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A 10,400-Molecular-Weight Membrane Protein Is Coded by Region E3 of Adenovirus

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Previous studies with adenovirus mutants have indicated that a 10,400-molecular-weight (10.4K) protein predicted to be coded by an open reading frame in region E3 of adenovirus functions to down regulate the epidermal growth factor receptor (C. R. Carlin, A. E. Tollefson, H. A. Brady, B. L. Hoffman, and W. S. M. Wold, Cell 57:135–144, 1989). We now demonstrate that the 10.4K protein is in fact synthesized in cells infected by group C adenoviruses. This was done by immunoprecipitation of 10.4K from cells infected by a variety of E3 mutants, using antisera against three different synthetic peptides corresponding to the predicted 10.4K sequence. The 10.4K protein was translated primarily from E3 mRNA *f*, as indicated by cell-free translation of mRNA purified by hybridization from cells infected with an RNA processing mutant that synthesizes predominantly mRNA *f*. The 10.4K protein was overproduced or underproduced in vivo, respectively, by mutants that overproduce or underproduce E3 mRNA *f*, also indicating that the 10.4K protein is translated primarily from mRNA *f*. The 10.4K protein migrated as two bands with apparent molecular weights of 16,000 and 11,000 (10 to 18% gradient gels); both bands contained 10.4K epitopes, as shown by Western blot (immunoblot). Only the 16K band was obtained by cell-free translation, suggesting that the 16K protein is the precursor to the 11K protein. The 10.4K protein is a membrane protein, as shown by cell fractionation experiments and as predicted from its sequence. The predicted 10.4K sequence as well as a putative N-terminal signal sequence and 30-residue transmembrane domain are conserved in adenovirus types 2 and 5 (group C) and in types 3, 7, and 35 (group B).

EGF-R, the receptor for epidermal growth factor (EGF), is a transmembrane protein localized on the plasma membrane (reviewed in reference 42). EGF-R is a member of the protein tyrosine kinase family of receptors and is the proto-oncogene for the v-*erb*B oncogene. Binding of EGF to the external ligand-binding domain of EGF-R leads to the activation of the protein tyrosine kinase activity of EGF-R. This results in autophosphorylation of EGF-R as well as phosphorylation of a number of cellular proteins, clustering of the EGF/EGF-R complex in coated pits, and internalization of the complex via endosomes that are transported to lysosomes, where the complex is degraded. This process triggers EGF signal transduction, which includes activation of cellular metabolism, induction of DNA synthesis, and mitosis. Two other growth factors, transforming growth factor α (32) and vaccinia virus growth factor (36), also bind to EGF-R and exert effects similar to EGF. Vaccinia virus growth factor appears to be responsible for the cell proliferative response to vaccinia virus (7), and this probably serves to activate cells for the efficient replication of vaccinia virus.

We have recently reported that EGF-R is down regulated during early stages of infection of human KB or A549 cells by human adenovirus type 5 (Ad5) or *rec*700, an Ad5-Ad2-Ad5 recombinant (10). Cell-surface EGF-R is internalized by endosome-mediated endocytosis and is degraded, presumably in lysosomes. There is no effect on the initial synthesis of EGF-R. Using a series of mutants that delete the various early transcription units, we mapped the gene(s) responsible for EGF-R down regulation to the E3 transcription unit. Then, using a series of mutants with mutations in region E3, we determined that mutants with lesions in an open reading frame (ORF) for a putative 10,400-molecular-weight (10.4K) protein (Fig. 1) were defective in down regulation of EGF-R. This finding indicated that the putative 10.4K protein played a role in down regulation of EGF-R. In addition to these genetic data, the 10.4K protein was predicted to exist on the basis of conservation of the sequence of the ORF in region E3 of Ad2 (18, 19), Ad5 (12), Ad3 (34), Ad7 (20), and Ad35 (14). Also, the 10.4K protein was predicted to be encoded by mRNA *f* as judged by the spliced structure of the mRNA (Fig. 1; 11, 12). In this report, we demonstrate that the 10.4K is actually synthesized in adenovirus-infected cells from mRNA *f*, and we provide an initial characterization of the protein.

MATERIALS AND METHODS

Cells and viruses. Virus stocks were prepared in suspension cultures of human KB cells and were titered on monolayers of human A549 cells. Procedures for the maintenance of KB cells, for adenovirus preparation, and for plaque assays have been described elsewhere (17). A549 cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum. H5/2*rec*700 is an Ad5-Ad2-Ad5 recombinant consisting of the Ad5 *Eco*RI A, Ad2 *Eco*RI D, and Ad5 *Eco*RI B fragments, map positions 0 to 76, 76 to 83, and 83 to 100, respectively (41). *rec*700 is the wild-type version for all mutants used in this study (Fig. 1). Mutants *dl*701, *dl*704 (3), *dl*708, *dl*712 (13), *dl*748, *dl*753 (5), *in*724, and *pm*760 (6) have been described. Construction of *dl*731, *dl*762, and *dl*763 will be described elsewhere (H. Brady, A. Scaria, and W. Wold, manuscript in preparation). *dl*759 was prepared by starting with the *Kpn*I A fragment (map position 71 to 94) of *rec*700 cloned at a *Kpn*I site that had been introduced at the original *Eco*RI site in pBR322. The *Eco*RI-*Nae*I fragment, nucleotides (nt) 2482 to 2801 in the E3 transcription unit of *rec*700, was excised from this clone, the *Eco*RI site was

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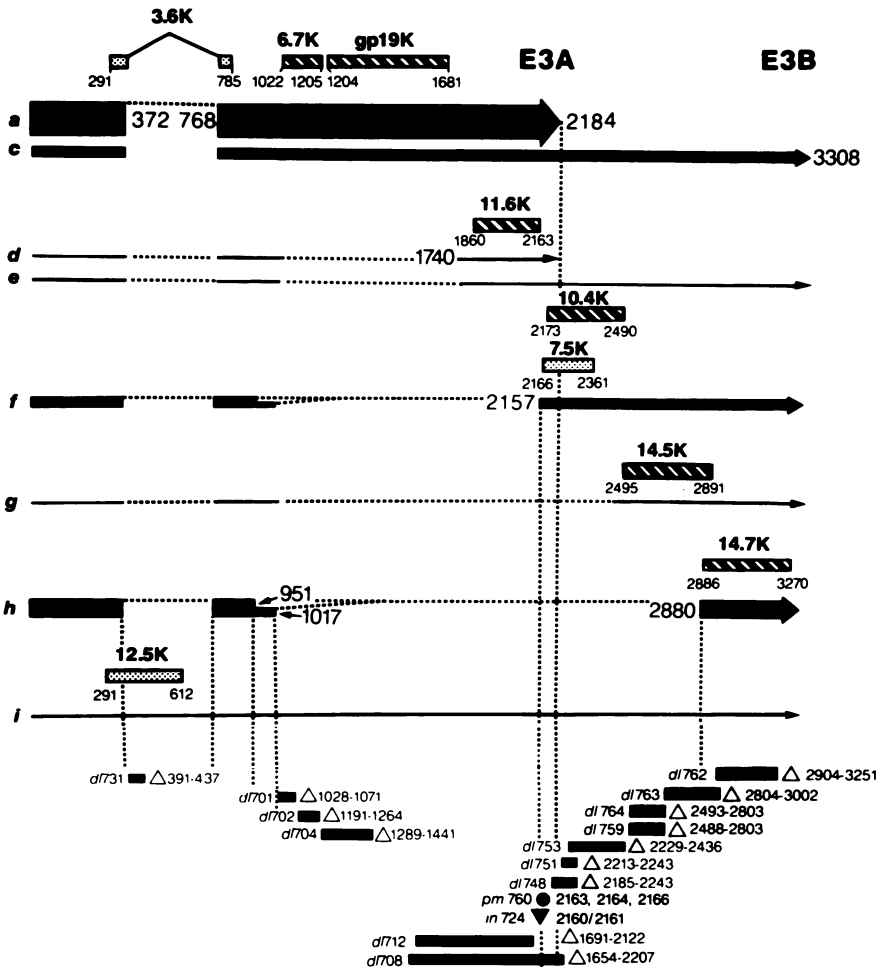

FIG. 1. Schematic illustration of region E3 of *rec*700. Arrows indicate the spliced structures of the mRNAs; the thickness of the arrows implies the relative abundance of the mRNA, and the dashed lines indicate introns. The middle exons in mRNAs *d* to *h* consist of either nt 768 to 951 or nt 768 to 1017 (B. Bhat and W. Wold, unpublished results). Bars above the arrows indicate proteins; hatched bars are proteins that have been identified in infected cells and mapped definitively to E3, namely, gp19K (22, 27, 28, 30, 33, 35, 39), 11.6K (40), 14.7K (29, 37, 38), 14.5K (Tollefson et al., Virology, in press), and 6.7K (41; J. Wilson-Rawls et al., unpublished results); stippled bars indicate proteins that are proposed to exist. Nt +1 is the transcription initiation site. *rec*700 is an Ad5-Ad2-Ad5 recombinant that has Ad2 sequences from nt −236 to 2437 in the E3 transcription unit. Numbers for the Ad2 transcription unit are used from nt 1 to 2437, and numbers for the Ad5 E3 transcription unit are used downstream of nt 2437 (see reference 12). Ad2 nt 2437 is equivalent to Ad5 nt 2482. Bars at the bottom indicate deletions in the virus mutants; the triangle indicates an insertion in *in*724, and the dot indicates the three-point mutations in *pm*760. The insertion in *in*724 is a 140-nt *Bgl*II-*Bam*HI fragment derived from nt 566 to 425 in the E3 transcription unit of Ad2; this fragment was cloned into a 6-base-pair *Bam*HI site that was inserted between nt 2160 and 2161 (6).

blunted by the DNA polymerase activity of the Klenow fragment, a 10-base-pair *Bam*HI linker (New England Bio-Labs, Inc., Beverly, Mass.) was ligated, and the plasmid was recircularized and cloned. The plasmid is called pKA(*dl*759). These manipulations removed the last amino acid and stop codon of 10.4K and resulted in a hybrid gene consisting of amino acids 1 to 90 of 10.4K, three amino acids (Arg-Asp-Pro) contributed by the *Bam*HI linker, and amino acids 104 to 132 of the 14.5K protein. The E3 deletion in pKA(*dl*759) was transferred to the adenovirus genome by the double-overlap recombination method (23) to produce *dl*759. This method will be described in detail elsewhere (Brady et al., in preparation). To produce *dl*764, pKA(*dl*759) was digested with *Bam*HI, and a duplex oligonucleotide (Operon Technologies, Alameta, Calif.) containing *Bam*HI sticky ends was cloned into the *Bam*HI site. The plasmid is called pKA(*dl*764). The oligonucleotide was designed to reconstruct the 3′ end of the 10.4K gene while preserving the deletion in the 14.5K gene. The sequence of the mutation, showing a portion of the oligonucleotide and a portion of the *Bam*HI linker (underlined), is 2434-CTCAGAATTCTTTAAGGATCCCGGGCGG-2808. The deletion in pKA(*dl*764) was built into the adenovirus genome by the double-overlap recombination method to produce *dl*764. The deletions in pKA(*dl*759), pKA(*dl*764), and all other plasmids that were the progenitors of virus mutants were confirmed by DNA sequencing. All viruses were plaque-purified once and then expanded into high-titer CsCl-banded stocks.

Generation of peptide antisera. Peptides were purchased from Multiple Peptide Systems, San Diego, Calif. (P77-91 and P68-80) or synthesized as described by Wold et al. (40) (P75-83). Peptide P77-91 has the sequence PQYRDRT IADLLRIL, corresponding to amino acids 77 to 91 at the C terminus of 10.4K of Ad2 (Fig. 2). Peptide P68-80 has the

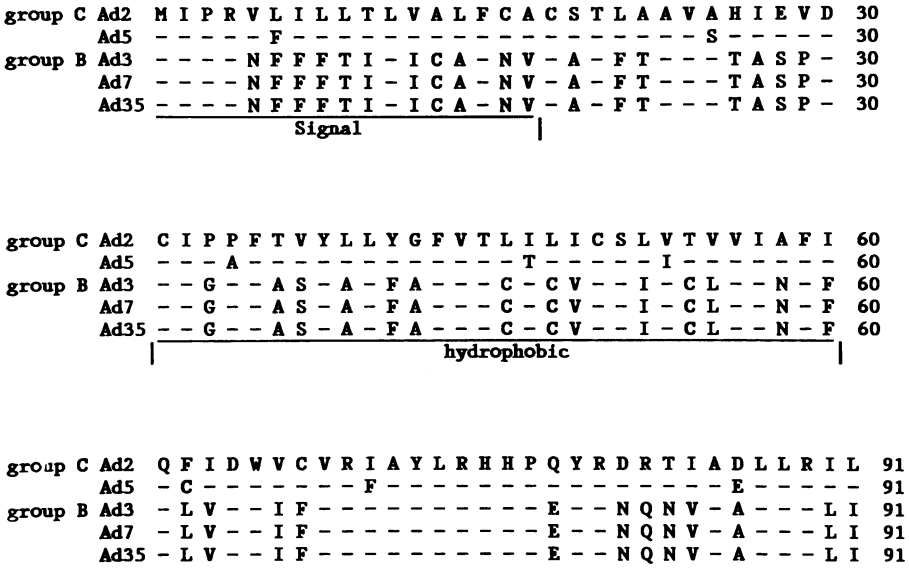

FIG. 2. Predicted sequences of the 10.4K protein from Ad2, Ad5, Ad3, Ad7, and Ad35. –, Amino acid is identical to that in Ad2.

sequence VRIAYLRHHPQYR. Peptide P75-83 has the sequence HHPQYRDRT. Peptides were coupled to keyhole limpet hemocyanin (KLH) (Calbiochem-Behring, La Jolla, Calif.) on a milligram-to-milligram basis, using bis(sulfosuccinimidyl)suberate for P68-80 and P77-91; P75-83 was coupled to KLH as previously described (40). The coupling reagents were obtained from Pierce Chemical Co., Rockford, Ill., and the methods used were those described by the manufacturer. For immunization, a 3-kg female New Zealand White rabbit was inoculated subcutaneously at multiple sites in the neck and back with the peptide-KLH conjugate (3 to 4 mg of peptide) in complete Freund adjuvant. Rabbits were similarly inoculated after 7 and 14 days with conjugate (3 to 4 mg of peptide) in incomplete Freund adjuvant and then boosted with conjugate (1.5 to 2 mg of peptide) in incomplete Freund adjuvant at biweekly intervals. Rabbits were bled from the ear after the fourth and subsequent injections.

In vivo protein labeling, immunoprecipitation, and SDS-PAGE. For the experiments shown in Fig. 3 to 6, KB cells (5.0×10^5/ml) were infected with 100 PFU of virus per cell, and early proteins were labeled from 7 to 12 h postinfection (p.i.) with 75 μCi of [^{35}S]Cys per ml (1,044 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) in Cys-free medium by the cycloheximide-enhanced procedure exactly as described previously (39, 40). In some experiments, proteins were labeled from 3 to 7 or 6 to 9 h p.i. without cycloheximide pretreatment. Cells were rinsed twice in cold phosphate-buffered saline (PBS) (pH 7.4) and lysed on ice with 0.5 ml of iso-hi-pH buffer (0.14 M NaCl, 1 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 8.5]) containing 0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were removed, and the supernatant (2×10^7 cpm) was analyzed by immunoprecipitation, using 5 μl of antiserum and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). Equal numbers of cells were infected, and we assume that the E3 proteins were labeled equally efficiently. For the peptide competition experiment (Fig. 4), 1 mg of peptide P77-91 was added to the immunoprecipitation reaction. Immunoprecipitates were rinsed six times with high-salt buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris hydrochloride [pH 7.4], 0.5% Nonidet P-40, 1% sodium deoxycholate) and then twice with 50 mM Tris hydrochloride (pH 6.8). Immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10 to 18% gradient gels (0.75 mm by 16 cm; acrylamide/*N*,*N*′-methylenebisacrylamide ratio, 29.2:0.8 [wt/wt]). All gels were fluorographed. ^{14}C-labeled molecular weight markers were obtained from Bethesda Research Laboratories, Gaithersburg, Md.

Cell fractionation and Western blot (immunoblot). Fractionation of cells into crude membrane and cytosol fractions was carried out with both [^{35}S]Cys-labeled and unlabeled cells. For the former, 100 ml of KB cells (3.5×10^5/ml) was infected with *pm*760 and labeled from 7.5 to 13.5 h p.i. by the cycloheximide-enhanced procedure as described above. Cells were collected, rinsed, and lysed by douncing (loose-fitting Wheaton pestle B) in 1 ml of lysis buffer (10 mM Tris hydrochloride [pH 7.2], 80 mM sucrose, 1 mM PMSF, 0.25 μg of leupeptin [Boehringer-Mannheim Biochemicals, Indianapolis, Ind.] per ml) until <5% of cells were intact. All steps were at 0 to 4°C. Nuclei were removed by centrifugation at $5,000 \times g$ for 5 min. The supernatant was centrifuged in a Ti50 rotor at $100,000 \times g$ 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 suspended in 1.5 ml of 10 mM Tris hydrochloride [pH 7.2]–10 mM EDTA–50 mM KCl–80 mM sucrose–1 mM PMSF–0.25 μg of leupeptin per ml and repelleted. The pellet, called the crude membrane fraction, was solubilized in Triton X-114 as described by Bordier (4). The cytosol and crude membrane fractions were analyzed by immunoprecipitation and SDS-PAGE.

For the unlabeled cells, 100 ml of KB cells was infected with *pm*760 and maintained in 25 μg of 1-β-D-arabinofuranosylcytosine per ml until 46 h p.i. Cytosol and crude membrane fractions were isolated as described above except that the membranes were solubilized in 0.3 ml of iso-hi-pH buffer containing 0.5% Nonidet P-40 and 1 mM PMSF. The protein concentration in the two fractions was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). A 20-μg sample of protein from each cytosol and membrane fraction was diluted into Laemmli buffer (62.5 mM Tris hydrochloride [pH 6.8], 10% glycerol,

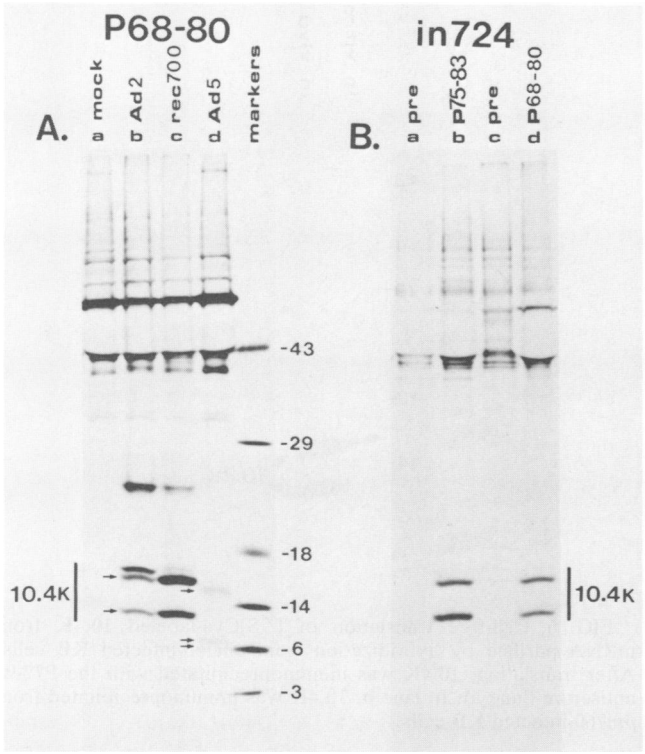

FIG. 3. Immunoprecipitation of [^{35}S]Cys-labeled 10.4K from virus-infected KB cells. (A) Cells were infected with Ad2, *rec*700, or Ad5, and immunoprecipitation was with the P68-80 antiserum. (B) Immunoprecipitation was carried out from *in*724-infected cells with the P75-83 or P68-80 antiserum or with sera taken from rabbits before immunization with these peptides (pre).

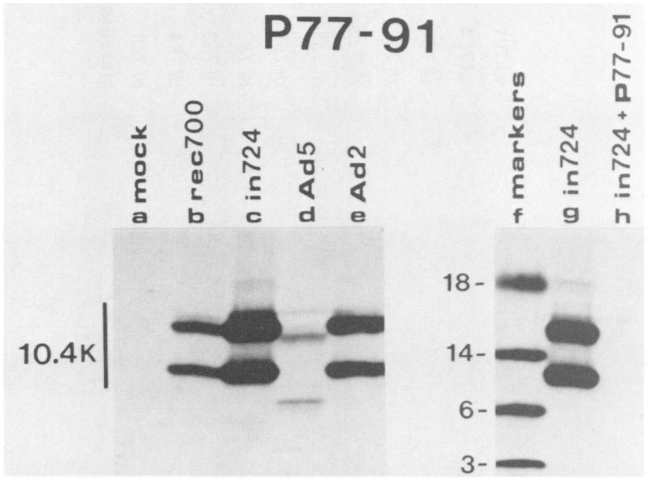

FIG. 4. Immunoprecipitation of [^{35}S]Cys-labeled 10.4K from KB cells infected with *rec*700, *in*724, Ad5, and Ad2. The P77-91 antiserum was used. In lanes g and h, immunoprecipitation was carried out with the P77-91 antiserum in the absence and presence, respectively, of 1 mg of the P77-91 peptide.

2% SDS, 5% β-mercaptoethanol, 0.00125% [wt/vol] bromophenol blue) and separated by SDS-PAGE on a 10 to 18% gradient SDS-polyacrylamide gel. The proteins were electrophoretically transferred (Trans-Blot SD; Bio-Rad) to nitrocellulose (NC; 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) in 25 mM Tris–192 mM glycine–20% methanol for 45 min at 150 mA. The blot was blocked with 1% bovine serum albumin (RIA grade; Sigma) in PBS for 1 h. Blots were probed with a 1:10 dilution of the 10.4K-specific P68-80 antiserum or the gp19K-specific Ad5 C-terminus peptide antiserum (39) in PBS plus 1% bovine serum albumin for 1 h. Blots were washed for 10-min intervals with PBS, PBS plus 0.1% Triton X-100, and then PBS again. Blots were incubated with ^{125}I-labeled *Staphylococcus* protein A (2×10^5 dpm/ml) in PBS for 1 h and then washed as before. Blots were dried in vacuum at 80°C for 30 min and then autoradiographed at −70°C, using X-ray intensifying screens. The protein A (Sigma) was labeled with ^{125}I (Dupont, NEN) by the chloramine T method (21).

Cell-free translation of hybridization-purified mRNA. KB cells were infected with *pm*760 and maintained in cycloheximide (10 μg/ml) from 3 to 13 h p.i., and cytoplasmic RNA was extracted as described elsewhere (11). For the preparation of DNA filters, the *rec*700 10.4K gene was engineered to have flanking *Bam*HI sites, and then the 10.4K *Bam*HI fragment (nt 2169 to 2492) was cloned into the *Bam*HI site of pGEM-1 (Promega Corp., Madison, Wis.). This plasmid was linearized with *Hin*dIII, and 5 μg of denatured DNA was immobilized on nitrocellulose filters (0.22-μm pore size; MSI/Fisher) as described previously (40). E3-specific mRNA was purified by hybridization (40), using 80 to 100 μg of cytoplasmic RNA and 5 μg of immobilized DNA on filters. The purified E3 mRNA from one filter was translated in a nuclease-treated rabbit reticulocyte extract (Promega), using [^{35}S]Cys as label. The 10.4K was immunoprecipitated (40) by using the P77-91 antiserum and analyzed by SDS-PAGE on 10 to 18% gradient gels, followed by fluorography.

RESULTS

Immunoprecipitation of the 10.4K protein from KB cells infected with *rec*700, Ad2, and Ad5, using antisera against peptides P77-91, P68-80, and P75-83. The sequence of the Ad2 version of 10.4K, as predicted from the DNA sequence (18, 19), is shown in Fig. 2. Antisera were prepared against synthetic peptides P77-91, P68-80, and P75-83, corresponding to amino acids 77 to 91, 68 to 80, and 75 to 83 in the predicted 10.4K of Ad2. The P68-80 antiserum immunoprecipitated two [^{35}S]Cys-labeled proteins of apparent molecular weights 11,000 and 16,000 from cells infected by Ad2 (Fig. 3A, arrows in lane b). The ca. 16.5K band (Fig. 3A, lanes b and c) was variable and was usually not detected (Fig. 3B, 4 to 6 and 8); as discussed below, this band may be the E3-14.5K protein. The ca. 25K band is probably the E3-gp19K protein, which is often nonspecifically coimmunoprecipitated by a variety of antisera. The 11K and 16K bands were also immunoprecipitated from *rec*700 (Fig. 3A, lane c), an Ad5-Ad2-Ad5 recombinant virus that contains the Ad2 version of 10.4K (and gp19K). The P68-80 antiserum also immunoprecipitated the Ad5 version of 10.4K; curiously, the Ad5 proteins migrated with apparent molecular weights of ca. 7,000 (a doublet) and 15,000 (Fig. 3A, arrows in lane d), even though the Ad2 and Ad5 proteins are both predicted to contain 91 amino acids (Fig. 2). These proteins were not obtained from mock-infected cells (Fig. 3A, lane a). The 11K and 16K proteins were immunoprecipitated from *in*724-infected cells by the P68-80 (Fig. 3B, lane d) and P75-83 (lane b) antisera but not by the corresponding preimmune sera (lanes a and c). The P77-91 antiserum gave essentially the same results with mock-, *rec*700-, *in*724-, Ad5-, and Ad2-infected cells (Fig. 4, lanes a to e), as did the

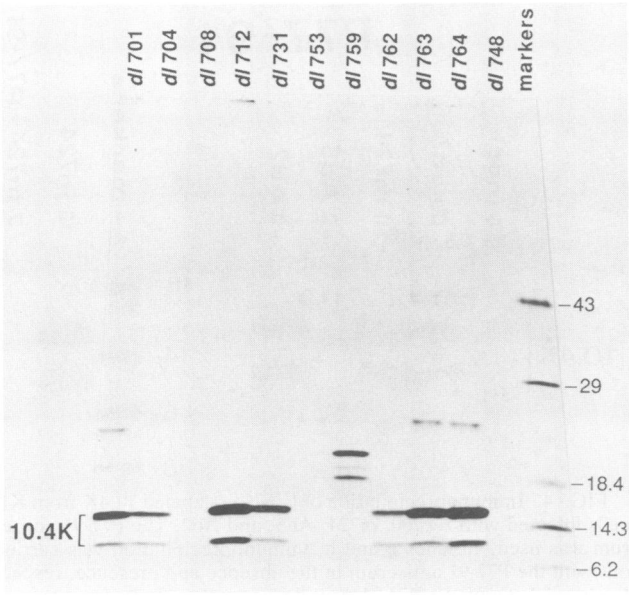

FIG. 5. Immunoprecipitation of [^{35}S]Cys-labeled 10.4K from KB cells infected with various E3 mutants. The antiserum used is against P77-91. *dl*708, *dl*753, and *dl*748 delete the 10.4K gene (see Fig. 1). *dl*759 deletes the C-terminal one amino acid of 10.4K and results in a fusion to amino acids 104 to 132 in the 14.5K protein. *dl*712, *dl*763, and *dl*764 overproduce 10.4K because they overproduce mRNA *f*, and *dl*762 underproduces 10.4K because it underproduces mRNA *f*.

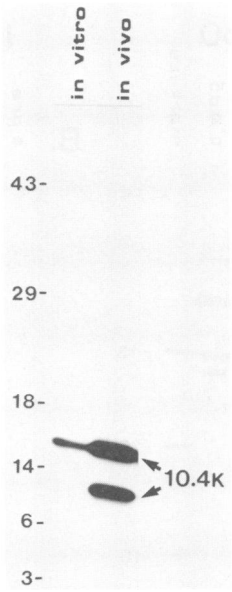

FIG. 6. Cell-free translation of [^{35}S]Cys-labeled 10.4K from mRNA purified by hybridization from *pm*760-infected KB cells. After translation, 10.4K was immunoprecipitated with the P77-91 antiserum (lane a). In lane b, 10.4K was immunoprecipitated from *pm*760-infected KB cells.

P68-80 antiserum (Fig. 3A). As discussed below, *in*724 overproduces 10.4K because it overproduces mRNA *f*, which encodes 10.4K. Immunoprecipitation of the 11K and 16K proteins by the P77-91 antiserum from *in*724-infected cells was blocked by competition with peptide P77-91, used to generate the antiserum (Fig. 4; compare lanes g and h), indicating that the immunoprecipitation was specific. These data indicate that the 11K and 16K proteins were immunoprecipitated specifically by antisera against the 10.4K peptides.

Mapping the 10.4K gene by using virus mutants. To obtain additional evidence that the 11K and 16K bands were specific to 10.4K, [^{35}S]Cys-labeled proteins were analyzed from cells infected by a variety of E3 mutants (Fig. 1). The P77-91 antiserum was used for immunoprecipitation. The 11K and 16K bands were obtained from cells infected by *dl*701, *dl*704, *dl*712, *dl*731, *dl*762, *dl*763, and *dl*764 (Fig. 5). These mutants all retain the 10.4K gene. In contrast, the 11K and 16K bands were not obtained from *dl*708, *dl*753, or *dl*748, mutants with deletions in the 10.4K gene. Proteins of about 18K, 19K, and 21K were obtained from *dl*759, whose deletion removes the C-terminal one amino acid of 10.4K and results in a fusion protein consisting of residues 1 to 90 of 10.4K fused to residues 104 to 132 of the 14.5K protein. An antiserum prepared against a synthetic peptide corresponding to residues 118 to 132 in 14.5K also immunoprecipitated the same proteins from *dl*759-infected cells (data not shown). These data strongly indicate that the 11K and 16K proteins are specific to the 10.4K ORF.

The 10.4K protein is translated from mRNA *f*. The 11K and 16K proteins were overproduced by *dl*712, *dl*763, and *dl*764 (Fig. 5). mRNA *f* is overproduced by *dl*712 (3, 13) and *dl*763 (Brady et al., in preparation). It is probably also overproduced by *dl*764 because *dl*764 has nearly the same deletion as *dl*759, and *dl*759 overproduces mRNA *f* (Brady et al., in preparation). The 11K and 16K proteins were also overproduced by *in*724 (Fig. 4; compare lanes b and c), another mutant that overproduces mRNA *f* (6). This finding indicates, as expected from the spliced structure of mRNA *f* (11), that 10.4K is translated from mRNA *f*. Consistent with this observation, the 11K and 16K proteins were underproduced by *dl*762 (Fig. 5), a mutant that underproduces mRNA *f* (Brady et al., in preparation). *dl*762 overproduces mRNA *a* and makes wild-type levels of mRNAs *c* and *i*, which also suggests that the 11K and 16K proteins are not substantially encoded by mRNA *a*, *c*, or *i*.

Cell-free translation of the 10.4K protein. The data presented so far strongly indicate that the 11K and 16K proteins are specific to the 10.4K ORF. To obtain additional proof, cell-free translation was carried out by using mRNA purified by hybridization from *pm*760-infected cells. The DNA used to select the mRNA contains essentially only the 10.4K gene; the DNA should select E3 mRNAs *c*, *e*, *f*, *i*, and perhaps *g*. However, the vast majority of mRNA selected should be mRNA *f* because *pm*760 is another RNA-processing mutant that makes predominantly mRNA *f* (6). A rabbit reticulocyte extract was used for translation, and the translation products were immunoprecipitated by using the P77-91 antiserum. A 16K band was obtained that comigrated with the 16K immunoprecipitated from *pm*760-infected cells (Fig. 6). We conclude that the 16K band corresponds to the 10.4K protein predicted to be encoded by the 10.4K ORF. Also, these results provide further evidence that 10.4K is coded by mRNA *f*.

The 11K and 16K proteins contain 10.4K-specific sequences, and they are localized in membrane fractions. If the 16K band corresponds to the primary translation product of 10.4K, what is the 11K band? It is possible that the 11K protein is a different cellular or viral protein that was immunoprecipitated together with 10.4K. A second possibility is that the

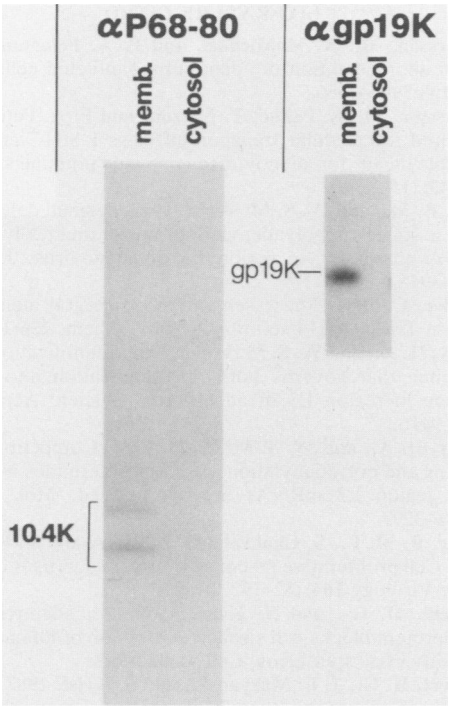

FIG. 7. Western blot of 10.4K and gp19K in crude membrane and cytosol fractions of *pm*760-infected KB cells. Antiserum to P68-80 was used for 10.4K, and antiserum to a peptide corresponding to the C-terminal 15 amino acids of gp19K (39) was used for gp19K.

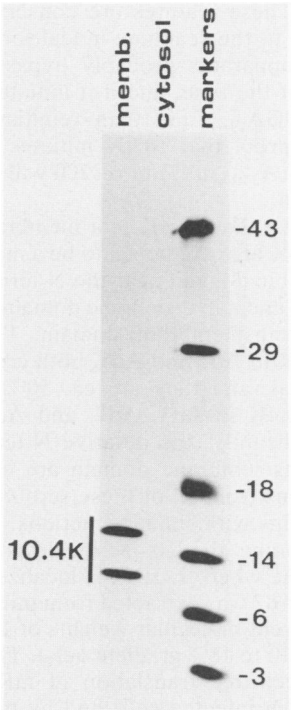

FIG. 8. Immunoprecipitation of 10.4K from [^{35}S]Cys-labeled membrane and cytosol fractions of *pm*760-infected KB cells. The P77-91 antiserum was used.

11K protein is derived from the 16K protein, presumably by proteolysis. To address these possibilities, a Western blot was carried out, using the P68-80 antiserum on proteins extracted from *pm*760-infected cells. Both the 11K and 16K bands reacted (Fig. 7), indicating that they both contain 10.4K epitopes. Similar results were obtained with the P77-91 antiserum (not shown). Thus, the 11K protein appears to be a posttranslational processing product of the 16K protein.

The predicted sequence of the 10.4K protein (Fig. 2) is strongly suggestive of a membrane protein. That is, there is a hydrophobic domain (residues 5 to 18) near the N terminus that is preceded by a positively charged residue (Arg at position 4). This is typical of N-terminal signal sequences that direct proteins into the membrane of the endoplasmic reticulum (25). Also, 10.4K has a second hydrophobic domain of 30 residues (31 to 60) that could be a transmembrane domain. Both 10.4K bands (11K and 16K) were found in the crude membrane fraction and not the cytosol fraction (Fig. 7). As a control, gp19K, which is known to be a membrane protein (27, 28, 30) localized primarily in the endoplasmic reticulum (26), was also shown to be localized exclusively in the same membrane extract as 10.4K (Fig. 7). Fractionation of [^{35}S]Cys-labeled cells into crude membrane and cytosol fractions, followed by immunoprecipitation with the P77-91 antiserum, also indicated that the 10.4K protein is localized exclusively in the membrane fraction (Fig. 8). Thus, 10.4K is a membrane protein.

DISCUSSION

We have shown that the 10.4K protein predicted from the ORF between the AUG at position 2173 (AUG_{2173}) and UAA_{2490} in *rec*700 is in fact synthesized in infected cells. (See the legend to Fig. 1 for an explanation of the nucleotide numbering system for *rec*700.) This was established by using three different peptide antisera, by using a variety of virus mutants with lesions throughout E3, and by cell-free translation of hybridization-purified mRNA.

The amount of 10.4K immunoprecipitated from cells infected by the various mutants strongly indicates that 10.4K is translated primarily from mRNA *f*. That is, 10.4K was overproduced by *dl*712, *in*724, *dl*763, and *dl*764, all mutants that overproduce mRNA *f*, and 10.4K was underproduced by *dl*762, a mutant that underproduces mRNA *f*. Also, 10.4K was translated in vitro by using mRNA purified from *pm*760-infected cells, and this is expected to be mostly mRNA *f*. However, we cannot exclude that 10.4K may also be translated in small part from mRNA *c*, *e*, or *i*. This same approach, i.e., correlating the amounts of protein produced with the amounts of mRNAs produced by our E3 pre-mRNA-processing mutants, has also indicated that gp19K is translated from mRNAs *a* and *c* and that 14.7K is translated from mRNA *h* (37).

The splicing pattern of mRNA *f* (11) also strongly suggests that 10.4K is coded by mRNA *f* (12). That is, the 3′ splice site at nt 2157 for the third exon of mRNA *f* is located 16 nt upstream of AUG_{2173} for 10.4K. This splicing pattern eliminates the 6.7K-, gp19K-, and 11.6K-coding sequence from the mRNA. In Ad2 (and *rec*700), there are three AUGs in the mRNA upstream of AUG_{2173}. These are AUG_{291}, which terminates after 33 codons at UAA_{785} after the 372-to-768 splice, AUG_{2158}, which terminates after 3 codons at UGA_{2167}, and AUG_{2166}, which could initiate a putative 7.5K protein in a different reading frame from 10.4K. AUG_{291} and AUG_{2166} are predicted by the Kozak rules (24) to be inefficient codons, whereas AUG_{2158} and AUG_{2173} are predicted

to be efficient. These features are conserved in Ad5 (12). Thus, according to the scanning model for translation (24), the translation apparatus probably bypasses AUG_{291} and AUG_{2166} most of the time, and if it initiates at AUG_{2158}, it terminates at UGA_{2167} and then reinitiates at AUG_{2173}. However, final proof that 10.4K initiates at AUG_{2173} and terminates at UAA_{2490} in E3 of *rec*700 will require sequencing of the protein.

We have shown that 10.4K is a membrane protein. The sequence of 10.4K also predicts it to be a membrane protein, with a hydrophobic domain near the N terminus, a putative signal sequence, and a hydrophobic domain at residues 31 to 60, a putative transmembrane domain. The predicted sequences of 10.4K in Ad2 and Ad5, both group C serotypes, are 92% identical, and they are ca. 50% identical to the sequences of 10.4K in Ad3, Ad7, and Ad35, all group B serotypes. Significantly, the putative N-terminal signal sequence and transmembrane domain are conserved. Thus, the 10.4K proteins from all of these serotypes are probably membrane proteins with similar functions. We do not know to which membrane the 10.4K localizes; it may be the plasma membrane where EGF-R is localized.

The 10.4K of Ad2 was extracted from infected cells as two bands with apparent molecular weights of 11,000 and 16,000 on SDS-PAGE (10 to 18% gradient gels). The 16K band was obtained after cell-free translation of mRNA purified by hybridization from infected cells and by translation of SP6 RNA (not shown), indicating that the 16K protein is the primary translation product and that the 11K protein is derived from it. If this processing step is proteolysis, then the cleavage site must be N terminal to residue 68 because the 11K and 16K bands were immunoprecipitated by antisera to P68-80, P75-83, and P77-91. The step could be cleavage of the putative N-terminal signal, but this occurs cotranslationally, and as such the 16K band would never be detected. We do not know whether 10.4K is posttranslationally modified in other ways. However, it is not Asn glycosylated because it does not contain N-X-(S,T) sites for Asn glycosylation.

The E3 proteins are turning out to have interesting functions. The 10.4K protein down regulates EGF-R (10). gp19K blocks transport of class I major histocompatibility antigens to the cell surface and protects adenovirus-infected cells against cytolysis by cytotoxic T lymphocytes (1, 2, 8, 9, 26, 31). gp19K also directly or indirectly reduces the infiltration of lymphocytes into adenovirus-infected areas in the lungs of cotton rats (15). The 14.7K protein protects adenovirus-infected cells against cytolysis by tumor necrosis factor (16). Further studies on these proteins will provide insights into EGF signal transduction and the antiviral defenses of the host. E3 has also been shown to express the 11.6K protein (40) as well as the 14.5K protein (A. Tollefson, P. Krajcsi, M. Pursley, L. Gooding, and W. S. M. Wold, Virology, in press) and the 6.7K protein (J. Wilson-Rawls, A. Tollefson, S. Saha, P. Krajcsi, and W. S. M. Wold, unpublished results) (Fig. 1). The 12.5K and 3.6K proteins are also probably expressed. These proteins are likely to be interesting as well.

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