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Enhancement of Transforming Potential of Human Insulinlike Growth Factor 1 Receptor by N-Terminal Truncation and Fusion to Avian Sarcoma Virus UR2 *gag* Sequence

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The human insulinlike growth factor 1 (hIGF-1) receptor (hIGFR) is a transmembrane protein tyrosine kinase (PTK) molecule which shares high sequence homology in the PTK domain with the insulin receptor and, to a lesser degree, the *ros* transforming protein of avian sarcoma virus UR2. To assess the transforming potential of hIGFR, we introduced the intact and altered hIGFR into chicken embryo fibroblasts (CEF). The full-length hIGFR cDNA (fIGFR) was cloned into a UR2 retroviral vector, replacing the original oncogene v-*ros*. fIGFR was able to promote the growth of CEF in soft agar and cause morphological alteration in the absence of added hIGF-1 to medium containing 11% calf and 1% chicken serum. The transforming ability of hIGFR was not further increased in the presence of 10 nM exogenous hIGF-1. The 180-kDa protein precursor of hIGFR was synthesized and processed into α and β subunits. The overexpressed hIGFR in CEF bound hIGF-1 with high affinity ($K_d = 5.4 \times 10^{-9}$ M) and responded to ligand stimulation with increased tyrosine autophosphorylation. The cDNA sequence coding for part of the β subunit of hIGFR, 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 tyrosine phosphorylation of the mature form of the fusion protein, P75, is sixfold higher in vitro and threefold higher in vivo than that of the native IGFR β subunit, P95. In conclusion, overexpression of the native or an altered hIGFR 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 phosphorylation.

Cellular proliferation and differentiation are normally regulated by extracellular polypeptide growth factors and hormones through their specific surface receptors. Many of these receptors are transmembrane proteins with an intrinsic protein tyrosine kinase (PTK) activity; they include receptors for insulin, insulinlike growth factor 1 (IGF-1), platelet-derived growth factor, monocyte colony-stimulating factor 1, and epidermal growth factor (66). These receptor-type PTKs (RPTKs) have similar molecular topologies. All possess an amino-terminal extracellular ligand-binding domain, a single transmembrane sequence, and a cytoplasmic domain bearing a PTK catalytic region which becomes activated upon ligand binding (70). Alterations of growth factor receptor activity can lead to subversion of normal growth control. Actually, some viral oncogene products have been found to be mutated versions of RPTKs; these include the products of *erbB* and *fms*, corresponding to the epidermal growth factor and colony-stimulating factor 1 receptors, respectively; recently, the *kit* protein was shown to correspond to the mast cell growth factor receptor (9, 21, 27a, 69). Other oncogenes, such as *ros*, *neu*, *met*, *ret*, and *trk*, are most likely also derived from RPTKs (reviewed in references 6, 27, and 70), indicating that tyrosine phosphorylation of proteins is important in the control of cell growth and differentiation.

IGF-1 receptor (IGFR), similar to the insulin receptor (IR), is synthesized as a single glycosylated polypeptide precursor of 180 kDa. The precursor is further processed into two polypeptides, an α subunit of 135 kDa and a β subunit of 95 kDa (29). The α subunit possesses the IGF-1-binding domain and is disulfide linked to the transmembrane β subunit, the cytoplasmic domain of which contains the catalytic region of PTK (13, 14). The mature IGFR, like IR, consists of two extracellular α and two transmembrane β subunits linked by disulfide bonds to form the β-α-α-β heterotetrameric receptor complex (56, 63, 65). The receptor undergoes phosphorylation on its β subunit upon binding of IGF-1 in both intact cells and cell lysate preparations (30, 43, 59, 63). Phosphorylation of IGFR occurs on both tyrosine and serine residues in living cells (63), whereas under in vitro conditions, autophosphorylation takes place exclusively on tyrosine residues (59). The autophosphorylation of β subunits appears to be catalyzed by the neighboring β-subunit kinase within the complex (5, 20). The major tyrosine residues that are involved in IGFR autophosphorylation are likely tyrosines 1131, 1135, and 1136 (7, 14), similar to those in IR (16, 18, 64).

Despite structural similarities between IGFR and IR, which share 84% amino acid identity in their PTK domains (65), their physiological functions are distinctive. Insulin primarily regulates rapid anabolic metabolism, including glucose uptake and lipid and glycogen synthesis, while IGF-1 appears to be one of the primary regulators of cell growth (57, 63). 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 was that of

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IR (43). 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$^{v\text{-}src}$ (38, 39). Moreover, many tumors have been found to secrete IGF-1, which has been suggested to be a potential growth promoter in human breast cancer (12, 28, 31, 46). IGFR expression has also been shown in benign and malignant breast tumors (12, 51–53). Almost all of the human breast cancer cell lines and tumor specimens tested were positive in IGFR expression (12, 52), and its level was shown to be higher in the cancer tissues than in adjacent normal tissues (51). These studies suggest that IGFR may be involved in cell transformation and tumorigenesis.

Avian sarcoma virus UR2 contains an oncogene, v-*ros*, which is a 5′-truncated version of its cellular counterpart c-*ros* coding for a growth factor receptor-like molecule with PTK activity (7, 19, 45, 47, 49, 62). The PTK domain of *ros* was found to have a closer homology with those of IR, IGFR, and *Drosophila* sevenless protein than with other members of the PTK family (4, 7, 44). In exploring the transforming potential of IR, we have fused the 5′-truncated human IR cDNA coding for a portion of the β subunit to the 5′ *gag* sequence in the UR2 genome and demonstrated that this *gag*-IR fusion protein was able to transform chicken embryo fibroblasts (CEF) (68). Moreover, further mutations of the *gag*-IR protein rendered it highly tumorigenic in vivo (54). 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 NIH 3T3 cells has been demonstrated to be able to induce transformation of these cells in the presence of hIGF-1, and the transformed NIH 3T3 cells could form tumors in athymic mice (35). 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 β-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.

MATERIALS AND METHODS

Cells and viruses. The preparation of CEF and colony formation assay of virus-infected CEF were done according to published procedures (26). The normal and transformed 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 (2, 48, 49, 67). Recombinant viruses were obtained by collecting culture medium from the transfected cells as soon as uniform transformation of CEF was obtained.

Recombinant plasmid construction. The starting plasmid was pMXIGFR (Fig. 1A), which contains the 4.4-kb full-length cDNA of the hIGFR gene, including about 500 bp of

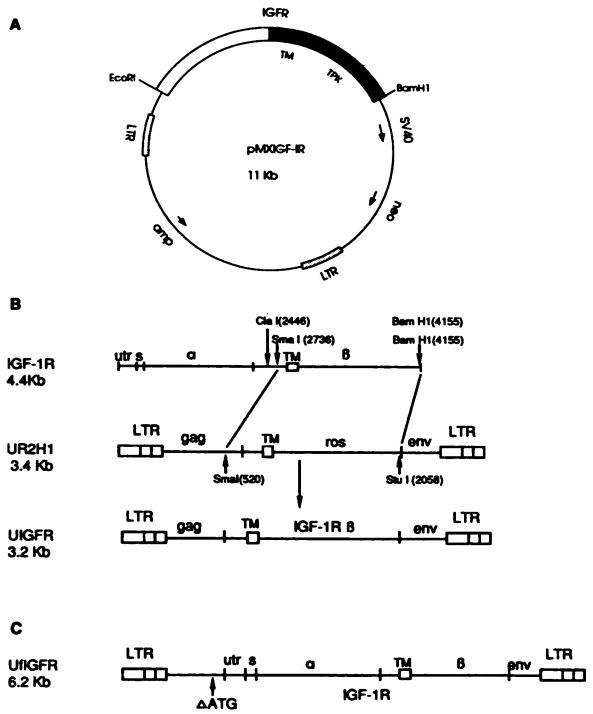

FIG. 1. Structures of plasmids pUIGFR and pUflGFR. (A) pMXIGFR. This plasmid contains the full-length cDNA of hIGFR cloned into the pMX plasmid vector downstream of the Moloney murine leukemia virus long terminal repeat (LTR). TM, transmembrane domain; SV40, simian virus 40. (B) Construction of pUIGFR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

5′ untranslated region (63). Restriction sites of the IGFR cDNA coding region were based on a map derived from the published cDNA sequence of IGFR (65). A 1,420-bp *Sma*I-*Bam*HI fragment of the IGFR cDNA (from nucleotide positions 2736 to 4155 [65]), coding for 36 aa of the extracellular region as well as the entire transmembrane and cytoplasmic domains of the IGFR β subunit, was freed from pMXIGFR and used for subcloning. pUR2H1 bearing the entire UR2 genome (47, 68) was digested completely with *Sma*I and *Stu*I to remove the v-*ros* coding sequence, and the large vector fragment was isolated. The 1.4-kb *Sma*I-*Bam*HI IGFR fragment was then blunt-end ligated in frame to the 5′ *gag* of the pUR2H1 vector DNA. The resulting plasmid containing the 3.2-kb 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 4.4-kb full-length IGFR cDNA was liberated from pMXIGFR by first cutting with *Eco*RI, filling the ends with Klenow enzyme, and digesting the plasmid with *Bam*HI. This 4.4-kb cDNA was inserted into *Sma*I-*Bam*HI-digested pUIGFR-ΔATG vector (see below). The resulting UR2 retroviral vector containing the full-length hIGFR cDNA was named pUflGFR. The plasmid

clone was confirmed by restriction enzyme analysis. The recombinant UIGFR is expected to encode a 516-aa *gag*-IGFR fusion protein containing 48 aa of UR2 p19 followed by 468 aa from human IGFR β subunit, whereas UfIGFR would encode the entire hIGFR precursor of 1,337 aa.

To prevent translation initiation from the UR2 p19 ATG codon and thus allow 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) (60). Two pairs of oligonucleotide primers were used: DLNco (5′-GGGACCATGGTATGTATAGGC-3′)/DLBgl3 (5′-TTCACTGCTAGATCTCCGGG-3′) for generating DNA fragments 5′ to the mutation site and DLBgl5 (5′-CCCGGA GATCTAGCAGTGAA-3′)/DLSph3 (5′-CTCACGCATGCT TGCGGCCT-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 200 μM each of the four deoxynucleotide triphosphates, 100 ng of each primer pair, 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 1,710-bp *Cla*I-*Bam*HI fragment (from nucleotide positions 2446 to 4155; Fig. 1B) (65) of the IGFR β-subunit cDNA sequence was cloned into bacterial expression vector pSJH57 (8). Briefly, pSJH57 was digested with *Cla*I and *Bam*HI, and then the *Cla*I-*Bam*HI 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 12 aa of lambda phage *c*II protein and the carboxyl 567 aa from the IGFR β subunit.

Antiserum production. The fusion protein S-I, encoded by pS-I, was purified as described previously (8). Purified protein (250 μg) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into four sites on the dorsal area of each New Zealand White rabbit (2.5 kg, female). The rabbits were given booster inoculations twice by the same route of injection with 150 μg of purified protein preemulsified with an equal volume of incomplete Freund's adjuvant. Bleeding was performed weekly starting 10 days after the second booster inoculation, and the antiserum was named αIB. Preparation and characterization of antiphosphotyrosine (αpTyr) antiserum will be described elsewhere (34a). The monoclonal αpTyr antibody used in this study was PY20 (ICN) (24).

DNA transfection and RNA analysis. Plasmids pUfIGFR and pUIGFR contain permuted proviral inserts. Before transfection, they were digested with *Hin*dIII to free the proviral inserts. The insert fragments were gel purified and briefly ligated (1 to 2 h) at 20°C to form nonpermuted proviral DNA. pUR2AV containing the helper viral genome was digested with *Sac*I to liberate the proviral insert. Transfection of viral DNA into CEF was done by a published procedure (37). Briefly, 7×10^5 CEF were seeded in 60-mm dishes; 18 h 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 (Aldrich Chemical Co., Milwaukee, Wis.) was then added. The culture was incubated for 6 h at 37°C with occasional shaking. At the end of this incubation period, the cells were treated with 2 ml of 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 two to three rounds of soft agar overlay. Analysis of RNA from transformed cells by RNA blotting and hybridization has been described elsewhere (48).

Biological assays. Cell transformation was monitored by observing morphological changes and by examining anchorage-independent growth of the transfected CEF (26). Tumorigenicity of the recombinant retroviruses was assayed as described previously (33, 54). Relative virus titers were measured either by determination of CFU in soft agar medium or by slot blotting of viral RNAs.

Protein analysis. Metabolic labeling of cells with [^{35}S]methionine or $^{32}P_i$, extraction of cellular proteins, immunoprecipitation, in vitro protein kinase assays, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (42) were done according to published procedures (18, 19, 22, 33). The procedure for treatment of UfIGFR virus-transformed cells with trypsin and subsequent protein analysis has been described elsewhere (32). Extraction and fractionation of membrane proteins were done as described previously (1, 23, 33). For the glycosylation inhibition experiment, cells were pretreated with 10 μg of tunicamycin per ml and 20 μM 2-deoxyglucose (Sigma Chemical Co.) for 2 h and then [^{35}S]methionine labeled in the presence of the inhibitors for 4 h 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 with Kodak X-Omat AR film and DuPont intensifying screens at −70°C.

Western immunoblotting. Western analysis was done as described previously (25, 33, 36), 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 3 h at 70 V or overnight at 35 V at room temperature. The filter was blocked at room temperature for 2 h with 5% nonfat dry milk in TTSS (10 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.9% NaCl, 0.02% sodium azide) and then subjected to binding with αIB (1:1,000 dilution) in 0.5% milk-containing TTSS for 6 h or longer at 4°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°C for 1 h. 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 (1:1,000 dilution; Sigma) for 2 h at 4°C. The filter was then washed, reblocked, labeled, and autoradiographed as described above. To reuse the PY20-blotted filter membrane for blotting with αIB antibody, the ^{125}I-labeled filter was incubated in elution buffer containing 0.2 M glycine-HCl (pH 2.0) (61) for 20 min at room temperature. This procedure was repeated twice. It was noticed that further elution with the above elution buffer did not reduce the signal 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 (1:1,000 dilution), 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 Tris-Glu buffer (25 mM Trizma base, 150 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose) and incubated with 18,000 cpm ^{125}I-IGF-1 (Amersham) and various amounts of unlabeled competitors (hIGF-1 [Collaborative Research, Inc.], insulin [Sigma], or calf serum [Hyclone]) for 2 h at 4°C in binding buffer (100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [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) (43, 63) 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 (43).

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 with Tris-Glu buffer, and serum-free F10 medium was added to the dishes; 15 h later, recombinant hIGF-1 (Collaborative Research) 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.

RESULTS

Transforming potential of the intact and 5'-truncated IGFR. The UfIGFR and UIGFR recombinant DNA inserts were freed from pUfIGFR and pUIGFR, respectively. 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 UfIGFR, while the retrovirus containing the extracellular 36 aa and the rest of the human IGFR β-subunit sequence was designated UIGFR. The transfected cells underwent morphological transformation about 2 weeks after initial addition of viral DNAs. The morphological change developed faster (1 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 effective than DNA transfection. The UfIGFR- and UIGFR-transformed CEF in monolayer culture assume an elongated shape (Fig. 2) similar to that of UR2- and UIR-transformed CEF (2, 68). 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 10 nM hIGF-I (Table 1). Cell transformation was also monitored by anchorage-independent growth (26). Both UfIGFR- and UIGFR-transformed CEF formed colonies in soft agar medium (Fig. 2), with a morphology similar to that of UIR-induced colonies but distinctive from that of Rous sarcoma virus- and UR2-induced colonies (68). 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 1). Addition of recombinant hIGF-1 at 10 to 100 nM did not significantly enhance the ability of colony formation of the UfIGFR-transformed CEF (Table 1 and data not shown). We suspected that the IGF-1 present in the 11% calf serum and 1% chicken serum was probably sufficient to support the anchorage-independent growth of the UfIGFR-transformed CEF; we therefore lowered the serum concentration fivefold 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 differences in colony-forming efficiency shown in Table 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 recombinant viruses are tumorigenic, equivalent amounts of the retroviruses were injected into wing webs of 1-day-old chicks. The injected chicks were observed for 2 to 3 months. No tumors were observed for UIGFR (three experiments) or UfIGFR (one experiment) (Table 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 ligand, the hIGFR-transformed CEF were incubated with labeled and unlabeled hIGF-1, insulin, or calf serum (Fig. 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 8.1×10^5 receptors per cell with an apparent K_d of 5.4×10^{-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 1). Untransfected CEF expressed either only background levels of IGF-1 receptors or very low affinity IGFR for human IGF-1 ligand. 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). It has been shown previously that chicken IGF-1 has 50% cross-reactivity with hIGF-1 (3), whereas bovine IGF-1 is identical to human IGF-1 in terms of amino acid sequence (3) and binding to hIGFR (55).

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) β subunits were detected by [^{35}S]Met labeling and immunoprecipitation with αIB, indicating that hIGFR was expressed

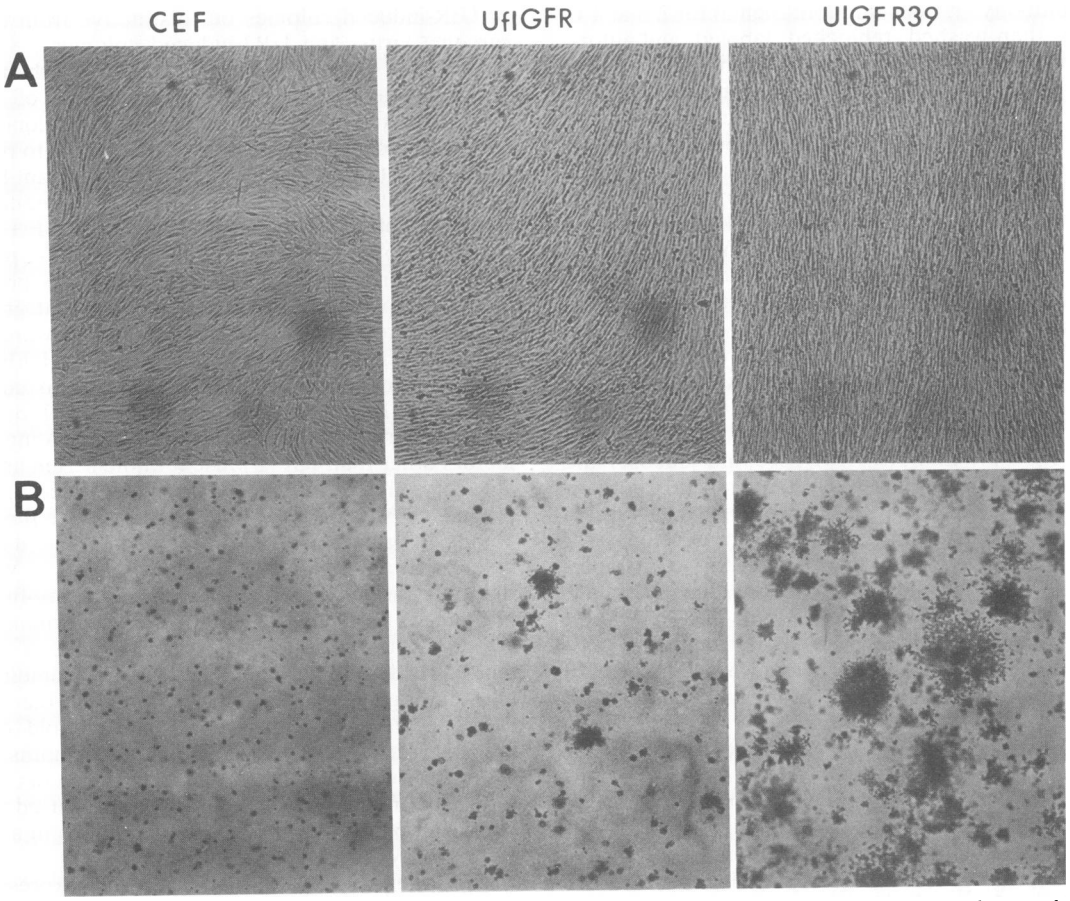

FIG. 2. CEF transformation by UflGFR and UIGFR. (A) Morphology of normal and transformed CEF on monolayer culture. CEF were transfected with proviral DNA from pUflGFR or pUIGFR together with pUR2AV and selected by overlaying the cell culture with soft agar medium (see Materials and Methods). Each photomicrograph 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 anchorage-independent growth of the cells (26). Shown are the colonies 2 weeks after plating of the cells in soft agar medium.

and properly processed in CEF (Fig. 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 β 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. 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. 5B). The elevated tyrosine phosphorylation in response to IGF-1 treatment indicates that the hIGFRs expressed in CEF are functionally active.

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) kDa were detected with antiserum αIB (Fig. 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 or phosphorylation. Since the UIGFR construct retains two potential asparagine-linked glycosylation sites in the remaining extracellular sequence of the β subunit of IGFR (65), the possibility of glycosylation was examined. A single 57-kDa protein band was observed after cells had been incubated with tunicamycin and 2-deoxyglucose and labeled with [^{35}S]methionine (Fig. 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 in Fig. 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 (50). 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. 6), although the three smaller species, P57, P60, and P64, were not separated well.

TABLE 1. Growth characteristics of transformed CEF

Expt	hIGF-1 (ng/ml)	Morphology	Anchorage-independent growth			Tumorigenicity[a]
			No. of colonies (%)[b]	Colony size (mm)	Latency (days)[c]	
A[d]						
CEF		Spindle	0			0/10
UIGFR		Fusiform	10^4 (2)	≥0.30	8–10	0/15
UflGFR		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 nM)	Spindle	0			
UflGFR	0	Fusiform	563 (0.56)	≥0.15	>14	
	77 (10 nM)	Fusiform	411 (0.41)	≥0.15	>14	

[a] Viruses equivalent to about 5×10^4 CFU were injected into each wing web of each 1-day-old chick. The chicks were observed for 2 months. Each result was derived from three injections for control medium and UIGFR virus, two injections for UR2 virus, and one injection for UflGFR virus.

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

[c] Time required for the appearance of colonies of 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 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 to 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 1 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 4 weeks after plating of the cells. The data represent averages from duplicate dishes.

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. 4). This minor 95-kDa band most likely represented the chicken IGFR β 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. 6). As for the kinase activity of intact IGFR, the 95-kDa β subunit of IGFR was autophosphorylated in vitro, whereas the ligand-binding α subunit was not (Fig. 6). The 180-kDa precursor was also phosphorylated, since it also contains the β subunit.

The phosphorylation profile of the IGFR proteins was also investigated in intact cells. Transformed CEF were metabolically labeled with $^{32}P_i$, and the cell lysates were immunoprecipitated with an αpTyr antibody; alternatively, unlabeled protein extracts were separated and immunoblotted with αpTyr 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. 7A and B). In Western blotting, P75 was sometimes resolved into a doublet, which probably reflected the heterogeneity of glycosylation or phosphorylation. Autophosphor-

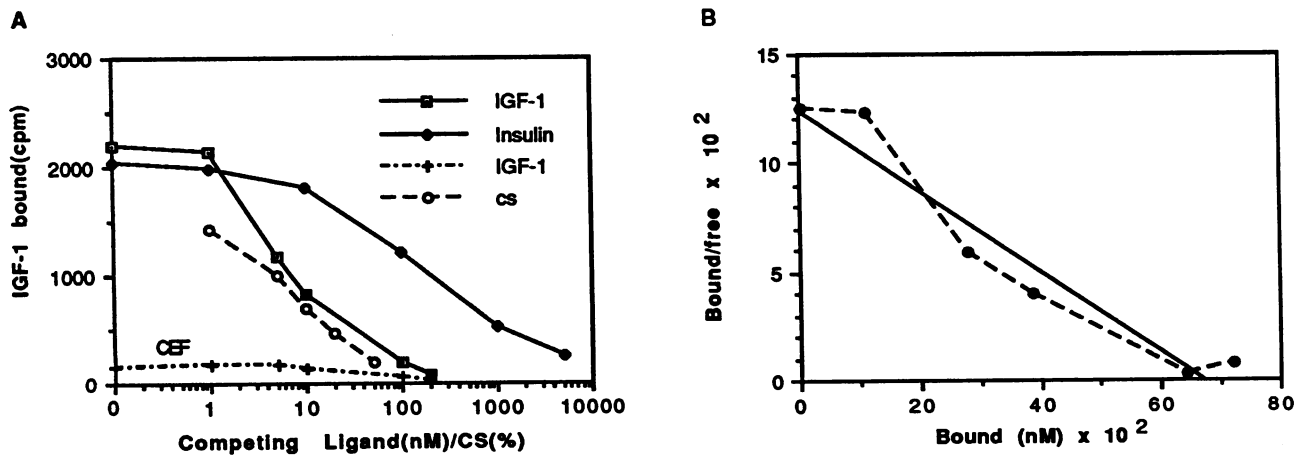

FIG. 3. Ligand binding to hIGFR expressed in CEF. (A) Duplicate wells of cells were incubated for 2 h at 4°C with ^{125}I-IGF-1 (18,000 cpm, 32 pM) and different amounts of unlabeled factors. Cells were washed, lysed, and counted as described in Materials and Methods. The averages of duplicate counts were used. Most of the averages were within a 5% difference. The competitor used for untransformed CEF (+) was hIGF-1 (0, 1, 5, 10, 100, and 200 nM); competitors used for hIGFR-transformed CEF were hIGF-1 (concentrations as stated above), insulin (0, 1, 10, 100, 1,000, and 5,000 nM), 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 plotting.

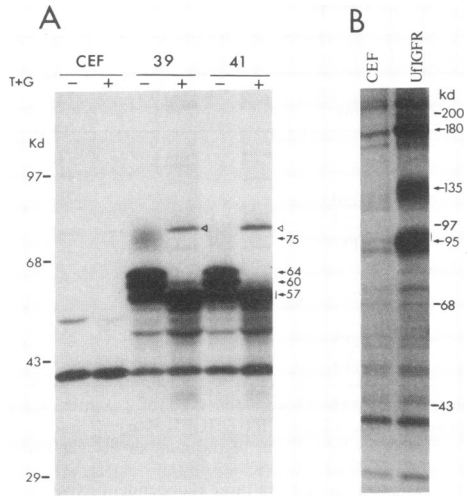

FIG. 4. Analysis of proteins from transformed CEF. (A) UIGFR-transformed CEF were treated with (+) or without (−) tunicamycin and 2-deoxyglucose (T+G) and labeled with [^{35}S]methionine (see Materials and Methods). Labeled protein extracts were immunoprecipitated with rabbit antiserum αIB raised against the IGFR β subunit. The immunoprecipitates were dissolved and analyzed on an 8% SDS-polyacrylamide gel. (B) UfIGFR-transformed CEF were labeled with [^{35}S]methionine without tunicamycin treatment, and protein extracts were immunoprecipitated and analyzed as described above. Protein size markers are shown in kilodaltons (kd). Arrows indicate IGFR proteins, with sizes represented in kilodaltons. The open triangle indicates the possible 78-kDa BiP protein (50); 39 and 41 are parallel UIGFR clones.

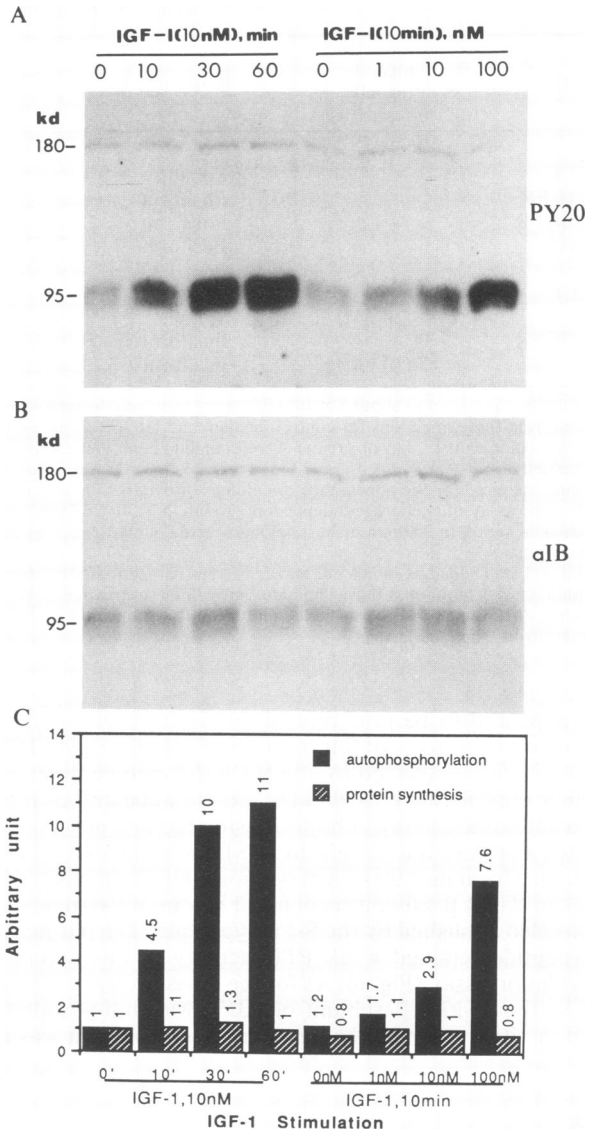

FIG. 5. Ligand stimulation of hIGFR expressed in UfIGFR-transformed CEF. Transformed CEF (10^6) were starved overnight in serum-free F10 medium. The cells were then incubated in serum-free F10 medium with 10 nM hIGF-1 for different lengths of time or with different amounts of IGF-1 for 10 min. The cell 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 αpTyr 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 95-kDa protein bands on the respective autoradiogram (C).

ylation of the 95-kDa IGFR β 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. 7C).

It has been shown that increased specific tyrosine phosphorylation of *gag*-IR proteins is correlated with the stronger transforming and tumorigenic activity of the protein encoded by the UIR19t virus (54). From in vitro autokinase and in vivo tyrosine phosphorylation experiments, we noticed that P75 was always phosphorylated to a greater extent than were the other three protein species (Fig. 6 and 7), although the physical amount of P75 was much less than those of the other three species. We therefore compared the specific tyrosine phosphorylation 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 αpTyr antibodies in parallel for determining in vivo specific tyrosine phosphorylation. To determine in vitro specific autophosphorylation, 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 2. P75 was preferentially phosphorylated in vitro at about sixfold-higher efficiency than were the other two UIGFR proteins. The specific tyrosine phosphorylation of P75 in vivo was about 2-fold higher than that of P60/64, 4- to 6-fold higher than that of P95 in vitro, and 2.5- to 3-fold greater than that of P95 in vivo, which is 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 95-kDa endogenous CEF IGFR β 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 $^{32}P_i$ labeling and αpTyr immunoblotting (Fig. 7).

Association of UIGFR proteins with the membrane fraction.

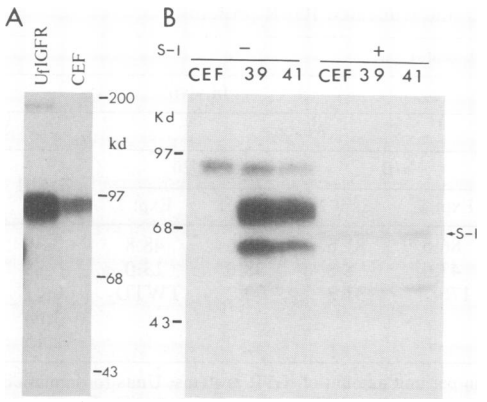

FIG. 6. Autophosphorylation of IGFR proteins in vitro. Total proteins were extracted from UfIGFR (A)- and UIGFR (B)-transformed CEF, and the extracts were immunoprecipitated with αIB antiserum. The in vitro kinase reaction was carried out with resuspended immunoprecipitates and analyzed as described previously (32). 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).

As the UIGFR gene retains the transmembrane domain of IGFR, it may be associated with the cellular membrane as is the case for P68$^{gag\text{-}ros}$ (32). 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. 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 (23, 33). P75 was detected in both light (Fig. 8A, lanes 7 to 9) and heavy (Fig. 8A, lanes 2 to 4) membrane fractions, but P60 and P64 appeared to be associated only with the heavy membrane fraction (Fig. 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 and 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.

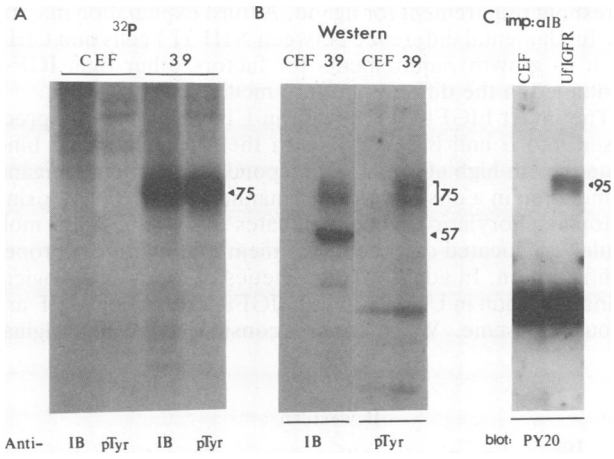

FIG. 7. Autophosphorylation of IGFR proteins in transformed CEF. (A) ^{32}P metabolic labeling. UIGFR-transformed CEF were starved for 2 h in P_i-free medium and then labeled with ^{32}P for 4 h. The cellular lysate was divided into two equal aliquots and immunoprecipitated with αIB or αpTyr antiserum. Resuspended immunoprecipitates were analyzed by 8% SDS-PAGE, and the gel was then treated with 1 N KOH at 55°C for 1 h to remove serine and threonine phosphorylation as described previously (32). (B) Immunoblotting. Cell lysates of UIGFR-transformed CEF were loaded in duplicate onto an 8% SDS-polyacrylamide gel. After separation, proteins were transferred to a nitrocellulose membrane. The membrane was cut into two parts, hybridized with αIB or αpTyr antiserum, respectively, and then labeled with ^{125}I-protein A. (C) Immunoprecipitation (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 immunoglobulin G heavy chain. Sizes of protein markers are indicated in kilodaltons.

It has been demonstrated that the P68$^{gag\text{-}ros}$ in UR2-transformed cells traverses the plasma membrane, with the p19 portion protruding extracellularly (32). This is a unique example among the *gag-onc* fusion proteins (33). 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 and P64 were unaffected (Fig. 8B), indicating that the P75 is indeed associated with and traverses the plasma membrane, whereas P60 and 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. 8B). Judging from the sizes of the P75 digestion products, it is most likely that the N-terminal sequence including 48 aa of UR2 p19 and 36 aa of the IGFR β 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 RPTK 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 (49), truncated hIR in UIR (68), and *erbB* in avian erythroblastosis virus (17). Since the specific tyrosine phosphorylation of P75 *gag*-IGFR is 4- to 6-fold higher in vitro and 2.5- to 3-fold higher in intact cells than those of P95 hIGFR-β, it is possible that the *gag*-IGFR fusion protein more efficiently transmits the signal in promoting transformation. Overexpressed native IGFR in CEF behaves like a transforming

TABLE 2. Specific tyrosine phosphorylation of intact and truncated IGFR proteins[a]

Protein	STP (arbitrary units)											
	In vitro[b]						In vivo[c]					
	Western (αIB)		Kinase		STP		Western				STP	
							αIB		PY20			
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
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	
P75/95[d]					6.2	4.07					2.93	2.47
P75/64[d]					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 for immunoblotting and in vitro autophosphorylation are defined as arbitrary 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 signal 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 amounts 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 amounts of cell extracts were analyzed by immunoblotting with αIB or αpTyr PY20. The intensities of protein bands were determined as for the in vitro assay.

[d] Values represent relative STP.

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 NIH 3T3 cells was ligand dependent and required a higher serum concentration (35). It was found that 10% calf serum competed for the overexpressed hIGFR as efficiently as did 15 nM hIGF-1 (Fig. 3), 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 (15) and chicken IGF-1 has 50% cross-reactivity with hIGF-1 (3), we cannot exclude the possibility that an autocrine 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 hIGF-1 ligand-binding capacities (Fig. 3). Another possible explanation for the discrepancy of ligand dependence between the earlier and our current studies might reside in the affinity of the receptors expressed in different cell systems; for instance, in our system, receptors with high affinity for their ligands might be expressed, which might decrease the threshold requirement for ligand. 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 β subunits with the expected sizes, bind ligands with high affinity, 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

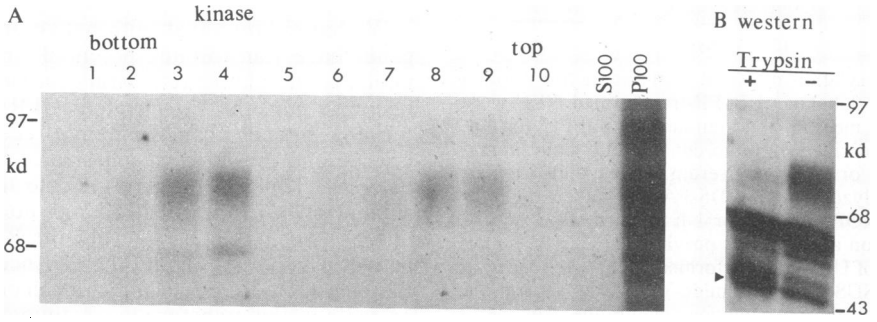

FIG. 8. Subcellular localization of *gag*-IGFR proteins. (A) UIGFR-infected CEF were homogenized before being subjected to differential ultracentrifugation. S100 and P100 represent the supernatant and pellet, respectively, of the postnuclear fraction after centrifugation at 100,000 × *g*. Proteins of the P100 fraction were further fractionated by sucrose gradient ultracentrifugation as described before (33). The sucrose solution was fractionated into 10 aliquots. Proteins from different fractions were immunoprecipitated with αIB, 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 (1,500 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 (32). Protein extracts from these cells were analyzed by immunoblotting with αIB. The arrowhead indicates possible degraded products of *gag*-IGFR P75.

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 the UR2 P68$^{gag\text{-}ros}$, in which the *gag* sequence has been shown to be essential for transforming activity (33). However, we cannot 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 UIR19t 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 (54, 54a). 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 36 aa can activate the tumorigenic potential of IGFR.

Four IGFR proteins were detected in the UIGFR-transformed cells. The 57-kDa protein is the unmodified *gag*-IGFR fusion polypeptide, and the 60-, 64-, 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 IGFR in the UIGFR construct. Experiments with tunicamycin confirmed the glycosylation of the P60, P64, and P75 fusion proteins. The significance of these differential posttranslational 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. 7). Accumulated evidence has also underscored the importance of membrane association of a PTK for its transforming activity. The transforming activity of the cytoplasmic PTK pp60$^{v\text{-}src}$ requires its association with the plasma membrane (10, 11, 40, 41). Membrane localization has also been shown to be necessary for the transforming activity of the RPTK oncoprotein v-*fms* (58). Our recent study also demonstrated that membrane association is necessary for the transforming function of P68$^{gag\text{-}ros}$ (34). Our data suggest that P75 is the only species that is transported to and traverses the plasma membrane (Fig. 8). Taken together, the results suggest that it is most likely that P75 is the active transforming protein in UIGFR-transformed CEF. However, we still cannot 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 or in the transmembrane region will be necessary to address this question.

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