The Adenovirus E3 10.4K and 14.5K Proteins, Which Function To Prevent Cytolysis by Tumor Necrosis Factor and To Down-Regulate the Epidermal Growth Factor Receptor, Are Localized in the Plasma Membrane

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The adenovirus type 2 and 5 E3 10,400- and 14,500-molecular-weight (10.4K and 14.5K) proteins are both required to protect some cell lines from lysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor. We have shown previously that both 10.4K and 14.5K are integral membrane proteins and that 14.5K is phosphorylated and O glycosylated. The 10.4K protein coimmunoprecipitates with 14.5K, indicating that the two proteins function as a complex. Here we show, using immunofluorescence and two different cell surface-labeling techniques, that both proteins are localized in the plasma membrane. In addition, we show that trafficking of each protein to the plasma membrane depends on concomitant expression of the other protein. Finally, neither protein could be immunoprecipitated from conditioned media, indicating that neither is secreted. Taken together, these results suggest that the plasma membrane is the site at which 10.4K and 14.5K function to inhibit cytolysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor.

The E3 transcription unit of adenovirus type 2 (Ad2) and Ad5 (subgroup C) is of considerable interest because it appears to encode proteins that are important, at least in part, for protecting virus-infected cells from the host immune system. E3 encodes seven proteins (reviewed in references 10, 58, and 59), two of which are termed 10.4K (49) and 14.5K (48) (their molecular masses being 10.4 and 14.5 kDa, respectively). The 10.4K and 14.5K proteins have been shown to have two functions. Apparently functioning as a complex, they can protect most mouse cell lines examined from lysis by tumor necrosis factor (TNF) (9). They also are both required to down-regulate the epidermal growth factor receptor (EGFR) from the surface of infected cells (4, 50). In addition, infection with an adenovirus mutant which overexpresses both proteins has been shown to cause down-regulation of the insulin and insulin-like growth factor 1 receptors as well as p185^{neu} (34).

Region E3 can be deleted without affecting virus replication (28). However, the 10.4K and 14.5K proteins have been found to be conserved in all human adenovirus serotypes sequenced to date. These include Ad3 (44), Ad7 (26), Ad11 (36, 37), and Ad35 (10.4K only) (7) (subgroup B); Ad2 (20, 21) and Ad5 (5) (subgroup C); Ad12 (46) (subgroup A); Ad40 (6) (subgroup F); and Ad9 (17) (subgroup D). This conservation across subgroups implies that these proteins play an important role in ordinary human infections. In the cotton rat model of adeno-

virus pathogenesis, wild-type subgroup C adenovirus causes a pneumonia very similar to that seen in humans (40). When cotton rats are infected with a virus with a deletion in the E3B portion of E3 (which encodes the 10.4K, 14.5K, and 14.7K proteins), an increased number of polymorphonuclear neutrophils is seen in the lungs, suggesting that some or all of these proteins are important in controlling the immune response to adenovirus infection (8).

The amino acid sequences of both 10.4K and 14.5K are shown in Fig. 1A. Full-length 10.4K has 91 amino acids. Approximately half of the 10.4K molecules have the signal sequence cleaved between Ala-22 and Ala-23 (30), and 10.4K can form dimers through disulfide bonding at Cys-31 (25). The primary translation product of Ad5 14.5K has 132 amino acids. It also has a cleaved signal sequence; however, the exact point of cleavage depends on the expression of 10.4K (32). The 14.5K protein is both phosphorylated on serine residues (33) and O glycosylated with mucin type oligosaccharides (32). The extent of both types of processing depends on the expression of 10.4K (32, 33). Both 10.4K and 14.5K are integral membrane proteins oriented with the C terminus in the cytoplasm (30, 31). The 10.4K protein is coimmunoprecipitated with 14.5K, suggesting that the two proteins function as a complex (9, 50). A diagram of the 10.4K-14.5K complex is shown in Fig. 1B. The idea that 10.4K and 14.5K exist as a complex is consistent with genetic data indicating that both proteins are required to prevent TNF cytolysis (9) and to down-regulate EGFR (50) in adenovirus-infected cells.

In order to understand how 10.4K and 14.5K function, it is necessary to know their intracellular localization. The 10.4K protein has been shown to be expressed on the surface of infected cells (25). In this report, we show that both 10.4K and 14.5K are expressed on the cell surface. In addition, we show that expression of both proteins is required for efficient

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FIG. 1. (A) Predicted amino acid sequences of the 10.4K protein from Ad2 and the 14.5K protein from Ad5. Amino acid position numbers are indicated above the sequences. Signal and transmembrane domains are underlined. (B) Model for complex formation between 10.4K and 14.5K. Both proteins are integral membrane proteins oriented with the C terminus in the cytoplasm (29, 30). The 10.4K protein exists in two forms, an uncleaved full-length product with two membrane-spanning regions (diagonal stripes) and a cleaved form with only one transmembrane region (29). The two forms of 10.4K are joined via a disulfide linkage at Cys-31 (24). The 14.5K protein has a cleaved signal sequence and a single membrane-spanning domain (30). It is both O glycosylated (31) and phosphorylated (32). The numbers correspond to those in panel A.

transport of either one out of the endoplasmic reticulum (ER) to the plasma membrane.

MATERIALS AND METHODS

Cells and viruses. Virus stocks were prepared in suspension cultures of human KB cells, and titers were determined on human A549 cells as described previously (12). Human A549 and A431 cells were maintained in Dulbecco modified Eagle medium (DME) with 10% fetal calf serum.

rec700 is an Ad5-Ad2-Ad5 recombinant whose genome consists of the Ad5 *EcoRI* A (map positions 0 to 76), Ad2 *EcoRI* D (map positions 76 to 83), and Ad5 *EcoRI* B (map positions 83 to 100) fragments (57). *rec700* is the parental virus of mutants *pm760*, *dl748*, *dl764*, and *sub765*. *pm760* overproduces E3 mRNA *f* (3) and consequently overproduces 10.4K (49) and 14.5K (48), which are encoded by mRNA *f*. *dl748* also overproduces mRNA *f* (2) and 14.5K (48), but it does not synthesize 10.4K because of a deletion in the 10.4K gene (49). *dl764* overproduces 10.4K (49), but it does not synthesize 14.5K because of a deletion in the 14.5K gene (48). *sub765* overproduces both 10.4K and 14.5K (60); the construction of this mutant will be described elsewhere.

In vivo protein labeling, cell fractionation, immunoprecipitation, and SDS-PAGE. KB cells (1.75×10^7 cells) were maintained as suspension cultures in Joklik-modified Dulbecco minimal essential medium containing 5% horse serum. Cells were infected with 150 PFU of virus per cell and labeled with 50 μ Ci of [35 S]Cys (Dupont, NEN Research Products, Boston, Mass.) per ml from 7 to 11 h postinfection (p.i.) by the cycloheximide (CH)-enhanced procedure de-

scribed previously (55, 56). Cells were maintained in CH (25 μ g/ml) from 3 to 7 h p.i. to enhance the accumulation of early mRNAs and, subsequently, early proteins after the removal of CH (16). Dialyzed serum was added to 5% at 1 h postlabeling. At 11 h p.i., cells were collected, washed once with phosphate-buffered saline (PBS) and twice with a solution of 10 mM Tris-HCl (pH 7.2), 80 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride. Cells were then resuspended in 500 μ l of the buffer described above and lysed by using a tight-fitting Dounce homogenizer (Wheaton pestle A). Nuclei were removed by centrifuging. Sucrose was added to the supernatant to a concentration of 50%, and membranes were separated by floating up through a 30 to 45% sucrose gradient in an SW41 rotor at $90,000 \times g$ for 18 h. Fractions were collected, and the membranes in each fraction were pelleted by centrifuging in a Ti50 rotor at 40,000 rpm for 1 h. Pellets were solubilized in 5% sodium dodecyl sulfate (SDS) and analyzed by immunoprecipitation with 10 μ l of antiserum per ml and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) as described previously (51). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (gel size, 0.75 mm by 16 cm; acrylamide/*N,N'*-methylenebisacrylamide ratio, 29.2:0.8 [wt/wt]). The gels were fluorographed. 14 C-labeled molecular weight markers were purchased from Bethesda Research Laboratories, Gaithersburg, Md.

To determine if 10.4K or 14.5K was secreted, KB cells were infected and labeled as described above. Cells (2.5×10^6) were collected in a microfuge (10 min at 4°C). The cell pellet was solubilized in 1% sodium deoxycholate, 0.5% Nonidet P-40 (NP-40), and 0.1% SDS. Proteins from both the pellet and supernatant were immunoprecipitated as described above.

The following rabbit polyclonal antisera were used. The P77-91 antiserum is directed against a synthetic peptide corresponding to residues 77 to 91 of the Ad2 version of 10.4K (49). The P118-132 antiserum is directed against a synthetic peptide corresponding to residues 118 to 132 of the Ad5 14.5K protein (48).

Pulse-chase analysis of 14.5K. KB cells (6×10^7) were infected at 50 (*pm760* and *dl748*) or 100 (*dl764* in the mixed infection) PFU of virus per cell and pulsed with 250 μ Ci of [35 S]Cys (Dupont, NEN) for 20 min as described previously (33). Cells were collected, rinsed three times with DME containing 5% horse serum, and resuspended in the same medium. Aliquots of 1.25×10^7 cells were taken after 15, 60, 120, and 480 min. Cells were lysed in 1.0 ml of iso-hi-pH buffer (51) containing 1% NP-40, 0.34 mg of phenylmethylsulfonyl fluoride per ml, 1 μ g of pepstatin A per ml, and 0.25 μ g of leupeptin per ml. The 14.5K protein was immunoprecipitated as described previously (51).

Lactoperoxidase-catalyzed cell surface iodination. A549 cells were plated onto 100-mm-diameter dishes and cultured in DME containing 10% fetal calf serum. The dishes were either mock infected or infected the following day with 100 PFU of virus per cell. CH was added to a concentration of 25 μ g/ml at 3 h p.i. At 6.5 h p.i., cells were released from CH by washing three times with DME containing 20 μ g of 1- β -D-arabinofuranosylcytosine (araC) per ml and cultured overnight in the presence of araC.

Iodinations were performed by the lactoperoxidase-catalyzing method as described previously (50). Cells were lysed and adenovirus proteins were immunoprecipitated as described above. Immunoprecipitates were analyzed by SDS-PAGE on 10 to 18% gradient gels. Transferrin receptor was immunoprecipitated with a monoclonal antibody (β 3/25; Boehringer Mannheim, Indianapolis, Ind.) and was analyzed on a 7.5% gel.

Surface biotinylation and blot analysis of the 10.4K and 14.5K proteins. A549 cells, maintained in DME, were plated onto 100-mm-diameter dishes at a density of 4×10^6 cells per dish. On the following day, cells were infected with adenovirus mutants at 100 PFU per cell except for *dl748*, which was used at 50 PFU per cell. Proteins were labeled from 5 to 9 h p.i. with 0.1 mCi of [35 S]Cys per dish. At 9 h p.i., cells were washed and incubated for 3 h in label-free DME plus 2% fetal calf serum. Whole-cell biotinylation was performed as described previously (15). Briefly, at 12 h p.i. cells were washed six times with PBS containing calcium and magnesium (PBS $^{++}$). Sulfo-NHS-biotin (Pierce Chemical Co., Rockford, Ill.) was added to a concentration of 1.5 mg/ml. The cells were then incubated on ice for 30 min. Excess biotin was removed by washing six times with cold 50 mM glycine in PBS $^{++}$. Infected, biotin-labeled cells were lysed in 1% NP-40. Adenovirus proteins were immunoprecipitated as described previously (51). The antiserum used to immunoprecipitate 14.7K was a rabbit polyclonal antiserum produced against a TrpE-14.7K fusion protein (51). The antiserum used to immunoprecipitate E1B-19K was a rabbit polyclonal antiserum produced against a C-terminal peptide of E1B-19K (11).

Immunoprecipitates were processed by two different procedures. In one, immunoprecipitates were split into two aliquots and electrophoresed on two different SDS-15% PAGE gels. One gel was fluorographed to visualize total labeled proteins. The other gel was analyzed by immunoblotting using the following procedure. The gel was equilibrated for 30 min in Towbin transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.4]) (53). Proteins were transferred to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) by using a semidry blot apparatus (Trans-Blot; Bio-Rad, Hercules, Calif.) at 150 mA for 30 min. The blots were blocked in 3% nonfat dry milk in PBS overnight at 4°C. Avidin conjugated to horseradish peroxidase (Boehringer Mannheim) was diluted 1:5,000 and was incubated with the blot for 1 h at room temperature. The blots were washed four times with PBS prior to one wash with PBS containing 0.3% Tween 20. The blots were developed by a chemiluminescent detection procedure (ECL; Amersham, Arlington Heights, Ill.). The blots

were stripped by incubation with 0.2 mM glycine-HCl (pH 2.5) at 80°C for 2 h, with one change of solution. The blots were blocked with 5% bovine serum albumin (BSA) and reprobed with a 1:100 dilution of the P77-91 antiserum directed against the C terminus of 10.4K for 1 h at room temperature. The blots were washed four times with PBS containing 0.5% BSA, were incubated with a 1:2,000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad), and were developed by the ECL procedure.

The second treatment of the immunoprecipitates involved dissociating the antigen-antibody complexes and reprecipitation with antibody as before or with streptavidin. Antigen-antibody complexes were dissociated as described previously (15) by boiling for 10 min in PBS containing 0.5% SDS and quenching with Triton X-100 to a final concentration of 0.4% SDS and 2% Triton X-100. One half of the sample was immunoprecipitated as before, and the other half was precipitated with streptavidin-agarose (Sigma) as described previously (15). Samples were analyzed by SDS-PAGE on 15% gels before they were fluorographed.

Indirect immunofluorescence of the 14.5K protein. A431 or A549 cells were plated onto glass coverslips in DME. Cells were either mock infected or infected with viruses and then were fixed as described previously (50) at the times indicated in the figure legends. For A431 cells infected with *rec700* (see Fig. 7), CH was added at 3 h p.i. to a concentration of 25 µg/ml. At 7 h p.i., cells were released from CH by washing three times in DME. The immunofluorescence assay was performed exactly as described previously (50), with a 1:250 dilution of the P118-132 antiserum specific for the 14.5K protein. Cells were fixed at 1, 2, and 3 h after CH release.

RESULTS

The E3 10.4K and 14.5K proteins are not secreted from infected cells. The patterns of 10.4K and 14.5K bands seen on SDS-PAGE are complex because both proteins undergo post-translational modification. Approximately half of the 10.4K protein molecules undergo posttranslational cleavage between residues 22 and 23 (30). The 14.5K protein is O glycosylated (32) and phosphorylated (33). The appearance of the 10.4K and 14.5K proteins extracted from cells infected with *pm760*, a mutant that overproduces both 10.4K and 14.5K, is shown in Fig. 2, lanes d and h. The 10.4K protein (lane h) migrates as two bands, upper and lower, which are indicated by the arrowheads. The upper band is the full-length primary translation product, and the lower band is the cleavage product. The upper band is more intensely labeled because the proteins were continuously labeled with [³⁵S]Cys, and the cleaved lower band lacks two cysteine residues that are present in the upper band. The 14.5K protein runs as a series of bands on SDS-PAGE, as seen in lane d (indicated by the small arrows). The uppermost band is the fully O-glycosylated and phosphorylated product (32). The lowermost band of 14.5K migrates slightly above the upper band of 10.4K. In addition, 10.4K coimmunoprecipitates with 14.5K (compare lanes d and h). The pattern of 14.5K bands produced by cells infected with *dl748*, a mutant which produces 14.5K but not 10.4K, is different from that seen with *pm760* (compare lanes b and d). The significance of this is discussed below.

It has been reported that 10.4K is not secreted into the medium during infection with an adenovirus which produces both 10.4K and 14.5K (24). As shown in Fig. 2, 10.4K was immunoprecipitated from extracts of cells infected with *pm760*, which produces both 10.4K and 14.5K (lane h), but 10.4K was not immunoprecipitated from conditioned medium from these cells (lane g). The same result was observed with cells infected with *dl764*, which produces 10.4K but not 14.5K (lanes f and e). The 14.5K protein was also immunoprecipitated from extracts of cells infected with *pm760* but not from the conditioned medium (lanes d and c). When cells were infected with *dl748*, which produces 14.5K but not 10.4K, the same result was observed, i.e., 14.5K was immunoprecipitated from the cell extract but not from the conditioned medium (lanes b and a). Both proteins could be immunoprecipitated from culture supernatants if the proteins were added exogenously (data not shown). These data confirm the previous

FIG. 2. Analysis of 10.4K and 14.5K in infected cells and in the medium from infected cells. KB cells were infected with 150 PFU of adenovirus mutants per cell and labeled with 50 µCi of [³⁵S]Cys per ml from 7 to 11 h p.i. after CH enhancement. Cells were collected by centrifugation and then were solubilized in 1% sodium deoxycholate, 0.5% NP-40, and 0.1% SDS. Both cells and supernatants were immunoprecipitated with antisera specific for 10.4K or 14.5K. Bands corresponding to 14.5K are indicated by arrows. The upper and lower 10.4K bands are indicated by arrowheads. Lanes: a, *dl748* (10.4K⁻ 14.5K⁺) supernatant, immunoprecipitated for 14.5K; b, *dl748* cells, immunoprecipitated for 14.5K; c, *pm760* (10.4K⁺ 14.5K⁺) supernatant, immunoprecipitated for 14.5K; d, *pm760* cells, immunoprecipitated for 14.5K; e, *dl764* (10.4K⁺ 14.5K⁻) supernatant, immunoprecipitated for 10.4K; f, *dl764* cells, immunoprecipitated for 10.4K; g, *pm760* supernatant, immunoprecipitated for 10.4K; h, *pm760* cells, immunoprecipitated for 10.4K; M, molecular weight standards (with sizes in kilodaltons indicated on the left).

report that 10.4K is not secreted during infection with a virus that produces both 10.4K and 14.5K (24). These data also show that 10.4K is not secreted in the absence of 14.5K, and that 14.5K is not secreted in either the presence or absence of 10.4K.

Posttranslational processing and stability of 14.5K depends on expression of 10.4K. As discussed above, the 14.5K protein is both O glycosylated and phosphorylated, and the extent of this posttranslational processing depends on the presence of the 10.4K protein (32, 33). In order to investigate this relationship more thoroughly and to examine the stability of 14.5K, a pulse-chase analysis was performed. After 15 min of chase, 14.5K from *pm760*, a mutant that expresses both proteins, exists as a series of bands (indicated by the arrows, Fig. 3, lane b). (The band at ca. 25 kDa is the E3 gp19K protein that often coimmunoprecipitates, presumably nonspecifically, when antisera to various E3 proteins are used). After 1 h, most of the 14.5K protein had been chased into the uppermost band (lane e), and the band pattern did not change significantly after 2 h (lane h) or 4 h (lane k) of chase. Furthermore, the amount of 14.5K obtained after 4 h was similar to that obtained after 1 h, indicating that 14.5K is quite stable.

The 10.4K protein was efficiently coimmunoprecipitated with the 14.5K protein at all periods of the chase. (In this particular experiment, the lower band of 10.4K migrated as a

FIG. 3. Pulse-chase analysis of the 14.5K protein. KB cells (6×10^7) were either mock infected, infected with 50 PFU of *pm760* or *dl748* per cell or with 100 PFU of *dl764* per cell in the mixed infection. *pm760* produces both 14.5K and 10.4K, *dl748* produces 14.5K but not 10.4K, and *dl764* produces 10.4K but not 14.5K. Infected cells were pulsed with 250 μ Ci [35 S]Cys for 20 min at 5 h p.i. and then were chased with complete medium. Extracts were made at the end of the chase periods indicated. The 14.5K protein was immunoprecipitated and analyzed on 10 to 18% gradient gels. Lanes: a, mock-infected (mock) cells; b, cells infected with *pm760*, chased for 15 min; c, *dl748*, chased for 15 min; d, *dl748* plus *dl764*, chased for 15 min; e, *pm760*, chased for 1 h; f, *dl748*, chased for 1 h; g, *dl748* plus *dl764*, chased for 1 h; h, *pm760*, chased for 2 h; i, *dl748*, chased for 2 h; j, *dl748* plus *dl764*, chased for 2 h; k, *pm760*, chased for 4 h; l, *dl748*, chased for 4 h; m, *dl748* plus *dl764*, chased for 4 h. MW, molecular weight (indicated in thousands by the numbers on the right). The 10.4K (arrowheads) and 14.5K (arrows) bands are indicated.

doublet; the explanation for this doublet is unknown.) Thus, the 10.4K-14.5K complex is stable.

Mutant *dl748* overproduces 14.5K but does not produce 10.4K (48, 49). After 15 min of chase, the 14.5K band pattern from *dl748* resembled that from *pm760* (Fig. 3, compare lane c with lane b). However, after 1 h of chase, the band pattern from *dl748* was markedly different from that of *pm760* (compare lanes f and e). With *dl748*, much less of the 14.5K protein had been chased into the uppermost band and an additional band (second from the top) was apparent. Also, the 14.5K protein of *dl748* was much less stable than 14.5K of *pm760* (compare lane i with lane h and lane l with lane k).

dl764 is a mutant that produces 10.4K but not 14.5K (48, 49). With a mixed infection of *dl764* with *dl748* (Fig. 3, lanes d, g, j, and m), the 14.5K band pattern resembled that of *pm760* rather than that of *dl748*. Thus, the 10.4K protein provided by *dl764* rescued the abnormal processing and stability of the 14.5K protein from *dl748*.

These results indicate that the presence of the 10.4K protein increases the efficiency with which the 14.5K protein is processed. In addition, 10.4K increases the stability of 14.5K.

Subcellular localization of both 10.4K and 14.5K depends on expression of both proteins. To examine the subcellular localization of both 10.4K and 14.5K, membranes from infected cells were fractionated on a sucrose gradient. The results are shown in Fig. 4. The early fractions represent primarily membranes of the ER and the Golgi apparatus, and the later fractions represent primarily plasma membrane (39). In cells infected with *dl764*, which produces 10.4K but not 14.5K, most of 10.4K was in fractions 1 and 2 (Fig. 4A). However, in cells infected with *pm760*, which produces both 10.4K and 14.5K, 10.4K shifted into fractions 2 to 6 (Fig. 4B), including the plasma membrane fractions (compare lanes 6 in

FIG. 4. Localization of 10.4K and 14.5K in cellular membrane fractions. KB cells (1.75×10^7 cells) were infected with 150 PFU of adenovirus mutants per cell and labeled from 7 to 11 h p.i. by the CH-enhanced procedure. At 11 h p.i., cells were homogenized and the membranes were fractionated through a sucrose gradient. Either the 10.4K (arrows) or 14.5K (bar) protein was immunoprecipitated from each fraction. Early fractions contain mostly intracellular membranes such as ER and Golgi apparatus. Late fractions contain mostly plasma membrane. (A) 10.4K immunoprecipitated from *dl764* ($10.4K^+ 14.5K^-$); (B) 10.4K *pm760* ($10.4K^+ 14.5K^+$); (C) 14.5K *dl748* ($10.4K^- 14.5K^+$); (D) 14.5K *pm760*; (E) gp19K immunoprecipitated from *pm760*. Protein sizes (in kilodaltons) are indicated on the left. Fraction numbers are indicated above the lanes.

Fig. 4A and B). The same result was seen for 14.5K. In cells infected with *dl748*, which produces 14.5K but not 10.4K, most of 14.5K was found in the ER and Golgi membranes (fractions 1 and 2 in Fig. 4C). In contrast, in cells infected with *pm760*, 14.5K was found in fractions 2 to 6 (Fig. 4D), with a large proportion of 14.5K in the plasma membrane fraction (lane 6). (The protein band running at ca. 14 kDa in Fig. 4C is not 10.4K, as is apparent from the fact that this protein was localized in a different fraction from 10.4K; the identity of this band is not known.) The E3 gp19K protein was also immunoprecipitated from the *pm760* fractions. As reported previously (39), gp19K was found mostly in the ER and Golgi fractions, with some in the plasma membrane fraction (Fig. 4E).

Localization of 14.5K was also investigated by indirect immunofluorescence assay. Cells were infected with *pm760*, which produces both 10.4K and 14.5K, or with *dl748*, which produces 14.5K but not 10.4K. Infected cells were stained with antibody to 14.5K at 6.5 h p.i. The results are shown in Fig. 5. With *pm760*, 14.5K was found in all cytoplasmic membranes, including the cell surface (the edges of the cells are crisply stained). This is consistent with 14.5K localization in the plasma membrane as suggested by the results of the membrane fractionation experiment shown in Fig. 4. With *dl748*, 14.5K was found only in the ER and Golgi fractions and not at the cell surface. These results are consistent with the data in Fig. 4 indicating that 10.4K is required for 14.5K to be transported efficiently to the cell surface. Note that 14.5K was excluded from the nucleus in cells infected with either *pm760* or *dl748*. We were not able to perform this experiment for 10.4K because none of our antisera recognize 10.4K efficiently in immunofluorescence assays.

To compare the localization of 14.5K at various times p.i.,

FIG. 5. Indirect immunofluorescence assay of 14.5K in A549 cells infected with *pm760* (10.4K⁺ 14.5K⁺) or *dl748* (10.4K⁻ 14.5K⁺). Cells were infected with 250 PFU of virus per cell and were fixed at 6.5 h p.i.

cells infected with adenovirus mutants were stained for 14.5K by indirect immunofluorescence at 7.5, 9.5, and 12 h p.i. The 14.5K protein was found at the edges of *pm760* (10.4K⁺ 14.5K⁺)-infected cells by 7.5 h p.i., but only in the ER and Golgi in *dl748* (10.4K⁻ 14.5K⁺)-infected cells, even as late as 12 h p.i. (Fig. 6). However, when 10.4K was provided by *dl764* (10.4K⁺ 14.5K⁻) in a mixed infection (*dl748* plus *dl764*), 14.5K was again seen at the edges of the cells by 7.5 h p.i. By 12 h p.i.,

the immunofluorescence pattern of 14.5K from the mixed infection was much more similar to that of *pm760* than that of *dl748*.

These data suggest that the ultimate location of 14.5K is the plasma membrane and that 10.4K is required for efficient transport of 14.5K to the plasma membrane.

Both 10.4K and 14.5K are expressed on the plasma membrane. The cell fractionation experiment (Fig. 4) and the immunofluorescence assay showing 14.5K staining at the cell surface (Fig. 5 and 6) provide strong evidence for localization of 10.4K and 14.5K in the plasma membrane. In order to examine the time course of expression of 14.5K at the surface of cells, infected cells were treated with and then released from CH so that a synchronous burst of 14.5K synthesis occurred. Cells were then fixed and stained for immunofluorescence at time points after release from CH. At 1 h after CH release, 14.5K was found mainly in the Golgi and ER (Fig. 7). By 2 h after release, 14.5K was just beginning to appear at the cell surface, and at 3 h postrelease, 14.5K staining was clearly at the edges of the cells. Again, these data indicate that 14.5K localizes to the plasma membrane.

To further demonstrate that both 10.4K and 14.5K are localized at the cell surface, they were labeled by two different cell surface-labeling techniques. A549 cells were infected with

FIG. 6. Indirect immunofluorescence assay of 14.5K in A549 cells infected with adenovirus mutants and fixed at 7.5, 9.5, or 12 h p.i. Cells were infected with 200 PFU of *pm760* (10.4K⁺ 14.5K⁺) or *dl748* (10.4K⁻ 14.5K⁺) per cell and with 100 PFU of *dl764* (10.4K⁺ 14.5K⁻) and *dl748* per cell in the mixed infection.

FIG. 7. Indirect immunofluorescence assay of 14.5K in A431 cells infected with *rec700* (10.4K⁺ 14.5K⁺). Cells were infected with 400 PFU of virus per cell, treated with CH from 3 to 7 h p.i., and fixed at 1, 2, or 3 h after the release of CH. mock, mock-infected cells.

rec700 (10.4K⁺ 14.5K⁺), *dl748* (10.4K⁻ 14.5K⁺), *dl764* (10.4K⁺ 14.5K⁻), and a mixture of *dl748* and *dl764*. Surface proteins were labeled with ¹²⁵I by the lactoperoxidase-catalyzing method. Adenovirus proteins were extracted, immunoprecipitated, and analyzed by SDS-PAGE. The results are shown in the top panel of Fig. 8. The lower band of 10.4K was iodinated in the *rec700* (lane b) and mixed (lane e) infections but not in cells infected with *dl764* alone (lane d). This is the expected result since, as shown in Fig. 4, 10.4K is not efficiently transported to the surface in the absence of 14.5K. Iodination of the upper band of 10.4K could not be detected. The 14.5K protein also was not efficiently iodinated (Fig. 8, lanes g, h, and j), possibly due to O glycosylation near the tyrosine available for iodination (see Discussion). However, note that the antiserum to 14.5K coimmunoprecipitated ¹²⁵I-labeled 10.4K (lower band) from the *rec700* (lane g) and the *dl748*-plus-*dl764* extracts (lane j). To control for general cell lysis, the E3 14.7K protein was immunoprecipitated from the same extracts. The 14.7K protein is localized in the nucleus and cytoplasm of infected cells (42, 52). No ¹²⁵I-labeled 14.7K protein was detected (lanes l to o), indicating that the cells had remained intact during the iodination procedure. As a positive control for this experiment, the transferrin receptor was immunoprecipitated from the same extracts; it was uniformly iodinated in all samples (Fig. 8, bottom panel).

Whole-cell biotinylation was also employed to demonstrate expression of 10.4K and 14.5K at the cell surface. Biotinylated proteins were detected by immunoprecipitation with antisera to 10.4K or 14.5K followed by blotting with avidin. Both 10.4K and 14.5K were efficiently produced in cells infected with a virus that produces both proteins (*rec700*) or with a virus that overproduces both proteins (*pm760*), as shown by the immunoprecipitation of proteins metabolically labeled with [³⁵S]Cys (Fig. 9A, lanes c through f). On blots probed with avidin, the lower band but not the upper band of immunoprecipitated 10.4K could be detected (Fig. 9B, lanes c and e); both 10.4K bands were immunoprecipitated and present on the blot, as shown when the blot was stripped and reprobed with the antiserum against 10.4K (Fig. 9C, lanes c and e). Note that the small amount of biotinylated 10.4K (lower band; Fig. 9B, lanes

FIG. 8. Cell surface iodination of adenovirus proteins. A549 cells were either mock infected (mock) or infected with adenovirus mutants in the presence of araC. At 26 h p.i., whole cells were iodinated and lysed with NP-40. The 14.5K protein was immunoprecipitated (top panel, lanes f to j) prior to clearing of the extract with Pansorbin and subsequent immunoprecipitation of 10.4K (lanes a to e) (arrowhead). Extracts were again cleared with Pansorbin, and the 14.7K protein was immunoprecipitated (lanes k to o). Extracts were again cleared with Pansorbin, and the transferrin receptor (Tf-R) was immunoprecipitated (bottom panel). Immunoprecipitates were analyzed on a 10 to 18% gradient gel (for adenovirus proteins, top panel) or on a 7.5% gel (for Tf-R, bottom panel). Lanes: b, g, and l, *rec700* (10.4K⁺ 14.5K⁺); c, h, and m, *dl748* (10.4K⁻ 14.5K⁺); d, i, and n, *dl764* (10.4K⁺ 14.5K⁻); e, j, and o, *dl748* plus *dl764*. Protein sizes (in kilodaltons) are indicated on the left.

c and e) correlated with the relatively small amounts of 10.4K seen when the antiserum to 10.4K was used (Fig. 9C, lanes c and e). The biotinylation of the lower but not the upper band of 10.4K is the expected result: since 10.4K has no extracellular lysines (Fig. 1A) (49), only a free N terminus would be available for biotinylation and it is highly likely that the N terminus of the lower band is located on the extracytoplasmic side of the membrane whereas the N terminus of the upper band is on the cytoplasmic side of the membrane (see Fig. 1B and Discussion).

Most of the 14.5K bands were also detected on the avidin blot (Fig. 9B, lanes d and f). Both bands of 10.4K were coimmunoprecipitated with 14.5K, as shown in the blot reprobed with anti-10.4K antiserum (Fig. 9C, lanes d and f). The lower but not the upper 10.4K band is seen on the avidin blot in the anti-14.5K immunoprecipitations (Fig. 9B, lanes d and f), again indicating that the lower but not the upper band of 10.4K is biotinylated. Interestingly, more biotinylated 10.4K was seen coimmunoprecipitating with 14.5K than was seen in the 10.4K immunoprecipitations (compare lanes b and d with lanes c and e). This suggests either that most of the 10.4K on the surface exists in a complex with 14.5K or that 10.4K not in a complex is less available for biotinylation.

To control for cell lysis, two other adenovirus proteins, E3 14.7K and E1B-19K, were immunoprecipitated and blotted by this procedure. Both are intracellular proteins (42, 52). Neither protein could be detected on an avidin blot (data not shown).

Biotinylated 14.5K was also detected by precipitation with

FIG. 9. Avidin blotting of biotinylated 10.4K and 14.5K. A549 cells were mock infected (mock) or infected with *rec700* (10.4K⁺ 14.5K⁺) or *pm760* (which overproduces both 10.4K and 14.5K). Cells were labeled from 5 to 9 h p.i. with 0.1 mCi of [³⁵S]Cys and then chased for 3 h in complete medium. At 12 h p.i., cells were biotinylated and lysed in 1% NP-40, and the 10.4K (▲) and 14.5K (←) proteins were immunoprecipitated. The immunoprecipitates were split and run on two different 10 to 18% gradient gels. One gel was fluorographed and exposed to film for autoradiography (A). The other gel was used for blotting with avidin (B). This blot was stripped and reprobed with anti-10.4K antibody (C). Lanes: a, mock, immunoprecipitated for 10.4K; b, mock, immunoprecipitated for 14.5K; c, *rec700*, 10.4K; d, *rec700*, 14.5K; e, *pm760*, 10.4K; f, *pm760*, 14.5K. Protein sizes (in kilodaltons) are indicated on the left.

streptavidin-agarose. In this experiment, radiolabeled, biotinylated proteins were first immunoprecipitated with antisera specific for 14.5K or E1B-19K, then were removed from the beads, and were either reimmunoprecipitated with the same antiserum for 14.5K (Fig. 10B) or E1B-19K (Fig. 10D) or reprecipitated with streptavidin-agarose (Fig. 10A and C). The 14.5K protein was biotinylated at the surface of cells when both 14.5K and 10.4K were produced in the same infection, as indicated by the reprecipitation of 14.5K by streptavidin-agarose; this result is seen for all the infections with viruses that make both proteins. These include *rec700*, *pm760*, and *sub765* (Fig. 10A, lanes b, c, and d) (compared with *rec700*, *pm760* and *sub765* overproduce 14.5K and 10.4K). Biotinylated 14.5K was also obtained from the coinfection with *dl764* (10.4K⁺ 14.5K⁻) and *dl748* (10.4K⁻ 14.5K⁺) (lane g). When cells were infected with *dl748* alone, 14.5K was not biotinylated (Fig. 10A, lane e), presumably because of the inefficient transport of 14.5K to the cell surface in the absence of 10.4K (Fig. 4).

The 14.5K protein was efficiently reimmunoprecipitated with the anti-14.5K antiserum from cells infected with *rec700*, *pm760*, *sub765*, or *dl748* plus *dl764* (Fig. 10B). Only a trace of 14.5K was obtained from *dl748* (Fig. 10B), because the proteins were chased for 3 h after the labeling period and 14.5K from *dl748* is unstable during a chase (see Fig. 3). Although 10.4K normally coimmunoprecipitates with 14.5K (Fig. 2 to 4, 8, and 9), 10.4K was not reimmunoprecipitated with 14.5K in this experiment (Fig. 10B). This was very likely because 10.4K was dissociated from 14.5K by boiling of the initial antigen-antibody complex in 0.5% SDS and then the reimmunoprecipitation being conducted in 0.4% SDS.

It is of interest that both the upper and lower bands of 10.4K were observed when the original 14.5K immunoprecipitates were reprecipitated with streptavidin-agarose (Fig. 10A). The 10.4K protein was present in this sample because 10.4K coimmunoprecipitated with 14.5K in the original 14.5K immunoprecipitation reaction. The data in Fig. 10B make it unlikely that the 10.4K seen in the streptavidin-agarose reprecipitation shown in Fig. 10A was derived from a complex with biotinylated 14.5K. Rather, it is highly probable that 10.4K was obtained because the lower band of 10.4K is biotinylated and thus was precipitated by streptavidin-agarose. The data in Fig. 9 indicate that the lower but not the upper band of 10.4K is biotinylated; the upper band was obtained in Fig. 10A because the upper and lower bands are joined by a disulfide bond between the cysteine residues at position 31 (25).

The E1B-19K protein was examined as a control for cell lysis during the labeling of the cells with biotin. Although E1B-19K was efficiently reimmunoprecipitated (Fig. 10D), it was not reprecipitated with streptavidin-agarose (Fig. 10C). The lack of biotinylation of E1B-19K indicates that cell lysis had not occurred.

We conclude from the data in Fig. 9 and 10 that 14.5K and the lower band of 10.4K can be biotinylated in intact cells and therefore they must be on the cell surface.

DISCUSSION

It has been shown previously that the adenovirus E3 10.4K and 14.5K proteins are both required in infected cells to down-regulate EGFR (4, 50) and to protect cells from lysis by TNF (9). Not only are both adenovirus proteins required for these functions, but they probably function as a complex, since 10.4K is coimmunoprecipitated with 14.5K (50). This report shows that 14.5K is less stable in the absence of 10.4K and that the processing of 14.5K expressed in the absence of 10.4K is much slower than in the presence of 10.4K. The upper band of 10.4K is also less stable in the absence of 14.5K and the rate of conversion of upper band to lower band may be slightly lower than when 14.5K is present (30). It has been shown previously that both glycosylation and phosphorylation patterns of 14.5K are different depending on concomitant expression of 10.4K (32, 33). This report also demonstrates that both 10.4K and 14.5K are localized on the plasma membrane, substantiating the model of 10.4K-14.5K complex localization at the cell surface shown in Fig. 1B. In addition, both the 10.4K and 14.5K molecules are required for efficient transport of the other protein to the cell surface. Lastly, neither 10.4K nor 14.5K is secreted.

Cell surface localization of 10.4K and 14.5K was shown by a number of techniques. First, when membranes from virus-infected cells were fractionated over a sucrose gradient, both proteins were found in abundance in the plasma membrane fraction only in those cells infected with a virus that expresses both proteins. In addition, the time course of 14.5K immunofluorescence shown in Fig. 6 indicates that 14.5K is transported to the plasma membrane much more efficiently when 10.4K is also expressed than in its absence.

Expression of both 10.4K and 14.5K at the plasma membrane was demonstrated by direct cell surface labeling. The lower band but not the upper band of 10.4K was efficiently iodinated in mutants expressing both proteins and in a mixed

FIG. 10. Streptavidin-agarose precipitation of biotinylated 14.5K. A549 cells were mock infected (mock) or infected with adenovirus mutants, labeled with [35 S]Cys from 5 to 9 h p.i., and then chased in complete medium for 3 h. At 12 h p.i., surface proteins were biotinylated and the cells were lysed in 1% NP-40. The 14.5K and E1B-19K proteins were immunoprecipitated sequentially and then removed from the beads by boiling in 0.5% SDS. One half of each sample was reimmunoprecipitated with either anti-14.5K (B) or anti-E1B-19K (D) antibodies; the other half was precipitated with streptavidin-agarose (A, 14.5K; and C, E1B-19K). The samples were analyzed on 15% gels. Lanes: a, mock; b, *rec700* ($10.4K^+$ $14.5K^+$); c, *pm760* ($10.4K^+$ $14.5K^+$, overproduced); d, *sub765* ($10.4K^+$ $14.5K^+$, overproduced); e, *dl748* ($10.4K^-$ $14.5K^+$); f, *dl764* ($10.4K^+$ $14.5K^-$); g, *dl748* plus *dl764*; M, molecular standards (with sizes in kilodaltons indicated on the right of each panel).

infection with *dl748* ($10.4K^-$ $14.5K^+$) and *dl764* ($10.4K^+$ $14.5K^-$). There are several possible explanations for the lack of efficient iodination of the upper band of 10.4K. One is that the lower band of 10.4K may be the major form found at the surface of infected cells; this is unlikely because the upper and lower forms of 10.4K are joined by a disulfide bond (25). A second is that since the upper band is the full-length product, the tyrosines at positions 38 and 41 (Fig. 1A) may be protected from iodination by the folding of this molecule; cleavage would then make the tyrosine available. A third is that participation in the complex with 14.5K may block iodination. A fourth explanation is that participation in an oligomer with upper or lower bands of 10.4K itself could block iodination. These last three possibilities are not mutually exclusive, as we do not yet know the exact identities of cleaved and processed products which participate in the 10.4K-14.5K complex. More efficient

iodination of the lower band of 10.4K than of the upper band is consistent with the previous report of 10.4K iodination (25).

We saw very little iodination of 14.5K, even though it has a tyrosine at position 42 (Fig. 1A) which should be available for iodination. However, 14.5K has serines at positions 46 and 49, one or both of which may be the site(s) at which 14.5K is O glycosylated (32). Therefore, it is possible that the carbohydrate inhibits efficient iodination of 14.5K. The 14.5K protein was efficiently biotinylated and could be detected by both avidin blotting and streptavidin-agarose precipitation. Mature 14.5K has a free N terminus (31) and lysines at positions 23 and 45 (Fig. 1A), one or more of which may be the sites of biotinylation. The 14.5K protein was also found at the cell surface only when both proteins were expressed, either in wild-type infections or in a mixed infection with *dl748* and *dl764*. The lower band but not the upper band of 10.4K was

also labeled with biotin. As predicted by Hoffman et al. (25) and shown by Krajcsi et al. (30), 10.4K is oriented with the C terminus in the cytoplasm. The upper band (the full-length translation product) has two membrane-spanning regions with the N terminus in the cytoplasm. The lower band is a product of proteolytic cleavage between the two membrane-spanning regions, resulting in an extracellular N terminus which is available for biotinylation. Since 10.4K has no lysines in the extracellular portion of the molecule, only the lower band should be biotinylated in whole cells. This is consistent with biotinylation of the 10.4K lower band seen in the avidin blot shown in Fig. 9.

Shuttling of membrane proteins from the ER to the plasma membrane is generally considered the default pathway of membrane protein trafficking, with retention in specific organelles requiring specific retention signals (41). Retention in the ER requires an ER retention signal such as is found on the adenovirus gp19K protein (38). Proteins that are folded incorrectly also are not transported out of the ER to the Golgi apparatus but are instead bound by the immunoglobulin heavy chain binding protein (BiP) and degraded (13). There are several reasons why both 10.4K and 14.5K could be required for efficient transport of the other protein to the plasma membrane. As mentioned, 10.4K forms a heterodimer with 14.5K. It is possible that the correct conformation of each protein is achieved only when the proteins are in their complexed form. In this case, uncomplexed protein would be seen as incorrectly folded and therefore a target for degradation. This model predicts that in the absence of one of the proteins, the stability of the other would be reduced; this is exactly what was observed for the 14.5K protein in Fig. 3. It is also possible that the individual 10.4K and 14.5K proteins contain ER retention signals that are masked upon complex formation, although this possibility seems less likely.

As has been discussed above, 10.4K and 14.5K are both required for down-regulation of EGFR and for protection of cells from lysis by TNF. The requirement for expression of both proteins for efficient transport to the plasma membrane suggests that the site of action for these proteins is at the plasma membrane. There are several possible targets for the activity of these proteins. One target is the receptors themselves. It has been suggested that the 10.4K protein could oligomerize with EGFR to trigger down-regulation (4). In addition, or instead of actually binding the receptor itself, the 10.4K-14.5K complex could activate the receptor causing signalling and down-regulation. Alternatively, the complex could interact with the coated pit or endosome formation machinery, causing EGFR to be internalized with or without concomitant activation of the receptor tyrosine kinase signalling activity. A likely target for this function is the AP-2 complex which is part of the coated pit. AP-2 is composed of α - and β -adaptins (~100 kDa each) and proteins of 50 and 16 kDa (1, 27, 43). The α -adaptin molecule has been shown to associate and coimmunoprecipitate with EGFR, and this association increases upon binding of epidermal growth factor to EGFR (45). The 10.4K-14.5K complex could facilitate the interaction between EGFR and α -adaptin in the absence of epidermal growth factor, thereby causing increased localization of EGFR in coated pits followed by down-regulation of the receptor. It has been proposed that the E3 proteins reroute constitutively recycling receptors to the liposomes where they are degraded. This rerouting is proposed to occur in an intracellular compartment, the multivesicular body (23). However, the actual mechanism by which the 10.4K and 14.5K proteins function remains unknown. Knowledge of the subcellular localization of

these proteins should allow us to think more clearly about the mechanism by which they may function.

Very little is known about the way in which TNF binding to its receptor causes cell lysis. Cytotoxicity is mediated through the cell surface type I 55-kDa TNF receptor (47). Recent studies indicate that the type I TNF receptor activates a neutral sphingomyelinase and an acidic sphingomyelinase which convert sphingomyelin to ceramide; ceramide then acts as a second messenger to mediate apoptosis (14, 19, 29). TNF is also able to activate the Ras-MAP kinase pathway (54), which can lead to activation of the 85-kDa cytosolic phospholipase A₂ (22, 35), an enzyme that may be essential for TNF cytolysis (18). Most of the proteins in these TNF signalling pathways function within or at the cytoplasmic surface of membranes. It is possible that the 10.4K-14.5K complex blocks TNF cytolysis by interfering with the function of one or more of the membrane-associated proteins that participate in TNF signalling.

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