57 in the UIGFR infected cells (see Chapter 3). The multiple bands seen in NM1 cell extracts are most likely due to differential phosphorylation of $P53^{NM1}$ since the N-linked glycosylation sites are deleted in NM1 and tunicamycin treatment of NM1 transformed cells did not result in the disappearance or altered mobility of these species (data not shown). Similar differential phosphorylation may also account for the heterogeneity of $P55^{NM2}$ and $P57^{NM4}$. However, the slower migrating species of NM3 proteins could be due to glycosylation and /or phosphorylation. When the extent of phosphorylation of the IGFR fusion proteins was normalized to the protein amounts as reflected in ^{35}S- signals, the specific autophosphorylation activity of $P53^{NM1}$ was about 4 fold higher than that of $P57\text{-}75^{UIGFR}$ (Fig. 4- 2), whereas the specific activities of $P54^{NM3}$ and $P57\text{-}75^{UIGFR}$ were similar. The specific autokinase activities of $P55^{NM2}$ and $P57^{NM4}$ were about 4 to 5 fold lower than that of $P57\text{-}75^{UIGFR}$. Intracellular tyrosine phosphorylation of the *gag*-IGFR proteins was examined by Western immunoblotting with antiphosphotyrosine antibody PY20 (ICN), and the signals were normalized to the protein amounts determined by Western blotting with αIB. $P53^{NM1}$ was about 2 fold more tyrosine phosphorylated than was $P57\text{-}75^{UIGFR}$ whereas proteins encoded by the other three mutant viruses had similar degrees of tyrosine phosphorylation (Fig. 4- 3). CEF transformed by each of the four NM mutants expressed comparable steady-state levels of transforming proteins as judged either by [^{35}S]methionine metabolic labeling or by immunoblotting (Fig. 4- 2A and 3A). This finding indicates that the different transforming and tumorigenic activities of the NM viruses were not due to expression levels of the transforming proteins. This conclusion was confirmed by [^{35}S]methionine pulse-chase labeling of transformed CEF which showed that $P53^{NM1}$ and $P57\text{-}75^{UIGFR}$ both had similar half-lives of about one hour (data not shown). Therefore, the increased autokinase specific activity in vitro and tyrosine phosphorylation in vivo may

correlate with the enhanced tumorigenic potential of NM1.

Oligomerization of mutant proteins. The mature form of UIGFR encoded *gag*-IGFR protein, P75, was shown to retain a similar transmembrane topology similar to that of native IGFR (see Chapter 3). NM1-encoded P53 was also found to be associated with the plasma membrane (unpublished data). To examine whether the truncated NM proteins exist as disulfide-linked oligomeric structures, transforming proteins of NM1 and UIGFR were ^{32}P labeled by in vitro autokinase assay and analyzed by SDS-PAGE under non-reducing condition. The electrophoretic mobility of the UIGFR and NM1 products shifted from monomer to dimer positions in the absence of reducing agent, suggesting that both proteins existed as disulfide bond linked dimers. This result demonstrates that the EC region of IGFR is not essential for the formation of covalently-linked dimers of those *gag*-IGFR proteins (Fig. 4-4). The covalent dimerization was not affected by the presence of a sulfhydryl alkylating reagent, iodoacetamide (10 mM), in the lysis buffer (data not shown). Therefore, it is unlikely that the covalent dimerization took place in vitro. In conclusion, both truncated IGFR fusion proteins form covalently linked dimers.

Association of PI 3-kinase activity with mutant proteins. Because of the involvement of PI 3-kinase in many PTK-mediated signaling pathways (see above), we examined the association of PI 3-kinase with the NM fusion proteins. 2-4 fold more PI 3-kinase activity was present in the receptor immunoprecipitates of extracts from IGFR transformed cells in comparison with normal CEF (Fig. 4- 5C, black bars), suggesting a specific association between *gag*-IGFR proteins and PI 3-kinases. The PI 3-kinase activity seen in normal CEF most likely reflects its association with the chicken IGFR which could also be recognized by the αIB antiserum (see Chapter 3). The amounts of PI 3-kinase activity recruited to the various virus-encoded mutant IGFR proteins were also compared. When PI 3-kinase activity was normalized to the *gag*-IGFR protein levels (Fig. 4- 5B), the UIGFR, NM1 and NM3 encoded IGFR proteins were found to have associated PI 3-kinase activities three- to four-fold higher than that of the full length fIGFR protein, whereas associated PI 3-

kinase activities of NM2 and NM4 proteins were similar to that of fIGFR (Fig. 4- 5C, hatched bars). The reaction conditions used in our assays were in the linear range of PI 3-kinase activity (Fig. 4- 5D). These results suggest that more abundant association of IGFR with the PI 3-kinase activity appears to correlate with the stronger transforming ability.

To further investigate the association of PI 3-kinase with the truncated IGFR proteins, cell lysates from UIGFR and NM1 transformed CEF were sedimented by ultracentrifugation through a continuous sucrose gradient (Fukui and Hanafusa, 1989). Peak PI 3-kinase activity associated with the UIGFR transforming proteins was detected in fractions 5 and 6 (Fig. 4- 6B), whereas peak UIGFR proteins were found in fractions 6 and 7 (Fig. 4- 6A). In NM1- transformed CEF, NM1 protein- associated PI 3-kinase activity peaked in fractions 4 and 5 (Fig. 4- 6D), while NM1 proteins peaked in fraction 6 (Fig. 4- 6C). Therefore, PI 3-kinase appeared to be associated with the heavier fractions of IGFR fusion proteins. Substantial amounts of PI 3-kinase activity remained in the supernatants of fractions 4 to 8 from both gradients after αIB immunoprecipitation as demonstrated by re-immunoprecipitation of the supernatants with antiserum against the 85KDa subunit of the PI 3-kinase (UBI) followed by PI 3-kinase assay (data not shown). These results indicated that only a small fraction of the PI 3-kinase molecules is associated with the truncated IGFR proteins. The association of PI 3-kinase with IGFR fusion proteins correlates with their transforming activity. However, this property alone is not solely responsible for the tumorigenicity of NM1 and NM3 since there was little difference among these two mutants and the non-tumorigenic UIGFR.

Tyrosine phosphorylation of cellular proteins. Since increased tyrosine kinase activity has been correlated with transformation and tumorigenicity induced by *gag*-IR and *gag*-IGFR (see Chapter 3; Poon et al, 1991;Wang et al, 1987) and (Fig. 4- 2), we examined whether specific cellular proteins are preferentially phosphorylated in cells transformed by the NM mutants. Tyrosine phosphorylation of an array of cellular proteins was found to be

increased in all transformed CEF (Fig. 4- 7). Proteins of 35, 120, 140, 160, and 170 seem to be preferentially tyrosine phosphorylated as detected by monoclonal antibody PY20. Immunoblotting with 4G10 monoclonal antibody (IgG2bK from UBI) detected a similar pattern of tyrosine phosphorylated cellular proteins (data not shown). Stronger tyrosine phosphorylation in NM3 and NM4 proteins was due to the fact that more fusion proteins were expressed in these particular cell cultures as determined by parallel immunoblotting with αIB antiserum (Fig. 4- 7). No significant quantitative difference in tyrosine phosphorylation of cellular proteins was observed among the *gag*-IGFR mutants.

DISCUSSION

Oncogenes with intrinsic protein tyrosine kinases, such as *erbB*, *fms*, *kit*, *met*, and *trk* are derived from the transmembrane receptors for epidermal growth factor (EGF), colony-stimulating factor 1 (CSF-1), mast cell growth factors, hepatocyte growth factors and nerve growth factors, respectively (Bottaro et al, 1991; Huang et al, 1990; Klein et al, 1991; Westermark and Heldin, 1988). Their oncogenic activity is activated by mutations that include 5' truncation, 3' deletion, and/or fusion to viral or cellular proteins (34,84). Tumorigenic v-*erbB* (Downward et al, 1984; Lax et al, 1985) and v-*ros* (Neckameyer et al, 1985, 1986) have almost complete truncation of the EC sequences of their corresponding proto-oncogenes. Truncation of most of the EC sequences of IR and IGFR and fusion of the remaining parts of the receptor molecules to viral *gag* resulted in activation of their potential to transform cells in culture but not to induce tumors in vivo (see Chapter 3,Wang et al, 1987). Further deletion of the entire IR EC sequence led to full-fledged transforming and tumorigenic activity of the IR fusion protein (Poon et al, 1991). In the present study, we show that removal of the EC 36 aa of IGFR in *gag*-IGFR similarly activates the protein's tumorigenic potential. Furthermore, the 20 aa sequence immediately upstream of the TM domain appears to be the sequence restricting the tumorigenic potential of the partially truncated IGFR. Although N-linked glycosylation is not required for cell transformation or tumorigenicity as demonstrated by NM1, it seems to have a positive effect on cell transformation when the EC 36 aa sequence is present since removal or mutation of the glycosylation sites weakened the transforming activities of NM2 and NM4. Our current results provide additional support to our previous finding that the EC sequence of IR has a negative effect on the PTK and transforming and tumorigenic activities of the *gag*-IR fusion protein (Poon et al, 1991). This study further suggests that the short stretch of sequence upstream of the TM domains of these receptors may be the critical negative regulatory regions. Removal of that region (20aa) from the *gag*-IGFR

protein relieved the inhibitory effect on its tumorigenic activity. Our results implicate that those EC sequences also play a role in the regulation of the activities of the native IR and IGFR.

The short EC sequence also affected the biochemical properties of the mutant IGFR proteins. NM1 and NM3 proteins displayed tyrosine kinase activities stronger than or similar to that of the parental *gag*-IGFR, whereas NM2 and NM4 proteins had 4 to 5 fold lower kinase activities. The elevated kinase activity in general correlates with the enhanced transforming activity of the mutant viruses. However, the in vivo tyrosine phosphorylation of the NM mutant proteins revealed only a small difference, which could be due to the possibility that the proteins are also phosphorylated in vivo by other cellular tyrosine kinases activated in the transformed cells, which could lessen the difference of tyrosine autophosphorylation among the transforming proteins. For instance, constitutively increased tyrosine phosphorylation of IGFR has been shown in *src*- transformed cells (Kozma et al, 1990).

Dimerization was proposed to be an early step of activation of receptors for EGF and platelet-derived growth factor (PDGF) leading to their intermolecular autophosphorylation (Cochet et al, 1988; Honegger et al, 1991; Lammers et al, 1990; Li and Schlessinger, 1991; Yarden and Schlessinger, 1987). Transphosphorylation between receptor molecules has also been suggested as a mechanism for IR activation (Balloti et al, 1989; Lammers et al, 1990). In this study, truncated IGFR fusion protein encoded by UIGFR and NM1 formed covalent dimers, indicating that oligomerization can be mediated by sequences other than the EC domain of IGFR. However, the possibility of disulfide linkage in the p19 region of the fusion proteins also exists. It is also noteworthy that complete removal of the EC sequence did not affect the membrane localization of the *gag*-IGFR proteins since $P53^{NM1}$ was found to be associated with the plasma membrane fraction as shown in the differential ultracentrifugation experiments (see Chapter 3, and data not shown).

PI 3-kinase has been suggested to be a common signaling component associated with several receptor and non-receptor PTKs (Bjorge et al, 1990;Fukui and Hanafusa, 1989;Kazklauskas and Cooper, 1990). Elevated levels of PI 3 phosphate were shown to correlate with cell transformation and oncogenesis by *src*, and polyomavirus middle T antigen (Ling et al, 1992; Macara et al, 1984; Sujimoto and Erickson, 1985; Whitman et al, 1985). In normal cells, stimulation with CSF-1, EGF, PDGF, or insulin through their cognate receptors is accompanied by increased receptor-associated PI 3-kinase activity (Auger et al, 1989; Bjorge et al, 1990; Endemann et al, 1990; Ruderman et al, 1990; Varticovski et al, 1989). We show that the PI 3-kinase activity is associated with the gag-IGFRs which have constitutively activated kinase activity. The PI 3-kinase activity in NM1 and NM3 protein immunoprecipitates was 3-4 fold higher than that recruited by fIGFR protein, whereas a similar amount of PI 3-kinase activity was associated with the weaker transforming proteins of fIGFR, NM2 and NM4. It seems that the IGFR associated PI 3-kinase activity correlates with the transforming potency of those mutants. However, the difference in PI 3-kinase activity associated with NM1 or NM3 versus UIGFR protein was not significant enough to account for their different tumorigenic activities. This finding suggests that the increased PI 3-kinase activity may be necessary but not sufficient for oncogenesis. It has been demonstrated that comparable levels of PI 3-kinase activity are associated with transforming and nontransforming *src* proteins suggesting that association with PI 3-kinase activity is not sufficient for cell transformation by these proteins (Fukui and Hanafusa, 1989, 1991; Fukui et al, 1989). It was noticed that the PI 3-kinase activity was associated with the heavier fractions of the *gag*-IGFR proteins, suggesting complex formation between the PI 3-kinase and IGFR. However, we could not discern whether PI 3-kinase was associated with monomeric or multimeric forms of the IGFR fusion proteins.

Expression of the truncated IGFR proteins resulted in an overall increase of tyrosine phosphorylation of cellular proteins. Most prominent being proteins of 35, 120, 140, 160 and 170kDa. However, neither qualitative nor quantitative differences in this

pattern were observed among the mutants. Cellular proteins of 120 and 240kDa have been previously characterized as common substrates for receptors of insulin, EGF, and IGF-I (Accili et al, 1986; Fanciulli et al, 1989; Kadowaki et al, 1987; Philips et al, 1987). An 185kDa protein has also been reported to be a common substrate for IR and IGFR (Kadowaki et al, 1987). Interestingly, another 185kDa protein (insulin receptor substrate 1) has been found to be increasingly phosphorylated on tyrosine residues in insulin stimulated cells and shown to be associated with the mitogenic signaling functions of the human IR (Chou et al, 1987; Shoelson et al, 1992; Sun et al, 1991; White et al, 1988). It is not clear whether the phosphorylated proteins detected in this study are related to those reported in the literature. The fact that the extent of overall tyrosine phosphorylation of cellular proteins does not necessarily reflect the potency of cell transformation underscores the possibility that phosphorylation of certain specific substrate(s) may be more relevant to the transformation.

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Table 4-1. Biological Properties of Mutant Viruses

Virus	CEF morphology	Anchorage-independent growth in soft agar[a]	Viral tumorigenicity[b]	
			Ratio[c]	Latency(wk)[d]
None (medium)	spindle	-	-[e]	
UIGFR	fusiform	++++	-	
NM1	fusiform	++++	21/22(5)	3-4
NM2	fusiform	+	0/9(2)	
NM3	fusiform	++	16/16(3)	3-4
NM4	fusiform	+	0/4(1)	
NM1t[f]	fusiform	ND	4/4(1)	4
NM3t[f]	fusiform	ND	5/5(1)	3

a. Performed exactly as described previously. The degree of growth ability was determined by estimating the number of colonies formed and latency of colony appearance. Plating density was 10^5 cells per ml of top soft agar medium. The assay was performed at least twice for each virus. ND: not done.

b. The amount of virus was estimated by slot blot analysis of RNA extracted from 5 ml of viral stock using an IGFR specific probe. On the basis of the intensity of hybridization signals as determined by densitometric scanning, an equivalent amount of virus in 0.1 ml of culture medium was injected into each wing web of 2 day old chicks.

c. Number of chickens with tumors/total number of chickens injected. Numbers in parentheses indicate numbers of independent injections performed. At least two different viral stocks obtained from independent DNA transfections were injected for NM1 and NM3.

d. Average length of time required for appearance of clearly visible tumors. W: week.

e. -, see table 3-1.

f. Viruses recovered from tumor tissues induced by NM1 and NM3, respectively.

Fig. 4-1. Structures of *gag*-IGFR mutant plasmids. (A) pBUIGFR-II, which contains a nonpermuted viral DNA insert and was used as a vector for construction of the NM mutants. Only the viral insert is shown. The N-terminal 49 aa of UR2 *gag* p19 and 36 aa of the EC sequence immediately upstream of the TM domain of IGFR are indicated. The blank box within the 36 aa region represents the 16 aa residues (aa 870-885) that include two N-linked glycosylation sites. The shaded box represents the 20 aa residues (aa 886-905) adjacent to the TM domain. LTR, long terminal repeat. (B) Plasmids with mutations in the EC 36-aa sequence. The nature of each mutation and expected sizes of *gag*-IGFR fusion proteins are indicated. Only the *gag*-IGFR fusion region of each plasmid is shown. The total number of amino acids and calculated molecular size of each mutant protein are indicated. x, mutation of the two N-linked glycosylation sites.

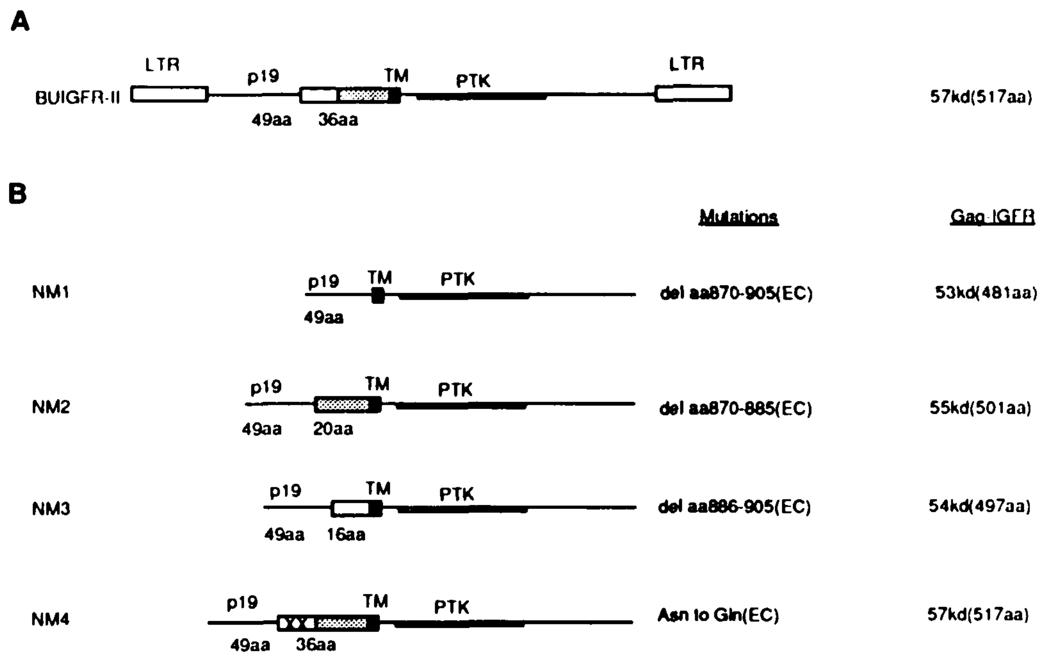


Fig. 4-2. Expression and in vitro tyrosine kinase activities of *gag*-IGFR proteins. CEF were metabolically labeled with[^{35}S]methionine for 4h. Cells were lysed with NP40 buffer (see "Materials and Methods"). Proteins in the lysates were quantitated with Bradford assay solution (Bio-Rad) and same amount of the cell lysates was immunoprecipitated with αIB antiserum. The immunoprecipitates were washed three times with the NP40 buffer. For each sample, one half of the immunoprecipitate was resuspended in Laemmli sample buffer and analyzed directly on an 8% SDS-PAGE gel (A) to determine amount of the *gag*-IGFR proteins, and the other half was washed once more with pre-kinase buffer (50 mM Tris-HCl, pH 8.0), and then resuspended in 20 μl of kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MnCl_2$) for the in vitro kinase reaction. The reaction was carried out in the presence of 10 μCi γ-^{32}P-ATP (6000 Ci/mmole, NEN) for 10 min at room temperature and terminated by adding 1 ml of NP40 buffer. The reaction mixtures were centrifuged and the precipitates were resuspended in the Laemmli sample buffer and analyzed on an 8% SDS-PAGE gel (B). ^{35}S labeled protein signals in panel A were amplified for 20 min with Amplify™ solution (Amersham) and visualized by fluorography, whereas proteins in panel B were visualized directly by autoradiography. Exposure times were 15h (A) and 5h (B). The exposure time was controlled so that the signals were in the linear range. Very little ^{35}S signal in panel B would be seen with a 5 h exposure time. UI: UIGFR. Molecular sizes indicated on the left.

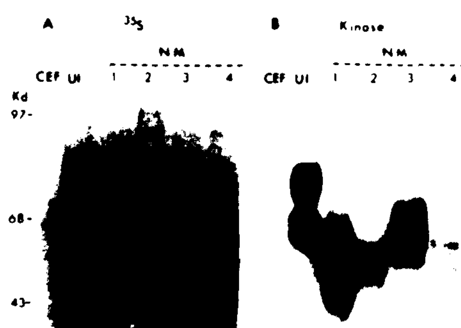


Fig. 4-3. Tyrosine phosphorylation of *gag*-IGFR proteins. CEF were lysed in NP40 buffer and equal amounts of cell lysates determined as described in the legend to Fig. 2. were immunoprecipitated with αIB. The immunoprecipitates were washed and resuspended in laemmli sample buffer. Equal amount were loaded into duplicate wells on 8% SDS-PAGE gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and hybridized with either αIB to determine protein amount (A) or PY20 to determine protein tyrosine phosphorylation (B). Exposure time was controlled in the linear range as described for Fig. 4-2.

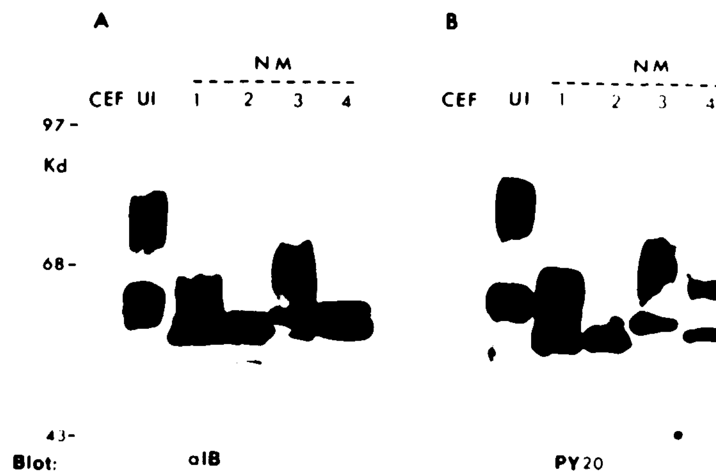


Fig. 4-4. Oligomerization of *gag*-IGFR proteins. Transformed CEF were lysed and cell lysates were immunoprecipitated with αIB. The kinase reaction was carried out with resuspended immunoprecipitates. After the kinase reaction, immunoprecipitates were divided into two parts, one half was resuspended in regular sample buffer containing 5% 2-mercaptoethanol (2-ME), and the other half was resuspended in sample buffer without 2-ME. Samples were boiled for 4 min and then separated on a 6% SDS-PAGE gel. Non-reduced protein bands were indicated as follows. black triangle: fIGFR protein; open triangles: UIGFR encoded transforming proteins; arrowhead: NM1-encoded transforming proteins.

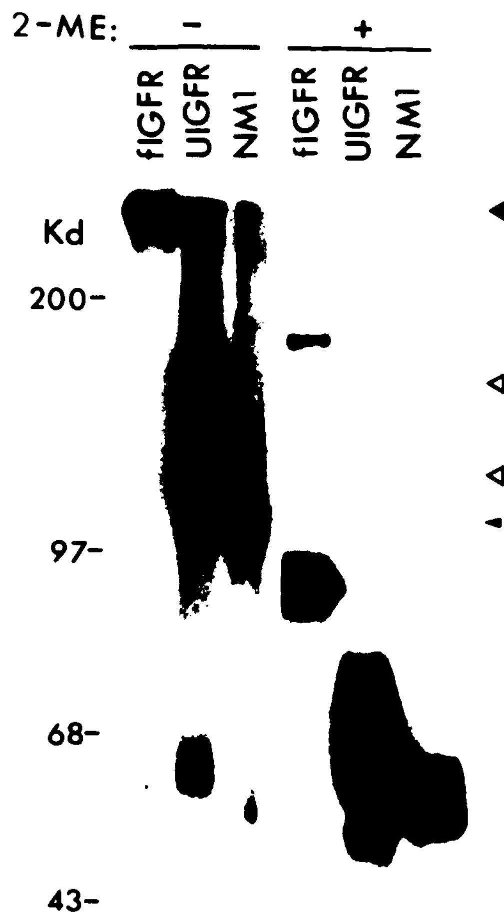


Fig. 4-5. Association of PI 3-kinase activity with *gag*-IGFR proteins. CEF were lysed and equal amount of cell lysates were immunoprecipitated with αIB. Immunoprecipitates were washed extensively (see "Materials and methods"). Half of the immuonoprecipitates were used for the PI 3-kinase assay, and PIP products were analyzed on thin layer chromatography plates (**A**). The other half were analyzed by immunoblotting with αIB to determine the amount of *gag*-IGFR proteins present in the immunoprecipitates. PIP and protein bands were visualized by autoradiography. Exposure time was controlled within the linear range of the autoradiography signals. Typical results of the PI 3-kinase assay (A) and parallel immunoblotting (B) are shown. (**C**) Plotting of normalized PI 3-kinase activity. ***Black bars***: relative PI 3-kinase activity normalized to the level of uninfected CEF. PI 3-kinase activity was represented by amount of PIP detected, which was determined by densitometric scanning of PIP signals on autoradiograms. ***Hatched bars***: relative PI 3-kinase activity normalized to the level of fIGFR. PI 3-kinase activity reflected by the intensity of PIP spots on the autoradiograms was normalized to the physical amount of each transforming protein. PI 3-kinase activity in immunoprecipitates from normal CEF lysates was deducted from those present in immunoprecipitates from transformed CEF lysates when the relative activity was calculated. The results represent averages of four experiments with standard errors ranging from 0.25 to 0.60. (**D**) Kinetics of the PI 3-kinase reaction. Cell lysates from 2 x 10^6 cells were immunoprecipitated with αIB. Immunoprecipitates were washed and the PI 3-kinase reaction was carried out for different times. PIP products were analyzed and visualized as described above. PI 3-kinase activity was represented by the amount of PIP product which was determined as described above. The densitometric scanning results were expressed as arbitrary units and plotted versus time (minutes).

Fig. 4-5.

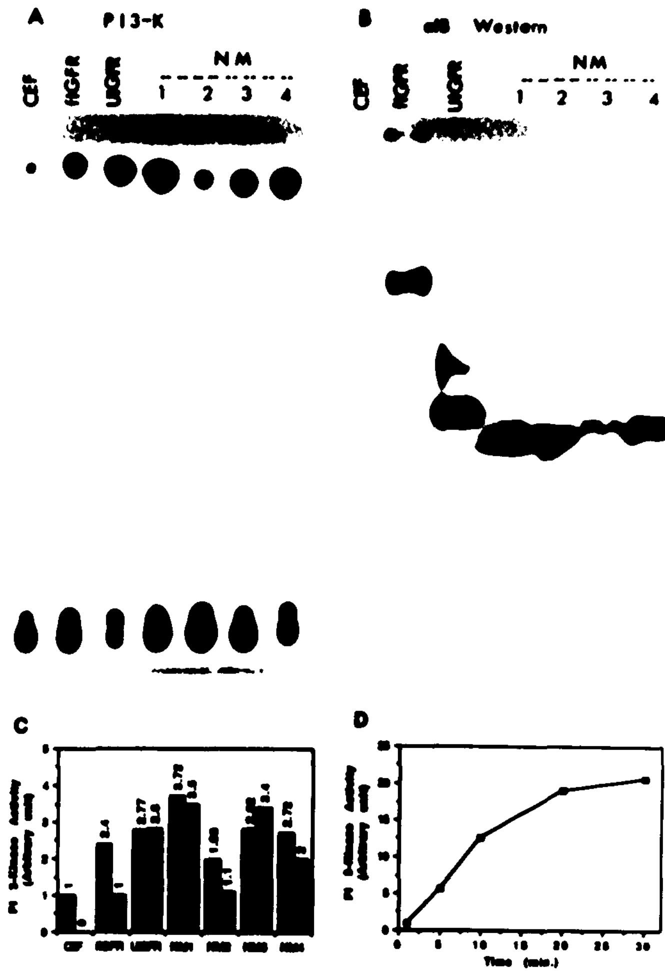


Fig. 4-6. Cosedimentation of PI 3-kinases with *gag*-IGFR proteins. UIGFR (**A** and **B**) and NM1 (**C** and **D**) -transformed CEF (10^7 cells) were lysed in 0.5ml of NP40 buffer. Cell lysates were centrifuged at 12,000xg for 10 min at 4°C to remove nuclei and protein aggregates. The supernatants were layered on top of a 5-20% sucrose gradient and sedimented by ultracentrifugation at 46,000 rpm in an SW 50.1 rotor for 7.5h at 4°C. The sucrose gradient was fractionated into 10 tubes, diluted with NP40 buffer and then immunoprecipitated with αIB. One-fifth of the αIB immunoprecipitates was used for the autokinase reaction, the other four-fifth of the immunoprecipitates was used for PI 3-kinase assay. Autokinase reaction products from each fraction were analyzed on an 8% SDS-PAGE gel (**A** and **C**); PI 3-kinase products were analyzed on thin layer chromatography plates (**B** and **D**). After collection of αIB immunoprecipitates, the supernatants in each fraction were re-immunoprecipitated with anti-p85 antiserum. Those immunoprecipitates were assayed for PI 3-kinase activity and analyzed as described above (result was described in the text).

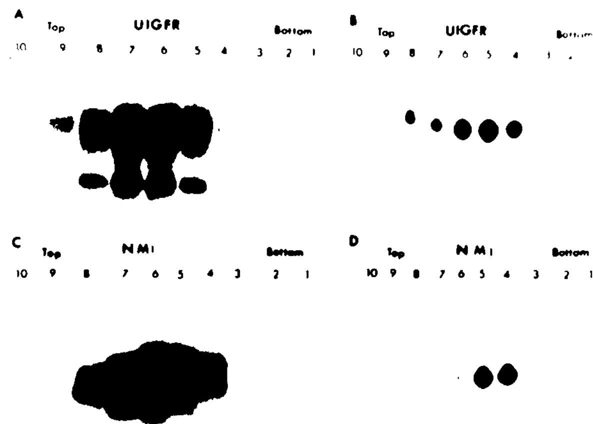
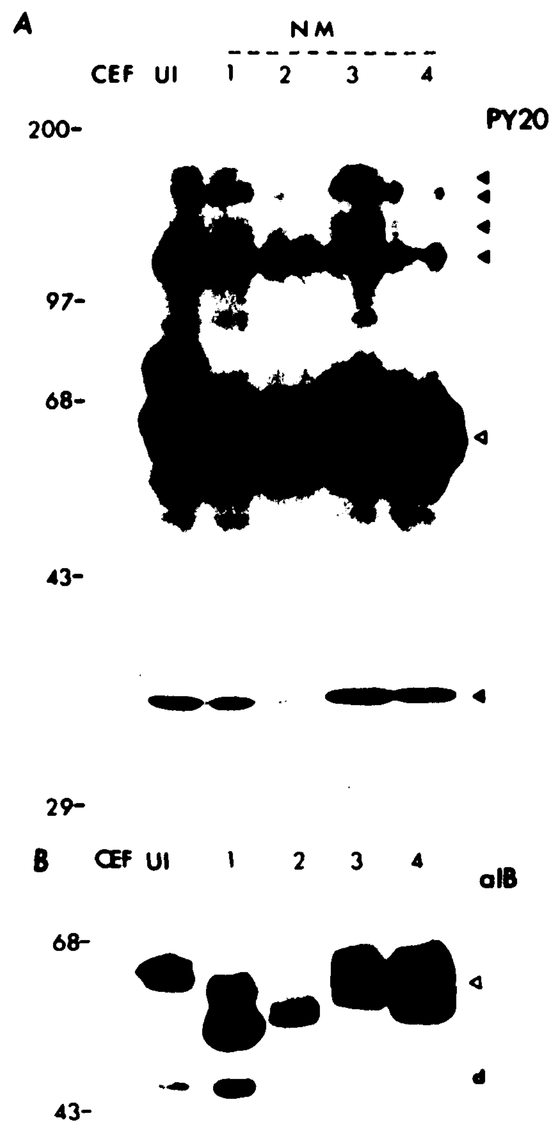


Fig. 4-7. Tyrosine phosphorylation of cellular proteins. CEF were incubated in medium containing 200 μM sodium orthovanadate for four hours before being subjected to protein extraction with buffer containing 10 mM Tris-HCl, pH7.5, 1% SDS, 1 mM Na_3VO_4, 1 mM sodium molybdate, 1% Trasylol (aprotonin, FBA pharmaceuticals), and 1mM PMSF. Cell lysates were boiled for one minute, then mixed with 2 times concentrated Laemmli sample buffer and boiled for another 4 min. The protein extracts were separated on an 8% SDS-PAGE, electrotransferred to nitrocellulose and immunoblotted with PY20 (**A**) or αIB (**B**). Immunoblotting of the protein extracts with 4G10 (IgG2bK from UBI) was done in parallel (not shown). Black triangles, preferentially tyrosine phosphorylated cellular proteins. open triangles, *gag*-IGFR proteins. d, degraded product of *gag*-IGFR. UI: UIGFR. Numbers 1, 2, 3, and 4 represent corresponding NM mutant. Sizes are indicated in kilodaltons.



Chapter 5

Distinctive Effects of the Carboxyl Terminal Sequence of the Human Insulinlike Growth Factor I Receptor on Its Biological Functions and Biochemical Properties

Abstract

We have shown previously that the extracellular sequences of the human insulin receptor (IR) and insulinlike growth factor I receptor (IGFR) have inhibitory effects on their protein tyrosine kinase (PTK) activities and their biological functions. To study the role of IGFR carboxyl terminal sequence in modulation of its PTK activity, biological function, and signal transduction, five mutants, CM1, CM2, CM3, CM4, and CM5, were constructed. These mutants contain increasing length of deletion of C-terminal amino acid sequence downstream of the putative IGFR tyrosine kinase domain. After transfection of plasmid DNA into primary chicken embryo fibroblasts (CEF), CM1, CM2, and CM4 caused morphological transformation of CEF, whereas no obvious morphological change was seen in CM3- and CM5- transfected CEF. When CM mutants were further characterized for their ability to promote anchorage-independent growth, CM1 and CM4 induced colony formation of transfected CEF in soft agar medium with a potency similar to that of parental UIGFR virus, CM2 was slightly weaker than UIGFR in this aspect. CM3- and CM5-transfected CEF failed to form colonies. Steady-state expression level of mutant IGFR proteins, PCM1, PCM2, PCM3, encoded by CM1, CM2, and CM3, respectively, were comparable to that encoded by UIGFR, CM4 had lower steady-state level of fusion protein

(PCM4) expression, and CM5 had remarkably lower expression level of PCM5 fusion protein. When in vitro autokinase activities and in vivo tyrosine phosphorylation of CM fusion proteins were compared with those of the parental P57-75^{UIGFR}, PCM1, PCM2, and PCM4 were similar to those of P57-75^{UIGFR}, whereas PCM3 and PCM5 had barely detectable in vitro kinase activity or in vivo tyrosine phosphorylation. PCM1 and PCM2 were able to associate with phosphatidylinositol (PI) 3-kinase, but PCM3 failed to do so. PI 3-kinase association with full-length IGFR (flGFR) was found to totally depend on ligand-induced activation of IGFR. The PI 3-kinase association was well correlated with autophosphorylation of IGFR. All of five CM mutant proteins were able to form disulfide bond-linked dimers.

We conclude that: 1. the most C-terminal 27 amino acids (aa1310-1337) of IGFR are dispensible, whereas the amino acid sequence 1250 to 1310 was critical for PTK activity and transforming ability of the gag-IGFR fusion protein; 2. PTK activity of gag-IGFR protein is essential for its biological function and signal transduction; 3. association of PI 3-kinase with IGFR requires its PTK activation and correlates with tyrosine phosphorylation; 4. the carboxyl terminal 27 aa is not essential for IGFR association with PI 3-kinase.

INTRODUCTION

Protein tyrosine kinase (PTK) receptors share significant amino acid sequence homology in their PTK domains, yet they have distinctive pathways for signal transduction (Hanks et al, 1988; Ullrich and Schlessinger, 1990; Cantley et al, 1991; Aaronson, 1991). The specificity of signal transduction by PTK receptors may involve sequences in the juxtamembrane region (Backer et al, 1991, 1992; Segatto et al, 1991; Kapeller et al, 1991;), kinase insert region (Fantl et al, 1992; Kashishian et al, 1992) and/or carboxyl terminal sequences following PTK domain. Carboxyl terminal sequences appear to be the most divergent regions among PTK receptors. Deletion of C- terminal sequences has been found to be a common mechanism for activation of proto-oncogenes like erbB, fms, ros, and kit (Downward et al, 1984; Coussens et al, 1986; Neckameyer et al, 1986; Besmer et al, 1986). The C-terminal deletion frequently includes important autophosphorylation sites.

The role of C-terminal tail of insulin receptor (IR) in signal transduction has been investigated in both rat-1 and Chinese hamster cells (CHO) by deletion of its C-terminal 43 aa (Maegawa et al, 1988; McClain et al, 1988; Myers et al, 1991). Removal of the 43 aa did not affect the tyrosine kinase activity of the mutant IR expressed in rat-1 cells; however, the mutant IR was less active in signaling biological effects such as glucose uptake and glycogen synthesis (Maegawa et al, 1988; McClain et al, 1988). In a separate study, a mutant IR lacking the same 43 aa functioned normally in CHO cells in the aspects of glycogen synthesis and interaction with cellular substrates PI 3-kinase and IRS-1; nontheless, the PTK activity of the mutant IR decreased 25% in comparison with that of wild type IR (Myers et al, 1991). In either case, DNA synthesis stimulated by the mutant receptor was unaffected. These studies suggest that C-terminus of IR is not essential for its mitogenic activity.

We have previously shown that the extracellular sequences of human IR and IGFR

have negatively regulatory effects on their tyrosine kinase activities and their biological functions (Wang et al, 1987; Poon et al, 1991; Liu et al, 1992). Both truncated PTK receptors have constitutively activated PTK activities and oncogenicities. However, no information regarding the role of IGFR C-terminus in modulation of its PTK activity and signal transduction is available. Even though the PTK domains of IR and IGFR have 84% identity, their carboxyl termini are highly divergent. This seems to suggest that their C-terminal sequences might have different roles in modulation of their PTK activities and signal transduction.

In this study we investigated the role of IGFR C-terminal sequence in modulating biological functions and biochemical properties of the IGFR PTK and show that the C-terminus contains sequence elements that have drastic effect on PTK activity and transforming ability of the gag-IGFR protein.

Results

Construction of IGFR carboxyl terminal mutants. The IGFR C-terminus contains 108 amino acid residues (Ullrich et al, 1986). To examine the role of the IGFR C-terminus in IGFR biological functions and biochemical properties, 88 aa were sequentially deleted by using oligonucleotide primers harboring the desired deletions (Fig. 5-1). CM1 contains a deletion of the most C-terminal 17 aa. CM2 lacks an additional 10 aa which contains a conserved YXXM motif involved in PI 3-kinase binding (Cantley et al, 1991). This 10 aa is highly conserved in human IR C-terminus (Ullrich et al, 1986). CM3 contains an extended deletion of another 20 aa (total of 47). This 20 aa sequence (aa 1291-1310) is the most divergent region among the C-termini of IGFR, IR, and IRR (Ullrich et al, 1986; Shier and Watt, 1989). CM4 has a further 20 aa deletion (aa 1271-1290) from CM3. CM5 lacks the C-terminal 88 aa. The 21 aa deletion (aa 1250-1270) specific for CM5 has two consecutive tyrosine residues (tyr-1250, tyr-1251) which are absent in human IR but highly

conserved in IRR (Shier and Watt, 1989). This 21 aa sequence is remarkably rich in charged amino acid residues.

Biological functions of IGFR CM mutants. 10 μg of plasmid pBUIGFR-II, pCM1, pCM2, pCM3, pCM4, or pCM5 (Fig. 5-1) was transfected into primary CEF together with 1 μg of Sac I-digested plasmid DNA of helper virus UR2AV (Neckameyer and Wang, 1984). Viruses, CM1, CM2, CM3, CM4, and CM5, recovered from culture medium, were named after the corresponding plasmid constructs. CM1, CM2, and CM4 caused morphological transformation of primary CEF about two to three weeks after DNA transfection (Fig. 5-2). The transformed CEF became morphologically elongated (fusiform). Neither CM3 nor CM5 caused obvious morphological change. The transforming activity of CM mutants was further assessed for their ability to promote anchorage-independent growth of transfected CEF. When compared with parental UIGFR, CM1 and CM4 had a similar potency to promote colony formation by transfected CEF in soft agar medium as that of the parental virus, while CM2 seemed to be slightly weaker than UIGFR; CM3 and CM5 failed to induce colony formation of transfected CEF (Fig. 5-1). To examine the biological activities of the CM mutants in vivo, recombinant retroviruses were injected into the wing-webs of one-day old chicks. None of the five CM viruses induced tumors up to 10 weeks after injection. Surprisingly, 60% of CM4 injected chickens (9 out of 15) developed tiny tumor nodules (about 5 mm^3) about 10 weeks after injection. The tumors expanded very slowly. The tumor-bearing chickens were physically healthy and were sacrificed around 16 weeks after injection. The cell transforming and weak tumorigenic activity of CM4 is surprising since CM3 with a deletion of only 47 aa has lost all the activity. Rigorous characterization including recloning and sequencing of the CM4 mutant (see "Materials and Methods") clearly excluded the possibility that unexpected mutation(s) was responsible for the recovery of CM4's transforming activity. Further mutation of CM4 mutant IGFR resulting in weak tumorigenicity in vivo seems unlikely, since viruses, CM4t, recovered from CM4-induced tumor tissue had a similar latency for tumor development. Besides, the CM4t

encoded-fusion IGFR had same mobility as that of CM4 mutant IGFR (data not shown). Therefore, the observed transforming activity of CM4 indeed reflects the nature of the construct and not due to additional mutations. This phenomenon of reactivation of the gag-IGFR biological activity from 47 aa deletion in CM3 to 67 aa deletion in CM4 will be discussed later.

Expression and kinase activity of CM mutant proteins. To explore the biochemical basis for the biological activities of CM mutant IGFR proteins, their in vitro kinase activities and tyrosine phosphorylation in living cells were characterized. The in vitro autokinase activities of CM1-, CM2-, and CM4- encoded fusion proteins were comparable with that of parental gag-IGFR proteins, while CM3 and CM5 encoded proteins had barely detectable autokinase activities (Fig. 5-3). Protein expression was examined by immunoblotting with αIB antiserum. CM1-, CM2- and CM3- transfected CEF had comparable steady-state levels of expression of gag-IGFR proteins with UIGFR-transformed CEF. CM4- transfected CEF seemed to have lower level of steady-state expression of fusion proteins than did parental UIGFR-transformed CEF, whereas CM5-transformed CEF had significantly lower steady-state level of expression of fusion proteins. The state of tyrosine phosphorylation of CM mutant proteins in vivo were assessed by immunoblotting with anti-phosphotyrosine antibody, 4G10 (UBI). In vivo tyrosine phosphorylation of the mutant proteins were generally consistent with their in vitro autokinase activities, namely, CM1, CM2 and CM4 had tyrosine phosphorylation comparable with that of P57-75^{UIGFR}, whereas CM3 and CM5 fusion proteins had negligible or undetectable level of tyrosine phosphorylation (Fig. 5-3). The data indicate that PTK activity and tyrosine phosphorylation of the gag-IGFR fusion proteins correlate well with their transforming activities. The carboxyl terminal 47 aa of IGFR, unlike the corresponding sequence of IR, is of primary importance for PTK activity of gag-IGFR.

Dimerization of CM mutant proteins. Mature IGFR forms a heterotetramer linked by disulfide bond. As discussed above, mutant IGFR with complete deletion of its

extracellular sequence was still able to form covalent dimers. The ability of dimerization of CM mutant proteins were characterized. The mobility of all of the five CM mutant proteins shifted to dimer position under non-reducing condition as did the P57-75^{UIGFR} (Fig. 5-4). Therefore, the loss of PTK and transforming activity of CM3 and CM5 is not due to their failure to dimerize. The data also indicate that truncation up to 88 aa of the C-terminal sequence did not affect the covalent dimerization of the gag-IGFR fusion proteins.

Association of PI 3-kinase with IGFR proteins. Association of PTK proteins with PI 3-kinase activity has been shown to be involved in cellular transformation by many PTK oncogenes (Cantley et al, 1991). Association of PI 3-kinase activity with N-terminal truncaded IGFR mutants described previously also correlated with their transforming ability. We therefore studied the PI 3-Kinase association with CM mutant proteins. After overnight starvation of transfected CEF in serum free F-10 medium, cells were lysed and cell lysates were immunoprecipitated with αIB. PI 3-Kinase activity in the immunoprecipitates were assayed. The amounts of mutant IGFR proteins present in the immunoprecipitate were monitored by western blotting with αIB. The result showed that CM1 and CM2 mutants had a comparable level of associated PI 3-kinase activity as that of P57-75^{UIGFR} (Fig. 5-5), whereas CM3 has negligible associated PI 3-kinase activity. NM1 had a greater amount of associated PI 3-kinase activity than did P57-75^{UIGFR}. Due to the low level of CM4 and CM5 fusion proteins in the cell lysates, it is not clear yet whether CM4 and CM5 fusion proteins are capable of binding PI 3-kinase.

The PTK positive CM1 and CM2 proteins are able to associate with PI 3-kinase activity whereas the PTK negative CM3 protein failed to do so. Thus, the association of PI 3-kinase activity with gag-IGFR proteins appears to require PTK activity. To further analyze this relationship in the native IGFR, the full-length IGFR overexpressed in CEF were characterized for its association with PI 3-kinase activity. The amount of PI 3-kinase activity associated with IGFR increased over 20-fold after stimulation of IGFR overexpressing CEF with recombinant human IGF-1 (Fig. 5-6A). Most of the anti-

phosphotyrosine antibody precipitated PI 3-kinase activity was also brought down by anti-IGFR antiserum (Fig. 5-6A), indicating that the observed PI 3-kinase activity was mostly associated with tyrosine phosphorylated IGFR proteins. Indeed, association of PI 3-kinase activity with IGFR correlated well with tyrosine phosphorylation of the receptor proteins (Fig. 5-6B). Most of the receptor proteins became tyrosine phosphorylated after IGF-1 stimulation (Fig. 5-6C). I conclude that association of PI 3-kinase activity with IGFR and its mutants (CM1, CM2, and CM3) requires PTK activity and correlates well with tyrosine phosphorylation of the IGFR proteins. Association of PI 3-kinase activity with CM1, CM2 and CM3 mutant proteins correlated with their ability to induce cellular transformation.

DISCUSSION

I have demonstrated that carboxyl terminal 88 aa of gag-IGFR fusion protein have remarkable effect on its biological function and biochemical properties. There are a total of 108 aa residues in the IGFR carboxyl terminus following the putative PTK domain (Ullrich et al, 1986). Deletion of the most C-terminal 27 aa (aa 1311-1337) did not have any significant effect on the transforming potency and autokinase activity of the gag-IGFR fusion protein as exemplified by CM1 and CM2. However, deletion of additional 20 aa (47 aa deletion) completely abolished tyrosine kinase activity and transforming ability of gag-IGFR fusion protein as demonstrated by CM3 mutant. The CM4 gag-IGFR mutant protein with 67 aa deletion in the C-terminus behaved very much like the parental P57-75^{UIGFR} with similar transforming ability and PTK activity. A more profound effect was seen in CM5 when a total of 88 aa were removed. CM5 encoded gag-IGFR protein (PCM5) lost transforming ability and tyrosine kinase activity. Besides, it seems that steady-state level of PCM5 was dramatically lower than that of other CM mutant proteins. These results suggest that the IGFR C-terminus plays an important role in modulating the biological function and biochemical properties of the receptor. First, the data indicate that the most C-terminal 27 aa are not essential for the biological and biochemical activities of IGFR. The amino acid sequence 1291 to 1310 is critical for the transforming and PTK activities of gag-IGFR fusion proteins. Further deletion of 20 aa (aa 1271-1290) in CM4 is able to recover the lost activity from CM3. These data suggest that the 20 aa sequence (1291-1310) is essential in maintaining a correct conformation of active IGFR and thus may be a sequence element of positive modulation, whereas the 20 aa sequence (1271-1290) may be a sequence element of negative modulation. Removal of this negatively modulatory sequence restored the gag-IGFR PTK activity and its transforming ability. Alternatively, deletion of the 20 aa (1271-1290) simply results in a compensatory effect on the misfolded CM3 protein such that an active conformation of the molecule is

restored. Secondly, the amino acid sequence adjacent to the putative PTK domain (aa 1250-1270) is indispensable for the IGFR tyrosine kinase activity. CM5 mutant protein was less abundant than any of the other four CM fusion proteins, even though the CM5 virus titer in culture medium seemed to be comparable to that of other four CM viruses as determined by slot-blotting analysis of viral RNA extracted from virions in culture fluid (data not shown). In addition, no gag-IGFR protein expression was ever detected when the C-terminal 108 aa was removed (CM6 mutant, data not shown). We speculate that this region (aa 1230-1270) might also be important for IGFR stability. The 21 aa deleted in CM5 are remarkably rich in acidic residues and contain two consecutive tyrosine residues (tyrosine 1250 and 1251). The twin tyrosine residues are absent in the corresponding region of IR (Ullrich et al, 1986). Interestingly, the corresponding sequence including the twin tyrosine in the C-terminus of IR-related receptor (IRR) was identical to that of IGFR (Shier and Watt, 1989). Whether these twin tyrosine residues have any role in regulating IGFR PTK remains to be elucidated. None of CM mutants showed acute tumorigenicity. CM4 induced small and slowly-growing tumor nodules three months after viral injection. Viruses recovered from the CM4-induced tumor tissues, CM4t, showed similar latency and potency of tumor induction when those viruses were reinjected into chicks. This biological result strongly argues against the possibility that the CM4-induced tumor was caused by additional mutation(s) in the CM4 viral genome during in vivo viral replication, since the spontaneously-arisen tumorigenic mutants isolated in our laboratory had significantly shorter latency of tumor induction and caused rapidly progressing tumors (Poon et al, 1991; Liu, D. and L-H. Wang, unpublished data). In addition, the CM4t-encoded gag-IGFR protein had a similar mobility as PCM4. Extensive recloning and sequencing of the CM4 plasmid clearly excluded the possibility that unexpected mutation was present in the gag-IGFR region. No biological or biochemical properties of the CM4 protein studied so far could be attributed to its weak tumorigenicity.

The CM3 mutant lacking C-terminal 47 aa seems structurally similar to an IR

mutant with a deletion of C-terminal 43 aa (Maegawa et al, 1988; McClain et al, 1988; Myers et al, 1991). The effects of the deletion were however totally different. CM3 lost PTK activity and transforming ability whereas the above IR mutant had nearly normal PTK and mitogenic activity (Maegawa et al, 1988; McClain et al, 1988; Myers et al, 1991). In a study employing a similar fusion protein, gag-IR, up to 70 aa of the carboxyl terminus of gag-IR protein could be deleted without affecting the PTK activity of the gag-IR fusion protein. In fact, the deletion enhanced the transforming activity of the mutant IR protein (Du, D., and L.-H. Wang, unpublished data). The 20 aa sequence (aa 1291-1310) deleted in CM3 is almost completely different from the corresponding sequence in IR. Between the C-terminal sequences of IR and IGFR, this region is the most divergent one. This difference in the primary structures of C-termini of the two PTK receptors may be in part responsible for their different biological functions. A recent study using EGFR/PDGFR chimera consisting of EGFR ligand binding domain and PDGFR cytoplasmic domain showed surprisingly similar observation (Seedorf et al, 1992). A mutant containing a 74 aa deletion in the PDGFR C-terminus of the chimera had low level tyrosine phosphorylation and completely lost oncogenic signaling potential, whereas an additional 6 aa deletion restored the receptor phosphorylation and wild-type level of transforming potential. Further deletion of 35 aa (115 aa deletion) yielded a receptor with nearly no PTK or biological activity. The biological and biochemical activities of those three chimeric mutants are very much similar to those of the mutants CM3, CM4, and CM5 characterized in this study. Although the two receptors are structurally very different, their carboxyl termini seem to have similar modulating effects on their biological and biochemical properties.

CM3 lost the ability to associate with PI 3-Kinase activity. Two possible explanations exist. First, CM3 has no detectable PTK activity nor tyrosine phosphorylation which are essential for PI 3-kinase association. Secondly, CM3 lost the structural motif essential for PI 3-kinase binding. Tyrosine kinase activity and tyrosine phosphorylation of

several PTK receptors have been shown to be necessary for PI 3-kinase binding (Kazlauskas and Cooper, 1990; Hu et al, 1992; Ruderman et al, 1991; Endemann et al, 1991). I demonstrate in this study that activated PTK activity of IGFR is essential for PI 3-kinase association and PI 3-kinase appears to only associate with tyrosine phosphorylated IGFR. It is therefore most likely that the inability of CM3 mutant to bind PI 3-Kinase is due to its loss of PTK activity and lack of tyrosine phosphorylation. My data also suggest that association of PI 3-Kinase with gag-IGFR fusion proteins correlated with their ability to induce cellular transformation. The 10 aa deleted in CM2 contained an amino acid sequence motif YXXM (aa 1316-1319) which was suggested to be a potential binding site for PI 3-Kinase (Cantley et al, 1991). My results show that this sequence motif is not essential for IGFR association with PI 3-kinase. This result is consistent with previous studies on the carboxyl terminus of IR which contains a similar YXXM motif that were not essential for IR association with PI 3-kinase (Myers et al, 1991; Backer et al, 1992). The sequence motif important for PI 3-kinase binding in IR was mapped to the juxtamembrane region which is also essential for interaction of IR with IRS-1, a major cellular substrate for IR PTK (Backer et al, 1991, 1992; Kapeller et al, 1991).

Oligomerization is an important mechanism for activation of EGFR and PDGFR (Cochet et al, 1988; Honegger et al, 1991; Lammers et al, 1990; Li and Schlessinger, 1991; Yarden and Schlessinger, 1987). However, kinase-deficient EGFR can still form ligand-dependent dimers (Gill et al, 1988). Similar to the N-terminal truncated gag-IGFR proteins, the gag-IGFR proteins encoded by the five CM mutants also formed disulfide-linked dimers. This result indicates that dimerization is not sufficient for activation of gag-IGFR PTK. Whether dimerization is necessary for gag-IGFR PTK activity is yet unknown.

Taken together the results from my previous study on N-terminal sequence and this study on C-terminal sequence, it appears that the extracellular sequence of IGFR has mainly inhibitory effect, while the C-terminal sequence has generally positive effect, on PTK activity and biological functions of the IGFR. The extracellular sequence of IR and

IGFR seem to have similar inhibitory role, but their carboxyl termini have different effects on their PTK activity and biological functions. A previous study on IR and IGFR chimera has shown that the specificity of their biological functions is determined by their intracellular domains (Lammers et al, 1989). The differential effects of C-terminal sequences of IR and IGFR on their functions elucidated in my study and in studies of others could be related to the divergence in their C-terminal primary amino acid sequences. Deletion of C-terminal sequences of IR and IGFR did not seem to affect their association with PI 3-kinase activity. It may be possible that, like IR, IGFR interacts with PI 3-kinase through its juxtamembrane region which contains well conserved tyrosine residues. Mutational analysis in this region would assist our understanding of IGFR signal transduction.

L(1230) EIISSIKEEM EPGFREVSFY YSEENKLPEP EELDLEPENM ESVPLDPSAS SSSLPLPDRH SGHKAENGPG PGVLVLRASF DERQPYAHMN GGRKNERALP LPQSSTC(1337)

Mutants	Mutation	Transformation	Tumorigenicity
UIGFR	gag-IGFR	+++	-
CM1	-17aa(1321-1337)	+++	-
CM2	-27aa(1311-1337)	++	-
CM3	-47aa(1291-1337)	-	-
CM4	-67aa(1271-1337)	+++	-/+
CM5	-88aa(1250-1337)	-	-

Fig. 5-1. Structure of carboxyl terminal mutants. The carboxyl terminal amino acid sequence (aa1230-1337) following the putative protein tyrosine kinase domain of human insulin-like growth factor I receptor was sequentially deleted (see "Materials and Methods"). Number and positions of amino acids deleted (indicated in the parentheses) in, and biological activity of, each mutant were shown. Activity of transformation (indicated by "+") of each mutant was judged by number of CEF colonies induced in soft agar medium. Tumorigenicity of the CM mutants was assayed as described in "Materials and Methods". "-" sign shown in "transformation" and "tumorigenicity" indicates no activity was observed.

Fig. 5-2. CEF transformation by CM mutant IGFRs. (A). Morphology of normal and transformed CEF on monolayer culture. CEF were transfected with proviral DNA from plasmids carrying CM mutant IGFRs together with pUR2AV and selected by overlaying the cell culture with soft agar medium(see "Materials and Methods"). Each photograph shows the transfected CEF about two weeks after transfection. (B). Recombinant virus-induced CEF colonies. Equal numbers of uniformly transformed CEF were plated in soft agar medium for assaying the anchorage-independent growth of the cells. Shown are colonies 3 weeks after plating of the cells in soft agar medium.

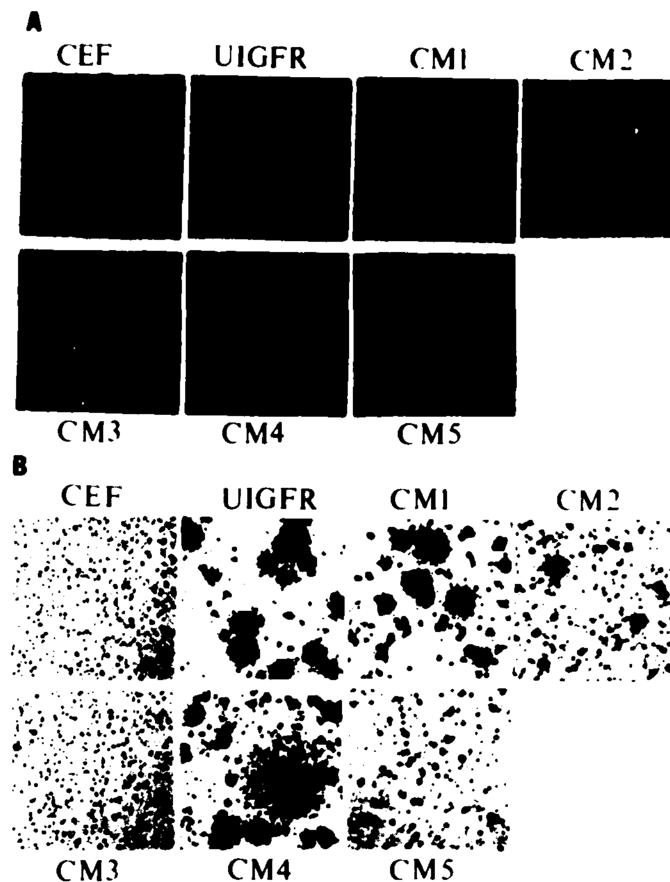


Fig. 5-3. Protein expression, autokinase activity, and in vivo tyrosine phosphorylation of CM mutant IGFR proteins. CEF were lysed in NP40 buffer and equal amounts of cell lysates were immunoprecipitated with αIB. The immunoprecipitates were washed and divided equally into three aliquots. One aliquot was used for in vitro kinase assay (B), the other two aliquots were analyzed either for protein expression of mutant IGFRs by immunoblotting with αIB antiserum (A) or for tyrosine phosphorylation of mutant IGFRs by immunoblotting with 4G10 (C). UI: UIGFR.

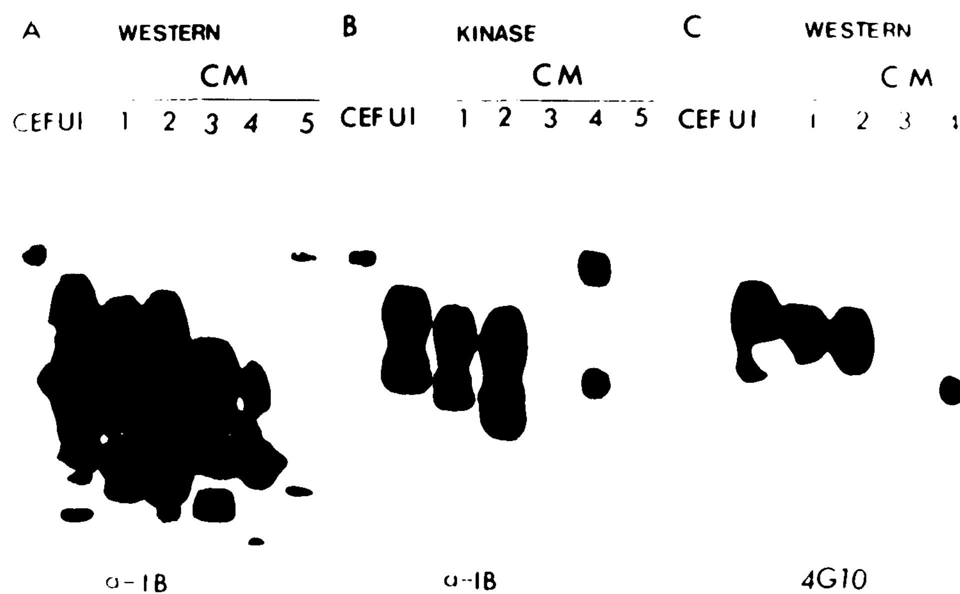


Fig. 5-4. Dimerization of CM mutant IGFR proteins. Transfected CEF were lysed with RIPA buffer (see "Materials and Methods"). Cell lysates were immunoprecipitated with αIB. The immunoprecipitates were divided into two parts, one half was resuspended in regular sample buffer containing (+) 5% 2-mercaptoethanol (2-ME), and the other half was resuspended in sample buffer without (-) 2-ME. Samples were boiled for 4 min and then separated on a 6% SDS-PAGE gel. Mutant IGFR proteins were detected by immunoblotting with αIB.

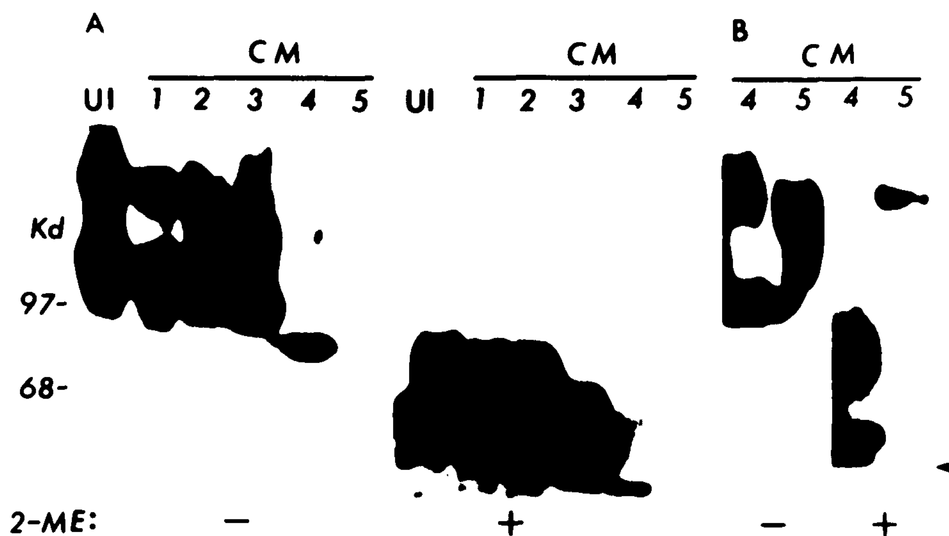


Fig. 5-5. Association of PI 3-kinase activity with CM mutant IGFR proteins. CEF were lysed and equal amount of cell lysates were immunoprecipitated with αIB. Immunoprecipitates were washed extensively (see "Materials and methods"). Half of the immuonoprecipitates were used for the PI 3-kinase assay, and PIP products were analyzed on thin layer chromatography plates (A). The other half were analyzed by immunoblotting with αIB to determine the amount of *gag*-IGFR proteins present in the immunoprecipitates (B). PIP and protein bands were visualized by autoradiography. Exposure time was controlled within the linear range of the autoradiography signals.

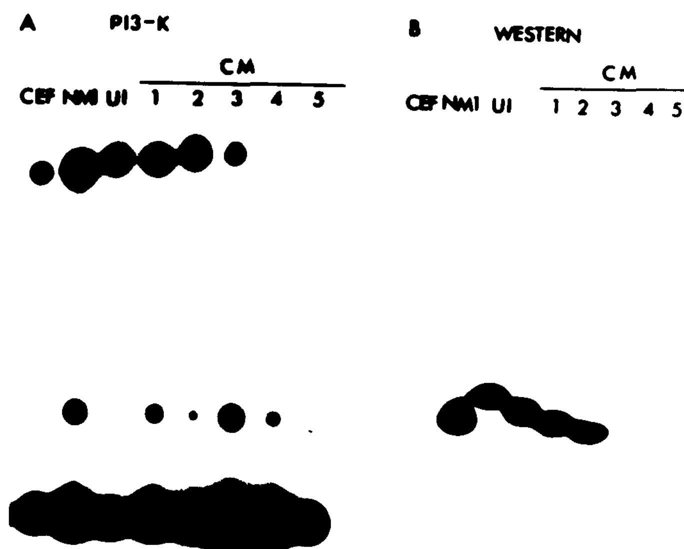


Fig. 5-6. Correlation of the activation of full length IGFR tyrosine kinase with its PI 3-kinase association. IGFR transformed CEF were starved overnight in serum-free F10 medium and stimulated with (+) or without (-) human IGF-1. One half of the cell lysates were immunoprecipitated with αIB, the other half were immunoprecipitated with PY20. Each immunoprecipitate was further divided into three aliquots. One aliquot was used for PI 3-kinase assay (A), the second aliquot was analyzed for IGFR tyrosine phosphorylation by immunoblotting with PY20 (B), and the third aliquot was analyzed for IGFR protein expression by immunoblotting with αIB (C).

A PI 3-K B WESTERN C WESTERN
IP α-IB PY20 α-IB PY20 α-IB PY20
+ - + - - + - + - + - + IGF-I
-180
-95
BLOT PY20 α-IB

Chapter 6 General Discussion

I. Sequence elements modulating biological function and biochemical properties of the human insulin-like growth factor I receptor.

Extracellular sequence has negative effects

Truncation of extracellular sequences (EC) is a common mechanism for oncogenic activation of PTK proto-oncogenes such as *erbB*, *ros* and *kit* and for activation of oncogenic potential of the human insulin receptor (see Chapter I). As a consequence, PTK activity of the oncogenes were dramatically increased. It seems that extracellular domain exerts negative effects on biological and biochemical activities of receptor PTKs. This thesis also shows that truncation of most of the IGFR EC sequence constitutively activates its PTK activity and liberates restraint on transforming potential of IGFR. My study further defines that a 20-aa stretch (WTDPVFFYVQAKTGYENFIH) immediately upstream of IGFR TM domain plays a major role in the inhibition of tumorigenic potential of IGFR. The constitutively activated PTK activity of the mutants may contribute to the activated tumorigenic potential of IGFR. Alternatively, the 20-aa sequence might be an immunogenic epitope. Removal of this epitope enables gag-IGFR- transformed cells to evade the normal immunosurveillence of the immune system and cause tumor formation in vivo. No significant homology with other polypeptides is found when the 20-aa sequence is compared with peptide database (SwissProt). However, the first nine (WTDPVFFYV) of the 20 aa are highly conserved in human IR (WTEPTYFYV). Deletion of a similar region containing the sequence in gag-IR also rendered the gag-IR mutant tumorigenic (Poon et al, 1991). It may be possible that the highly conserved sequence is the core region involved in modulation of the gag-IGFR tumorigenicity. When the EC sequence of IGFR is partially or completely truncated, the IGFR PTK activity is significantly increased. Two

mechanisms may be responsible for the increased PTK activity. First, removal of ligand binding domain locks the PTK in the active conformation irreversibly. Therefore, the unchecked IGFR PTK can send its biological signal constitutively to the nucleus through a distinctive yet ill-defined pathway, ultimating in deregulated cell growth. Second, deletion of EC sequence, especially the short sequence immediately upstream of the transmembrane domain dramatically alters conformation of IGFR PTK domain. This new conformation of tyrosine kinase domain lowers *Km* of the enzyme molecule for intracellular ATP and other substrates, and also makes autophosphorylation sites of the kinase molecule more accessible. As a result, the growth signal is amplified and more efficiently delivered, leading to accelerated cell proliferation. Studies on mutants of insulin receptor (IR) seem to support this hypothesis. IR mutants with mutation in its juxtamembrane region was shown to have high *Km* for ATP and less active PTK activity (Backer et al, 1991). Crystal structure of IGFR PTK domain will provide invaluable information in this regard.

C-terminal sequence has positive effects

Study on IGFR C-terminal mutants provides additional information on the structure-function relationship of this receptor. Deletion of the most C-terminal 27 aa does not seem to have any significant effect on IGFR PTK activity. However, deletion of 47 aa has a dramatic effect--inactivation of IGFR PTK activity. Removal of additional 20 aa (total 67 aa deletion) restored the PTK activity. Deletion of 88 aa of the C-terminus abolished the PTK activity of IGFR again. These results clearly indicate that the C-terminal sequence from 1250-1310 has remarkable modulating effects on IGFR PTK activity. Deletion of the C-terminal sequence (aa 1250-1337) either impairs or maintains but does not enhance IGFR PTK activity. Therefore, C-terminal sequence of IGFR has generally positive effects on its PTK activity. Truncation of C-terminus is a common feature for several oncogenes. v-*erb*B contains a deletion of C-terminal 34 aa; v-*fms* lacks C-terminal 40 aa, v-*ros* lacks

C-terminal 39 aa, whereas v-*kit* lost 49 aa in its C-terminus. Deletion of 43 aa in human IR did not affect its PTK and mitogenic activity, and deletion of 70 aa further boosts the transforming activity of gag-IR. By and large, deletion of C-terminal sequences in those proto-oncogenes and in IR either activates or has no effect on their PTK activity. Thus, the positive effects of IGFR C-terminus on its PTK activity seem to be rather unique among receptor PTKs studied so far.

PTK activity is essential for IGFR biological function

Increased PTK activity correlated with strong transforming and tumorigenic activity of UIGFR and NM1, while loss of PTK activity of CM3- encoded gag-IGFR protein correlated with inability of CM3 to cause cell transformation. These results clearly suggest that PTK activity of IGFR plays a pivotal role in its biological function.

II. IGFR signal transduction through PI 3-kinase

PI 3-kinase is associated with and activated by EGFR, PDGFR, IR and Polyoma middle T (Cantley et al, 1991; Koch et al, 1991; Endemann et al, 1990; Ruderman et al; 1990). My study also demonstrates that PI 3-kinase is associated with IGFR. Association of PI 3-kinase with IGFR is remarkably increased by IGF-1 stimulation. IGFR PTK activation and autophosphorylation are well correlated with the increased PI 3-kinase association. Strongly transforming mutants, NM1 and UIGFR, have greater amount of associated PI 3-kinase activity, whereas non-transforming CM3 lost ability to associate with PI 3-kinase activity. Therefore, association of PI 3-kinase with IGFR correlates with its biological functions, suggesting that PI 3-kinase is involved in IGFR signal transduction. However, C-terminal YXXM motif (YAHM) of IGFR is not essential for its PI 3-kinase association as shown by the CM2 mutant protein.

III. Future exploration in IGFR signal transduction

With the PTK-active and PTK-negative IGFR mutants with differential biological activities, it is now possible to further explore the IGFR signaling pathway. Discovery of the SH2 domain has led to tremendous advances in our understanding of cellular signaling events after receptor PTK activation. SH2-containing proteins like GAP and PLC-γ have been shown to be involved in EGFR, PDGFR, CSF-1R, and c-kit signal transduction (see chapter I). It is worthwhile to study whether GAP and PLC-γ are also involved in IGFR signaling pathway, and to look for and compare specific cellular substrates for IR, IGFR and Ros.

IRS-1, the major insulin receptor substrate, is involved in IR mitogenic effect. Although it contains neither SH2-domain nor catalytic sequence, it has multiple tyrosine phosphorylation sites which are YMXM or YXXM motifs important for binding SH2-containing proteins such as PI 3-kinase 85kd subunit (Sun et al, 1991). It is most likely that IGFR also signals through IRS-1 or a similar molecule.

SH2-containing proteins like SHC and GRB2/ASH have been implicated in EGFR and PDGFR signal transduction and are related to cell transformation (Lowenstein et al, 1992; Matuota et al, 1992; Pelicci et al, 1992). It will be interesting to study whether they are also involved in IGFR mitogenic signaling pathway. Recently, I molecularly cloned murine homolog of the human GRB2/ASH. Their amino acid sequence is almost identical. Preliminary study showed that murine GRB2 expressed in bacteria could associate with human IGFR in vitro (data not shown). Further analysis of this group of molecules should prove to be fruitful in probing the pathway of IGFR signal transduction.

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