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The 10,400- and 14,500-Dalton Proteins Encoded by Region E3 of Adenovirus Form a Complex and Function Together To Down-Regulate the Epidermal Growth Factor Receptor

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In adenovirus-infected cells, the epidermal growth factor receptor (EGF-R) is internalized from the cell surface via endosomes and is degraded, and the E3 10,400-dalton protein (10.4K protein) is required for this effect (C. R. Carlin, A. E. Tollefson, H. A. Brady, B. L. Hoffman, and W. S. M. Wold, Cell 57:135–144, 1989). We now report that both the E3 10.4K and E3 14.5K proteins are required for this down-regulation of EGF-R in adenovirus-infected cells. Down-regulation of cell surface EGF-R was demonstrated by results from several methods, namely the absence of EGF-R autophosphorylation in an immune complex kinase assay, the inability to iodinate EGF-R on the cell surface, the formation of endosomes containing EGF-R as detected by immunofluorescence, and the degradation of the metabolically [^{35}S]Met-labeled fully processed 170K species of EGF-R. No effect on the initial synthesis of EGF-R was observed. This down-regulation was ascribed to the 10.4K and 14.5K proteins through the analysis of cells infected with *rec*700 (wild-type), *dl*748 (10.4K$^-$, 14.5K$^+$), or *dl*764 (10.4K$^+$, 14.5K$^-$) or coinfected with *dl*748 plus *dl*764. Further evidence that the 10.4K and 14.5K proteins function in concert was obtained by demonstrating that the 10.4K protein was coimmunoprecipitated with the 14.5K protein by using three different antisera to the 14.5K protein, strongly implying that the 10.4K and 14.5K proteins exist as a complex. Together, these results indicate that the 10.4K and 14.5K proteins function as a complex to stimulate endosome-mediated internalization and degradation of EGF-R in adenovirus-infected cells.

The human adenovirus (Ad) genome is expressed in a temporally regulated manner beginning with expression of the immediate-early genes in the E1A transcription unit (for a review, see reference 16). The E1A 289R protein then transactivates the delayed-early transcription units, E1B, E2, E3, E4, and LI (early). These ca. 28 early proteins carry out a variety of functions that prepare the cell for the efficient synthesis of viral DNA and proteins. Viral DNA replication begins at about 7 h postinfection (p.i.), and then the infection moves into the late stage; late genes encode mainly virion structural proteins.

One of the events which occurs early after infection is the down-regulation of the epidermal growth factor receptor (EGF-R) (11). EGF-R is a transmembrane glycoprotein localized in the plasma membrane, and it is a member of the protein tyrosine kinase class of membrane receptors (for reviews, see references 39 and 46). EGF is a polypeptide growth factor. Binding of EGF to the extracellular ligand binding domain stimulates the intrinsic protein tyrosine kinase activity of EGF-R which is located in the cytoplasmic portion of EGF-R; this results in autophosphorylation of EGF-R on Tyr residues as well as Tyr phosphorylation of a variety of cellular proteins. The EGF/EGF-R complex clusters in clathrin-coated pits which are internalized into endosomes, and the complex is transported to lysosomes, where it is degraded. This process results in EGF signal transduction which in general activates cellular metabolism and eventually induces DNA synthesis and mitosis. The protein tyrosine kinase activity of EGF-R is essential for EGF signal transduction.

Ad infection mimics many aspects of EGF-mediated down-regulation of EGF-R. Specifically, cell surface EGF-R is internalized via endosomes and degraded, presumably in lysosomes (11). This was shown previously (11) by using several methods, including (i) autophosphorylation of EGF-R in an immune complex kinase assay, (ii) cell surface iodination using lactoperoxidase-catalyzed iodination, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), (iii) EGF binding, (iv) metabolic labeling, and (v) an immunofluorescence assay for endosomes. Degradation of EGF-R was demonstrated by pulse-chase experiments. No effect on the initial synthesis of EGF-R was detected. By using virus mutants with deletions in E1A, E1B, E3, and E4, the Ad function responsible for EGF-R down-regulation was mapped to E3 (11). Then, by using a panel of E3 deletion mutants, the E3 10,400-dalton protein (10.4K protein) was shown to be required for EGF-R down-regulation (11). The 10.4K protein was identified in Ad-infected cells and was shown to be a cytoplasmic membrane protein translated from E3 mRNA *f* (37). EGF-R was down-regulated in a mouse cell line expressing the 10.4K protein from a retrovirus vector and in mouse HERc cells (cells stably transfected with human EGF-R) acutely infected with the 10.4K-protein-expressing retrovirus vector, indicating that the 10.4K protein can function autonomously in this system (18).

The E3 14.5K protein is also translated, at least in part, from E3 mRNA *f* (Fig. 1) (36). Like the 10.4K protein, the 14.5K protein is a cytoplasmic membrane protein (21a). The cotranslation of the 10.4K and 14.5K proteins from the same mRNA suggests that there might be an evolutionary functional relationship between the two proteins (36). In this communication, we show that both the 10.4K and 14.5K proteins are required to down-regulate EGF-R in Ad-infected cells. We also show that the 10.4K protein coimmu-

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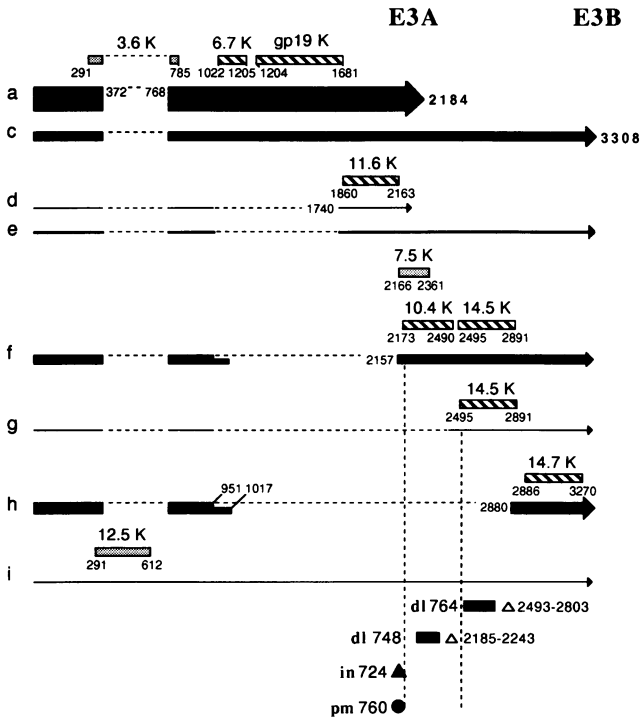

FIG. 1. Schematic illustration of region E3 of *rec*700. Arrows indicate the spliced structures of the mRNAs; the thickness of the arrows reflects the relative abundance of the mRNAs, and the broken lines indicate introns. Bars above the arrows indicate proteins; hatched bars are proteins that have been identified, and stippled bars are proteins that are proposed to exist. Nucleotide (nt) +1 is the transcription initiation site. *rec*700 is an Ad5-Ad2-Ad5 recombinant that has Ad2 sequences from nt −236 to 2437 in the E3 transcription unit. In *rec*700, numbers for the Ad2 E3 transcription unit are used from nt 1 to 2437, and numbers for the Ad5 E3 transcription unit are used downstream of nt 2437 (14). Ad2 nt 2437 is equivalent to Ad5 nt 2482. Bars at the bottom indicate the deletions in *dl*748 and *dl*764. The triangle indicates the 140-bp insertion in *in*724 between nt 2160 and 2161 (4). The large dot indicates the three point mutations in *pm*760 at nt 2163, 2164, and 2166.

noprecipitates with the 14.5K protein, strongly suggesting that the two proteins exist as a complex and consistent with the proposal that they function in concert.

MATERIALS AND METHODS

Cells and viruses. Viruses were prepared in suspension cultures of human KB cells and titers were determined on human A549 cells as described previously (17). A549 cells were maintained in Dulbecco's modified Eagle medium with 10% fetal calf serum.

H5/2*rec*700 is an adenovirus type 5 (Ad5)-Ad2-Ad5 recombinant whose genome consists of the Ad5 *Eco*RI A (map position 0 to 76), Ad2 *Eco*RI D (76 to 83), and Ad5 *Eco*RI C (83 to 100) fragments (45). *rec*700 is the parental virus for the mutants used in this study (Fig. 1). Mutants *dl*748 (3), *in*724, *pm*760 (4), and *dl*764 (37) have been described previously.

In vivo protein labeling, immunoprecipitation of the 10.4K and 14.5K proteins, and SDS-PAGE. KB cells (10^7 total cells, 5×10^5 cells per ml) were infected with 50 PFU of virus per cell, and early proteins were labeled from 7 to 12 h p.i. with 100 μCi of [^{35}S]Cys per ml (ca. 1,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) in Cys-free medium by the cycloheximide-enhanced procedure exactly as described previously (43, 44). Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS) (pH 7.4) and lysed on ice with 0.5 ml of coimmunoprecipitation buffer (0.15 M NaCl, 40 mM Tris-HCl [pH 8.0], 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, and 0.1% SDS containing 1 mM phenylmethylsulfonyl fluoride [PMSF]). Nuclei were removed by centrifugation, and the supernatant (2×10^7 cpm) was analyzed by immunoprecipitation with 5 μl of antiserum and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). Immunoprecipitates were rinsed seven times with high-salt buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.4], 0.5% NP-40, 1% sodium deoxycholate) and then twice with 50 mM Tris-HCl (pH 6.8). Immunoprecipitates were analyzed by SDS-PAGE on 10 to 18% gradient gels (0.75 mm by 16 cm; acrylamide/*N*,*N*′-methylenebisacrylamide ratio, 29.2:0.8, wt/wt). All gels were fluorographed. ^{14}C-labeled molecular weight markers were purchased from Bethesda Research Laboratories, Gaithersburg, Md.

The following rabbit polyclonal antisera were used. The P77-91 antiserum is directed against synthetic peptide P77-91, corresponding to residues 77 to 91 in the 91-residue 10.4K protein of Ad2 (37). The P19-34 and P118-132 antisera are directed against synthetic peptides P19-34 and P118-132, respectively, corresponding to residues 19 to 34 and 118 to 132, respectively, in the 132-residue 14.5K protein of Ad5 (36). The TrpE-14.5K antisera are directed against a TrpE-14.5K fusion protein synthesized in *Escherichia coli*; the fusion protein contains the complete Ad5 14.5K protein sequence (36).

Immunoblot analysis of the 10.4K/14.5K protein complex. KB cells (100 ml, 3×10^5 cells per ml) were infected with 300 PFU of *dl*748, *dl*764, or *pm*760 per cell. At 7 h p.i., 1-β-D-arabinofuranosylcytosine (araC) was added to a concentration of 20 μg/ml. At 12 h p.i., cells were collected, washed with cold PBS, and lysed on ice for 15 min with 1 ml of 10 mM Tris-HCl (pH 8.0)–0.15 M KCl–1.5 mM $MgCl_2$–1 mM dithiothreitol–0.1 mM PMSF-0.5% NP-40. Nuclei were removed by centrifugation, and 0.4 ml of the cytosol was subjected to immunoprecipitation with the P118-132 antiserum specific to the 14.5K protein. Immunoprecipitates equivalent to 1.2×10^6 cells per gel lane were electrophoresed on 10 to 18% gradient SDS-PAGE gels and then electroblotted onto Immobilon polyvinylidene difluoride membranes as described previously (42). Blots were probed either with the P118-132 antiserum to the 14.5K protein or with the P77-91 antiserum specific to the 10.4K protein. The bound antibodies were visualized by using ^{125}I-labeled protein A. Protein A (Sigma) was iodinated with Na^{125}I (ICN Radiochemicals, Inc., Irvine, Calif.) by the chloramine T method (19).

Immune complex protein tyrosine kinase assay. KB cells (10^7 cells, 5×10^5 cells per ml) were either mock infected or infected with 150 PFU per cell of virus. In mixed infections, 150 PFU per cell of each virus was used. Cells were harvested at 24 h p.i. and lysed at 4°C for 15 to 30 min in 0.5 ml of SB buffer (0.1 M Tris-HCl [pH 6.8], 2 mM EDTA, 15% glycerol, 0.034 mg of PMSF per ml, 0.25 μg of leupeptin per ml, 1 μg of pepstatin A per ml) containing 1% NP-40 (8). The nuclei were removed by centrifugation. The leupeptin and pepstatin were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and the PMSF was from Sigma. Autophosphorylation of EGF-R in the immune complex was carried out as described previously (9, 11). All steps were at 4°C. EGF-R was immunoprecipitated with ascites obtained

from mice inoculated with the EGF-R-specific monoclonal antibody-producing cell line EGF-R1 (41). For each kinase reaction, 10 μl of the EGF-R1 ascites were incubated with protein A-Sepharose beads (Sigma) for 1 h on ice. The beads containing adsorbed antibody were washed three times in STN buffer (0.15 M NaCl, 10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 0.034 mg of PMSF per ml, 0.25 μg of leupeptin per ml, 1 μg of pepstatin A per ml, and 0.25% NP-40). The KB cell extracts were precleared with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) and then incubated for 30 to 45 min with the protein A-Sepharose beads containing bound antibody. The beads were washed three times in STN buffer, and the kinase reaction was carried out with the beads in 50 μl of 30 mM NaCl–50 mM $MgCl_2$–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4)–10% glycerol–50 μCi of [γ-^{32}P]ATP (Dupont, NEN) for 5 min. The beads were washed three times in STN buffer containing 0.45 M NaCl, twice in STN buffer, and once in SB buffer lacking glycerol. ^{32}P-labeled EGF-R was dissociated from the immune complex by boiling in ESB buffer (2% SDS, 0.1 M dithiothreitol, 80 mM Tris-HCl [pH 6.8], 1.6 mM EDTA, 12% glycerol, 0.02% bromphenol blue), and it was analyzed by SDS-PAGE on 7.5% gels.

Lactoperoxidase-catalyzed cell surface iodination. KB cells (10^7 cells, at 5×10^5 cells per ml) were infected with 100 to 150 PFU of virus per cell; in mixed infections, each virus was added at 150 PFU of virus per cell. At 10 h p.i., cells were collected, washed three times in cold PBS, and resuspended in 1 ml of cold PBS containing 5 mM glucose, and cell surface proteins were labeled with ^{125}I (20). The following reagents were added: 1 mCi of Na^{125}I (Dupont, NEN), lactoperoxidase (20 mg/ml final concentration) (Calbiochem-Behring), and glucose oxidase (0.1 U/ml final concentration) (Boehringer Mannheim), and the sample was incubated for 10 min at 20°C. One milliliter of PBI (PBS with 0.2 g of NaI per ml) containing 0.034 mg of PMSF per ml, 0.25 μg of leupeptin per ml, and 1 μg of pepstatin A per ml) was added, and then the cells were washed five times with PBI. Cells were lysed in SB buffer containing 1% NP-40, and ^{125}I-labeled EGF-R or transferrin receptor was immunoprecipitated and analyzed by SDS-PAGE on 7.5% gels. A monoclonal antibody (β3/25 from Boehringer Mannheim) to transferrin receptor was used.

^{125}I-EGF binding. A549 cells, maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, were plated into 35-mm dishes at a density of 10^6 cells per dish. The following day, cells were either mock infected or infected with 50 PFU of virus per cell; in mixed infections, 50 PFU of each virus per cell was used. At 18 h p.i., cells were washed three times with warm binding buffer (Dulbecco's modified Eagle medium containing 10 mM HEPES [pH 7.4] and 0.2% radioimmunoassay-grade bovine serum albumin), and EGF binding was assayed as described previously (8). Receptor-grade EGF (Sigma) was labeled with ^{125}I (19). Cells were incubated with 5 ng of ^{125}I-EGF (4.2×10^4 cpm/ng) in 0.5 ml of binding buffer for 1 h at 37°C with periodic agitation. Cells were washed twice with binding buffer, washed twice in binding buffer lacking bovine serum albumin, solubilized in 1 ml of 1 N NaOH, and counted in a Beckman Model 310 gamma counter. Nonspecific binding, which was <10% of specific binding, was determined by using 5 ng of ^{125}I-EGF plus 5 μg of unlabeled EGF; this was subtracted from the specific binding counts.

Metabolic labeling of EGF-R with [^{35}S]Met. KB cells (10^7 cells, 5×10^5 cells/ml) were either mock infected or infected with 150 PFU per cell of virus (150 PFU per cell of each virus in mixed infections) and were labeled with 250 μCi of Tran^{35}S-label (≥70% [^{35}S]Met, 1,112 Ci/mmol, ≤15% [^{35}S]Cys; ICN) from 7 to 11 h p.i. in Met-free Cys-free Dulbecco's modified Eagle medium containing 20 μg of araC per ml. Cells were washed twice in Joklik modified minimal essential medium containing 5% horse serum and then incubated for 3 h in this medium. Cells were lysed, and EGF-R was immunoprecipitated and analyzed by SDS-PAGE as described above for the kinase reaction.

Indirect immunofluorescence of EGF-R. A549 cells growing on glass coverslips were either mock infected or infected with 250 PFU of virus per cell, or with 150 PFU of each virus per cell in the mixed infection, and were fixed for immunofluorescence at 6.5 h p.i. One coverslip of cells was treated with 10 ng of receptor grade EGF (Sigma) per ml 40 min before fixation. Cells were fixed in methanol (−20°C) for 8 min, rehydrated with PBS, and then stained with a 1:200 dilution of an antipeptide antiserum to EGF-R (see below). The second antibody was goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (Organon Teknika, Malvern, Pa.). Coverslips were mounted in polyvinyl alcohol medium containing 1 mg of *p*-phenylenediamine (Sigma) per ml. Photographs were taken on Kodak T-Max 400 film on a Nikon epifluorescence microscope equipped with a 60× Plan apo objective lens. Film was processed by using Diafine developer (Acufine, Inc., Chicago, Ill.).

Peptide P1172-1186 with the sequence CBYLRVAPQSSE FIGA, corresponding to the C-terminal 15 amino acids of EGF-R (the Cys is not part of the EGF-R sequence), was purchased from Multiple Peptide Systems (San Diego, Calif.). This peptide has been shown previously to be immunogenic (24). The peptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring) on an equal-weight basis by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by the manufacturer (Pierce Chemical Co., Rockford, Ill.). Rabbits were immunized with the peptide conjugate as described previously (37).

RESULTS

The E3 10.4K and E3 14.5K proteins are both required for endosome-mediated internalization and degradation of EGF-R in Ad-infected cells. Cell surface EGF-R begins to decline in abundance by about 4 h p.i. of KB cells by group C Ads, and the receptor is undetectable by about 8 h p.i. (11). Figure 2 shows the status of EGF-R in uninfected KB cells and in KB cells infected with *rec*700, *dl*748, or *dl*764. *rec*700 is a wild-type virus in this context, *dl*748 synthesizes the E3 14.5K protein (36) but not the E3 10.4K protein (37), and *dl*764 synthesizes the 10.4K protein (37) but not the 14.5K protein (36). The assay used is an immune complex kinase assay in which cells are lysed with detergent, EGF-R is immunoprecipitated, and a protein tyrosine kinase reaction is carried out in the immune complex by using [γ-^{32}P]ATP (8). Under these conditions, EGF-R autophosphorylates or transphosphorylates. ^{32}P-labeled EGF-R is then extracted from the immune complex and analyzed by SDS-PAGE. As shown in Fig. 2, a strong band of EGF-R was obtained from mock-infected KB cells, as expected (11). EGF-R was undetectable in extracts from *rec*700-infected cells, also as expected (11). This down-regulation (i.e., the absence) of EGF-R in *rec*700 is due to the internalization and degradation of EGF-R in Ad-infected cells (11). In *dl*748 and *dl*764 infections, a strong band of EGF-R comparable to that from mock-infected cells was obtained, indicating that the absence of either the 10.4K or the 14.5K protein abrogates

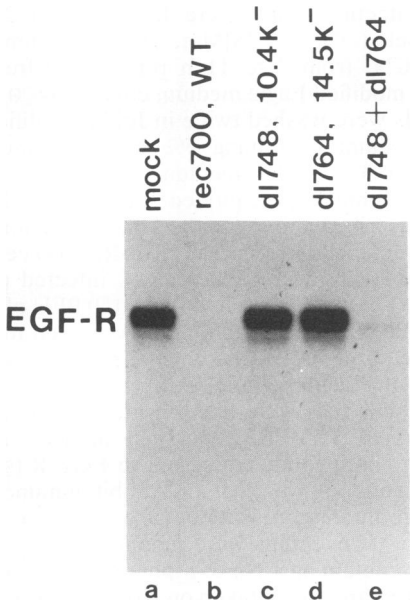

FIG. 2. Immune complex protein tyrosine kinase assay of cells infected with mutants. KB cells were either mock infected or infected with mutants. At 24 h p.i., cells were lysed with NP-40, EGF-R was immunoprecipitated, and a kinase reaction was carried out in the immune complex by using [γ-^{32}P]ATP as label. The ^{32}P-labeled autophosphorylated EGF-R was extracted from the immune complex and analyzed by SDS-PAGE on 7.5% gels. Lanes: a, Mock-infected cells; b, cells infected with 150 PFU per cell of *rec*700, a wild-type (WT) virus in this assay; c, cells infected with 150 PFU per cell of *dl*748, which synthesizes the 14.5K but not the 10.4K protein; d, cells infected with 150 PFU per cell of *dl*764, which synthesizes the 10.4K but not the 14.5K protein; e, cells coinfected with 150 PFU per cell each of *dl*748 and *dl*764.

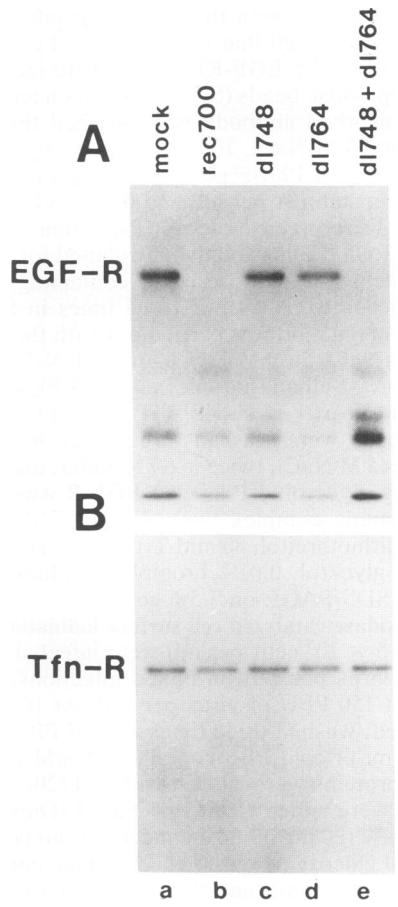

FIG. 3. Down-regulation of radioiodinated EGF-R on the cell surface. KB cells were mock infected (lane a), infected with 150 PFU per cell of *rec*700 (lane b), *dl*748 (lane c), or *dl*764 (lane d), or coinfected with 150 PFU per cell each of *dl*748 and *dl*764 (lane e). At 10 h p.i., cell surface proteins were iodinated with lactoperoxidase and Na^{125}I. Cells were lysed with NP-40, and EGF-R (A) or transferrin receptor (Tfn-R) (B) was immunoprecipitated and analyzed by SDS-PAGE.

down-regulation of EGF-R. However, in a mixed infection with *dl*748 and *dl*764, EGF-R was nearly completely down-regulated (Fig. 2); this indicates that the mutants complement each other and that both the 10.4K and 14.5K proteins are required to down-regulate EGF-R under these conditions.

If cell surface EGF-R has been internalized in response to the Ad proteins, then it should be unavailable for cell surface iodination. Accordingly, KB cells were either mock infected or infected with various mutants, and at 10 h p.i., the cell surface proteins were labeled with ^{125}I by using lactoperoxidase. EGF-R was immunoprecipitated and analyzed by SDS-PAGE. ^{125}I-labeled EGF-R was obtained from mock-infected cells (Fig. 3A, lane a) but not from *rec*700-infected cells (lane b). This is similar to earlier results (11). The ^{125}I-labeled EGF-R band was observed with *dl*748 (Fig. 3, lane c) and *dl*764 (lane d) but not with the mixed infection of *dl*748 and *dl*764 (lane e). This indicates that both the 10.4K and 14.5K proteins are required to internalize cell surface EGF-R. Equal amounts of ^{125}I-labeled transferrin receptor were immunoprecipitated from these same extracts (Fig. 3B), indicating that in each case, the cells had been iodinated efficiently and that the down-regulation of EGF-R that was observed was not a general effect on cell surface receptors.

If cell surface EGF-R has been internalized, then it should be unavailable for binding EGF. Thus, an EGF binding experiment was carried out. KB cells were infected with Ad mutants in the presence of araC, and binding of ^{125}I-labeled EGF was assayed at 24 h p.i. Mock-infected cells and cells infected with *dl*748 or *dl*764 bound 13,890, 14,960, and 14,790 cpm, respectively, of ^{125}I-EGF, whereas cells infected with *rec*700 or *dl*748 plus *dl*764 bound 1,890 and 1,800 cpm, respectively, of ^{125}I-EGF (Fig. 4). These results are consistent with those in Fig. 2 and 3 and indicate that both the 10.4K and 14.5K proteins are required for internalization of cell surface EGF-R.

When cells are treated with EGF, the EGF-EGF-R complex is internalized via endosomes. This also appears to be true in Ad-infected cells, and the 10.4K protein is required for this process (11). To address whether both the 10.4K and 14.5K proteins are required, A549 cells were mock infected, treated with EGF, or infected with mutant viruses and analyzed by immunofluorescence by using an antipeptide antiserum to EGF-R. A striking punctate staining pattern was observed in cells treated with EGF (Fig. 5B) but not in untreated cells (Fig. 5A). The small bright dots within the cells are endosomes containing EGF-R (1, 11). A similar punctate staining pattern was seen in cells infected with *rec*700 (panel C) or with *dl*748 plus *dl*764 (panel F) but not in mock-infected cells (panel A) or in cells infected with *dl*748

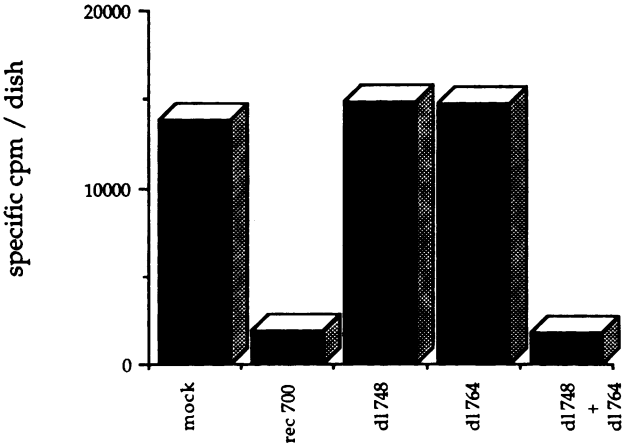

FIG. 4. ^{125}I-EGF binding to cells. A549 cells were mock infected, infected with 50 PFU per cell of *rec*700, *dl*748, or *dl*764, or coinfected with 50 PFU per cell each of *dl*748 and *dl*764. At 18 h p.i., cells were washed, incubated with ^{125}I-EGF, washed again, and solubilized in NaOH, and the counts per minute bound were determined. The specifically bound counts bound were 13,890 ± 1,810 for mock infections, 1,890 ± 130 for *rec*700 infections, 14,960 ± 1,580 for *dl*748 infections, 14,790 ± 1,540 for *dl*764 infections, and 1,800 ± 350 for *dl*748-plus-*dl*764 infections.

(panel D) or *dl*764 (panel E) alone. Inasmuch as the punctate staining pattern seen in EGF-treated cells was indistinguishable from that seen in infected cells, we presume that the punctate staining in infected cells represents endosomes containing EGF-R. Given this, we conclude that both the 10.4K and 14.5K proteins are required for the formation of EGF-R-containing endosomes in Ad-infected cells, and the 10.4K and 14.5K proteins appear to mimic EGF in this respect.

EGF induces internalization and degradation of EGF-R. The data in Fig. 2 through 5 strongly indicate that EGF-R is also degraded in Ad-infected cells. To address this point more directly, a pulse-chase experiment was carried out. Mock-infected or mutant-infected cells were metabolically labeled with Tran^{35}S-label (≥70% [^{35}S]Met) from 7 to 11 h p.i. and then chased for 3 h in excess unlabeled Met. [^{35}S]Met-labeled EGF-R was immunoprecipitated and analyzed by SDS-PAGE. Two bands, of 170K and 160K, were obtained from mock-infected cells (Fig. 6, lane a). The 160K band is the biosynthetic precursor, whereas the 170K band is the fully glycosylated and phosphorylated form found on the cell surface (10, 27). The 170K band was much more abundant in the experiment in Fig. 6, lane a, because most of the 160K band had been chased into the 170K band. The 170K form was nearly absent in cells infected with *rec*700 (lane b), whereas the 160K band was at the same abundance as in mock-infected cells. The near absence of the 170K moiety in *rec*700-infected cells is consistent with the results in Fig. 2 through 5 which indicate that the mature form of the cell surface receptor is degraded. The continued presence of the 160K species indicates that Ad infection has no effect on the initial synthesis of EGF-R. These results and conclusions are in accordance with previous data (11). *dl*748 and *dl*764 gave a pattern of 160K and 170K species similar to that of mock-infected cells, and the coinfection with *dl*748 plus *dl*764 gave a pattern similar to that of infection with *rec*700 (Fig. 6). We conclude that both the 10.4K and 14.5K proteins are required to stimulate endosome-mediated internalization and degradation of cell surface EGF-R.

The 10.4K protein coimmunoprecipitates with the 14.5K protein. The above results indicate that the 10.4K and 14.5K proteins function in concert to down-regulate EGF-R. In accordance with this conclusion, we show below that the 10.4K protein coimmunoprecipitates together with the 14.5K protein, strongly suggesting that the proteins may exist as a complex. *in*724-, *dl*748-, or *dl*764-infected KB cells were labeled with [^{35}S]Cys, and the 10.4K and 14.5K proteins were immunoprecipitated and analyzed by SDS-PAGE. *in*724 overproduces both the 10.4K and 14.5K proteins, *dl*748 overproduces the 14.5K protein but does not make the 10.4K protein, and *dl*764 overproduces the 10.4K protein but does not make the 14.5K protein (36, 37). With *dl*748, approximately six bands (ca. 15 to 18K) corresponding to the 14.5K protein were immunoprecipitated with the P118-132 peptide antiserum directed against the C-terminal 15 amino acids of the 14.5K protein (Fig. 7, lane a). This pattern of 14.5K bands is characteristic of *dl*748 (36). With *dl*764, two strong bands corresponding to the 10.4K protein were immunoprecipitated with the P77-91 peptide antiserum directed against the C-terminal 15 amino acids of the 10.4K protein (lane d). The top 10.4K band is the primary translation product which is processed into the bottom band (21a, 37). When the *in*724 extract was immunoprecipitated by using the 14.5K-protein antiserum, both 14.5K and 10.4K proteins were observed (lane b). Thus, the 10.4K protein was very efficiently coimmunoprecipitated with the 14.5K protein. Interestingly, when this same *in*724 extract was immunoprecipitated with the 10.4K-protein antiserum, the 10.4K protein but not the 14.5K protein was observed (lane c). Thus, whereas the 10.4K protein was coimmunoprecipitated with the P118-132 antiserum to the 14.5K protein, the reverse was not true with the antiserum to the 10.4K protein. Note that the 10.4K protein was not observed with *dl*748 (lane a), which is expected because the gene is deleted in *dl*748.

To prove that the two bands being coimmunoprecipitated with the 14.5K protein are in fact the 10.4K protein, unlabeled protein extracts from *dl*748-, *pm*760-, or *dl*764-infected cells were immunoprecipitated by using the P118-132 antiserum to the 14.5K protein; then the immunoprecipitates were electrophoresed on SDS-PAGE gels, electroblotted onto an Immobilon membrane, and probed with either the P118-132 antiserum to the 14.5K protein or the P77-91 antiserum to the 10.4K protein. As expected, the 14.5K protein was detected in *dl*748- (Fig. 8A, lane a) and *pm*760- (lane b) but not *dl*764- (lane c) infected cells. The 10.4K protein was detected in *pm*760- (Fig. 8B, lane b) but not in *dl*748- (lane a) or *dl*764- (lane c) infected cells; this is the anticipated result because *dl*748 does not make the 10.4K protein, *pm*760 makes both the 10.4K and 14.5K proteins, and *dl*764 does not make the 14.5K protein. In other experiments (data not shown) using in-frame deletion mutants in the 10.4K gene, the two 10.4K bands that were coimmunoprecipitated with the 14.5K-protein antiserum varied in size as expected from the deletion. We conclude that the 10.4K protein efficiently coimmunoprecipitates with the 14.5K protein.

The data in Fig. 7 and 8 strongly suggest that the 10.4K and 14.5K proteins exist as a complex in vivo. Roughly equivalent amounts of both proteins were coimmunoprecipitated, suggesting that the 14.5K/10.4K complex may be roughly 1:1. (Excluding the possible cleavage of N-terminal signal sequences, both proteins have 5 Cys residues.) The putative 10.4K/14.5K complex also appears to be very stable, because the extractions and immunoprecipitations

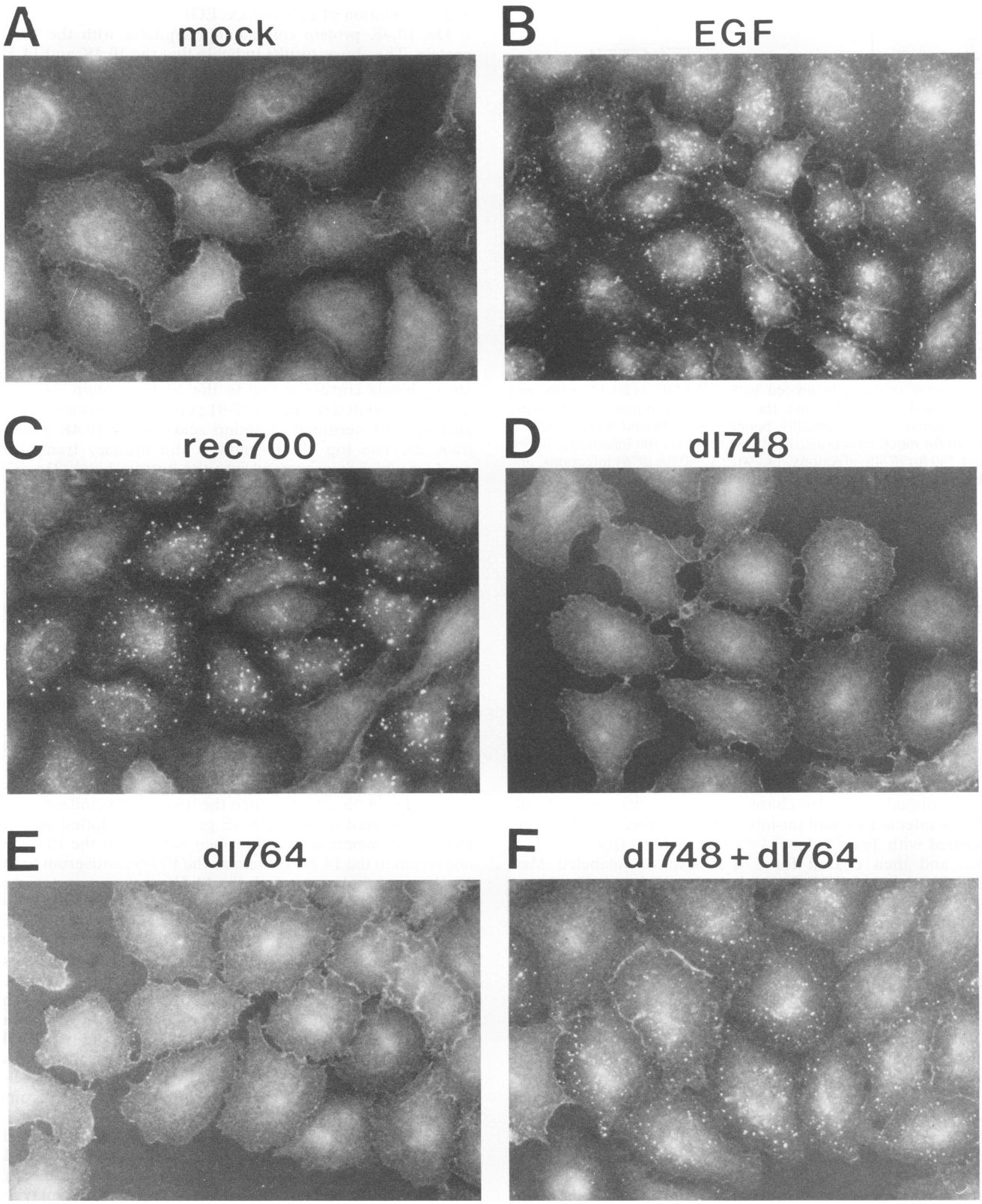

FIG. 5. Immunofluorescence of EGF-R in A549 cells infected with mutants or treated with EGF. (A) Mock-infected A549 cells. (B) A549 cells treated with 10 ng of EGF per ml 40 min prior to fixation. (C) Cells infected with *rec*700, a wild-type virus in this context. (D) Cells infected with *dl*748, a mutant which synthesizes the 14.5K but not the 10.4K protein. (E) Cells infected with *dl*764, a mutant which synthesizes the 10.4K but not the 14.5K protein. (F) Cells coinfected with *dl*748 and *dl*764.

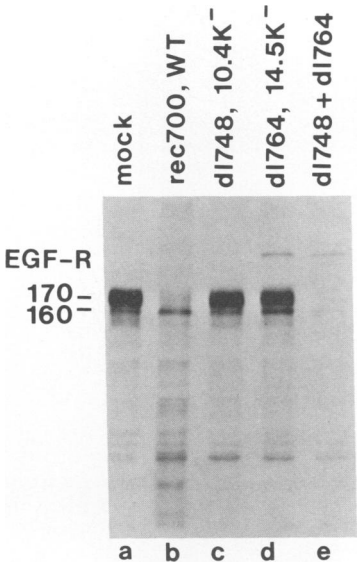

FIG. 6. Analysis of pulse-chase [^{35}S]Met-labeled EGF-R in mutant-infected cells. KB cells were mock infected (lane a), infected with 150 PFU per cell of *rec*700 (lane b), *dl*748 (lane c), or *dl*764 (lane d), or coinfected with 150 PFU per cell each of *dl*748 and *dl*764 (lane e). Cells were metabolically labeled with [^{35}S]Met from 7 to 11 h p.i., washed, and chased for 3 h in excess unlabeled Met, and then EGF-R was immunoprecipitated and analyzed by SDS-PAGE on 7.5% gels. WT, wild type.

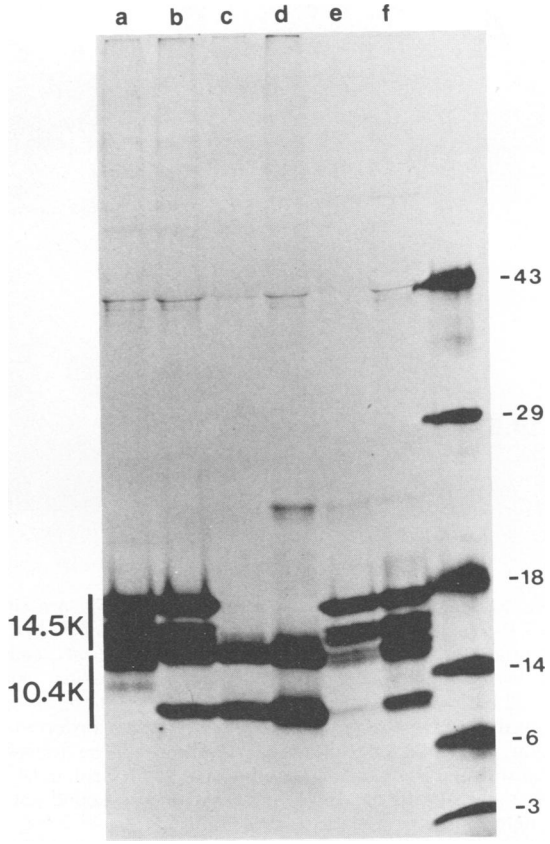

FIG. 7. Coimmunoprecipitation of the 10.4K protein with the 14.5K protein. Mutant-infected KB cells (50 PFU per cell) were labeled from 7 to 11 h p.i. with [^{35}S]Cys, lysed with NP-40, subjected to immunoprecipitation, and analyzed on 10 to 18% gradient SDS-PAGE gels. Lanes: a, cells infected with *dl*748, immunoprecipitated with the P118-132 antiserum to the 14.5K protein; b, cells infected with *in*724, immunoprecipitated with P118-132 antiserum; c, cells infected with *in*724, immunoprecipitated with the P77-91 antiserum to the 10.4K protein; d, cells infected with *dl*764, immunoprecipitated with P77-91 antiserum; e, KB cells were coinfected with 50 PFU of *dl*748 and *dl*764 per cell, labeled with [^{35}S]Cys, and immunoprecipitated using the P118-132 antiserum to the 14.5K protein; f, the *dl*748- and *dl*764-infected extracts used for lanes a and d were mixed in vitro and then immunoprecipitated with the P118-132 antiserum to the 14.5K protein. Counts (2×10^7) were used for the immunoprecipitations.

shown in Fig. 7, lanes a through d, were carried out in a stringent coimmunoprecipitation buffer containing 0.15 M NaCl–1% NP-40–0.5% sodium deoxycholate–0.1% SDS, and the immunoprecipitates were washed seven times in buffer containing 0.5 M NaCl–0.5% NP-40–1% sodium deoxycholate.

We have reported that the 10.4K protein is translated from E3 mRNA *f* (37) and that much or most of the 14.5K protein that is made in cells is also translated from mRNA *f* (36). These conclusions were based, among other considerations, on the observation that virus mutants that overproduce mRNA *f* also overproduce the 10.4K and 14.5K proteins. Presumably, translation of the 14.5K protein is reinitiated on mRNA *f* after termination of translation of the 10.4K protein. This possible scenario raises the question of whether the 10.4K/14.5K complex forms immediately upon translation from the same mRNA molecule. To address this question, cells were coinfected with *dl*748 (10.4K$^-$, 14.5K$^+$) and *dl*764 (10.4K$^+$, 14.5K$^-$), and the [^{35}S]Cys-labeled proteins were immunoprecipitated with the P118-132 antiserum to the 14.5K protein. The 10.4K protein was coimmunoprecipitated (Fig. 7, lane e). Thus, the 10.4K and 14.5K proteins need not be translated from the same mRNA molecule for the putative complex to form.

The 10.4K and 14.5K proteins are both membrane proteins, probably transmembrane proteins because they have hydrophobic domains of >20 amino acids (21a, 36, 37). This raises the question of whether the proteins must be embedded in membranes in order for the complex to form. To address this, cells were infected with *dl*748 or *dl*764, and the proteins were labeled with [^{35}S]Cys, extracted into coimmunoprecipitation buffer, mixed in vitro, and analyzed by immunoprecipitation with the 10.4K- and 14.5K-protein antisera. Once again, both the 14.5K and 10.4K proteins were observed with the 14.5K-protein antiserum (Fig. 7, lane f). Therefore, detergent-solubilized 10.4K and 14.5K proteins are able to form a complex in vitro.

The immunoprecipitations in Fig. 7 and 8 were done with the P118-132 antiserum specific to residues 118 to 132 at the extreme C terminus of the 14.5K protein (see also Fig. 9, lane d). The 10.4K protein was also coimmunoprecipitated when antisera to a TrpE-14.5K fusion protein were used (Fig. 9, lanes a and b) or when the P19-34 antiserum, specific to residues 19 to 34 in the 14.5K protein, was used (lane c). (In lanes a and b, the antisera were from two different rabbits immunized with the TrpE-14.5K fusion protein.) Thus, the putative 10.4K/14.5K complex must form such that residues 19 to 34 near the N terminus (residue 19 is probably at or near the extreme N terminus after cleavage of the putative N-terminal signal sequence) and residues 118 to 132 at the C terminus are accessible to the 14.5K-protein antisera.

Immunoprecipitation of [^{35}S]Cys-labeled 10.4K protein

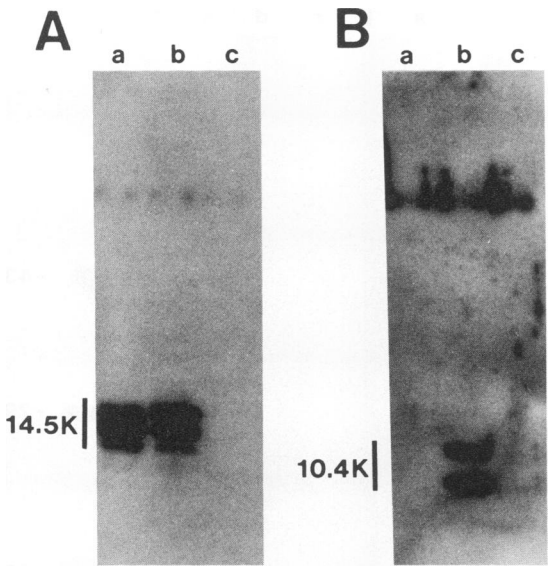

FIG. 8. Immunoblot analysis of the 10.4K/14.5K protein complex. KB cells were infected with 300 PFU per cell of *dl*748 (lane a), *pm*760 (lane b), or *dl*764 (lane c). At 12 h p.i. in araC, cells were lysed with NP-40, subjected to immunoprecipitation using the P118-132 antiserum to the 14.5K protein, electrophoresed on 10 to 18% gradient SDS-PAGE gels, and then electrotransferred to Immobilon polyvinylidene difluoride membranes. The transfer was probed with the P118-132 antiserum to the 14.5K protein (A) or the P77-91 antiserum to the 10.4K protein (B). The bound antibodies were detected with ^{125}I-labeled protein A.

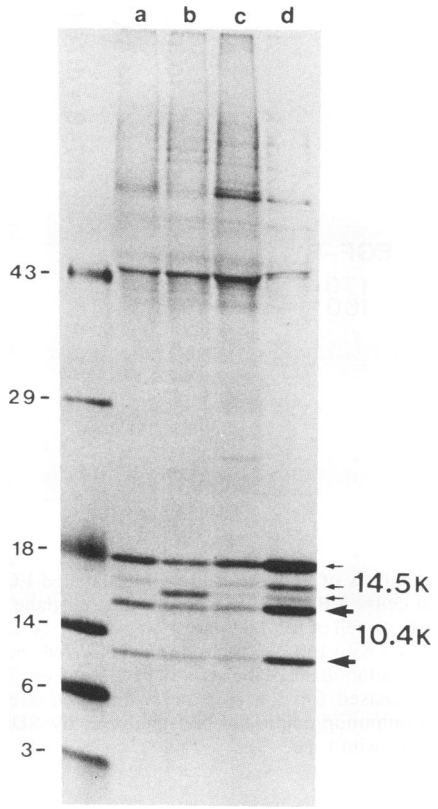

FIG. 9. Coimmunoprecipitation of the 10.4K protein with the 14.5K protein by using the P118-132 antiserum (lane d), the P19-34 antiserum (lane c), or the TrpE-14.5K-protein antisera (lanes a and b). In lanes a and b, antisera from two different rabbits were used. KB cells were infected with 50 PFU per cell of *in*724, labeled with [^{35}S]Cys from 7 to 12 h p.i. by the cycloheximide-enhanced procedure, lysed with NP-40, extracted into 0.5 ml of 0.14 M NaCl–1 mM $MgCl_2$–10 mM Tris-HCl (pH 8.5)–1 mM PMSF–0.5% NP-40, and subjected to immunoprecipitation. Immunoprecipitates were electrophoresed on 10 to 18% gradient SDS-PAGE gels. The three small arrows indicate bands corresponding to the 14.5K protein, and the two large arrows indicate bands corresponding to the 10.4K protein.

with preimmune sera (37), antisera to the E3 6.7K protein (42), or antisera to the E3-coded 14.7K or gp19K proteins (unpublished results) has never been observed. Therefore, coimmunoprecipitation of the 10.4K protein is specific to 14.5K-protein antisera, and it is not the result of nonspecific association of the 10.4K protein with immunoglobulin G or protein A.

DISCUSSION

We have shown by using Ad mutants that the E3 10.4K and E3 14.5K proteins are both required to down-regulate EGF-R in Ad-infected human cells. Down-regulation in this context means that cell surface EGF-R was internalized in an endosome-mediated pathway and degraded, presumably in lysosomes. EGF-R was not down-regulated when cells were infected with mutants that lack either the 10.4K or the 14.5K protein, but it was down-regulated when cells were coinfected with *dl*748 (10.4K$^-$, 14.5K$^+$) and *dl*764 (10.4K$^+$, 14.5K$^-$). Down-regulation was demonstrated by results of several different methods, namely (i) the absence of EGF-R autophosphorylation in an immune complex kinase assay, (ii) the inability to iodinate EGF-R on the cell surface, (iii) the lack of binding of ^{125}I-labeled EGF, (iv) the formation of endosomes containing EGF-R as detected by immunofluorescence, and (v) the degradation of the [^{35}S]Met-labeled fully processed 170K species of EGF-R in a pulse-chase experiment. No effect on the 160K immature species of EGF-R was observed, suggesting that 10.4K and 14.5K proteins do not affect the initial synthesis of EGF-R.

We have also presented strong evidence that 10.4K and 14.5K proteins exist as a complex in vivo. Both proteins are cytoplasmic membrane proteins as implied by their predicted sequence (14) and as demonstrated experimentally (21a, 37). We have shown here that the 10.4K protein is very efficiently coimmunoprecipitated from extracts of Ad-infected cells with antisera to the 14.5K protein. Coimmunoprecipitation was observed when detergent-solubilized extracts from cells infected with a 10.4K- or 14.5K-protein-negative mutant were mixed in vitro, indicating that the putative complex can form in vitro and that it does not require intact membranes. The complex is very stable, because it was not dissociated by a solution containing 1% NP-40–1% sodium deoxycholate–0.1% SDS or by 0.5 M NaCl. The stoichiometry of the complex appears to be roughly 1:1 as judged by the intensity of the 10.4K and 14.5K bands after coimmunoprecipitation and after coimmunoprecipitation and immunoblotting. The complex was observed with antisera to a TrpE-14.5K fusion protein as well as to two synthetic peptides corresponding to residues 19 to 34 and 118 to 132, respectively, in the 14.5K protein; this suggests that the extreme N terminus (after cleavage of the putative N-terminal signal) and the extreme C terminus of the 14.5K protein may not be involved in the complex because they were accessible to the

antipeptide sera. The complex was not observed with antipeptide sera to residues 77 to 91 in the 10.4K protein (Fig. 7) or to residues 68 to 80 or 75 to 83 (37); this may suggest that these regions of the 10.4K protein are involved in the complex and that the epitopes are not accessible to the sera. However, further work is needed to determine which regions in the 14.5K and 10.4K proteins are involved in complex formation.

Other lines of evidence also link the 10.4K and 14.5K proteins. First, both proteins are translated from E3 mRNA *f* (36, 37), which suggests a functional relationship (the 14.5K protein is also encoded in part by other mRNAs). Coimmunoprecipitation was observed by using extracts from cells coinfected with 10.4K- and 14.5K-protein-negative mutants; this indicates that the 10.4K and 14.5K proteins need not be translated from the same mRNA molecule in order for the complex to form. Second, the pattern of 14.5K bands is different with mutants that either contain or lack the 10.4K protein (compare lanes a and b in Fig. 7; see also reference 36).

Hoffman et al. (18) showed that the 10.4K protein expressed from the DOL murine retrovirus vector could down-regulate EGF-R in two acutely infected murine cell lines expressing the human EGF-R. The authors analyzed EGF-R down-regulation by using the immune complex kinase assay, the EGF binding assay, and the immunofluorescence assay for endosomes containing EGF-R. They also detected a 75% reduction in EGF binding in the psi-cre packaging cell line for the DOL 10.4K-protein retrovirus vector. These studies indicated that the 10.4K protein could act in the absence of other Ad proteins to down-regulate EGF-R under the conditions used (18). In the studies reported here using Ad-infected cells, however, we observed an absolute requirement for both the 10.4K and 14.5K proteins to down-regulate EGF-R. This was true at both early (7 to 11 h) and late (24 h) periods p.i. It is not clear why there is this difference between the retrovirus and Ad systems. Perhaps the 10.4K protein is the primary functional component of the putative 10.4K-14.5K complex and the 14.5K protein plays only an auxiliary role, such as facilitating the transport of the 10.4K protein to the correct cellular organelle. In the retrovirus system, perhaps sufficient 10.4K protein is synthesized and transported for the protein to function independently of the 14.5K protein. Alternatively, it is possible that there is a fundamental difference between Ad-infected and retrovirus-infected cells. For example, one of the other Ad proteins could modify the cell such that both the 10.4K and 14.5K proteins are required to act on EGF-R, whereas in the retrovirus case, this modification does not occur. It is also possible that 10.4K protein might act in concert with one of the retrovirus proteins to down-regulate EGF-R. Whatever the explanation, it is likely that this difference between the retrovirus and Ad systems will be instructive with regard to the mechanism of action of the 10.4K and 14.5K proteins. In this connection, it is important to keep in mind that Ad-infected cells is the natural milieu in which the 10.4K and 14.5K proteins function.

We previously reported that *dl*763, a mutant that makes the 10.4K protein and a truncated version of the 14.5K protein lacking residues 104 to 132 at the C terminus, was able to down-regulate EGF-R as determined by using the immune complex kinase assay (11). We noted that this did not exclude the possibility that the 14.5K protein could be involved together with the 10.4K protein in down-regulating EGF-R, i.e., that the N-terminal 103 residues of the 14.5K protein might be sufficient for the 14.5K protein to function (11). Indeed, we constructed *dl*764 to ascertain whether the 14.5K protein was involved. In our subsequent experiments with *dl*763, we have generally found that it has a mutant phenotype for EGF-R down-regulation as determined by using the immune complex kinase assay, the metabolic labeling assay, the cell surface iodination assay, and the immunofluorescence assay for endosomes (unpublished results). Thus, it may be that *dl*763 has a partial phenotype.

We have few clues at this point as to the mechanism of action of the 10.4K/14.5K complex of proteins. Both are cytoplasmic membrane proteins, but the membrane organelle to which they localize and in which they function is not known. It is reasonable to suppose that they are transmembrane proteins which localize to the plasma membrane, where they interact with EGF-R, but this remains to be demonstrated.

There are now three known classes of viral proteins that act on EGF-R. One class is represented by the vaccinia virus growth factor (VGF), which is synthesized by vaccinia virus (2, 5, 33, 35, 38). Related proteins are synthesized by other poxviruses, including Shope fibroma virus (13), molluscum contagiosum virus (29), and myxomavirus (40). These proteins are related in sequence to EGF and transforming growth factor alpha (TGF-α). Like EGF and TGF-α, VGF (12) and presumably the other poxvirus VGF-like proteins are derived from larger precursors and are secreted from the cell. VGF mimics EGF and TGF-α by binding to the extracellular domain of EGF-R and inducing at least some aspects of EGF signal transduction, including activation of the protein tyrosine kinase activity of EGF-R, stimulation of DNA synthesis in quiescent human fibroblasts, and colony formation in soft agar (21, 35, 38). Chemically synthesized VGF (23) and Shope fibroma virus growth factor (22) also bind to EGF-R and stimulate DNA synthesis and colony formation. The second class is represented by the 44-residue E5 transforming protein of bovine papillomavirus. E5 is a membranous nonsecreted protein which appears to up-regulate EGF-R activity by inhibiting the internalization and degradation of EGF-R and accordingly increasing the persistence of EGF-R on the cell surface (26). E5 has also been reported to exert a similar effect on the receptor for colony-stimulating factor type 1, which, like EGF-R, is a member of the protein tyrosine kinase class of receptors (26). The third class is represented by the Ad 10.4K/14.5K complex of nonsecreted cytoplasmic membrane proteins. Thus, three classes of DNA viruses encode proteins that act on EGF-R, suggesting that such proteins might be a general feature of DNA viruses.

What are the functions of the VGF, E5, and 10.4K and 14.5K proteins in the life cycle of their respective viruses? As noted above, VGF and the related poxvirus proteins have EGF-like activities, i.e., they bind to and induce tyrosine phosphorylation of EGF-R, and they are mitogenic in quiescent cells. Also, studies with VGF-negative virus mutants indicate that the cell-proliferative response to vaccinia virus is mediated by VGF (7), and VGF has been shown to stimulate epidermal regeneration when applied topically (34). Thus, as suggested previously (e.g., in references 7, 22, and 40), it is likely that VGF serves to activate infected quiescent cells and also cells neighboring the initial site of infection so that they can more readily synthesize the large amounts of DNA, RNA, and proteins that are required for efficient virus multiplication. The E5 protein may similarly activate the infected cell by reducing down-regulation of EGF-R (26). The 10.4K and 14.5K proteins mimic EGF in stimulating down-regulation of EGF-R, which, at first

glance, might seem opposite to the putative role of VGF and E5. However, it is possible that, as for EGF and VGF, the initial effect of the 10.4K and 14.5K proteins may be to activate the protein tyrosine kinase activity of EGF-R (this has not yet been demonstrated). Thus, the 10.4K and 14.5K proteins may serve the same putative role as VGF, namely, to activate quiescent cells for efficient virus multiplication (11). In this respect, the 10.4K and 14.5K proteins may synergize with the region E1A-coded 243R protein which stimulates DNA synthesis in quiescent cells (30, 31). If this is the role of the 10.4K and 14.5K proteins, then we would not expect them to be absolutely essential for virus replication in vivo, and indeed, deletion of these genes does not preclude Ad multiplication in the lungs of hamsters (28) or cotton rats (15). The VGF gene also is not essential, although VGF-negative mutants exhibit decreased virulence (6).

It is also possible that the 10.4K and 14.5K proteins exert a negative effect on the activities of EGF-R (11). Activated macrophages secrete TGF-α, which is thought to be important in wound repair (25, 32); by removing EGF-R from the cell surface, the 10.4K and 14.5K proteins could preclude any antiviral effects that TGF-α may have.

Further studies on the 10.4K and 14.5K proteins, in particular to determine whether they activate the protein tyrosine kinase activity of EGF-R and whether they influence any other cell surface receptors, will be of great interest.

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