Structurally Related Class I and Class II Receptor Protein Tyrosine Kinases Are Down-regulated by the Same E3 Protein Coded for by Human Group C Adenoviruses

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Abstract. Receptor tyrosine kinases (RTKs) are grouped into subcategories based on shared sequence and structural features. Human group C adenoviruses down-regulate EGF receptors, which are members of the class I family of RTKs, during the early stages of infection. Adenovirus appears to utilize a nonsaturable intracellular pathway since it causes EGF-R down-regulation even in cells that significantly overexpress EGF-R. Adenovirus-induced down-regulation is mediated by a small hydrophobic molecule coded for by the E3 early transcription region that has recently been localized to plasma membrane. Here we examine intracellular trafficking of other RTKs in adenovirus-infected cells, to better understand the molecular basis for the action of the E3 protein. Although $p185^{c\text{-}neu}$, which is a class I RTK closely related to the EGF receptor, is down-regulated in cells expressing physiological concentrations of this molecule, it is not down-regulated in tumor cell lines that significantly overexpress $p185^{c\text{-}neu}$. Cell surface receptors for insulin and IGF1, which are class II RTKs, are also reduced in cells expressing the E3 protein, although to a slightly lesser extent than the EGF receptor. Moreover, whereas EGF receptors are degraded between 3- and 9-h postinfection, insulin and IGF1 receptors are degraded between 6- and 12-h postinfection under identical conditions. In contrast to the class I and class II RTKs, there is no difference in the expression of the class III receptors for PDGF and aFGF in cells infected with a virus with an intact E3 region versus a virus mutant with an internal deletion in the relevant E3 gene. These results suggest that the E3 protein provides an internalization and degradative sorting signal for some class I and class II RTKs, although down-regulation of class II RTKs is somewhat less efficient. Molecular recognition of class I and class II RTKs during adenovirus infection may not be due strictly to amino acid structure, however, since EGF-R but not $p185^{c\text{-}neu}$ is down-regulated in cells where it is significantly overexpressed.

PROTEIN transport and sorting are essential processes for many cellular functions. Understanding molecular interactions that regulate intracellular protein traffic is therefore a fundamental question in cell biology. The subject of this study is an integral membrane protein synthesized by group C human adenoviruses that regulates trafficking of the EGF receptor (EGF-R).[1] EGF-Rs are rapidly cleared from the cell surface and degraded in cells expressing this protein (7, 25). Adenovirus appears to use a nonsaturable intracellular pathway, since it causes EGF-R down-regulation even in cells that significantly overexpress EGF-R (26). Although stability of nascent EGF-Rs is not affected, it is unclear whether EGF-Rs in the biosynthetic pathway actually reach the cell surface, or are diverted to a degradative pathway from a post-Golgi compartment (26). The adenovirus protein responsible for this effect is coded for by the E3 early region (7, 52), and has an approximate molecular weight of 13.7 kD. Several lines of evidence suggest that the 13.7-kD protein has two membrane-spanning regions, and that the NH_2- and COOH termini both reside in the cytosol (27, 33). The NH_2-terminal membrane-spanning region is cleaved in the ER, giving a product of approximate molecular weight of 11.3 kD. This reaction is incomplete, however, and both proteins are detected during early infection (27, 33). At present, it is unclear which is the bioactive form of the molecule.

The EGF-R is one member of a family of cellular receptors with intrinsic tyrosine kinase activity. Ligand activation of the receptor catalytic domain is essential for signal transduction (reviewed in reference 55). Although all receptor tyrosine kinases (RTKs) have homologous cytosolic tyrosine kinase catalytic domains (55), members of this protein family have been grouped into subcategories based on other shared sequence and structural features. EGF-R is a prototypic class I RTK, consisting of a single polypeptide chain

1. *Abbreviations used in this paper*: aFGF-R, aFGF receptor; EGF-R, EGF receptor; IGF1-R, IGF1 receptor; IN-R, insulin receptor; PDGF-R, PDGF receptor; pfu, plaque-forming units; pi, postinfection; RTK, receptor tyrosine kinase; Tfn-R, transferrin receptor.

and a cysteine-rich extracellular ligand-binding domain (56). Other class I RTKs include the proto-oncogene p185$^{c\text{-}neu}$ (also called *erb*B2) (11), and a recently identified molecule called *erb*B3 (35). Class II RTKs are disulfide-linked heterotetramers comprised of two α- and two β-subunits; the cysteine-rich α-subunits are extracellular and bind ligand, whereas intrinsic tyrosine kinase activity resides in membrane-bound β-subunits. Receptors for insulin (57) and insulin-like growth factor 1 (IGF1) (58) fall into this category. Class III RTKs are characterized by an extracellular ligand-binding domain with immunoglobulin-like repeats, and a cytosolic tyrosine kinase domain interrupted by a hydrophilic insertion of variable length. Class III RTKs include receptors for PDGF (62), colony stimulating factor-1 (12), and FGF (18).

In most cases, ligand-bound RTKs are internalized through clathrin-coated pits (3, 43), although IN-Rs also enter the endocytotic pathway via non-coated micropinocytotic invaginations in some cell types (22). Numerous studies have shown that RTK cytoplasmic domains contain signals important for efficient internalization (8, 13, 44, 45, 51). Moreover, interactions between adjacent cytoplasmic domains may also contribute to the structure of internalization signals, since class I and class III RTKs form oligomers after binding ligand (55). Once inside the cell, the post-endocytotic itinerary of RTKs is variable. EGF-Rs bound to EGF, for example, are mostly sorted to lysosomes (3), whereas transforming growth factor α/EGF-R complexes are more likely to recycle to the cell surface (20). Postendocytotic trafficking pathways are also cell-type dependent. For instance, although EGF-Rs recycle in many cell types (19, 46), there is little if any recycling of this molecule in human fibroblasts (47).

Based on limited sequence similarity between the COOH-terminal cytoplasmic region of 13.7 and 11.3 kD and the EGF-R juxtamembrane domain, we have suggested that the E3 protein causes down-regulation by associating with unoccupied receptors to mimic ligand-induced receptor oligomerization (7, 26). It is also possible that viral protein-receptor aggregates are stabilized by interactions between α-helical domains in the plasma membrane (27, 39). Since receptor oligomerization is a common feature of RTKs, the purpose of this study was to determine whether other receptors were similarly regulated during adenovirus infection. Results presented here show that the 13.7- and 11.3-kD proteins cause accelerated turnover of p185$^{c\text{-}neu}$ in cells that express physiological amounts of this protein, but not in tumor cell lines that significantly overexpress p185$^{c\text{-}neu}$. IN-Rs and IGF-1 receptors (IGF1-Rs) are also down-regulated, while PDGF-Rs and aFGF receptors (aFGF-Rs) are not. We therefore conclude that several members of the RTK protein family contain information making them targets for adenovirus-mediated down-regulation. Molecular recognition of class I and class II RTKs during adenovirus infection may not be due strictly to amino acid structure, however, since EGF-R but not p185$^{c\text{-}neu}$ is down-regulated in cells where it is significantly overexpressed.

Materials and Methods

Cell Lines, Transfections, and Viruses

Human hepatocellular carcinoma-derived N-PLC/PRF/5 and Hep 3B cells, and human fetal lung-derived WI-38 cells, were maintained using MEM supplemented with 10% FBS and 2 mM glutamine. Human breast carcinoma-derived SK-BR-3 and MDA-MB-453 cells were grown in McCoy's 5a medium and L15 medium, respectively; both media were supplemented with 10% FBS and 2 mM glutamine. COS-7 cells (21) were maintained in DME supplemented with 10% FBS and 2 mM glutamine. COS-7 cells were transfected with eukaryotic expression vectors containing either the entire coding sequence of the human EGF-R (56), or of the *bek* FGF-R (18; gift of M. Jaye, G. Crumley, and J. Schlessinger, Rhone-Phoulenc Rorer, King of Prussia, PA), using the *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered calcium phosphate method (32). Virus stocks were propagated in spinner cultures of human KB cells maintained with Joklik's-modified MEM supplemented with 5% horse serum and 2 mM glutamine, and titres were determined by plaque assay using human embryonic kidney 293 cells (24) grown in DME supplemented with 10% FBS and 2 mM glutamine. *in*724 is an adenovirus type 5-adenovirus type 2-adenovirus type 5 recombinant insertion mutant with increased distance between the 3′ splice site of the E3 transcript that encodes the 13.7-kD protein (i.e., message *f*), and the polyadenylation site for the major E3 transcript seen in wild-type virus (message *a*) (10); consequently message *f* is overproduced at the expense of message *a* (4). *dl*753 is a virus mutant with an internal deletion of 207 nucleotides in the 13.7-kD open reading frame (4), and cells infected with *dl*753 do not produce 13.7-kD related proteins (52). Most acute infections were carried out in the presence of arabinofuranosyl-cytosine (20 μg/ml) to enhance early viral protein synthesis (31).

Cross-linking Studies

Cells were incubated with ^{125}I-labeled EGF (8×10^6 cpm/ml, ~sp act = 2×10^8 cpm/μg of protein) in serum-free MEM supplemented with 0.1% (wt/vol) BSA for 15 min at 37°C. Receptor-grade mouse EGF (Toyobo Biochemicals, Osaka, Japan) was labeled with ^{125}I (carrier-free, >350 mCi/ml; New England Nuclear, Wilmington, DE) using the chloramine-T method. Cells were rinsed twice with PBS, and then incubated with 2 mM disuccinimidyl suberate (Pierce, Rockford, IL) in 0.1 M Hepes, pH 7.4, supplemented with 120 mM NaCl, 5 mM KCl, 8 mM glucose, and 1.2 mM $MgSO_4$, for 15 min at room temperature. Disuccinimidyl suberate was quenched by a 5-min incubation with 0.05 M Tris, pH 7.4, at room temperature. Cell lysates prepared using 1% (wt/wt) NP-40 in 0.1 M Tris, pH 6.8, supplemented with 15% (wt/wt) glycerol, 2 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin A, were separated by SDS-PAGE (38); detergents and protease inhibitors were from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Cell Labeling and Immunoprecipitation

Cells were labeled with L-[^{35}S]cysteine (200 μCi/ml; >600 Ci/mmol, New England Nuclear) in cysteine- and serum-free medium as described in figure legends. Enzymatic cell surface labeling with ^{125}I was carried out using lactoperoxidase (Calbiochem-Novabiochem, La Jolla, CA). Cell lysates for immunoprecipitation with receptor antibodies were prepared using 1% NP-40 as described above. Lysates for immunoprecipitation with rabbit synthetic peptide antisera specific for E3 proteins were prepared using 1% (wt/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) SDS in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM PMSF, and 1 μM each of leupeptin and pepstatin A; preparation and characterization of the rabbit antisera are described in references 52 and 53. Immunoprecipitations were carried out with antibodies absorbed onto protein A–Sepharose CL-4B beads (Sigma Chemical Co., St. Louis, MO). The mAb EGF-R1 is specific for human EGF-R (5, 59). The IR-1 (37) and OKT-9 (50) mAbs are specific for human insulin receptor (IN-R) and human transferrin receptor (Tfn-R), respectively, and were obtained from ATCC. IGF1-R and human pp185$^{c\text{-}neu}$ mAbs were purchased from Oncogene Sciences (Manhasset, NY). The PDGF receptor (PDGF-R) was immunoprecipitated with a rabbit polyclonal synthetic peptide antiserum (gift of J. Huang, St. Louis University Medical School, St. Louis, MO). Proteins were eluted from beads by boiling in Laemmli buffer, and separated by SDS-PAGE (38). Gels containing L-[^{35}S]cysteine-labeled proteins were processed for fluorography using En^{3}Hance (New England Nuclear, Boston, MA).

Immunoblot Analysis

Proteins were resolved by SDS-PAGE, and transferred to nitrocellulose electrophoretically according to Towbin et al. (54). Primary and secondary incubations were with the viral protein-specific rabbit antiserum described above (1:50) for 18 h at 4°C, and ^{125}I-labeled protein G (1×10^6 cpm/ml)

for 4 h at room temperature, respectively. Protein G (Calbiochem-Novabiochem) was radiolabeled by the chloramine-T method. A solution of 10 mM Tris, pH 7.4, supplemented with 0.9% (wt/vol) NaCl, 1% (wt/vol) sodium azide, 0.5% (wt/vol) Tween-20, and 1 mM EDTA was used to block and wash blots; reagents were diluted with the same buffer supplemented with 3% fatty acid–free BSA (Sigma Chemical Co.).

Indirect Immunofluorescence

Cells were seeded on coverslips and infected 24 h later using 200 plaque-forming units (pfu) per cell. Coverslips were washed three times with PBS and then fixed with 3.7% paraformaldehyde (EM Reagents; Fort Washington, PA) for 10 min at room temperature. Cells were permeabilized during a second 10-min incubation with 0.2% Triton X-100. Primary staining was carried out for 1 h at 37°C in a humidified chamber using a mAb from Oncogene Sciences Inc. (Manhasset, NY) specific for adenovirus 2 E1A proteins, diluted 1:100. Secondary staining with FITC-coupled goat anti-mouse Ig (Cappel Products, Malvern, PA) diluted 1:500, was for 30 min at 37°C. Reagents were diluted using PBS supplemented with 10% FBS and 0.1% sodium azide. Immunofluorescence was viewed using a Multiphot microscope (Nikon, Melville, NY) equipped with epi-fluorescence optics.

Phosphor Storage Autoradiography

Ionizing radiation from autoradiograms was stored on a phosphor screen with a linear dynamic range covering five orders of magnitude, and re-emitted by exposing the screen to an 88-μm-diam helium-neon laser beam (32; PhosphorImager™, Molecular Dynamics Inc., Sunnyvale, CA). Digitized images were analyzed using the ImageQuant™ software package (Molecular Dynamics). This program averages five measurements of light emission for each pixel location, to give a pixel value which is proportional to the amount of stored radiation.

Radioligand Binding Assays

Receptor grade mouse EGF and human recombinant aFGF (29; gift of M. Jaye) were radioiodinated by the chloramine-T method as described above. Receptor grade porcine ^{125}I-labeled insulin (2,200 Ci/mmol) was purchased from New England Nuclear. Total binding was measured using 5 ng/ml ^{125}I-labeled ligand, and non-specific binding was measured using the same amount of radiolabeled ligand plus 500 ng/ml nonradioactive ligand. Cells bound with ^{125}I-aFGF were pretreated for 15 min with 5 U/ml heparin (Upjohn, Kalamazoo, MI) on ice, to block low affinity binding to cell surface heparin sulfate proteoglycans (41). Cells were incubated with ligand for 30 min at 37°C, or for 45 min at 4°C, and solubilized with 1 M NaOH. For ^{125}I-labeled EGF binding, incubations and washes were carried out using serum-free MEM supplemented with 0.02 M Hepes, pH 7.4, and 0.1% BSA; the same solution adjusted to pH 8.0 was used to measure ^{125}I-labeled insulin binding. All determinations were made in duplicate. Radioactivity was measured in a Cobra Auto-Gamma Counter (Packard Instrs., Meriden, CT).

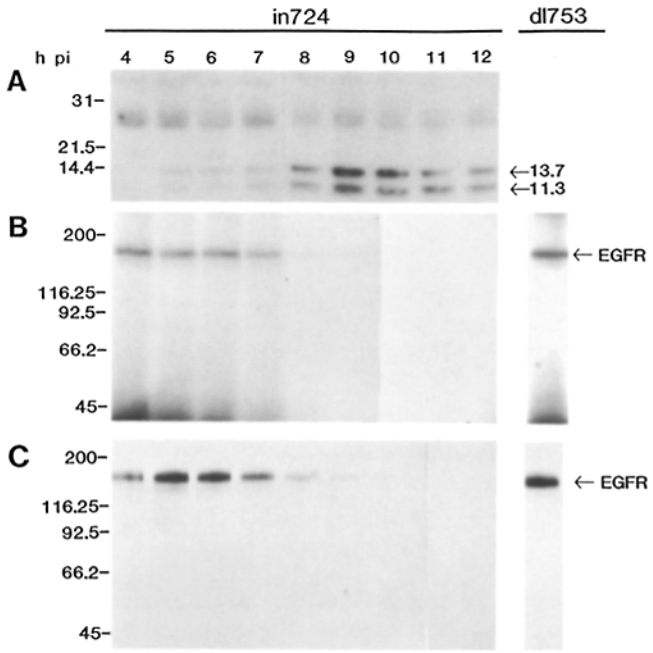

Figure 1. 13.7- and 11.3-kD viral protein expression, and EGF-R internalization and degradation in adenovirus-infected N-PLC/PRF/5 cells. Cells were infected with a virus mutant which overexpresses 13.7 and 11.3 kD (*in*724) using 200 pfu/cell, and harvested at hourly intervals pi. (*A*) Cell lysates were immunoprecipitated using a rabbit antiserum specific for the 13.7- and 11.3-kD proteins. Immunoprecipitates were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose, and immunoblots were probed with the same rabbit antiserum, followed by ^{125}I-labeled protein G; the second reagent also stains IgG light chains from the rabbit antiserum. (*B*) ^{125}I-EGF was cross-linked to *in*724-infected cells using disuccinimidyl suberate, and cell lysates were analyzed by SDS-PAGE; the lane to the extreme right shows results for cells infected with an E3 deletion mutant (*dl*753), and cross-linked with ^{125}I-labeled EGF at 12-h pi. (*C*) Lysates from cells which had been prelabeled for 1 h with L-[^{35}S]cysteine before being infected with *in*724 were immunoprecipitated with EGF-R1; EGF-R stability at 12-h pi in cells infected with *dl*753 is shown at the extreme right. Molecular weight standards: myosin, 200,000; β-galactosidase, 116,250; phosphorylase B, 92,500; BSA, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

Results

13.7- and 11.3-kD E3 Protein Expression during Adenovirus-induced Down-regulation of the Class I RTK EGF-R

Replicate dishes of human hepatocellular carcinoma-derived N-PLC/PRF/5 cells (6) were infected with a virus mutant which overproduces the 13.7- and 11.3-kD proteins (*in*724), so that time courses for viral protein expression and EGF-R down-regulation could be measured simultaneously. The 13.7- and 11.3-kD proteins were first detectable at 5-h postinfection (pi) (Fig. 1 *A*). The steady increase in 13.7- and 11.3-kD protein expression is due in part to the fact that early viral protein expression was enhanced in this experiment with arabinofuranosyl-cytosine (31). In addition, newly synthesized protein accumulates over time since the viral proteins are stable (27). EGF-R down-regulation was measured by examining the cell surface for receptor molecules cross-linked to ^{125}I-labeled EGF (Fig. 1 *B*), and by looking for evidence of increased EGF-R turnover in infected cells which had been prelabeled with L-[^{35}S]cysteine (Fig. 1 *C*). Loss of EGF-R from the cell surface was first detectable between 6 and 7 h pi, and was followed by increased EGF-R degradation ~1 h later. In contrast, there was no change in EGF-R binding capacity or stability when cells were infected using a virus mutant with a internal deletion in the 13.7-kD open reading frame (*dl*753). These data suggest that there is an interval of ~1 to 2 h between the onset of 13.7- and 11.3-kD protein expression, and EGF-R down-regulation. Considering that the 13.7- and 11.3-kD proteins both originate in the ER (27), this lag probably represents the time required for intracellular transit of the viral proteins to the cell surface, where the viral proteins are known to localize (27). These results suggest that 13.7- and 11.3-kD proteins act directly in the plasma membrane, or by causing translocation

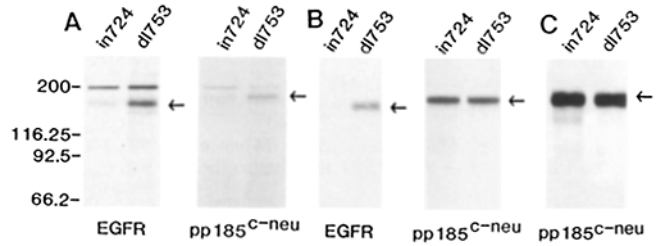

Figure 2. p185$^{c\text{-}neu}$ stability during adenovirus infection. Cells were prelabeled with L-[^{35}S]cysteine for 1 h, and then infected with a virus mutant which overexpresses 13.7 and 11.3 kD (*in*724) or a virus mutant with an internal deletion in the 13.7-kD open-reading frame (*dl*753), using 200 pfu/cell. Cell lysates were prepared at 18-h pi. Lysates were immunoprecipitated with mAbs specific for EGF-R (*A* and *B*) or p185$^{c\text{-}neu}$ (*A*, *B*, and *C*). Aliquots containing equal cpm were immunoprecipitated from cells infected with the two viruses. (*A*) WI-38 cells; (*B*) SK-BR-3 cells; (*C*) MDA-MB-453 cells.

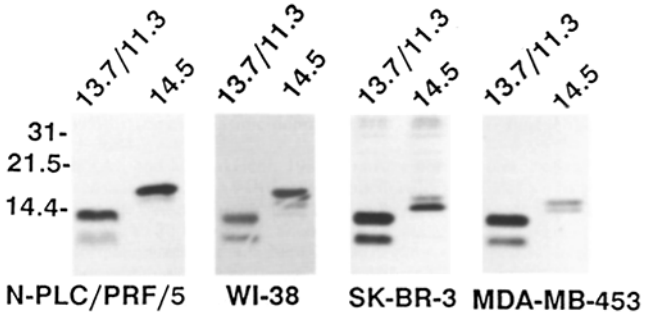

Figure 3. Synthesis of E3-encoded viral proteins following infection with a group C adenovirus. Cells listed in the figure were infected with the virus mutant *in*724 using 200 pfu per cells, and then labeled with L-[^{35}S]cysteine for 5 h starting at 7-h pi for all cells except WI-38, which were labeled starting at 12-h pi. Cell lysates were immunoprecipitated using rabbit antisera specific for either the 13.7- and 11.3-kD proteins; or a second protein encoded by the E3 region referred to as 14.5 K (53).

of sequestered cellular proteins that mediate internalization and degradation of unoccupied EGF-Rs.

Stability of the Class I RTK p185$^{c\text{-}neu}$ in Adenovirus-infected Cells Expressing the E3 13.7- and 11.3-kD Proteins

Expression of the p185$^{c\text{-}neu}$ proto-oncogene during adenovirus infection was examined in normal human diploid fibroblasts and two breast carcinoma-derived cell lines, SK-BR-3 and MDA-MB-453. Compared to human fibroblasts, p185$^{c\text{-}neu}$ is produced at high levels in the SK-BR-3 and MDA-MB-453 cell lines, due to gene amplification and mRNA overexpression (34). There are reports that the adenovirus immediate early E1A protein down-regulates p185$^{c\text{-}neu}$ by transcriptional repression (61). To circumvent the E1A effect, we therefore measured stability of molecules synthesized before infection. When WI-38 cells were prelabeled with L-[^{35}S]-cysteine and then infected with adenovirus, the amount of EGF-R and p185$^{c\text{-}neu}$ present at 24-h pi was significantly reduced in cells expressing the E3 13.7- and 11.3-kD proteins, compared to cells infected with the 13.7-kD deletion mutant (Fig. 2 *A*). In contrast, there was essentially no difference in p185$^{c\text{-}neu}$ protein abundance in either SK-BR-3 (Fig. 2 *B*) or MDA-MB-453 (Fig. 2 *C*) cells infected with the same two viruses. EGF-Rs were, however, down-regulated in the SK-BR-3 cells infected with *in*724, suggesting that the E3 protein was being expressed. Since MDA-MB-453 cells lack EGF-Rs, we also examined infected cells directly for evidence of early viral protein expression. When MDA-MB-453 cells were fixed and stained using a mAb specific for the adenovirus immediate early E1A proteins at 8-h pi, for example, there was intense nuclear immunofluorescence indicative of E1A expression in essentially all of the cells (data not shown). Moreover, all of the cell lines which were examined for p185$^{c\text{-}neu}$ expression synthesized the 13.7- and 11.3-kD proteins, as well as a second E3-encoded protein referred to as 14.5K which is not deleted in *dl*753 (58), shortly after viral infection (Fig. 3). These results suggest that p185$^{c\text{-}neu}$ is down-regulated by the E3 protein, unless it is significantly overexpressed. This is in contrast to previous results showing that adenovirus uses a non-saturable pathway when down-regulating EGF-Rs (26; also see Fig. 8 *A*).

Cell Surface Expression of the Class II RTK Receptors for Insulin and IGF1 in Adenovirus-infected Cells Expressing the E3 13.7- and 11.3-kD Proteins

We first asked whether IN-R or IGF1-R expression was affected by adenovirus, by examining cell surface expression of these two molecules in N-PLC/PRF/5 cells. Cells were infected with *in*724 or *dl*753, and then labeled enzymatically with ^{125}I at 20-h pi. When immunoprecipitates formed using receptor-specific mAbs were compared, surface expression of the receptors for insulin and IGF-1 was diminished in cells expressing 13.7 and 11.3 kD compared to cells infected with the 13.7-kD deletion mutant (Fig. 4). IN-R and IGF1-R surface expression in *in*724-infected cells was not, however, reduced to the same extent as EGF-R. Since cell surface IGF1-Rs are difficult to measure accurately with radiolabeled ligand due to the presence of abundant low-affinity IGF1 binding proteins (2), receptor down-regulation was esti-

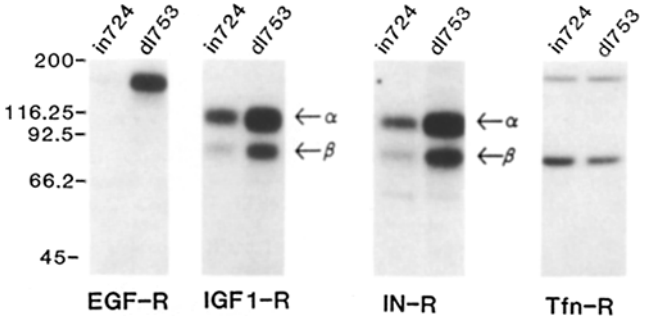

Figure 4. Cell surface expression of IN-R and IGF1-R during adenovirus infection. N-PLC/PRF/5 cells were infected with *in*724 or *dl*753 using 200 pfu/cell. Intact monolayers were labeled enzymatically with ^{125}I at 20-h pi. Cell lysates were immunoprecipitated with mAbs specific for EGF-R, IN-R, IGF1-R, and Tfn-R. Aliquots containing equal cpm were immunoprecipitated from cells infected with the two viruses. In addition to the extracellular IN-R and IGF1-R α-subunits, the β-subunits are also labeled in this protocol due to the presence of several tyrosine residues in the extracellular domain of each of these subunits (57, 58; see also reference 37).

Table I. Quantitation of ^{125}I-labeled Receptor Proteins in Fig. 5 by Phosphor Storage Autoradiography

Receptor	*dl*753*	*in*724*	Normalized *in*724‡	% down-regulation§
				(%)
EGFR	534 ± 16	77 ± 2.4	54	90
INR				
α-subunit	117 ± 8.8	51 ± 1.0	36	69
β-subunit	73 ± 1.4	35 ± 2.6	24	67
IGF1R				
α-subunit	680 ± 16	296 ± 15	207	70
β-subunit	288 ± 10	123 ± 9.5	86	70
TfnR	143 ± 47	203 ± 16	142	–

* Volume integration of pixel values in a defined area with background subtracted. Pixel values are arbitrary units that are proportional to the amount of radiation stored and re-emitted from phosphor screens. Values in the table represent the mean of three independent determinations for each band ±SEM, and have been multiplied by 10^{-3}.
‡ Raw values for *in*724 were normalized assuming equivalent TfnR expression in cells infected with either virus (×0.70).
§ 100 − (normalized value for *in*724 − raw value for *dl*753).

mated using phosphor storage autoradiography (30) to quantitate immunoprecipitates of ^{125}I-labeled receptor proteins. Results for the gel shown in Fig. 4 are given in Table I. The value obtained for the band representing Tfn-R in cells infected with *dl*753 was ~30% less than what was measured for cells infected with *in*724. Values for the other proteins were therefore normalized assuming equivalent Tfn-Rs, since Tfn-R expression is not affected by adenovirus (7). Based on this analysis, cell surface EGF-R expression in cells expressing the 13.7- and 11.3-kD proteins was reduced by ~90%, compared to a 70% reduction for IN-R and IGF1-R. Even without normalization, IN-R and IGF1-R surface expression was down-regulated after infection with *in*724 by more than 50%. Similar results were obtained in three independent experiments with N-PLC/PRF/5 cells, and in a second human hepatocellular carcinoma-derived cell line Hep 3B (not shown).

IN-R and IGF1-R Protein Stability during the Early Stages of Adenovirus Infection

IN-R and IGF1-R protein stability was examined by comparing receptor expression in cells prelabeled with L-[^{35}S]cysteine for 1 h and then infected with adenovirus. Whereas EGF-R degradation occurred between 3- and 9-h pi in cells expressing the 13.7- and 11.3-kD proteins, increased turnover of the IN-R was most evident between 6- and 12-h pi (Fig. 5). In contrast, there was essentially no turnover of EGF-R or IN-R in cells infected with the 13.7-kD deletion mutant. Similar results were obtained when IGF1-R stability during adenovirus infection was examined (Fig. 5). Tfn-R stability was measured as a control for general membrane protein stability; while there was some Tfn-R turnover at 15-h pi, it occurred to an essentially equivalent extent regardless of which virus was used for infection. It therefore appears that loss of surface IN-Rs and IGF1-Rs during the early stages of adenovirus infection is due to increased turnover of these molecules. Degradation of class II RTKs, however, occurs 2 to 3 h after EGF-R degradation in cells expressing the 13.7- and 11.3-kD proteins.

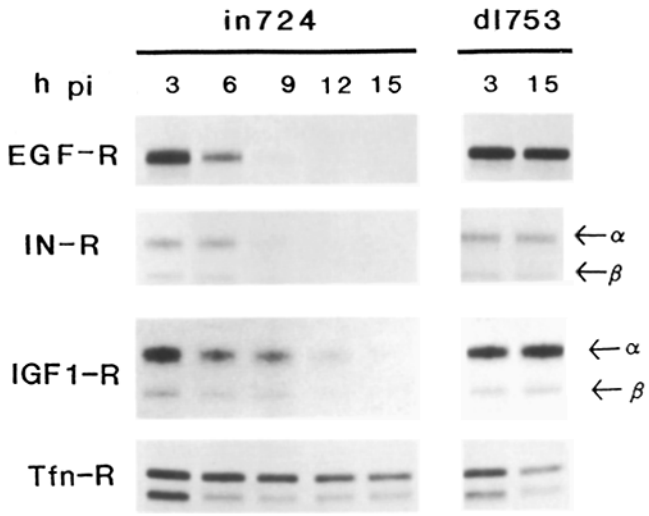

Figure 5. Comparison of EGF-R, IN-R, IGF1-R, and Tfn-R stability during adenovirus infection. N-PLC/PRF/5 cells were labeled with L-[^{35}S]cysteine for 1 h, and then infected with *in*724 or *dl*753, using 200 pfu/cell. Cell lysates prepared at the times indicated in the figure were immunoprecipitated for EGF-R, IN-R, IGF1-R, or Tfn-R.

One possible explanation for these results is that class II RTKs are somehow "dragged" into a degradative pathway when EGF-Rs are down-regulated in cells expressing the 13.7- and 11.3-kD proteins. We therefore asked whether IN-R surface expression was regulated by adenovirus if EGF-Rs were down-regulated by ligand before infection. This was achieved by stimulating uninfected N-PLC/PRF/5 cells with a saturating concentration of nonradioactive EGF (100 ng/ml) for 1 h, and then replenishing the medium with the same concentration of EGF every 3 h during subsequent infection. ^{125}I-labeled EGF binding capacity was reduced by >90% in cells infected with *dl*753 receiving this treatment for 18 h (Fig. 6, *top*). When a trace amount of ^{125}I-labeled EGF was added to infected cells 18-h pi, as expected EGF-R binding capacity was reduced by >90% in cells infected with *in*724, compared to cells infected with *dl*753 (Fig. 6, *top*); there was no significant reduction in binding by *in*724-infected cells treated with saturating amounts of nonradioactive EGF. When IN-R binding capacity was measured in cells infected with *in*724, the reduction in surface IN-Rs was essentially the same regardless of whether cells had been stimulated with nonradioactive EGF to down-regulate EGF-R independent of adenovirus infection (Fig. 6, *bottom*). The slight difference in ^{125}I-labeled insulin binding by *dl*753-infected cells plus and minus EGF was not statistically significant. Taken together with results showing that EGF-R and IN-R are degraded with slightly different kinetics during adenovirus infection (Fig. 5), these findings suggest that adenovirus-mediated down-regulation of EGF-R and IN-R are independent events.

Expression of Class III RTKs during Adenovirus Infection

The class III RTK PDGF-R is expressed by a variety of cells of mesodermal origin, including human diploid fibroblasts. We therefore infected human fetal lung-derived WI-38 cells with a wild-type group C recombinant adenovirus which ex-

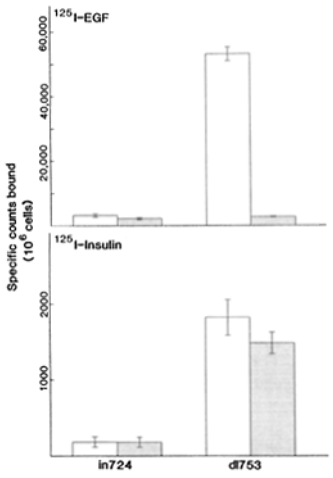

Figure 6. ^{125}I-EGF and ^{125}I-insulin binding by N-PLC/PRF/5 cells infected with adenovirus. Cells were infected with *in*724 or *dl*753, using 200 pfu/cell (□, −EGF). Duplicate dishes were treated with 100 ng/ml nonradioactive EGF for 1 h prior to infection, and every 3 h thereafter (▨, +EGF). Binding assays were carried out at 18 h pi; non-specific binding was measured using a 500-fold excess of nonradioactive ligand, and was subtracted from total binding to give specific binding. All measurements were done in duplicate; the SEM bars in the figure are sums of SEMs for total and non-specific binding.

presses 13.7 and 11.3 kD (*rec*700), and the 13.7-kD deletion mutant (*dl*753). When infected monolayers were labeled enzymatically with ^{125}I at 20-h pi, EGF-R was clearly down-regulated in cells expressing the 13.7- and 11.3-kD proteins (Fig. 7). There was, however, essentially no difference in cell surface PDGF-R expression in WI-38 cells infected with either virus. Experiments to measure RTK stability during adenovirus infection showed that 13.7- and 11.3-kD expression also caused turnover of EGF-R but not PDGF-R (data not shown).

Another member of the class III RTK family, the FGF-R, was also examined for down-regulation during adenovirus infection. In these experiments, COS-7 cells were transfected with a eukaryotic expression plasmid containing the cDNA for human *bek* FGF-R regulated by the human CMV promoter to allow for transient expression, and then infected with adenovirus 48 h later. COS-7 cells were used because they lack detectable levels of endogenous FGF-R (unpublished result), and also are efficient hosts for human adenoviruses since SV-40 T antigen provides a helper function for adenovirus growth in monkey cells (31). To demonstrate the feasibility of this approach, we first transfected COS-7 cells with a human wild-type EGF-R cDNA also regulated by the human CMV promoter. When cells transfected with the EGF-R cDNA were infected with adenovirus, EGF-Rs were clearly down-regulated in cells expressing the E3 protein despite being overexpressed compared to the endogenous monkey EGF-R (Fig. 8 A). In COS-7 cells transfected with a plasmid coding for *bek* FGF-R (18), the specific cpm bound by cells infected with the virus mutant that overproduces 13.7 and 11.3 kD was not significantly different than the value obtained for cells infected with the 13.7-kD deletion mutant (Fig. 8 *B*). In this experiment, endogenous EGF-Rs were also measured to control for any adverse effects on the function of the E3 protein due to transfection. Similarly, endogenous EGF-Rs but not transiently expressed *bek* FGF-Rs were degraded in COS-7 cells expressing the 13.7- and 11.3-kD proteins (data not shown). Taken together with results for PDGF-R, these experiments show that the 13.7- and 11.3-kD proteins do not down-regulate either of these class III RTKs.

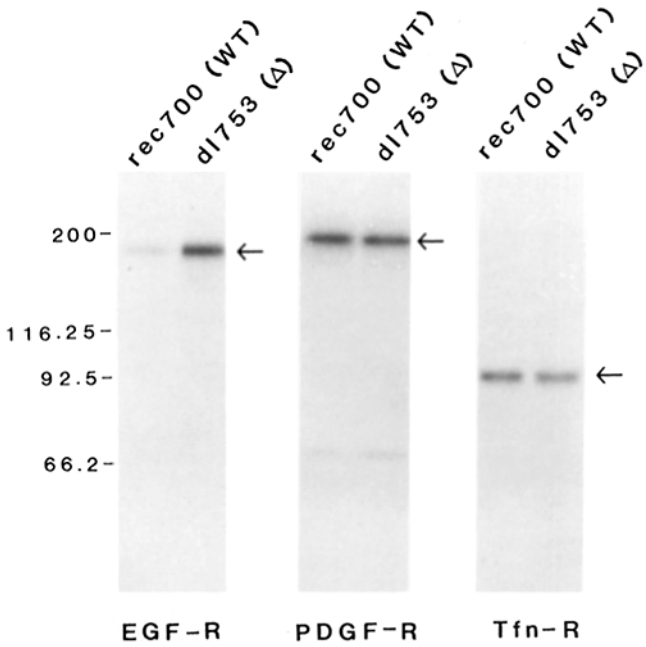

Figure 7. Cell surface expression of the PDGF-R during adenovirus infection. WI-38 cells were infected with the wild-type group C virus *rec*700 or *dl*753, using 200 pfu/cell. Cells were enzymatically labeled with ^{125}I at 20-h pi, and cell lysates were immunoprecipitated with mAbs specific for EGF-R or Tfn-R, or a rabbit polyclonal antiserum specific for PDGF-R; aliquots containing equal cpm were immunoprecipitated from cells infected with the two viruses.

Discussion

We show here that in addition to EGF-R, p185$^{c\text{-}neu}$, IN-R, and IGF1-R enter a degradative pathway during the early stages of adenovirus infection independent of ligand. Down-regulation of these RTKs is linked to expression of an adenovirus region E3-encoded 13.7-kD protein that localizes to the plasma membrane (27). A second proteolytically derived 11.3-kD protein is also expressed at the cell surface (27, 33), and it is currently unclear which form of the molecule is bioactive. We have shown previously that the kinetics of EGF-R down-regulation is positively regulated by the amount of viral protein expressed (26), suggesting that there may be a stoichiometric relation between these two molecules. Based on the subcellular localization of the 13.7- and 11.3-kD proteins (27) and the short interval between E3 protein synthesis and RTK degradation (Fig. 1), it is likely that these viral proteins act directly in the plasma membrane. Local changes in the density of an accessory molecule have been shown to be sufficient to initiate acetylcholine receptor clustering at neuromuscular junctions during synaptogenesis (48). Perhaps the E3 proteins studied here behave similarly, although there must still be a mechanism that accounts for common recognition of class I and class II RTKs. Although EGF-Rs are better targets for adenovirus-mediated down-regulation than the class II RTKs, the ability of these molecules to be down-regulated by a common mechanism further distinguishes them from the class III RTKs examined here. At present, the molecular basis of this specificity is not clear. We have noted previously that there is limited sequence similarity between the COOH-terminal cytoplasmic region of 13.7- and 11.3-kD and the EGF-R juxtamembrane domain

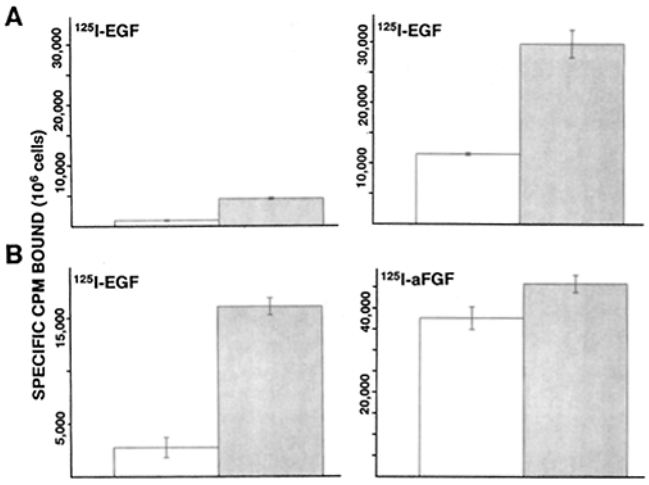

Figure 8. ^{125}I-ligand binding by COS-7 cells infected with adenovirus following transfection with cDNAs coding for human EGF-R or bek FGF-R. (*A*) Untransfected COS-7 cells (*left*) or COS-7 cells transfected with a cDNA encoding the wild-type human EGF-R (*right*) were infected with *in*724 (□) or *dl*753 (□) were assayed for ^{125}I-labeled EGF binding capacity. Assays are shown using the same scale to illustrate that the transfected cDNA is overexpressed compared to endogenous monkey EGF-Rs. (*B*) COS-7 cells transfected with a cDNA coding for the *bek* FGF-R were infected with *in*724 (□) or *dl*753 (□), and assayed for ^{125}I-labeled EGF by endogenous EGF-Rs, or ^{125}I-aFGF by transiently expressed aFGF-Rs. Transfected cells were infected 48 h after DNA was added, and assayed for ligand binding 18- to 20-h pi. All measurements were done in duplicate, and SEM bars are indicated in the figure.

(7). Close examination of the corresponding regions of the IN-R and IGF1-R did not reveal any significant sequence homology or shared predicted secondary structure (not shown). We are currently using a molecular approach to more precisely identify which regions in class I and class II RTKs are required for adenovirus-mediated down-regulation.

Internalization recognition signals have been identified in the cytoplasmic tails of several receptors that associate with clathrin-coated pits independent of ligand binding, such as the low-density lipoprotein receptor (9). These signals are characterized by a tyrosyl residue preceded by a cluster of amino acids frequently found in a β-turn (36). It is generally thought that tyrosine recognition signals bind receptors to clathrin-associated proteins. Receptors for EGF and IN, on the other hand, concentrate in clathrin-coated pits after binding ligand (3, 43). Although tyrosine recognition signals are conserved in most RTKs, the only one linked to ligand-induced internalization to date maps to the region surrounding Tyr-960 in the juxtamembrane region of the IN-R β-subunit; IN-R mutants with phenylalanine substituted for Tyr-960 are still internalized, but at a rate that is much lower than for wild-type IN-R (1). The three consensus tyrosine recognition signals located near the EGF-R COOH-terminus (9) are nonessential for ligand-induced internalization (i.e., reference 13). Interestingly, one of the tyrosyl residues in the cytoplasmic tails of the 13.7- and 11.3-kD proteins lies in a predicted β-turn (27). This raises the possibility that the 13.7- and 11.3-kD proteins anchor class I and class II RTKs to clathrin-associated proteins, although it is not currently known whether adenovirus utilizes a clathrin-dependent or -independent pathway. Association with the adenovirus proteins may also unmask previously unidentified cryptic internalization signals shared by class I and class II RTKs. In either case, viral protein–RTK complexes might be stabilized by helix–helix interactions in the plasma membrane (27, 39). It is also possible that sequestered cellular proteins which cause unoccupied RTKs to be down-regulated are translocated to the plasma membrane following 13.7- and 11.3-kD expression.

Interestingly, although EGF-R and p185$^{c\text{-}neu}$ are closely related class I RTKs, p185$^{c\text{-}neu}$ is not a target for 13.7- and 11.3-kD–mediated down-regulation in cells where it is overexpressed. If the endocytotic pathway used by adenovirus is saturable and rate limiting, there is the possibility that p185$^{c\text{-}neu}$ down-regulation is not easy to detect since the cells used in this study significantly overexpress p185$^{c\text{-}neu}$. This seems unlikely, however, since EGF-R down-regulation by adenovirus infection in cells which have very high levels of that molecule is very efficient (27), despite lowered rates of EGF-R internalization in these cells during stimulation with EGF (60). In contrast to EGF-R (15), p185$^{c\text{-}neu}$ is constitutively active in a variety of cells where it is overexpressed (14), including those studied here (unpublished data). p185$^{c\text{-}neu}$ is also constitutively active in transfected NIH 3T3 cells overexpressing chimeric RTKs with EGF-R extracellular domains (16). It may therefore be that the mechanism of action utilized by the 13.7- and 11.3-kD proteins is not compatible with class I RTKs that have high levels of kinase activity.

In contrast to most viral proteins that regulate subcellular routing of RTKs, the E3 proteins studied here are not secreted growth factors that recognize RTK ligand-binding domains (26). One other viral protein has been identified, the E5 transforming protein of bovine papillomavirus (BPV-E5) (17), that has similar structural properties. Like the E3 proteins (27), BPV-E5 is a disulfide-linked dimer found in cell membranes including the plasma membrane (28). It has recently been shown that BPV-E5 associates with the channel protein component of vacuolar H^+-ATPases (23). This raises the possibility that BPV-E5 regulates endosomal pH, so that recycling is favored at the expense of postendocytotic sorting to lysosomes. This would account for the observation that in contrast to adenovirus-infected cells, ligand-bound RTKs are activated but not degraded in cells transfected with BPV-E5–containing plasmids (40, 42).

The purpose served by 13.7- and 11.3-kD expression during adenovirus infection is unclear. Although group C adenoviruses primarily infect pulmonary epithelial cells, there can be widespread dissemination in severe infections, particularly to liver (49). Considering that the E3 transcription region is not obligatory for viral replication (31), RTK down-regulation in these tissues is probably not a signal to increase metabolism in nonproliferating cells during an acute infection. Recent evidence that adenovirus down-regulates EGF-Rs without enhancing intrinsic autophosphorylating activity is consistent with this interpretation (Carlin, C. R., S. Felder, and P. H. Hoffman, manuscript submitted for publication). Alternative possibilities are that there is some benefit derived that helps counter host antiviral defense mechanisms, or that establishes host-tissue tropisms of different adenoviruses. It is also possible that the 13.7- and 11.3-kD proteins are expressed periodically when latent virus is reactivated. If IN-R expression is affected in vivo, it is interesting to consider whether intermittent

adenovirus reactivation is a factor in some cases of episodic glucose intolerance. Since viral proteins often usurp functions of cellular counterparts, the unique structure of these adenovirus proteins suggest that there may be alternative pathways for intracellular RTK trafficking. Understanding physiological states when expression of a similar ligand-independent pathway is beneficial may help explain why these E3 proteins have evolved in human adenoviruses.

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