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# The Adenovirus E3 14.5-Kilodalton Protein, Which Is Required for Down-Regulation of the Epidermal Growth Factor Receptor and Prevention of Tumor Necrosis Factor Cytolysis, Is an Integral Membrane Protein Oriented with Its C Terminus in the Cytoplasm

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**We previously reported that the adenovirus type 5 E3 14.5-kilodalton protein (14.5K) forms a complex with E3 10.4K and that both proteins are required to down-regulate the epidermal growth factor receptor in adenovirus-infected human cells. Both proteins are also required to prevent cytolysis by tumor necrosis factor of most mouse cell lines infected by adenovirus mutants that lack E3 14.7K. The E3 14.5K amino acid sequence suggests that 14.5K is an integral membrane protein with an N-terminal signal sequence for membrane insertion. Here we show that 14.5K was found exclusively in cytoplasmic membrane fractions. Radiochemical sequencing of 14.5K indicated that the N-terminal signal sequence is cleaved predominantly between Cys-18 and Ser-19. With a mutant that does not express 10.4K, cleavage occurs predominantly between Phe-17 and Cys-18, indicating that the presence or absence of 10.4K affects the signal cleavage site. 14.5K was extracted into the detergent phase with Triton X-114, it remained associated with membranes after extraction with $Na_2CO_3$ at pH 11.5, and it was partially protected by membranes from proteinase K digestion; these observations indicate that 14.5K is an integral membrane protein. Proteinase K digestion followed by immunoprecipitation with antipeptide antisera directed against the N or C terminus of mature 14.5K indicated that 14.5K is oriented in the membrane with its N terminus in the lumen and its C terminus in the cytoplasm. Thus, 14.5K is a type I bitopic membrane protein. Previous studies indicated that 10.4K is also an integral membrane protein oriented with its C terminus in the cytoplasm. Altogether, these findings suggest that cytoplasmic membranes are the site of action when 10.4K and 14.5K down-regulate the epidermal growth factor receptor and prevent tumor necrosis factor cytolysis.**

The early proteins of adenovirus (Ad), which are initially synthesized prior to viral DNA replication, are of great interest because they exert their functions on key regulatory aspects of the cell and host. Recently, considerable information has been obtained about the proteins derived from the early E3 transcription unit (reviewed in references 17, 47, and 48). Six E3 proteins have been identified to date: the 6.7-kilodalton protein (6.7K) (43), gp19K (34), 11.6K (45), 10.4K (38), 14.5K (37), and 14.7K (40, 42). Little is known about 6.7K and 11.6K, but functions have been ascribed to the other proteins.

gp19K (also called E19) is a transmembrane glycoprotein, localized in the endoplasmic reticulum, which prevents cytolysis by Ad-specific (35) and alloreactive (1, 6) cytotoxic T lymphocytes. It does so, at least in part, by forming a complex with class I antigens of the major histocompatibility complex, blocking their transport to the cell surface, and thereby preventing recognition of the cell by cytotoxic T lymphocytes. gp19K has a sequence near its C terminus by which gp19K and the gp19K-class I complex are retained in the endoplasmic reticulum (12, 24, 29).

E3 14.7K protects Ad-infected mouse cells from cytolysis by tumor necrosis factor (TNF) (14, 23). TNF is a multifunctional immunoregulatory protein that has antiviral properties. Ad-infected cells are rendered sensitive to TNF cytolysis by proteins encoded by the E1A transcription unit (9, 10). Cells stably transfected with the 14.7K gene are protected from TNF cytolysis, indicating that 14.7K can function in the absence of other Ad proteins (22). 14.7K appears to be a general inhibitor of TNF cytolysis because it functions in 13 of 15 mouse cell lines tested and because it prevents TNF cytolysis when cells are sensitized to TNF by expression of E1A, by inhibition of protein synthesis, or by treatment with cytochalasin E or when cells are spontaneously sensitive to TNF cytolysis (16). Remarkably, two other E3 proteins, 10.4K and 14.5K, functioning in concert can also prevent TNF cytolysis when mouse cells are infected with Ad mutants that lack 14.7K (15). 10.4K and 14.5K are able to block TNF cytolysis in 11 of 15 mouse cell lines tested. 10.4K coimmunoprecipitates with 14.5K, strongly suggesting that the proteins exist as a complex in vivo (39). Yet another Ad protein, 19K, encoded by the E1B transcription unit, prevents TNF cytolysis of Ad-infected human cells (13). E1B 19K does not prevent TNF cytolysis of mouse cells. Thus, there are three "sets" of Ad proteins that can prevent TNF cytolysis, E3 14.7K, E3 10.4K and 14.5K, and E1B 19K. It has been hypothesized (17, 47, 48) that gp19K functions in vivo to prevent Ad-infected cells from being killed by the cytotoxic T-lymphocyte branch of the immune system and that E3 14.7K, E3 10.4K and 14.5K, and E1B 19K function to prevent killing by the TNF-macrophage branch of the immune system.

The 10.4K-14.5K complex of proteins also has another function, namely, to down-regulate cell surface expression

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of the epidermal growth factor receptor in Ad-infected cells (7, 39). It is not known whether this function of 10.4K and 14.5K is related to their anti-TNF function.

To understand how 10.4K and 14.5K function, we have begun to characterize these proteins. 10.4K migrates as two bands, upper and lower, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38). Both bands are integral membrane proteins (21, 25). Half of the upper band is processed into the lower band by cleavage between Ala-22 and Ala-23 of a signal sequence for membrane insertion, and the other half of the upper band remains uncleaved (25). Both the upper and the lower 10.4K bands are anchored in the membrane by an apolar domain in the central portion of the molecule; the C termini of both bands extend into the cytoplasm (25). The upper band also is probably anchored in the membrane by a signal anchor sequence near the N terminus. In the present communication, we show that 14.5K is also an integral membrane protein oriented in the membrane with its C terminus in the cytoplasm.

## MATERIALS AND METHODS

**Cells and viruses.** Virus stocks were prepared in suspension cultures of human KB cells, and their titers were determined on human A549 cells as described previously (18). The *rec*700 virus is an Ad type 5 (Ad5)-Ad2-Ad5 recombinant whose genome consists of the Ad5 *Eco*RI-A (map positions 0 to 76), Ad2 *Eco*RI-D (map positions 76 to 83), and Ad5 *Eco*RI-B (map positions 83 to 100) fragments (46). The *rec*700 virus is the parental virus of mutants *pm*760, *dl*748, and *dl*764. *pm*760 is a mutant that overproduces E3 mRNA *f* (5) and consequently overproduces E3 10.4K (38) and E3 14.5K (37), which are encoded by mRNA *f*. *dl*748 is a mutant that also overproduces E3 mRNA *f* (4) and E3 14.5K (37), but it does not synthesize E3 10.4K (38). *dl*764 overproduces 10.4K (38), but it does not synthesize 14.5K (37).

**In vivo protein labeling, cell fractionation, immunoprecipitation, and SDS-PAGE.** Human KB cells ($10^7$ cells total; $5 \times 10^5$ cells per ml) were infected with 150 PFU of virus per cell, and early proteins were labeled with 75 μCi of [$^{35}$S]Cys per ml (ca. 1,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass., or ICN Radiochemicals, Inc., Irvine, Calif.) at 7 to 11 h postinfection by use of the cycloheximide-enhanced procedure as described previously (44, 46). After the labeling was done, the cells were rinsed in cold phosphate-buffered saline, suspended in 0.5 ml of 0.15 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM phenylmethylsulfonyl fluoride (PMSF)–0.25 μg of leupeptin per ml, lysed by use of a loosely fitting Dounce homogenizer (Wheaton pestle B), and fractionated into cytosol and crude membrane fractions (see Fig. 2) as follows. Nuclei were removed by centrifugation at 5,000 × *g* for 5 min, and the supernatant was centrifuged in a 50 Ti rotor at 100,000 × *g* for 1 h. The supernatant (the cytosol) was removed, and the pellet was rinsed once with 0.1 M NaCl and once with distilled $H_2O$. The pellet was solubilized in 50 μl of 10% Nonidet P-40–5% sodium deoxycholate–1% SDS and diluted to 500 μl with $H_2O$.

The cytosol and solubilized crude membrane fractions were analyzed by immunoprecipitation with 5 μl of antiserum and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) as described previously (40). The antiserum used in all experiments but one (see Fig. 10) was a polyclonal rabbit antiserum against P118-132, a synthetic peptide corresponding to residues 118 to 132 in the Ad5 version of 14.5K (37).

Immunoprecipitates were analyzed by SDS-PAGE on 10 to 18% gradient gels (0.75 mm by 16 cm; acrylamide–*N*,*N*′-methylenebisacrylamide, 29.2:0.8, wt/wt). The gels were fluorographed. $^{14}$C-labeled molecular weight markers were obtained from Bethesda Research Laboratories (Gaithersburg, Md.).

**Cell-free translation of pGEM-14.5K RNA.** Plasmid pGEM-14.5K was prepared by cloning the *Eco*RI-*Bgl*II fragment (nucleotides 2482 to 2900) in E3 of *rec*700 or Ad5 (8) between the *Eco*RI and *Bam*HI sites of pGEM-7Zf(−) (Promega Corp., Madison, Wis.). The resulting plasmid was linearized with *Xho*I, and 14.5K-specific RNA was synthesized by use of SP6 bacteriophage RNA polymerase in accordance with the manufacturer's instructions. The in vitro-transcribed pGEM-14.5K RNA (1 μg) was translated in a nuclease-treated rabbit reticulocyte extract (Promega) with [$^{35}$S]Cys (1,000 Ci/mmol) as the label and in the presence of 1.8 equivalents of dog pancreas microsomes (Promega) per 25 μl of translation mixture. 14.5K was immunoprecipitated as described previously (45). In brief, the sample was adjusted to 5% SDS, boiled for 2 min, diluted to a final concentration of 0.5% SDS, and subjected to immunoprecipitation and then to SDS-PAGE.

**Protein extraction with Triton X-114 and N-terminal sequencing of 14.5K radiolabeled in vivo and in vitro.** KB cells (40 ml; $5 \times 10^5$ cells per ml) were infected with 100 PFU of *pm*760 or *dl*748 per cell and labeled with 300 μCi of [2,3,4,5-$^3$H]Pro (86 Ci/mmol; ICN) at 8 to 10 h postinfection in DME lacking serum by use of the cycloheximide-enhanced procedure. Cells were collected, rinsed, and further incubated for 2.5 h in label-free DME containing 5% horse serum. *pm*760- and *dl*748-infected cells were similarly labeled with 300 μCi of [4,5-$^3$H]Ile (95 Ci/mmol; NEN) in Ile-free DME (serum free) and incubated for 2.5 h in DME containing serum and three times the normal amount of Ile. Finally, *pm*760- and *dl*748-infected cells were similarly labeled with [$^{35}$S]Cys in Cys-free medium and chased for 2.5 h in complete medium.

*dl*748-infected cells were rinsed and lysed on ice in 800 μl of 0.14 M NaCl–1 mM $MgCl_2$–10 mM Tris-Cl (pH 8.5)–0.5% Nonidet P-40–1 mM PMSF–0.25 μg of leupeptin per ml–1 μg of pepstatin per ml. After removal of the nuclei by centrifugation, the supernatant was subjected to immunoprecipitation with the P118-132 antiserum as described previously (37).

A different lysis procedure was used for the *pm*760-infected cells to avoid coimmunoprecipitation of the 10.4K protein. Cells were suspended in buffer C (0.15 M NaCl, 10 mM Tris-Cl [pH 7.4], 1 mM PMSF, 0.25 μg of leupeptin per ml, 1 μg of pepstatin per ml), and the cellular membranes were solubilized on ice with 1% (final concentration) Triton X-114 (3). The sample was subjected to phase separation as described by Bordier (3). In brief, after removal of the particulate matter by centrifugation, the sample was layered over a sucrose cushion, warmed to 30°C to separate the phases, and centrifuged at room temperature. The detergent phase (an oily droplet at the bottom of the centrifuge tube) was diluted with about 10 volumes of buffer C containing 2.5% SDS. The sample was boiled for 5 min and diluted 1:12 in immunoprecipitation buffer, and immunoprecipitation was carried out as described previously (37).

For both the *dl*748 and the *pm*760 [$^3$H]Pro- and [$^{35}$S]Cys-labeled samples, 14.5K was eluted from the protein A beads with 300 μl of 50 mM $NH_4HCO_3$ (pH 7.5)–0.1% SDS–0.2% β-mercaptoethanol–50 μg of bovine serum albumin per ml in

a boiling water bath for 5 min. A portion (5 to 10%) of each sample was analyzed for purity by SDS-PAGE, and the remainder was subjected to N-terminal sequencing in a Beckman 890C spinning-cup sequencer as described elsewhere (28).

pGEM-14.5K RNA was transcribed in 50 μl of rabbit reticulocyte lysate pretreated for 15 min at 37°C with 1.25 U of citrate synthase (Sigma)–1 mM oxaloacetic acid (Sigma) to prevent acetylation of the N terminus of 14.5K (30). The label was [$^3$H]Leu. 14.5K was immunoprecipitated (45) and subjected to SDS-PAGE, and the dried gel was autoradiographed without fluorography. The two bands corresponding to 14.5K were excised from the gel in one piece and eluted into 5 ml of 50 mM $NH_4HCO_3$–0.1% SDS–0.2% β-mercaptoethanol–50 μg of bovine serum albumin per ml for 1 day at 37°C with slow mixing. After repeated lyophilization, 14.5K was sequenced on an Applied Biosystems model 477A gas-liquid-phase protein sequencer equipped with an on-line Applied Biosystems model 120A phenylthiohydantoin amino acid analyzer in the protein sequencing facility in the Department of Biochemistry and Molecular Biology, St. Louis University Medical Center.

**Extraction of 14.5K-containing membranes with $Na_2CO_3$ at pH 11.5.** For 14.5K synthesized in vivo, KB cells ($10^7$ cells total; $3.5 \times 10^5$ cells per ml) were infected with *pm*760 and proteins were labeled at 7 to 11 h postinfection with [$^{35}$S]Cys by use of the cycloheximide-enhanced procedure. Cells were collected, rinsed once in cold phosphate-buffered saline and twice in 80 mM sucrose–10 mM Tris-Cl (pH 7.2), and lysed in 0.5 ml of the latter buffer by Dounce homogenization with a loosely fitting Wheaton homogenizer (pestle B). Nuclei were removed by centrifugation at 5,000 × *g* for 5 min. The supernatant containing the cytosol and crude membrane fractions was extracted with $Na_2CO_3$ (pH 11.5) as described by Perara and Lingappa (31). In brief, half the sample was adjusted to 0.1 M $Na_2CO_3$ (pH 11.5), and the other half was adjusted to 0.25 M sucrose–5 mM $MgCl_2$–1 mM dithiothreitol–50 mM triethanolamine–0.1 M KCl. A portion of each sample was centrifuged in a Beckman 50 Ti rotor for 1 h at 4°C and 48,000 rpm. The supernatant of the $Na_2CO_3$-treated sample was neutralized with glacial acetic acid. Pellets were rinsed with water and solubilized in 5% SDS. After dilution to 0.5% SDS, 14.5K was immunoprecipitated from the supernatants and solubilized pellets and analyzed by SDS-PAGE.

For 14.5K synthesized in vitro, pGEM-14.5K RNA was translated in the presence of microsomes with [$^{35}$S]Cys as the label and 12.5 μl of the translation mixture was extracted with $Na_2CO_3$ or was used as a non-$Na_2CO_3$ control as described above. As controls, pGEM-gp19K RNA transcribed from a pGEM-7Zf(+) clone containing the *Hin*dIII-*Xho*I fragment (nucleotides 1045 to 2180) in E3 of Ad2 (8, 20) and β-lactamase RNA (Promega) were also processed in a similar manner.

**Proteinase K digestion of dog pancreas microsomes containing 14.5K.** pGEM-14.5K RNA was translated in the presence of dog pancreas microsomes with [$^{35}$S]Cys to label 14.5K, and 50 μl of the translation mixture was centrifuged. The pellet was rinsed with 100 μl of 50 mM Tris-Cl (pH 7.5) and resuspended in 70 μl of the same buffer. Aliquots (16 μl) of this sample were incubated on ice for 90 min with or without the addition of 2 μl of proteinase K (10 mg/ml; Bethesda Research Laboratories) or 2 μl of detergent mixture (10% Nonidet P-40, 5% sodium deoxycholate, 1% SDS). Samples to be treated with proteinase K were adjusted to 10 mM $CaCl_2$ just prior to the addition of proteinase K because 14.5K was partially degraded in the presence of $CaCl_2$ even without the addition of the protease. Proteinase K was preincubated for 15 min at 37°C to degrade contaminating lipases (19). Digestion was stopped by the addition of an equal amount of 2× Laemmli buffer and 1 μl of 33 mM PMSF. The samples were immediately placed in a boiling water bath for 5 min and analyzed by SDS-PAGE.

**Orientation of 14.5K in the membrane.** pGEM-14.5K RNA was translated for 60 min at 30°C in the presence of dog pancreas microsomes with [$^{35}$S]Cys as the label. The sample (100 μl) was adjusted to 10 mM $CaCl_2$ by the addition of 1.1 μl of 1 M $CaCl_2$ (pH 7.8) and split in half. Proteinase K (1.25 μl of 10-mg/ml proteinase K in 50 mM Tris-Cl [pH 7.5]–10 mM $CaCl_2$) was added to half the sample, and the other half was treated with proteinase K buffer. The samples were incubated for 90 min on ice, 2 μl of 0.1 M PMSF was added, and the samples were centrifuged. Pellets were rinsed with 100 μl of 50 mM Tris-Cl (pH 6.8)–1 mM PMSF and resuspended in 80 μl of the same buffer. Twenty microliters was mixed with 60 μl of 2× Laemmli buffer, and the mixture was boiled for 3 min. The remainder of the sample (60 μl) was mixed with 60 μl of 3.3% SDS and boiled for 3 min, and 40-μl aliquots were immunoprecipitated with different antisera in a final volume of 500 μl. Antisera to P19-34 and P118-132, corresponding to residues 19 to 34 and 118 to 132, respectively, in the 14.5K protein, were used (37). Immunoprecipitates were analyzed by SDS-PAGE on 10 to 18% gradient gels.

## RESULTS

**14.5K is found in crude membrane fractions.** The predicted sequence of 14.5K (Fig. 1) suggests that it is an integral membrane protein. To determine whether this is so, we metabolically labeled *pm*760-infected KB cells with [$^{35}$S]Cys, lysed them by Dounce homogenization, and isolated and analyzed the cytosol and crude membrane fractions by immunoprecipitation using an antiserum to 14.5K. *pm*760 is a mutant that overproduces both E3 14.5K and E3 10.4K (37, 38). 14.5K was found exclusively in the crude membrane fraction (Fig. 2). 10.4K was coimmunoprecipitated with 14.5K, indicating that 10.4K and 14.5K form a complex in vivo (39). As expected, 10.4K was coimmunoprecipitated with 14.5K from the crude membrane fraction (Fig. 2; the middle and lowest bands are the two forms of 10.4K).

Given that 14.5K and 10.4K form a complex, we considered it possible that 14.5K might not associate with membranes in the absence of 10.4K. Accordingly, 14.5K from *dl*748-infected cells was analyzed as described above; *dl*748 is a deletion mutant that overproduces 14.5K but does not synthesize 10.4K (37, 38). 14.5K was found in the crude membrane fraction but not in the cytosol fraction (Fig. 2). Therefore, 14.5K can associate with membranes in the absence of 10.4K.

14.5K migrates as multiple bands in SDS-PAGE, indicating that it undergoes posttranslational modification (Fig. 2). Note that the pattern of 14.5K bands obtained from *dl*748 is quite different from the pattern obtained from *pm*760. With *pm*760, there is a major upper band and two minor bands with increased mobilities. The dark middle band is the uncleaved form of 10.4K. With *dl*748, about six bands, three major and three minor, are apparent. Thus, the presence or absence of 10.4K affects the posttranslational modification of 14.5K.

**14.5K is cleaved predominantly between Cys-18 and Ser-19**

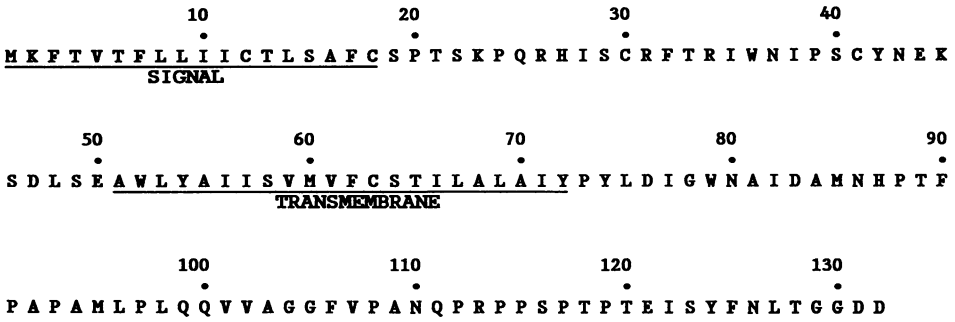


FIG. 1. Sequence of the 14.5K gene of Ad5. The DNA sequence of the 14.5K gene was determined by Cladaras and Wold (8).

**in the presence of 10.4K and between Phe-17 and Cys-18 in the absence of 10.4K.** Radiochemical sequence analyses were carried out to determine whether the hydrophobic domain near the N terminus of 14.5K is a signal sequence that is cleaved. KB cells were infected with *pm*760 or *dl*748, the proteins were labeled with [$^3$H]Pro or [$^3$H]Ile, and 14.5K was immunoprecipitated (see Materials and Methods). To avoid coimmunoprecipitation of 10.4K from the *pm*760 sample, we boiled the sample in 2.5% SDS and carried out immunoprecipitation with 0.2% SDS. Analysis of a portion of the samples indicated that the [$^3$H]Ile-labeled 14.5K samples from *dl*748 (Fig. 3A, lane a) and *pm*760 (lane c) were essentially free of contaminating proteins, as were the [$^3$H]Pro-labeled samples from *dl*748 (Fig. 3B, lane a) and *pm*760 (lane b).

The remainder of the immunoprecipitated 14.5K samples was subjected to N-terminal sequencing without further purification. With the [$^3$H]Pro-labeled sample from *pm*760, peaks were observed at cycles 2 and 6 (Fig. 4, top); these are the results expected if 14.5K is cleaved between Cys-18 and Ser-19 (Fig. 1). With the [$^3$H]Ile-labeled sample from *pm*760, peaks were observed at cycles 10, 17, and 20 (Fig. 4, bottom); again, these results are expected if cleavage occurs between Cys-18 and Ser-19 (Fig. 1). However, the cleavage peaks were not as distinct as usually observed. For example, with the [$^3$H]Ile-labeled sample, the peak at cycle 10 tailed

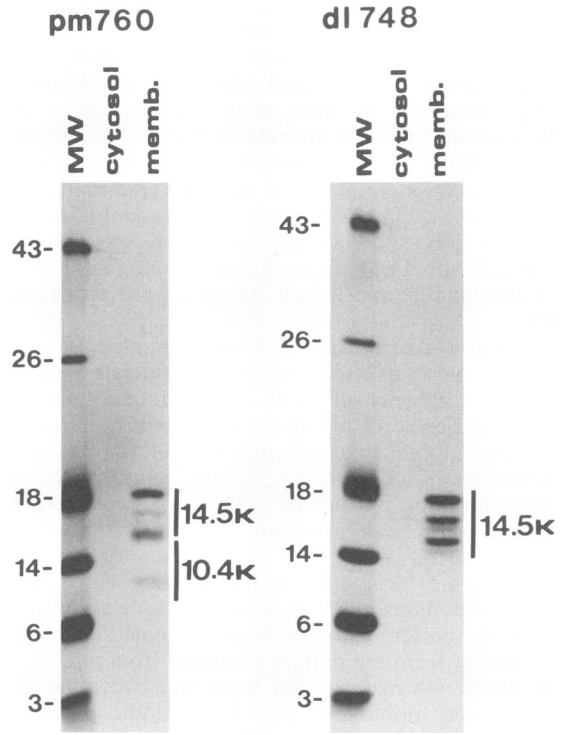


FIG. 2. Membrane association of 14.5K. *pm*760- or *dl*748-infected KB cells were labeled with [$^{35}$S]Cys, the cells were lysed with a Dounce homogenizer, and cytosol and crude membrane (memb.) fractions were collected and analyzed by immunoprecipitation with antisera to 14.5K. Numbers at left are molecular weight markers.

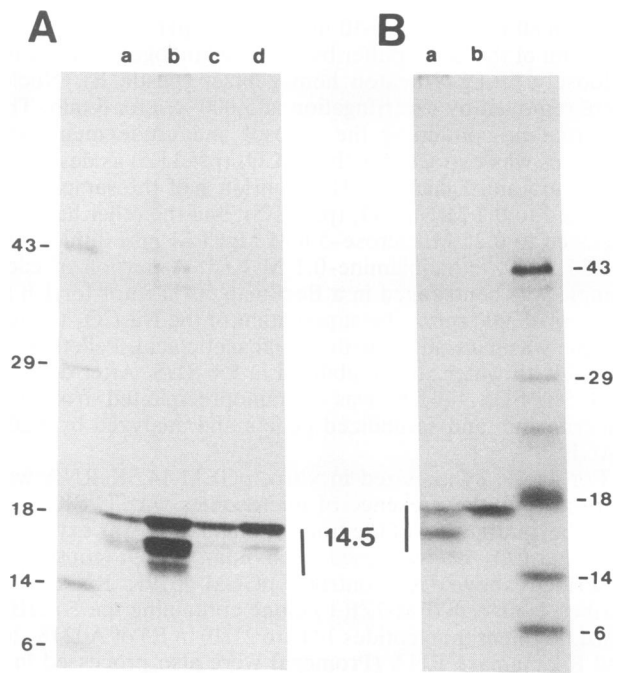


FIG. 3. Presence of 14.5K in the detergent phase after extraction with Triton X-114 and purity of the samples used for N-terminal sequencing. (A) 14.5K from *dl*748- or *pm*760-infected KB cells was metabolically labeled with [$^3$H]Ile or [$^{35}$S]Cys. The *pm*760-infected samples were subjected to Triton X-114 extraction and phase separation as described in Materials and Methods. Lanes: a, *dl*748, with [$^3$H]Ile as the label; b, *dl*748, with [$^{35}$S]Cys as the label; c, *pm*760, with [$^3$H]Ile as the label; d, *pm*760, with [$^{35}$S]Cys as the label. The N-terminal radiochemical sequences of the samples in lanes a and c are shown in Fig. 4 and 5 (lower panels). (B) Same as panel A but with [$^3$H]Pro as the label. Lanes: a, *dl*748; b, *pm*760. The N-terminal radiochemical sequences of these samples are shown in Fig. 4 and 5 (upper panels). Numbers on outsides of panels are molecular weight markers.

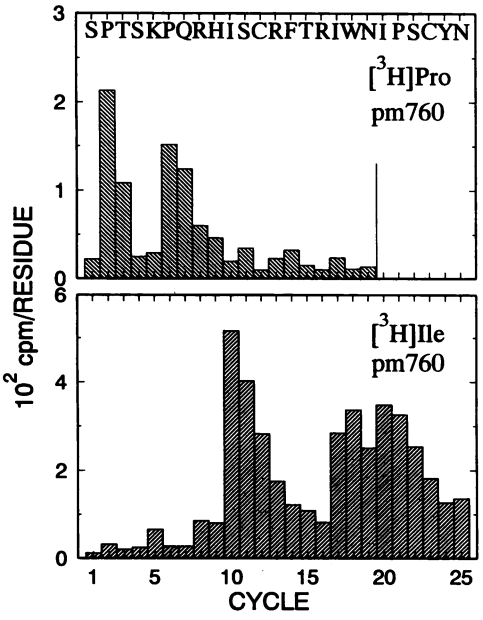


FIG. 4. N-terminal radiochemical sequence of [$^3$H]Pro- or [$^3$H]Ile-labeled 14.5K extracted from *pm*760-infected KB cells. The purities of the [$^3$H]Pro- and [$^3$H]Ile-labeled samples are shown in Fig. 3B, lane b, and Fig. 3A, lane c, respectively. Samples were applied to a spinning-cup sequencer together with 0.5 mg of apomyoglobin and 3 mg of Polybrene. The residue in each cycle was dissolved in 50% methanol; half was mixed with scintillation fluid and used to determine the radioactivity present. Part of the remainder of the residue from selected cycles was analyzed for phenylthiohydantoin amino acids that resulted from the carrier apomyoglobin. Shown is the total radioactivity in the residue for each cycle. The radioactivity applied to the sequencer was as follows: [$^3$H]Pro, 7,700 cpm; [$^3$H]Ile, 18,100 cpm.

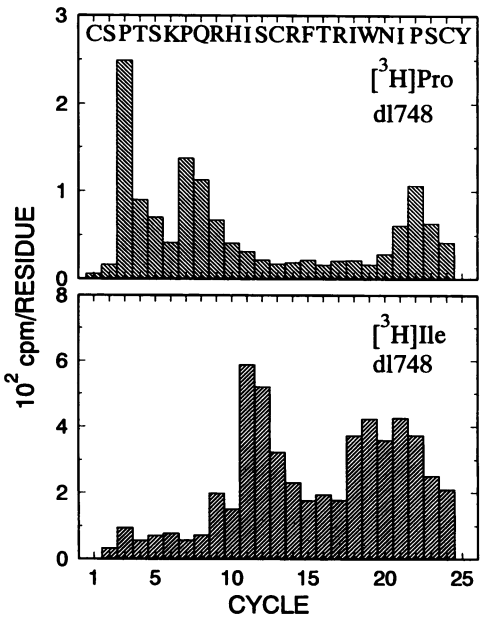


FIG. 5. N-terminal radiochemical sequence of [$^3$H]Pro- or [$^3$H]Ile-labeled 14.5K extracted from *dl*748-infected KB cells. The purities of the [$^3$H]Pro- and [$^3$H]Ile-labeled samples are shown in Fig. 3B, lane a, and Fig. 3A, lane a, respectively. The sequence analysis was as described in the legend to Fig. 4. The radioactivity applied to the sequencer was as follows: [$^3$H]Pro, 4,800 cpm; [$^3$H]Ile, 26,000 cpm.

significantly and the residues from cycles 17 and 18 and from cycles 20 and 21 had nearly equal counts. Part of this apparent asynchrony in the sequence probably resulted from incomplete reactions during the Edman degradations, which may occur if the polypeptide is not fully solubilized during the coupling or cleavage steps. However, the asynchrony also suggests that cleavage of the signal sequence may be imprecise (see *dl*748 results below). We interpret the results shown in Fig. 4 to indicate that the majority of 14.5K from *pm*760 was cleaved predominantly between Cys-18 and Ser-19; some cleavage may also have occurred between Phe-17 and Cys-18 and perhaps also at other nearby positions.

With the *dl*748 samples, the cleavage site was predominantly between Phe-17 and Cys-18; i.e., [$^3$H]Pro peaks were observed at cycles 3, 7, and 22 (Fig. 5, top), and [$^3$H]Ile peaks were observed at cycles 11, 18, and 21 (Fig. 5, bottom). Again, there appeared to be heterogeneity in the cleavage site, as almost equal [$^3$H]Pro counts were observed in cycles 7 and 8 and almost equal [$^3$H]Ile counts were observed in cycles 18 and 19 and in cycles 21 and 22. That the sequence asynchrony did not result from malfunctioning of the sequencer was determined by analysis of the carrier apomyoglobin, which was also present during these sequencing reactions.

We conclude that 14.5K from *pm*760 and 14.5K from *dl*748 are cleaved primarily between Cys-18 and Ser-19 and between Phe-17 and Cys-18, respectively, but that a minority of molecules are cleaved one or two residues upstream or downstream of these sites. Since 14.5K from *pm*760 and 14.5K from *dl*748 are cleaved predominantly at different positions, it appears that the presence or absence of 10.4K affects the signal cleavage site.

**14.5K is extracted into the detergent phase with Triton X-114.** Bordier (3) has shown that when Triton X-114 is used to solubilize cellular membranes and the solubilized material is subjected to temperature-dependent phase separation, integral membrane proteins with an amphiphilic nature are partitioned into the detergent phase, whereas hydrophilic proteins are excluded from this phase. Accordingly, we used extraction and phase separation with Triton X-114 for *pm*760-infected cells to address whether 14.5K is an integral membrane protein. Indeed, 14.5K labeled with [$^3$H]Ile (Fig. 3A, lane c), [$^{35}$S]Cys (Fig. 3A, lane d), or [$^3$H]Pro (Fig. 3B, lane b) was immunoprecipitated from the detergent phase. This result indicates that 14.5K is an integral membrane protein. 10.4K has also been shown to be partitioned into the Triton X-114 detergent phase (21).

**14.5K remains associated with membranes after extraction at pH 11.5.** To obtain further evidence that 14.5K is an integral membrane protein, we used the alkaline extraction procedure of Fujiki et al. (11). These authors showed that when membranes are extracted with $Na_2CO_3$ at pH 11.0 to 11.5, peripheral membrane proteins are extracted, membrane vesicles are converted to membrane sheets, thereby releasing lumenal proteins, but integral membrane proteins remain associated with membranes. *pm*760-infected cells were labeled with [$^{35}$S]Cys, and the crude membrane pellet was isolated. Half the sample was treated with $Na_2CO_3$ (pH 11.5), a portion was removed, and the membranes were centrifuged to produce pellet and soluble fractions. The other half of the sample was similarly processed but without

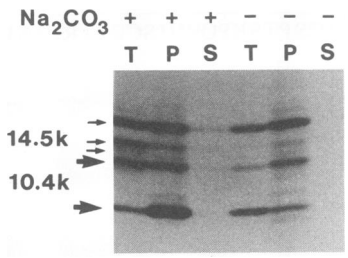


FIG. 6. Continued association of 14.5K synthesized in vivo with membranes after extraction with $Na_2CO_3$ at pH 11.5. [$^{35}$S]Cys-labeled crude membrane fractions were prepared from *pm*760-infected KB cells. Half of each fraction was treated with $Na_2CO_3$ (pH 11.5) (+), and half was left untreated (−). A portion of each half was removed (T, for total), and the remainder was centrifuged. The supernatant (S) was collected, and the membranes in the pellet (P) were solubilized in 5% SDS. After dilution of the pellet fraction to 0.5% SDS, 14.5K was immunoprecipitated from the T, S, and P samples.

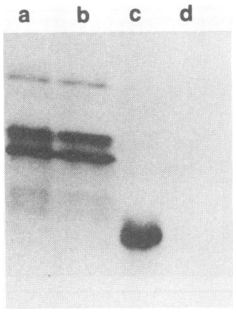


FIG. 8. Partial protection of 14.5K by membranes from proteinase K digestion. pGEM-14.5K RNA was translated in the presence of microsomes with [$^{35}$S]Cys as the label. The microsomes were pelleted, suspended in buffer with or without detergent, digested or not digested with proteinase K, and analyzed by SDS-PAGE. Lanes: a, no detergent, no proteinase K; b, detergent, no proteinase K; c, no detergent, proteinase K; d, detergent, proteinase K.

$Na_2CO_3$ extraction. All fractions were analyzed by SDS-PAGE. Nearly all of 14.5K (Fig. 6, small arrows) remained associated with the pellet fraction, indicating that all the forms of 14.5K are integral membrane proteins. The two bands corresponding to 10.4K (Fig. 6, thick arrows) were coimmunoprecipitated from the pellet fraction, confirming similar earlier results (25) and consistent with the conclusion that 10.4K is an integral membrane protein.

As a means of obtaining more data on the membrane association properties of 14.5K, pGEM-14.5K RNA, transcribed in vitro from a pGEM clone containing the 14.5K gene, was translated in a nuclease-treated rabbit reticulocyte extract in the presence of dog pancreas microsomes. Under these conditions, two major bands corresponding to 14.5K were observed, and both of these became associated with the microsomal membranes (Fig. 7, 8, and 9). The lower band is the primary translation product, and the upper band arises by phosphorylation of 14.5K in the reticulocyte extract (26). To determine where translation initiated in vitro, we translated pGEM-14.5K RNA in the absence of microsomes using [$^3$H]Leu as a label. 14.5K was immunoprecipitated and analyzed by N-terminal radiochemical sequencing. Radioactive peaks were observed at cycles 1, 8, and 9 (Fig. 10). If 14.5K initiates at the equivalent of AUG-2495 in the E3 transcription unit of Ad5 (8), then Leu should be found at cycles 8 and 9 (Fig. 1 and 10). We conclude that 14.5K initiates translation in pGEM-14.5K RNA at the equivalent of AUG-2495. The peak at cycle 1 is due to wash-through of the sample (a predegradation cycle was not performed).

To determine whether 14.5K translated from pGEM-14.5K RNA in the presence of microsomes is an integral membrane protein, we extracted the microsomes with $Na_2CO_3$ at pH 11.5. Both bands of 14.5K remained with the membrane pellet fraction and not the soluble fraction extracted by $Na_2CO_3$ (Fig. 7). When this same experiment was attempted with gp19K, a known integral membrane protein (33), this protein also remained in the membrane pellet fraction, as expected (Fig. 7). In contrast, when this experiment was performed with β-lactamase, a lumenal protein, β-lactamase was extracted into the soluble fraction, as expected (Fig. 7). These results further indicate that 14.5K is an integral membrane protein.

**14.5K is partially protected by membranes from protease**

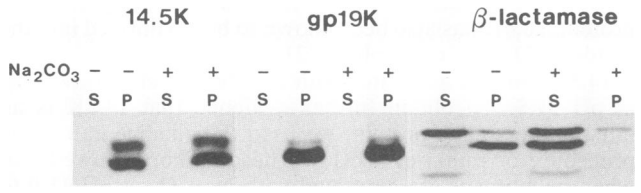


FIG. 7. Continued association of 14.5K synthesized by cell-free translation of pGEM-14.5K RNA in the presence of dog pancreas microsomes with membranes after extraction with $Na_2CO_3$ at pH 11.5. After translation, [$^{35}$S]Cys-labeled 14.5K was treated (+) or not treated (−) with $Na_2CO_3$ (pH 11.5) and then centrifuged. The supernatant (S) was removed, and the membranes in the pellet (P) were solubilized in 5% SDS. After dilution of the membrane fraction to 0.5% SDS, the S and P fractions were analyzed by SDS-PAGE. RNAs encoding gp19K or β-lactamase were translated in vitro and processed as described for pGEM-14.5K RNA.

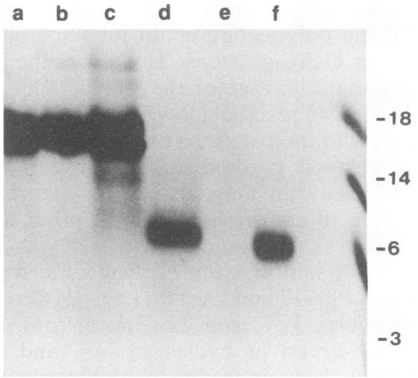


FIG. 9. Orientation of 14.5K in the membrane with the C terminus accessible and the N terminus inaccessible to proteinase K digestion. [$^{35}$S]Cys-labeled 14.5K was translated in vitro in the presence of microsomes. The microsomes were pelleted, suspended in proteinase K buffer, digested or not digested with proteinase K, and analyzed by immunoprecipitation and SDS-PAGE. Lanes: a, no proteinase K, P19-34 antiserum; b, no proteinase K, P118-132 antiserum; c, no proteinase K, pelleted, solubilized, and electrophoresed membranes; d, same as in lane c but with proteinase K; e, proteinase K, P118-132 antiserum; f, proteinase K, P19-34 antiserum. Numbers at right are molecular weight markers.

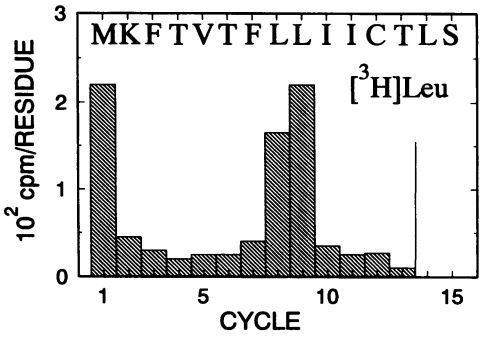


FIG. 10. N-terminal radiochemical sequence of 14.5K translated in vitro from pGEM-14.5K RNA. pGEM-14.5K RNA was translated for 10 min in the absence of microsomes with [$^3$H]Leu as the label. [$^3$H]Leu-labeled 14.5K was immunoprecipitated and sequenced. Analysis of a portion of the sample by SDS-PAGE prior to sequencing indicated that the sample was free of contaminating proteins (data not shown).

**digestion.** If 14.5K is an integral membrane protein, then it should be partially protected by membranes from digestion with protease. Accordingly, 14.5K was translated in vitro in the presence of microsomes, and a portion of the sample was digested with proteinase K in the presence or absence of detergent to solubilize the membranes. 14.5K was not observed in the detergent-treated, proteinase K-digested sample (Fig. 8, lane d), indicating that extensive proteolysis of 14.5K had occurred. In contrast, a strong band of increased mobility was seen after proteinase K digestion in the absence of detergent (lane c), indicating that a portion of 14.5K was resistant to proteolysis. This band was not observed in the absence of proteinase K digestion (lanes a and b). Thus, 14.5K is partially protected from proteolysis by microsomal membranes.

**14.5K is oriented in the membrane with its C terminus in the cytoplasm.** Further experiments were carried out to determine the orientation of 14.5K in the membrane. 14.5K was translated in vitro in the presence of dog pancreas microsomes, digested with proteinase K, immunoprecipitated with antipeptide antisera directed against the N or C termini of mature 14.5K, and analyzed by SDS-PAGE. A band with a molecular weight of about 6,000 was immunoprecipitated with the antiserum specific for residues 19 to 34 in 14.5K (Fig. 9, lane f). This band had a mobility similar to that of the band obtained by simple pelleting of the microsomes (lane d). No band was detected with the antiserum directed against residues 118 to 132 in 14.5K (lane e). Both the N- and C-terminus-specific antisera immunoprecipitated full-length 14.5K in the absence of proteinase K digestion (lanes a and b). Thus, 14.5K appears to be oriented in the membrane with its C terminus in the cytoplasm, accessible to proteinase K, but with its N terminus protected from proteinase K, probably because the N terminus is in the lumen of the endoplasmic reticulum.

## DISCUSSION

The predicted sequence of 14.5K is consistent with 14.5K being an integral membrane protein (Fig. 1). The N-terminal apolar domain of 14.5K is typical of signals for membrane insertion, i.e., a basic residue (Lys) near the N terminus and a hydrophobic central core of at least seven residues (36, 41). This apolar domain in 14.5K is almost certainly a signal for membrane insertion because, as we have shown here, 14.5K is found exclusively in crude membrane fractions and the signal sequence is cleaved. For *pm*760, which expresses both 14.5K and 10.4K, cleavage occurred predominantly between Cys-18 and Ser-19. This cleavage site is in accord with the "−3/−1" rule, which states that the residue at position −1 (Cys-18) must be small, that the residue at position −3 (Ala-16) must not be aromatic, charged, or polar, and that Pro must be absent from positions −3 through −1 (32, 41). Also, with this rule, the region near the cleavage site is hydrophilic and often deviates from a potential α-helix configuration; this is the case for 14.5K (Lys is at position 23 and Pro is at position 20). For *dl*748, which does not express 10.4K, cleavage occurred predominantly between Phe-17 and Cys-18. This cleavage site deviates to some extent from the −3/−1 rule because Phe-17 at position −1 is not small. Nevertheless, this cleavage site is in accord with the sequence data for both the [$^3$H]Pro- and the [$^3$H]Ile-labeled samples. For both the *pm*760 and the *dl*748 samples, the sequence data suggest that a minority of the molecules were cleaved one or two residues upstream or downstream of the major cleavage site.

14.5K was shown to be an integral membrane protein by several methods. First, it was found in the detergent phase after extraction with Triton X-114, a property of integral membrane proteins (3). Second, it remained associated with the membrane pellet after extraction with $Na_2CO_3$ at pH 11.5. Only integral membrane proteins, not peripheral or lumenal membrane proteins, remain associated with membranes after alkali extraction (11). Third, 14.5K translated in vitro was partially protected by microsomal membranes from proteinase K digestion.

When 14.5K in microsomal membranes was digested with proteinase K and immunoprecipitated with antipeptide antiserum specific for residues 19 to 34 at the N terminus or residues 118 to 132 at the C terminus, a protein with a molecular weight of ca. 6,000 was detected with the former antiserum and no protein was detected with the latter antiserum. Thus, the C-terminal but not the N-terminal region was accessible to proteolysis, indicating that the C terminus extends into the cytoplasm and the cleaved N terminus extends into the lumen. This conclusion is also consistent with the size of the proteinase K digestion product (molecular weight of 6,000). If one assumes that residues 51 to 72 are the transmembrane domain, the Pro at residue 73 is probably not included because transmembrane domains usually are α-helices. Thus, after the signal sequence is cleaved and proteinase K digests the C-terminal sequences near the membrane, about 54 residues remain; these have a molecular weight of nearly 6,000. Altogether, these observations indicate that 14.5K is a typical type I bitopic integral membrane protein (2, 36).

When 10.4K was analyzed by methods similar to those described here for 14.5K (25), we concluded that the lower band of 10.4K is a type I bitopic membrane protein with a cleaved N-terminal signal sequence. Thus, the lower 10.4K band and 14.5K have similar configurations in the membrane. The upper band of 10.4K also has its C terminus in the cytoplasm, but the N-terminal signal sequence is uncleaved. We postulated that this band of 10.4K has its N terminus in the cytoplasm, that the hydrophobic domain at residues 5 to 25 spans the membrane, that ca. residues 26 to 34 loop into the lumen, that the hydrophobic domain at ca. residues 35 to 60 spans the membrane, and that residues 61 to 91 extend into the cytoplasm (25). It will be interesting to determine the nature of the interactions among 14.5K and the two forms of 10.4K.

The difference in the cleavage site in 14.5K in the presence and absence of 10.4K documents another way in which 10.4K affects the properties of 14.5K. 14.5K migrates as multiple bands in SDS-PAGE because it is both phosphorylated (26) and O glycosylated (27). The band heterogeneity of 14.5K is more pronounced in the absence of 10.4K than in the presence of 10.4K (Fig. 2) (37). Also, in the presence of 10.4K only phosphoserine can be detected in 14.5K, whereas in the absence of 10.4K phosphothreonine can be detected as well (26). A 10.4K effect on the properties of 14.5K is not surprising, considering that 10.4K is coimmunoprecipitated with 14.5K, implying that the two proteins exist as a complex. However, the mechanism by which 10.4K affects the cleavage site in 14.5K is unclear. Given that the cleavage of signal sequences is generally cotranslational (36), it would appear that the nascent polypeptide chain of 14.5K is able to interact with 10.4K during the translation of 14.5K.

10.4K and 14.5K are both required, and apparently function as a complex, to down-regulate the cell surface epidermal growth factor receptor (7, 39) and to prevent TNF cytolysis (15) in Ad-infected cells. It is not known whether these functions of 10.4K and 14.5K are related or different. Regardless, these clearly are membrane-related phenomena, considering that 10.4K and 14.5K are integral membrane proteins. It seems likely that 10.4K and 14.5K exert their effect at the plasma membrane, as this is where the epidermal growth factor receptor and the TNF receptors are located, but this remains to be determined.

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## REFERENCES

1. **Andersson, M., A. McMichael, and P. A. Peterson.** 1987. Reduced allorecognition of adenovirus 2 infected cells. J. Immunol. **138**:3960–3966.
2. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77**:1496–1500.
3. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604–1607.
4. **Brady, H. A., and W. S. M. Wold.** 1987. Identification of a novel sequence that governs both polyadenylation and alternative splicing in region E3 of adenovirus. Nucleic Acids Res. **15**:9397–9416.
5. **Brady, H. A., and W. S. M. Wold.** 1988. Competition between splicing and polyadenylation reactions determines which adenovirus region E3 mRNAs are synthesized. Mol. Cell. Biol. **8**:3291–3297.
6. **Burgert, H.-G., J. L. Maryanski, and S. Kvist.** 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proc. Natl. Acad. Sci. USA **84**:1356–1360.
7. **Carlin, C. R., A. E. Tollefson, H. A. Brady, B. L. Hoffman, and W. S. M. Wold.** 1989. Epidermal growth factor receptor is down-regulated by a 10,400 MW protein encoded by the E3 region of adenovirus. Cell **57**:135–144.
8. **Cladaras, C., and W. S. M. Wold.** 1985. DNA sequence of the E3 transcription unit of adenovirus 5. Virology **140**:28–43.
9. **Duerksen-Hughes, P., W. S. M. Wold, and L. R. Gooding.** 1989. Adenovirus E1A renders infected cells sensitive to cytolysis by tumor necrosis factor. J. Immunol. **143**:4193–4200.
10. **Duerksen-Hughes, P. J., T. W. Hermiston, W. S. M. Wold, and L. R. Gooding.** 1991. The amino-terminal portion of CD1 of the adenovirus E1A proteins is required to induce susceptibility to tumor necrosis factor cytolysis in adenovirus-infected mouse cells. J. Virol. **65**:1236–1244.
11. **Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow.** 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. **93**:97–102.
12. **Gabathuler, R., and S. Kvist.** 1990. The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus type 2 consists of three separate amino acid segments at the carboxy terminus. J. Cell Biol. **111**:1803–1810.
13. **Gooding, L. R., L. Aquino, P. J. Duerksen-Hughes, D. Day, T. M. Horton, S. Yei, and W. S. M. Wold.** 1991. The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. J. Virol. **65**:3083–3094.
14. **Gooding, L. R., L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold.** 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53**:341–346.
15. **Gooding, L. R., T. S. Ranheim, A. E. Tollefson, L. Aquino, P. Duerksen-Hughes, T. M. Horton, and W. S. M. Wold.** 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. **65**:4114–4123.
16. **Gooding, L. R., I. O. Sofola, A. E. Tollefson, P. Duerksen-Hughes, and W. S. M. Wold.** 1990. The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. J. Immunol. **145**:3080–3086.
17. **Gooding, L. R., and W. S. M. Wold.** 1990. Molecular mechanisms by which adenoviruses counteract antiviral immune defenses. Crit. Rev. Immunol. **10**:53–71.
18. **Green, M., and W. S. M. Wold.** 1979. Preparation of human adenoviruses. Methods Enzymol. **58**:425–435.
19. **Hansen, W., P. D. Garcia, and P. Walter.** 1986. *In vitro* protein translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the prepro-α-factor. Cell **45**:397–406.
20. **Hérissé, J., G. Courtois, and F. Galibert.** 1980. Nucleotide sequence of the EcoRI D fragment of adenovirus 2 genome. Nucleic Acids Res. **8**:2173–2192.
21. **Hoffman, B. L., A. Ullrich, W. S. M. Wold, and C. R. Carlin.** 1990. Retrovirus-mediated transfer of an adenovirus gene encoding an integral membrane protein is sufficient to down-regulate the receptor for epidermal growth factor. Mol. Cell. Biol. **10**:5521–5524.
22. **Horton, T. M., T. S. Ranheim, L. Aquino, D. I. Kusher, S. K. Saha, C. F. Ware, W. S. M. Wold, and L. R. Gooding.** 1991. Adenovirus E3 14.7K protein functions in the absence of other adenovirus proteins to protect transfected cells from tumor necrosis factor cytolysis. J. Virol. **65**:2629–2639.
23. **Horton, T. M., A. E. Tollefson, W. S. M. Wold, and L. R. Gooding.** 1990. A protein serologically and functionally related to the group C E3 14,700-kilodalton protein is found in multiple adenovirus serotypes. J. Virol. **64**:1250–1255.
24. **Jackson, M. R., T. Nilsson, and P. A. Peterson.** 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. **9**:3153–3162.
25. **Krajcsi, P., A. E. Tollefson, C. W. Anderson, A. R. Stewart, C. R. Carlin, and W. S. M. Wold.** The E3-10.4K protein of adenovirus is an integral membrane protein that is partially cleaved between $Ala_{22}$ and $Ala_{23}$ and has a $C_{cyt}$ orientation. Virology, in press.
26. **Krajcsi, P., and W. S. M. Wold.** The adenovirus E3-14.5K protein which is required for prevention of TNF cytolysis and for down-regulation of EGF receptor contains phosphoserine. Virology, in press.
27. **Krajcsi, P., and W. S. M. Wold.** Unpublished data.
28. **Lees-Miller, S. P., and C. W. Anderson.** 1989. The human double-stranded DNA-activated protein kinase phosphorylates the 90-kDa heat-shock protein, hsp90α, at two $NH_2$-terminal threonine residues. J. Biol. Chem. **264**:17275–17280.
29. **Pääbo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson.** 1987. A short sequence in the COOH-terminus makes an adenovirus

membrane glycoprotein a resident of the endoplasmic reticulum. Cell **50**:311–317.
30. **Palmiter, R. D.** 1977. Acetylation of proteins synthesized in cell free systems. J. Biol. Chem. **252**:8781–8783.
31. **Perara, E., and V. R. Lingappa.** 1985. A former amino terminal signal sequence engineered to an internal location directs translocation of both flanking protein domains. J. Cell Biol. **101**: 2292–2301.
32. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391–409.
33. **Persson, H., M. Jansson, and L. Philipson.** 1980. Synthesis and genomic site for an adenovirus type 2 early glycoprotein. J. Mol. Biol. **136**:375–394.
34. **Persson, H., H. Jornvall, and J. Zabielski.** 1980. Multiple mRNA species for the precursor to an adenovirus-encoded glycoprotein: identification and structure of the signal sequence. Proc. Natl. Acad. Sci. USA **77**:6349–6353.
35. **Rawle, F. C., A. E. Tollefson, W. S. M. Wold, and L. R. Gooding.** 1989. Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. J. Immunol. **143**:2031–2037.
36. **Singer, S. J.** 1990. The structure and insertion of integral proteins in membranes. Annu. Rev. Cell Biol. **6**:247–296.
37. **Tollefson, A. E., P. Krajsci, M. H. Pursley, L. R. Gooding, and W. S. M. Wold.** 1990. A 14,500 MW protein is coded by region E3 of group C human adenoviruses. Virology **175**:19–29.
38. **Tollefson, A. E., P. Krajcsi, S. Yei, C. R. Carlin, and W. S. M. Wold.** 1990. A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. J. Virol. **64**:794–801.
39. **Tollefson, A. E., A. R. Stewart, S. Yei, S. K. Saha, and W. S. M. Wold.** 1991. 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. J. Virol. **65**:3095–3105.
40. **Tollefson, A. E., and W. S. M. Wold.** 1988. Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. J. Virol. **62**:33–39.
41. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
42. **Wang, E. W., M. O. Scott, and R. P. Ricciardi.** 1988. An adenovirus mRNA which encodes a 14,700-$M_r$ protein that maps to the last open reading frame in region E3 is expressed during infection. J. Virol. **62**:1456–1459.
43. **Wilson-Rawls, J., S. K. Saha, P. Krajcsi, A. E. Tollefson, L. R. Gooding, and W. S. M. Wold.** 1990. A 6700 MW membrane protein is encoded by region E3 of adenovirus type 2. Virology **178**:204–212.
44. **Wold, W. S. M., C. Cladaras, S. L. Deutscher, and Q. S. Kapoor.** 1985. The 19-kDa glycoprotein coded by region E3 of adenovirus: purification, characterization, and structural analysis. J. Biol. Chem. **260**:2424–2431.
45. **Wold, W. S. M., C. Cladaras, S. C. Magie, and N. Yacoub.** 1984. Mapping a new gene that encodes an 11,600-molecular-weight protein in the E3 transcription unit of adenovirus 2. J. Virol. **52**:307–313.
46. **Wold, W. S. M., S. L. Deutscher, N. Takemori, B. M. Bhat, and S. C. Magie.** 1986. Evidence that AG*U*AU*AUG*A and CC*A*A-G*AUG*A initiate translation in the same mRNA in region E3 of adenovirus. Virology **148**:168–180.
47. **Wold, W. S. M., and L. R. Gooding.** 1989. Adenovirus region E3 proteins that prevent cytolysis by cytotoxic T cells and tumor necrosis factor. Mol. Biol. Med. **6**:433–452.
48. **Wold, W. S. M., and L. R. Gooding.** 1991. Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions. Virology **184**:1–8.