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

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We reported previously that an N-terminally truncated insulinlike growth factor I receptor (IGFR) fused to avian sarcoma virus UR2 *gag* p19 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 (aa 870 to 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 to 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 transfected chicken embryo fibroblasts, while NM2 and NM4 had weaker transforming potential than did the original UIGFR virus. 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 fourfold higher than that of the parental $P57\text{-}75^{UIGFR}$, whereas NM2 and NM4 proteins exhibited four- to fivefold-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, 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.

Normal receptor-type protein tyrosine kinases (PTKs) are involved in control of cell growth, and altered versions of these receptor-type PTKs are responsible for oncogenic transformation (9, 34, 78). Accumulated evidence has clearly indicated that protein tyrosine phosphorylation plays a crucial role in cellular signal transduction, growth control, and oncogenesis. Recently, several signaling proteins have been shown to be physically associated with activated receptor and nonreceptor PTKs. They include phospholipase C-γ (53, 59, 81), Ras GTPase-activating protein (2, 3, 18, 37, 40, 50), and phosphatidylinositol (PI) 3-kinase (7, 10, 14, 24, 26, 39, 44, 87), all of which contain sequences homologous to the SH2 of *src* (*src*-homology domain 2) (21, 22, 64, 68, 71, 72, 76, 80). The SH2 domain has been shown to be involved in the interaction with the PTKs in the process of signal transduction (3, 9, 42, 54, 58, 60). Mutations in the SH2 region have been shown to cause dramatic changes in biochemical properties and biological functions of those SH2-containing proteins (27, 54, 56, 57).

Insulin receptor (IR), a member of the receptor-type PTK family, was shown to harbor the potential for cell transformation and tumorigenesis (66, 83). Overexpression of IR induces ligand-dependent transformation of fibroblast and ovarian cells (28). Insulin stimulation results in an increased IR-associated PI 3-kinase activity, suggesting the involvement of PI 3-kinase in IR signal transduction (20, 67).

Human insulinlike growth factor I receptor (IGFR), like IR, is a transmembrane protein molecule with intrinsic PTK activity (15, 16). The tyrosine kinase is activated upon insulinlike growth factor I binding, resulting in rapid autophosphorylation on tyrosine residues and stimulation of cell growth (15, 16). However, little is known about the intracellular events following receptor activation.

Given the sequence homology among IR, IGFR, and the oncogene *ros* product, a receptorlike PTK (11, 55, 61), we examined whether IGFR exhibits transforming and tumorigenic activities and demonstrated that overexpressed IGFR was able to transform primary chicken embryo fibroblasts (CEF) (49). Moreover, truncation of most of the extracellular (EC) sequence of IGFR and fusion of the remaining β subunit to avian sarcoma virus UR2 *gag* p19 significantly enhanced the protein's transforming potential (49). How-

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ever, 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 the *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.

MATERIALS AND METHODS

Cells and viruses. The preparation of CEF and colony formation assay of virus-infected CEF were done according to published procedures (29). 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 (5, 61–63, 82). 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 previously described (30). 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 (49) 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 to 10 h later and used as the virus stock.

Recombinant plasmid construction. In general, DNA fragments containing deletions or specific mutations were generated by polymerase chain reaction (PCR) performed in a programmable thermocycler (Perkin Elmer-Cetus), using synthesized oligonucleotide primers. The conditions were the same as described previously (49). In all cases, the template used for PCR was plasmid pUIGFR (49). The subsequent cloning procedure was done according to standard protocols (69). Oligonucleotide primers used in PCR had the following sequences (nucleotide and amino acid positions for UR2 [63] and IGFR [77] are numbered according to the published sequences [63, 77]; 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′; and 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 to 905) was generated by PCR, using primers DL5 and Sph3. The PCR product was digested with *Sma*I and *Sph*I and then reinserted into the *Sma*I-*Sph*I-digested pBUIGFR-II vector (see below). This plasmid was named pNM1.

Sequence encoding aa 870 to 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 to 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*I-*Bam*HI and *Bam*HI-*Sph*I, respectively, and ligated into *Bgl*II-*Sph*I-digested pBUIGFR-II vector. This construct, named pNM3, contains artificial *Bgl*II and *Bam*HI 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 *Eco*RI 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.

The prototype vector, pBUIGFR-II, containing a nonpermuted viral genome was constructed as follows. pUIGFR (49) was digested with *Nhe*I and *Eco*RI, and the fragment containing the viral DNA was isolated and ligated into *Spe*I-*Eco*RI-cut pBluescript SK(+) (Stratagene); the resulting plasmid, pBUIGFR, contains a permuted proviral genome with a long terminal repeat located upstream of the *gag*-IGFR fusion gene. An *Nru*I-*Kpn*I 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. The PCR fragments containing the mutations in the four plasmids were confirmed by sequencing.

Antibodies. Production and characterization of rabbit antiserum αIB, specific for the IGFR β subunit, have been described elsewhere (49). Monoclonal antiphosphotyrosine antibody PY20 was purchased from ICN; 4G10 (immunoglobulin G2bK) was purchased from Upstate Biotechnology, Inc. (UBI). 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, biological assays, and protein analysis. The plasmids (10 μg) containing nonpermuted proviral genomes were used directly for transfection into CEF. The transfection procedure and selection for transformed CEF were done according to published protocols (35, 38, 49). Biological assays for cellular transformation and tumorigenicity as well as analysis for protein expression and tyrosine kinase activity were done as described previously (19, 35, 49). Prior to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), proteins were resuspended in Laemmli sample buffer containing 2-mercaptoethanol (49). To examine whether the truncated IGFR fusion protein can form covalent dimers, 2-mercaptoethanol was omitted from the sample buffer as described previously (47).

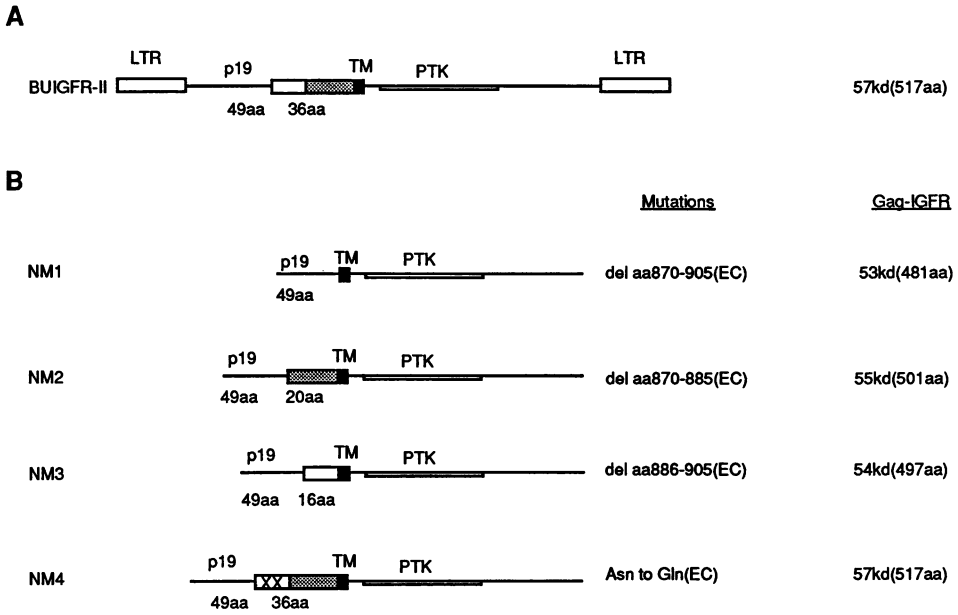

FIG. 1. Structures of *gag*-IGFR mutant plasmids. (A) pBUIGFR-II, which contains a nonpermuted viral DNA insert and was used as a vector for construction of the NM mutants. Only the viral insert is shown. The N-terminal 49 aa of UR2 *gag* p19 and 36 aa of the EC sequence immediately upstream of the TM domain of IGFR are indicated. The blank box within the 36-aa region represents the 16 aa residues (aa 870 to 885) that include two N-linked glycosylation sites. The shaded box represents the 20 aa residues (aa 886 to 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, mutations of the two N-linked glycosylation sites.

PI 3-kinase assay. The PI 3-kinase assay was done essentially as described previously (24, 26), with slight modification. Transformed CEF were lysed in Nonidet P-40 (NP-40) buffer (20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 100 mM NaF, 50 mM sodium PP_i). After clearing of the cell lysates by centrifugation at 12,000 × *g* for 10 min, the supernatant was incubated with αIB antiserum (1:1,000 dilution) for 2 h at 4°C. Then 15 μl of protein A-agarose beads (Repligen) was added, and the mixture was incubated for 1 h at 4°C. The immunoprecipitates were washed as described previously (24, 26), and the washed beads were resuspended in 25 μl of TGN buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5 mM EGTA). Ten micrograms of the substrate PI (20 μg/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. Premixed [γ-^{32}P]ATP (10 μCi per assay; NEN) and $MgCl_2$ (final concentration, 20 mM) were then added, and the mixture was incubated at room temperature for 10 min. Formation of PI 3-phosphate (PIP) was linear during this period (see Fig. 5D). PIP was extracted and analyzed on a thin-layer chromatography Silica Gel 60 plate (Merck) exactly as described previously (24).

Sucrose gradient sedimentation. Cell lysates were prepared as described above, layered onto 5 to 20% continuous sucrose gradients, and centrifuged in a Beckman SW50.1 rotor at 46,000 rpm for 7.5 h as described previously (24). The gradients were divided into 10 fractions, and each fraction was diluted with NP-40 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 (Bethesda Research Laboratories) were also sedimented through parallel 5 to 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 SDS–8% polyacrylamide gels.

RESULTS

Construction of mutants. We have shown previously that UIGFR virus containing a *gag*-IGFR fusion gene is strongly transforming in vitro but cannot induce tumors in vivo (49). A *gag*-IR-containing virus, UIR, was found to become tumorigenic only after deletion of the EC 44 aa upstream of the IR transmembrane (TM) domain (66). 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. 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. 1; see Materials and Methods for details).

In previous studies (49, 66, 83), vectors containing permuted proviral DNA were used. To facilitate DNA transfection and increase the transfection efficiency, plasmid vector

TABLE 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 (49). 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 indicates 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.

[e] —, see reference 49.

[f] Viruses recovered from tumor tissues induced by NM1 and NM3, respectively (see Materials and Methods).

pBUIGFR-II containing a nonpermuted proviral genome was constructed (Fig. 1A).

Biological properties of mutant UIGFR retroviruses. Ten micrograms of plasmid pBUIGFR-II, pNM1, pNM2, pNM3, or pNM4 was transfected into primary CEF together with 1 μg of *Sac*I-digested plasmid DNA of the helper virus UR2AV (62). pBUIGFR-II- and pNM1-transfected CEF were morphologically transformed about 2 weeks after transfection. The procedure included three to four passages and two soft agar overlays to promote growth of the transformed cells. The other three mutant-transfected CEF developed uniform morphological transformation about 3 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 (29). All four mutants induced colony formation in soft agar medium (data not shown) with morphologies similar to those of UIGFR- and UIR-induced colonies (49, 83). 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 1). Our earlier study has shown that UIGFR cannot induce tumors in vivo even though it causes obvious cell transformation in vitro (49). 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 1). The NM1 and NM3 viruses were found to induce tumors about 3 weeks after virus injection (Table 1). No tumors developed in chicks injected with the NM2 or NM4 virus 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 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.

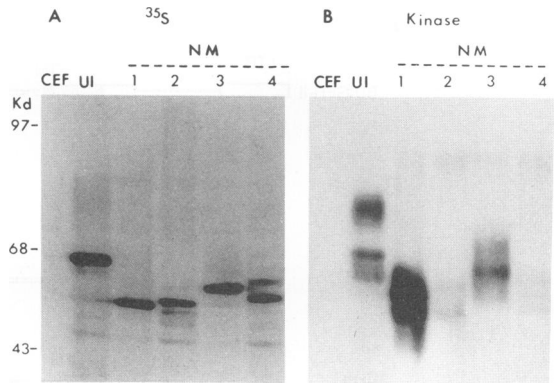

FIG. 2. Expression and in vitro tyrosine kinase activities of *gag*-IGFR proteins. CEF were metabolically labeled with [^{35}S]methionine for 4 h. Cells were lysed with NP-40 buffer (see Materials and Methods). Proteins in the lysates were quantitated with Bradford assay solution (Bio-Rad), and same amount of the cell lysates was immunoprecipitated with αIB antiserum. The immunoprecipitates were washed three times with the NP-40 buffer. For each sample, one half of the immunoprecipitate was resuspended in Laemmli sample buffer and analyzed directly on an SDS-8% polyacrylamide 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 of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) for 10 min at room temperature and terminated by adding 1 ml of NP-40 buffer. The reaction mixtures were centrifuged, and the precipitates were resuspended in Laemmli sample buffer and analyzed on an SDS-8% polyacrylamide 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 15 h (A) and 5 h (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 are on the left.

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. 2A). Several larger 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 75 kDa in addition to the expected P57 in UIGFR-infected cells (49). 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

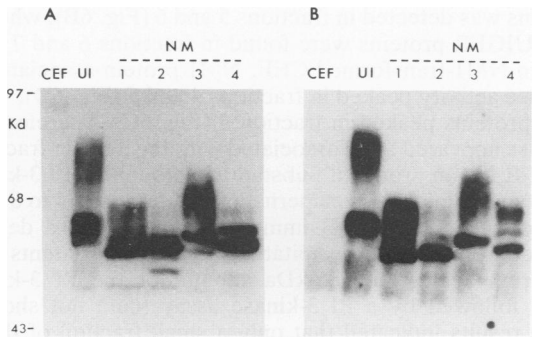

FIG. 3. Tyrosine phosphorylation of *gag*-IGFR proteins. CEF were lysed in NP-40 buffer, and equal amounts of cell lysates determined as described in the legend to Fig. 2 were immunoprecipitated with αIB. The immunoprecipitates were washed and resuspended in Laemmli sample buffer. Equal amounts were loaded into duplicate wells on an SDS–8% polyacrylamide 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 for Fig. 2. UI, UIGFR.

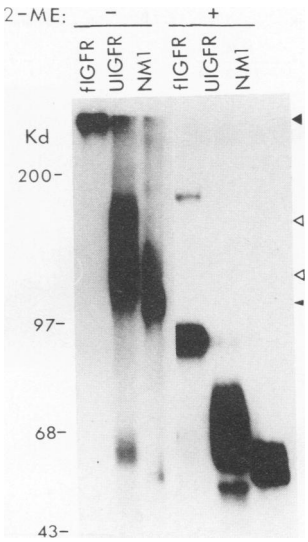

FIG. 4. Oligomerization of *gag*-IGFR proteins. Transformed CEF were lysed, and cell lysates were immunoprecipitated with αIB. The kinase reaction was carried out with resuspended immunoprecipitates. After the kinase reaction, immunoprecipitates were divided into two parts; one half was resuspended in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

also account for the heterogeneity of $P55^{NM2}$ and $P57^{NM4}$. However, the slower-migrating species of NM3 proteins could be due to glycosylation 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 fourfold higher than that of $P57\text{-}75^{UIGFR}$ (Fig. 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 four- to fivefold 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 twofold 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. 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. 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 half-lives of about 1 h (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 transmembrane topology similar to that of native IGFR (49). 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 an in vitro autokinase assay and analyzed by SDS-PAGE under nonreducing conditions. The electrophoretic mobility of the UIGFR and NM1 products shifted from monomer to dimer positions in the absence of a 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). 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. Two- to fourfold more PI 3-kinase activity was present in the receptor immunoprecipitates of extracts from IGFR transformed cells in comparison with normal CEF (Fig. 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 (49). 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. 5B), the UIGFR-, NM1-, and NM3-encoded IGFR proteins were found to have associated PI 3-kinase activities three- to fourfold 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. 5C, hatched bars). The reaction condi-

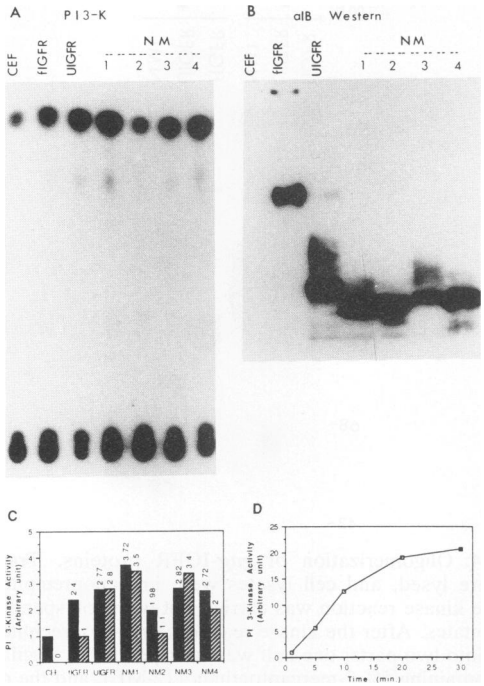

FIG. 5. Association of PI 3-kinase activity with *gag*-IGFR proteins. CEF were lysed, and equal amounts of cell lysates were immunoprecipitated with αIB. Immunoprecipitates were washed extensively (see Materials and Methods). Half of the immunoprecipitates were used for the PI 3-kinase assay, and PIP 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 2×10^6 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).

tions used in our assays were in the linear range of PI 3-kinase activity (Fig. 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 (24). Peak PI 3-kinase activity associated with the UIGFR transforming proteins was detected in fractions 5 and 6 (Fig. 6B), whereas peak UIGFR proteins were found in fractions 6 and 7 (Fig. 6A). In NM1-transformed CEF, NM1 protein-associated PI 3-kinase activity peaked in fractions 4 and 5 (Fig. 6D), while NM1 proteins peaked in fraction 6 (Fig. 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 reimmunoprecipitation of the supernatants with antiserum against the 85-kDa subunit of the PI 3-kinase (UBI) followed by a 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 is not solely responsible for the tumorigenicity of NM1 and NM3, since there was little difference among these two mutants and the nontumorigenic 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 (49, 66, 83) (Fig. 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. 7). Proteins of 35, 120, 140, 160, and 170 kDa seem to be preferentially tyrosine phosphorylated, as detected by monoclonal antibody PY20. Immunoblotting with monoclonal antibody 4G10 (immunoglobulin G2bK; 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. 7). No significant quantitative difference in tyrosine phosphorylation of cellular proteins was observed among the *gag*-IGFR mutants.

DISCUSSION

Oncogenes with intrinsic PTKs, such as *erbB*, *fms*, *kit*, *met*, and *trk*, are derived from the transmembrane receptors for epidermal growth factor (EGF), colony-stimulating factor 1, mast cell growth factors, hepatocyte growth factors, and nerve growth factors, respectively (8, 33, 41, 84). 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* (17, 46) and v-*ros* (61, 63) 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 (49, 83). Further deletion of the entire IR EC sequence led to full-fledged transforming and tumorigenic activity of the IR fusion protein (66). 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,

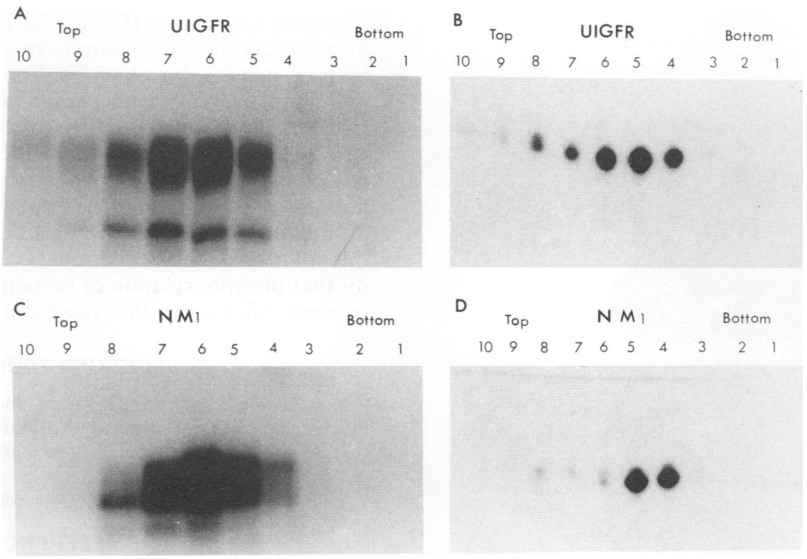

FIG. 6. Cosedimentation of PI 3-kinases with *gag*-IGFR proteins. UIGFR (A and B)- and NM1 (C and D)-transformed CEF (10^7 cells) were lysed in 0.5 ml of NP-40 buffer. Cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C to remove nuclei and protein aggregates. The supernatants were layered on top of a 5 to 20% sucrose gradient and sedimented by ultracentrifugation at 46,000 rpm in an SW50.1 rotor for 7.5 h at 4°C. The sucrose gradient was fractionated into 10 tubes, diluted with NP-40 buffer, and then immunoprecipitated with αIB. One-fifth of the αIB immunoprecipitates was used for the autokinase reaction; the other four-fifths was used for the PI 3-kinase assay. Autokinase reaction products from each fraction were analyzed on an SDS–8% polyacrylamide 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 reimmunoprecipitated with anti-p85 antiserum. Those immunoprecipitates were assayed for PI 3-kinase activity and analyzed as described in the text.

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, transforming, and tumorigenic activities of the *gag*-IR fusion protein (66). 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 (20 aa) from the *gag*-IGFR protein relieved the inhibitory effect on its tumorigenic activity. Our results imply that those EC sequences also play a role in 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 four- to fivefold-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 (43).

Dimerization was proposed to be an early step of activation of EGF and plate-derived growth factor receptors leading to their intermolecular autophosphorylation (13, 31, 45, 47, 88). Transphosphorylation between receptor molecules has also been suggested as a mechanism for IR activation (6, 45). Since both of the truncated IGFR fusion proteins encoded by UIGFR and NM1 formed covalent dimers, it appears 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 differential ultracentrifugation experiments (49; data not shown).

PI 3-kinase has been suggested to be a common signaling component associated with several receptor and nonreceptor PTKs (7, 24, 39). Elevated levels of PIP were shown to correlate with cell transformation and oncogenesis by *src* and polyomavirus middle T antigen (48, 51, 73, 87). In normal cells, stimulation with colony-stimulating factor 1, EGF, platelet-derived growth factor, or insulin through their cognate receptors is accompanied by increased receptor-associated PI 3-kinase activity (4, 7, 20, 32, 37, 39, 52, 53, 67, 74, 79, 88). 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 three- to fourfold 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

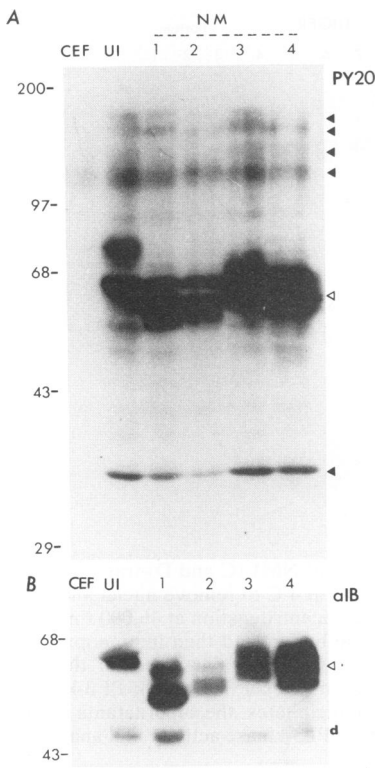

FIG. 7. Tyrosine phosphorylation of cellular proteins. CEF were incubated in medium containing 200 μM sodium orthovanadate for 4 h before being subjected to protein extraction with buffer containing 10 mM Tris-HCl (pH 7.5), 1% SDS, 1 mM Na_3VO_4, 1 mM sodium molybdate, 1% Trasylol (aprotonin; FBA Pharmaceuticals), and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were boiled for 1 min, then mixed with 2× Laemmli sample buffer, and boiled for another 4 min. The protein extracts were separated on an SDS–8% polyacrylamide gel, electrotransferred to nitrocellulose, and immunoblotted with PY20 (A) or αIB (B). Immunoblotting of the protein extracts with 4G10 (immunoglobulin G2bK; 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 the corresponding NM mutants. Sizes are indicated in kilodaltons.

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 (24–26). It was noticed that the PI 3-kinase activity was associated with the heavier fractions of the *gag*-IGFR proteins, suggesting complex formation between 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, the most prominent being proteins of 35, 120, 140, 160, and 170 kDa. However, neither qualitative nor quantitative differences in this pattern were observed among the mutants. Cellular proteins of 120 and 240 kDa have been previously characterized as common substrates for receptors of insulin, EGF, and insulinlike growth factor I (1, 23, 36, 65). An 185-kDa protein has also been reported to be a common substrate for IR and IGFR (36). Interestingly, another 185-kDa protein (insulin substrate receptor 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 (12, 70, 75, 85). 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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