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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

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We have reported that the E3 14,700-dalton protein (E3 14.7K protein) protects adenovirus-infected mouse C3HA fibroblasts against lysis by tumor necrosis factor (TNF) (L. R. Gooding, L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold, Cell 53:341–346, 1988). We have also observed that the E1B 19K protein protects adenovirus-infected human but not mouse cells against TNF lysis (L. R. Gooding, L. Aquino, P. J. Duerksen-Hughes, D. Day, T. M. Horton, S. Yei, and W. S. M. Wold, J. Virol. 65:3083–3094, 1991). We now report that, in the absence of E3 14.7K, the E3 10.4K and E3 14.5K proteins are both required to protect C127 as well as several other mouse cell lines against TNF lysis. The 14.7K protein can also protect these cells from TNF in the absence of the 10.4K and 14.5K proteins. This protection by the 10.4K and 14.5K proteins was not observed in the C3HA cell line. These conclusions are based on ^{51}Cr release assays of cells infected with virus E3 mutants that express the 14.7K protein alone, that express both the 10.4K and 14.5K proteins, and that delete the 14.7K in combination with either the 10.4K or 14.5K protein. The 10.4K protein was efficiently coimmunoprecipitated together with the 14.5K protein by using an antiserum to the 14.5K protein, suggesting that the 10.4K and 14.5K proteins exist as a complex in the infected mouse cells and consistent with the notion that they function in concert. Considering that three sets of proteins (E3 14.7K, E1B 19K, and E3 10.4K/14.5K proteins) exist in adenovirus to prevent TNF cytolysis of different cell types, it would appear that TNF is a major antiadenovirus defense of the host.

Evidence that adenovirus (Ad) may encode several proteins that allow it to escape immune surveillance is accumulating. One such protein is gp19K, coded by the E3 transcription unit, which is retained in the membrane of the endoplasmic reticulum (ER) by virtue of a specific ER retention signal located at its C terminus (12, 31, 32). gp19K binds to certain class I antigens of the major histocompatibility complex, thereby retaining them in the ER and blocking their transport to the cell surface (reviewed in references 21, 33, 48). This in turn prevents lysis of gp19K-expressing cells by Ad-specific cytotoxic T lymphocytes (CTL) (34) and by alloreactive CTL (2, 6). Accordingly, the function of gp19K may be to protect Ad-infected cells in the host from being killed by CTL. It has also been suggested that gp19K may be involved in suppressing production of cytokines which attract lymphocytes, because there is an increase in the lymphocyte and macrophage/monocyte inflammatory response in the lungs of cotton rats infected with Ad mutants that lack gp19K (16).

Although CTL are believed to be a primary means by which the host counteracts virus infections, tumor necrosis factor (TNF) appears to be another means. TNF is an immunoregulatory protein secreted by macrophages and lymphocytes (reviewed in reference 3). TNF inhibits the replication of a number of DNA and RNA viruses in cultured cells, and it lyses cells infected by certain viruses (reviewed in reference 24). We have reported that in mouse C3HA and NIH 3T3 fibroblasts infected with group C Ads, expression of the E1A 289R or 243R protein renders cells susceptible to TNF lysis (15). Deletion of a portion of conserved region 1 of region E1A abrogates the ability of E1A to induce sensitivity to TNF, as determined by ^{51}Cr release assays in mouse C3HA cells infected with Ad double mutants that lack region E3 and that have deletions in E1A (14). E1A-expressing mouse (1, 9, 11, 42) and rat (29) cell lines are spontaneously sensitive to TNF cytolysis, but certain E1A-expressing rat and human cells are not sensitive to TNF (42). Whereas E1A induces sensitivity to TNF, the 14,700-M_r protein (14.7K protein) encoded by region E3 (41) protects mouse cells against TNF cytolysis (19). This was established by analysis of cells infected by Ads that do not express the 14.7K protein (19). In Ad-infected cells, the 14.7K protein prevents TNF cytolysis regardless of whether cells are rendered susceptible to TNF by expression of E1A, inhibition of protein synthesis, or treatment with cytochalasin E or whether the cells are spontaneously sensitive to TNF cytolysis; this indicates that the 14.7K protein is a general inhibitor of TNF cytolysis (20).

The 14.7K protein prevents TNF cytolysis in nearly all mouse cell lines analyzed. Cells become susceptible to TNF cytolysis when treated with inhibitors of protein synthesis or cytochalasin E or when infected by 14.7K protein deletion mutants; cells stably transfected with the 14.7K protein gene expressed from a bovine papillomavirus vector are not lysed by TNF under these conditions, indicating that the 14.7K protein can function in the absence of other Ad proteins to prevent TNF cytolysis (24). Representative serotypes from Ad groups A, B, D, and E have an anti-TNF function similar

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to that described above for group C Ads, indicating that this is a general property of Ads in these groups (25).

We have recently observed that a second Ad protein, the 19K protein encoded by region E1B, can protect human cells from TNF cytolysis (18). This was established by analyzing cell lysis in human embryo lung cells (HEL-299), ME-180 cervical carcinoma cells, and four other human cell lines infected by Ads that lack both the E1B 19K protein and region E3 (i.e., the E3 deletion in H5*dl*309). In contrast to the E3 14.7K protein, the E1B 19K protein did not protect six different mouse cell lines against TNF lysis. HEL-299 and ME-180 cells were not lysed by TNF when infected by a mutant (H2*dl*250) that lacks the E1B 19K protein but retains E3, indicating that one or more of the E3 proteins can block TNF lysis of human cells in the absence of the E1B 19K protein.

In addition to the 14.7K protein and gp19K, region E3 has been shown to encode the 6.7K (45), 11.6K (46), 10.4K (39), and 14.5K (38) proteins. In the present communication we report that, in the absence of the 14.7K protein, both the 10.4K and 14.5K proteins are required to protect many mouse cell lines against lysis by TNF. In these mouse cell lines, the 10.4K protein was coimmunoprecipitated with the 14.5K protein by using antiserum to the 14.5K protein, suggesting that the 10.4K and 14.5K proteins exist as a complex in mouse cells and consistent with the idea that they function in concert. However, the putative 10.4K-14.5K protein complex did not protect the C3HA cell line against lysis by TNF. Thus, there are now three sets of proteins that can protect against TNF, depending on the cell type: E3 14.7K protein in nearly all mouse cells, E1B 19K protein in human cells, and E3 10.4K/14.5K proteins in many mouse cells. Most interesting, the E3 10.4K protein is required to downregulate the epidermal growth factor receptor (EGF-R) (7, 23), and recent data indicate that the E3 14.5K protein is required together with the 10.4K protein to downregulate EGF-R in Ad-infected cells (40). This raises the possibility that there may be a relationship between the ability of the E3 10.4K and 14.5K proteins to act on EGF-R and their ability to protect against cytolysis by TNF.

MATERIALS AND METHODS

Mouse cell lines. NIH 3T3, contact-inhibited fibroblasts, and C127, a clone derived from an RIII mouse mammary tumor, were obtained from the American Type Culture Collection (ATCC). C3HA is a 3T3-like line derived from C3H mouse embryo fibroblasts (17). Cell line 10T1/2, contact-inhibited C3H fibroblasts, and simian virus 40 (SV40)-transformed 10T1/2 (SV-10T1/2) were obtained from J. Pipas (University of Pittsburgh, Pittsburgh, Pa.) (36). LPVBETK1-C115, C3H-derived LMTK$^-$ cells transfected with the entire SV40 early region and expressing wild-type SV40 T antigen (35), were obtained from S. Tevethia (University of Pennsylvania, Hershey). The MCA line is derived from a methylcholanthrene-induced fibrosarcoma in C57BL/6 mice and was obtained from D. Adams (Duke University, Durham, N.C.). Mel-B16 is a cultured explant of the C57BL/6 B16 melanoma, obtained from the Jackson Laboratory (Bar Harbor, Maine). All cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, Utah).

Viruses. Viruses were grown in suspension cultures of human KB cells and banded in CsCl, and their titers were determined on human A549 cells as described before (22).

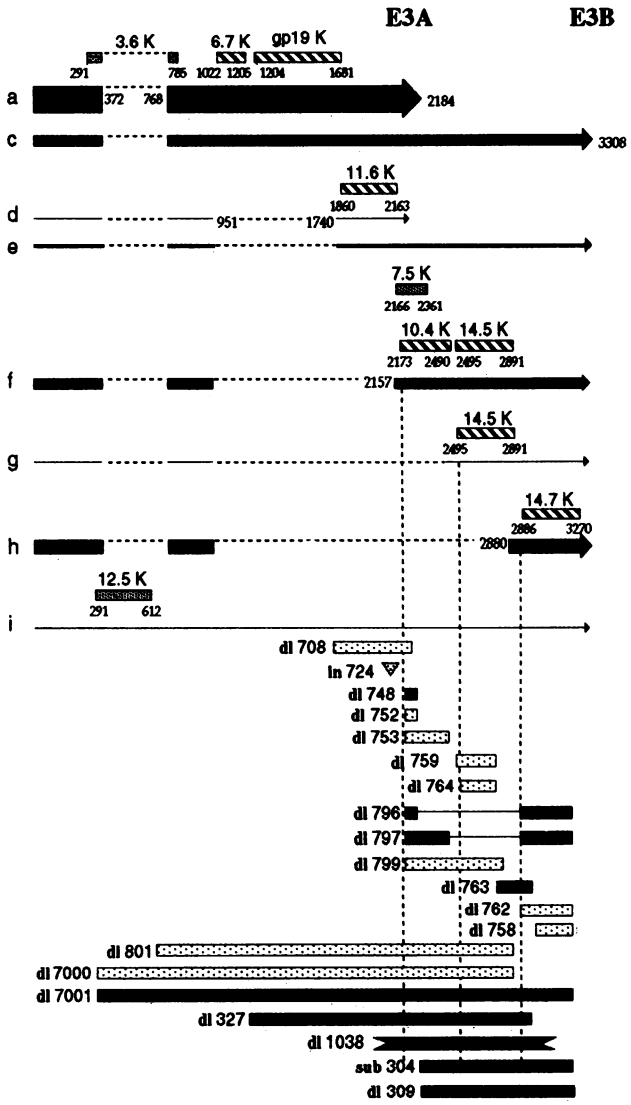

FIG. 1. Schematic illustration of region E3 of *rec*700. Arrows indicate the spliced structures of the mRNAs; solid parts are exons, dashed parts are introns, and the thickness implies the relative abundance. Nucleotide (nt) +1 is the transcription initiation site. E3A and E3B refer to polyadenylation sites. The bars above the arrows indicate proteins; hatched bars are proteins that have been identified in infected cells, and stippled bars are proteins that are proposed to exist. Bars at the bottom indicate the deletions in the virus mutants. C127 cells infected with mutants shown as stippled bars were not lysed by TNF, whereas those infected with mutants shown as solid bars were lysed by TNF. *in*724 has a 140-bp insertion between nt 2160 and 2161.

All the mutants with 700 or 7000 numbers were derived from *rec*700, an Ad5-Ad2-Ad5 recombinant whose genome consists of the Ad5 *Eco*RI-A fragment (map position 0 to 76), Ad2 *Eco*RI-D (76 to 83), and Ad5 *Eco*RI-B (83 to 100) (47). The E3 transcription unit and the E3 genes deleted in the mutants are shown in Fig. 1. *dl*708 (Δ1654 to 2207) (13), *dl*752 (Δ2229 to 2243), *dl*753 (Δ2229 to 2436), *dl*748 (Δ2185 to 2243) (4), *in*724 (5), and *dl*764 (Δ2493 to 2803) (39) have been described. *in*724 has a 140-bp insertion between nucleotides (nt) 2160 and 2161. Construction of *dl*758 (Δ3003 to 3251),

*dl*759 (Δ2488 to 2803), *dl*762 (Δ2904 to 3251), and *dl*763 (Δ2804 to 3002) will be described elsewhere (3a), as will construction of *dl*7000 and *dl*7001 (33a). *dl*7001 lacks all the E3 genes, and *dl*7000 lacks all the E3 genes except that for the 14.7K protein. *dl*796 and *dl*797 were constructed by combining the deletions in *dl*762 with those in *dl*752 and *dl*753, respectively; that is, the pED plasmid (Ad2 *Eco*RI-D in pBR322) containing the *dl*752 or *dl*753 deletion was cleaved with *Eco*RI and then ligated between the *Eco*RI-A and *Eco*RI-B fragments of *dl*762 DNA as described earlier except that proteinase K-digested *dl*762 DNA was used instead of terminal protein DNA (47). Plaques were picked, screened for the correct mutation by Hirt analysis, plaque purified, and expanded into CsCl-banded virus stocks.

H5*sub*304 (26), H5*dl*309 (27), and H5*dl*327 (isogenic with H5*dl*324 [37]) were obtained from T. Shenk (Princeton University, Princeton, N.J.). H2*dl*801 (8, 43) was obtained from G. Ketner (Johns Hopkins University, Baltimore, Md.), as was H2/5*dl*1038, an Ad5-Ad2 recombinant (19). Ad2$^+$ND2 is a nondefective Ad2-SV40 hybrid virus that has a portion of E3 deleted and replaced by an insert of SV40 DNA (28); this was obtained from A. Lewis (National Institutes of Health [NIH], Bethesda, Md.).

Protein labeling, immunoprecipitation, and SDS-PAGE. For the experiments shown in Fig. 6A, B, and D, C3HA or C127 cells were plated at 2×10^6 cells per 100-mm dish. At 6 h postplating, cells were infected with 400 PFU of virus per cell. Cells were labeled from 12 to 21 h postinfection (p.i.) with 40 μCi of [^{35}S]cysteine ([^{35}S]Cys; 1,153 Ci/mmol; Du Pont, NEN Research Products, Boston, Mass.) per ml in 3 ml of Cys-free DMEM. Cells were collected by trypsinization, rinsed twice in cold phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and then lysed on ice with 0.8 ml of iso-hi-pH buffer (0.14 M NaCl, 1 mM $MgCl_2$, 10 mM Tris-Cl [pH 8.0]) containing 0.5% Nonidet P-40 (NP-40) and 1 mM PMSF. The nuclei were removed by centrifugation, and the supernatants (5×10^6 cpm) were analyzed by immunoprecipitation with 5 μl of antiserum and protein A-Sepharose (Sigma Chemical Company, St. Louis, Mo.). The rabbit antisera were P77-91 (39) and P118-132 (38), directed against synthetic peptides corresponding to the C-terminal 15 amino acids of the 10.4K and 14.5K proteins, respectively, and a TrpE-14.7K protein antiserum specific for the 14.7K protein (41). Immunoprecipitates were rinsed six times with high-salt buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris-Cl [pH 7.4], 0.5% NP-40, 1% sodium deoxycholate) and then twice with 50 mM Tris-Cl (pH 6.8). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% to 18% gradient gels (0.75 mm by 16 cm; acrylamide/*N*,*N*'-methylenebisacrylamide ratio, 29.2:0.8 [wt/wt]). ^{14}C-labeled molecular weight markers were obtained from Bethesda Research Laboratories, Gaithersburg, Md. All gels were fluorographed.

For the experiment shown in Fig. 6C, C3HA and C127 cells were infected with 1,000 PFU of *dl*748 or *dl*763 or with 1,500 PFU of *dl*796 or *dl*797 per cell. For the coinfections, cells were infected with 500 PFU of *dl*748 plus 1,000 PFU of *dl*763 per cell, with 1,500 PFU of *dl*796 plus 1,000 PFU of *dl*763 per cell, or with 1,500 PFU of *dl*797 plus 1,000 PFU of *dl*763 per cell. High PFUs were used because *dl*796 and especially *dl*797 underproduce the 14.5K protein and because human Ads do not infect mouse cells as readily as they do human cells. Cells were labeled from 12 to 21.5 h p.i. with 50 μCi of Tran^{35}S-Label (1,046 Ci/mmol; 70% [^{35}S]methionine ([^{35}S]Met), 15% [^{35}S]Cys; ICN Biomedicals, Inc., Irvine, Calif.) per ml in Met-free DMEM. Proteins were extracted, and supernatants (3×10^7 cpm) were analyzed by immunoprecipitation and SDS-PAGE as described above.

Assay of TNF cytotoxicity (^{51}Cr release). The ^{51}Cr release assay of TNF cytotoxicity was performed as described previously (19). Briefly, mouse target cells were mock infected or infected with 100 to 300 PFU of virus per cell. At 18 h p.i., $Na_2{}^{51}CrO_4$ (200 μCi per 60-mm culture dish; Du Pont, NEN) was added, and cells were incubated at 37°C. Six hours later (24 h p.i.), the cells were rinsed and then placed in regular medium containing human recombinant TNF (hrTNF) (13×10^6 U/mg of protein) (the generous gift of Cetus Corporation, Emeryville, Calif.). ^{51}Cr release was measured 18 h later. When cycloheximide (CHI) was used, the CHI (25 μg/ml, final concentration) was added together with TNF. Results are expressed as percent specific lysis, which equals 100 times ^{51}Cr cpm released from cells in the presence of TNF minus ^{51}Cr cpm released in the presence of medium alone (spontaneous release) divided by ^{51}Cr cpm released in 1 N HCl (maximum release) minus ^{51}Cr cpm released in medium alone. Spontaneous release averaged about 30%. In all experiments, infected cells were plated onto glass slides, fixed, and stained with anti-Ad5 serum (ATCC, Rockville, Md.) to confirm the percentage of cells infected. Cells were at least 95% infected in all experiments reported here.

RESULTS

The E3 14.7K protein is the only E3 protein that is required to protect Ad-infected C3HA cells against lysis by TNF. As described previously (19), neither uninfected nor Ad5-infected C3HA cells were lysed by TNF, but those infected with *dl*748 or *dl*758 were lysed (Fig. 2A). *dl*748 is an RNA processing mutant that does not synthesize detectable mRNA *h* (4) or 14.7K protein (19, 41). *dl*758 deletes only the E3 14.7K protein gene (Fig. 1). These results demonstrate that Ad infection renders C3HA cells susceptible to cytolysis by TNF and that the 14.7K protein is required to protect them from cytolysis by TNF. Ad-infected C3HA cells are sensitized to TNF cytolysis by expression of E1A (14, 15).

Treatment of many cell types with CHI to inhibit protein synthesis renders them susceptible to cytolysis by TNF (30). This is also true for C3HA cells (20). CHI-treated C3HA cells infected with Ad5 were protected against TNF, whereas those infected with *dl*748 or *dl*758 were not protected (Fig. 2C). This result indicates that the 14.7K protein can protect C3HA cells against TNF cytolysis when the cells are sensitized to TNF by treatment with CHI.

Either the E3 14.7K protein or the E3 10.4K plus E3 14.5K proteins are required to protect Ad-infected C127 cells against lysis by TNF. As we proceeded to screen other mouse cell lines for their response to TNF when infected by Ad E3 deletion mutants, we noticed a second phenotype, exemplified by C127 cells, which was different from that observed in C3HA cells. Neither uninfected nor *rec*700-infected C127 cells were lysed by TNF, whereas cells infected with *dl*327 were lysed (Fig. 2B). Thus, as with C3HA cells, Ad infection sensitizes C127 cells to TNF cytolysis, and E3 is required to prevent TNF cytolysis. However, in contrast to C3HA cells, cells infected with *dl*758 were not lysed by TNF (Fig. 2B). C127 cells were sensitized to TNF by treatment with CHI, and infection with *rec*700 but not with *dl*327 blocked TNF cytolysis (Fig. 2D). But again, in contrast to C3HA cells, cells infected with *dl*758 were not lysed by TNF (Fig. 2D). These results indicate that an E3 protein(s) other than the 14.7K protein can protect C127 cells against TNF cytolysis.

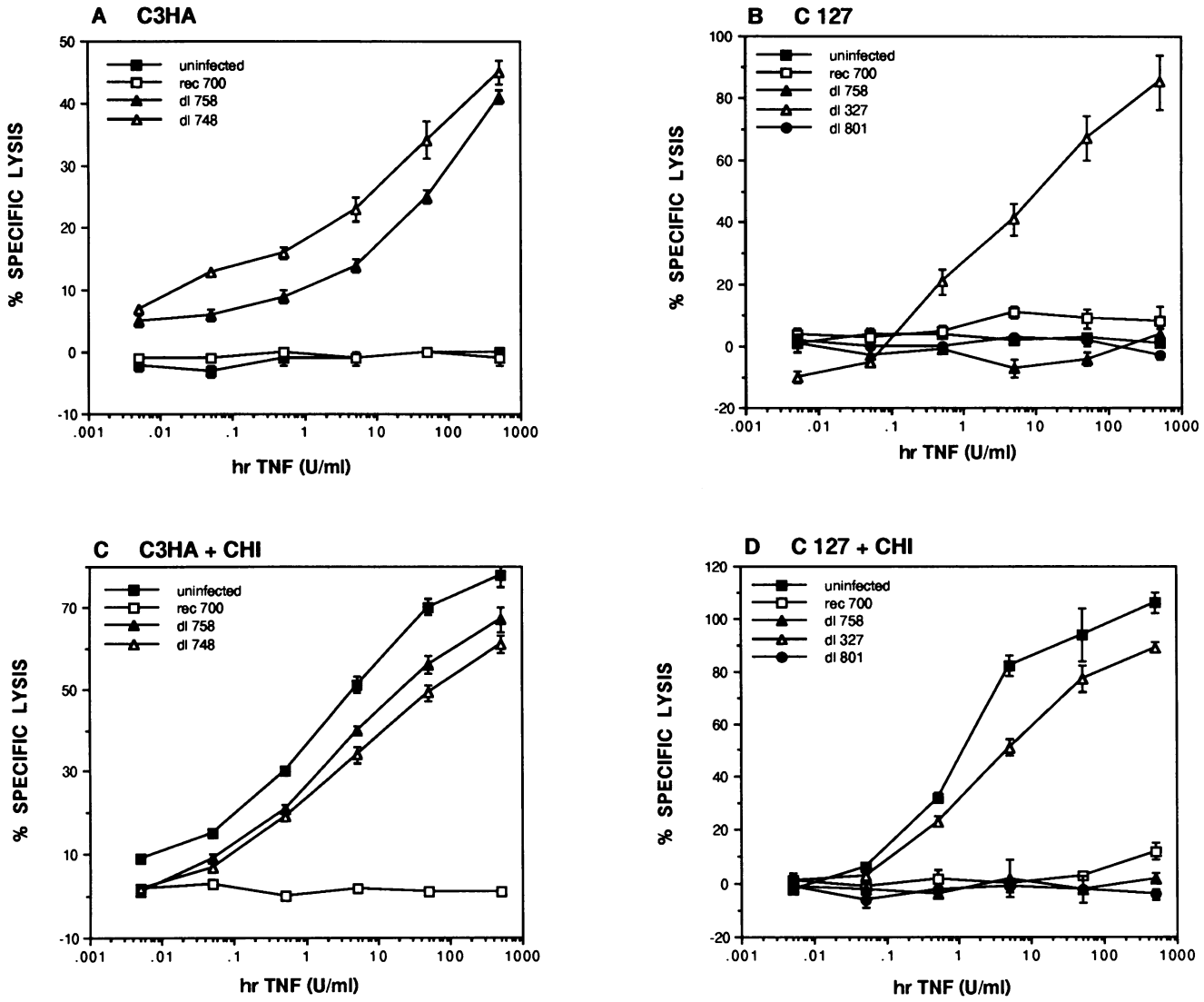

FIG. 2. The 14.7K protein is the only Ad protein that can protect C3HA cells against TNF cytolysis, but either the 14.7K protein or one or more of the other E3 proteins can protect C127 cells against TNF cytolysis. C3HA and C127 cells were mock infected with Ad E3 mutants as indicated, and TNF cytotoxicity was measured by a ^{51}Cr release assay as described in Materials and Methods. In panels C and D, CHI (25 μg/ml) was added at the same time that hrTNF was added.

To determine whether the 14.7K protein can protect C127 cells from TNF lysis in the absence of other E3 proteins, *dl*801 was analyzed. *dl*801 deletes all E3 genes except that for the 14.7K protein and the putative 12.5K protein gene (Fig. 1), and it expresses the 14.7K protein (19, 41, 43). *dl*801-infected C127 cells were not killed by TNF (Fig. 2B), and *dl*801 prevented TNF lysis in the presence of CHI (Fig. 2D). Therefore, the 14.7K protein can protect C127 cells against TNF cytolysis. (The putative 12.5K protein cannot protect because C127 cells infected with *dl*327, which should express the putative 12.5K protein, were lysed by TNF [Fig. 2B]).

In order to determine which E3 protein(s) can block TNF cytolysis of C127 cells in the absence of the 14.7K protein, it was necessary to analyze mutants that lack the 14.7K protein and that also lack one of the other E3 proteins (the TNF lysis phenotypes of the mutants analyzed are depicted in Fig. 1). An important mutant in this respect is *dl*763, which deletes the C-terminal 29 residues in the 14.5K protein (the 14.5K protein from Ad5 has 132 residues) and the N-terminal 39 codons in the 14.7K protein gene; neither the 14.5K nor the 14.7K protein was detectable in *dl*763-infected C127 or C3HA cells (see Fig. 6A and 6C). As shown in Fig. 3, C127 cells infected with *dl*763 were lysed by TNF; this implicates the 14.5K protein as a protein that is required to protect C127 cells against lysis by TNF in the absence of the 14.7K protein. As expected from this conclusion, C127 cells were lysed by TNF when infected with *sub*304 (deletes the 10.4K, 14.5K, and 14.7K protein genes), *dl*309 (identical to *sub*304 except it has an undefined mutation that destroys the *Xba*I site in the 6.7K protein gene), and *dl*1038 (deletes the 11.6K, 10.4K, 14.5K, and 14.7K protein genes) (Fig. 3). C127 cells infected with *dl*327 were lysed by TNF, whereas those infected with *dl*758 (deletes the 14.7K protein gene only) were not lysed (Fig. 2B and 3). C127 cells were also

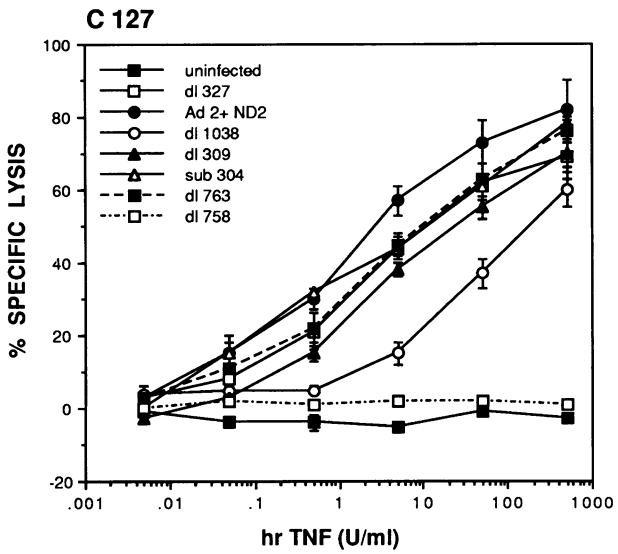

FIG. 3. The 14.5K protein is required to protect C127 cells against TNF cytolysis in the absence of the 14.7K protein. C127 cells were infected with Ad mutants, and TNF cytolysis was measured by ^{51}Cr release.

lysed by TNF when infected with Ad2^{+}ND2 (which has an imprecisely defined deletion similar to that of *dl*327), indicating that lysis occurs with Ad2 E3 deletion mutants as well as with Ad5 mutants (Fig. 3).

Additional mutants were analyzed as shown in Fig. 4A and B. Again, C127 cells were lysed by TNF when infected with *dl*327 or *dl*763. Again, they were not lysed when infected with *dl*758 or *dl*762, which delete the 14.7K protein gene only. They were not lysed when infected with *dl*752 (deletion in the 10.4K protein gene), *dl*753 (deletes the 10.4K protein gene), *dl*759 (deletes the 14.5K and the 3′ end of the 10.4K protein gene), *dl*764 (deletes the 14.5K protein gene), or *dl*799 (deletes the 10.4K and 14.5K protein genes) because all these mutants synthesize the 14.7K protein, which provides protection against TNF.

As noted in the introduction, the 14.5K protein functions in concert with the 10.4K protein to downregulate EGF-R in Ad-infected human cells, and the 10.4K protein coimmunoprecipitates with the 14.5K protein in extracts from human cells. Accordingly, we examined whether the 10.4K protein might function together with the 14.5K protein to prevent TNF cytolysis of C127 cells. Three mutants relevant to this question were analyzed. *dl*748 has a frameshift deletion in the 10.4K protein gene. This deletion is also in the E3A polyadenylation signal, and it results in the nearly exclusive synthesis of E3 mRNA *f* (with a deletion in the 10.4K protein-coding sequences) (4). Thus, *dl*748 synthesizes large amounts of the 14.5K protein (see Fig. 6C), which is coded by mRNA *f*, and it does not synthesize detectable amounts of the 14.7K protein (which is coded by mRNA *h*) (19) or the 10.4K protein (39). C127 cells infected with *dl*748 were lysed by TNF as efficiently as were cells infected with *dl*763 (deletes the 14.5K and 14.7K protein genes) (Fig. 5A). Thus, although the 14.5K protein is required to prevent TNF cytolysis in the absence of the 14.7K protein, abundant expression of the 14.5K protein is insufficient to prevent TNF cytolysis. Importantly, when C127 cells were coinfected with *dl*748 and *dl*763, cells were not lysed by TNF

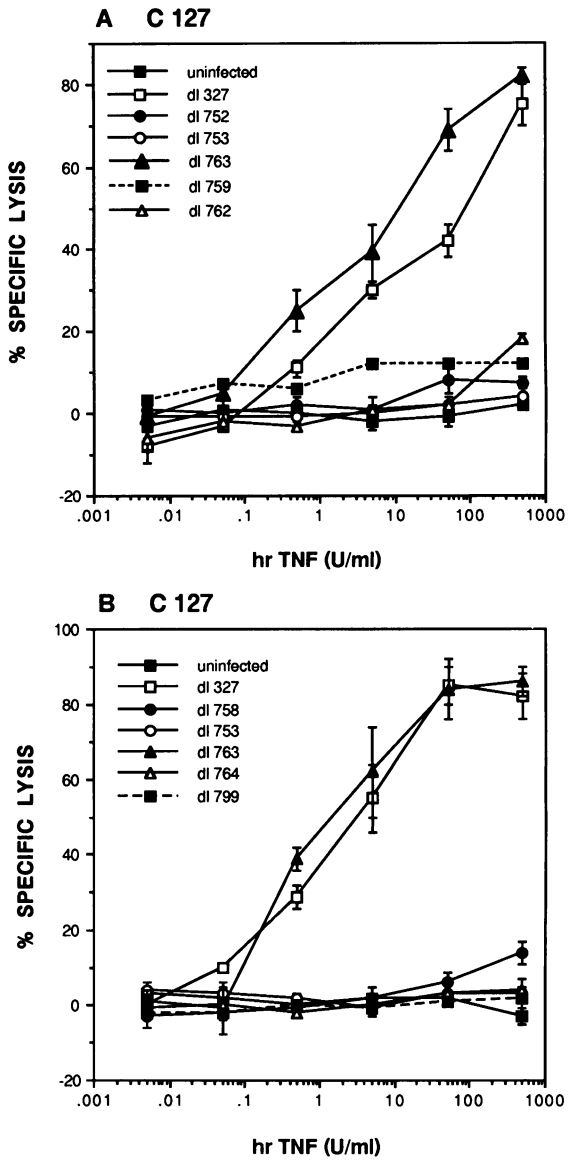

FIG. 4. The 14.7K protein protects C127 cells against TNF cytolysis in the absence of the 10.4K or 14.5K protein. C127 cells were infected with Ad mutants, and TNF cytolysis was measured by ^{51}Cr release.

(Fig. 5A). Thus, the two mutations complement each other in TNF protection, indicating that both the 10.4K and 14.5K proteins are required to prevent TNF cytolysis in the absence of the 14.7K protein (*dl*748 provides the 14.5K protein, *dl*763 provides the 10.4K protein, and neither mutant provides the 14.7K protein; Fig. 6A to C).

This conclusion is supported by *dl*796, which is a double mutant that has the 10.4K protein gene deletion mutation of *dl*752 and the 14.7K protein gene deletion of *dl*762 (nearly the entire 14.7K protein gene is deleted). *dl*796 synthesizes the 14.5K but not the 10.4K or 14.7K protein (Fig. 6A to C). C127 cells infected with *dl*796 were lysed by TNF (Fig. 5B). However, cells coinfected with *dl*796 and *dl*763 were not lysed by TNF (Fig. 5B), again indicating that the mutations are complementary and that both the 10.4K and 14.5K

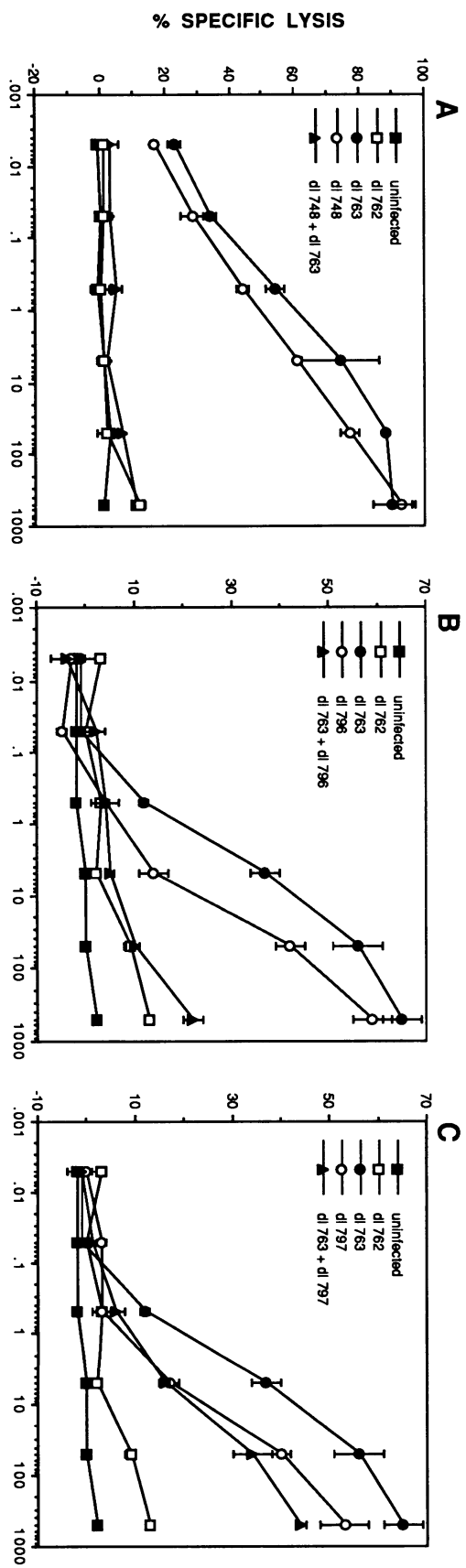

FIG. 5. Both the 10.4K and 14.5K proteins are required to protect C127 cells against TNF cytolysis in the absence of the 14.7K protein. (A) Cells were infected at a multiplicity of infection of 200 PFU per cell. (B and C) Cells were infected with the following amounts of virus (per cell): *dl*762, 200 PFU; *dl*763, 800 PFU; *dl*796, 2,500 PFU; *dl*797, 5,000 PFU; *dl*763 plus *dl*796, 400 and 1,250 PFU, respectively; *dl*763 plus *dl*797, 400 and 2,500 PFU, respectively. Cytolysis was measured as ^{51}Cr release after an 18-h incubation.

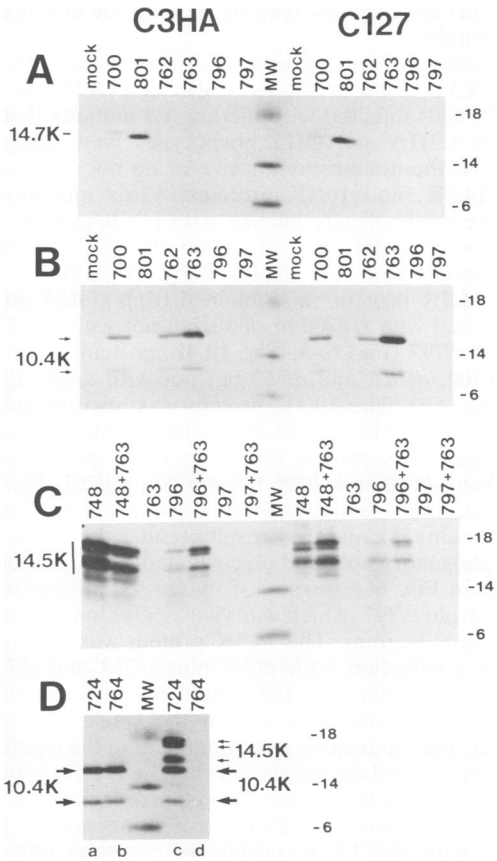

FIG. 6. E3 deletion mutants synthesize the 10.4K, 14.5K, and 14.7K proteins as expected in C3HA and C127 cells, and the 10.4K protein coimmunoprecipitates with the 14.5K protein. (A, B, and C) C3HA and C127 cells were mock infected or infected with Ad E3 mutants as indicated, and ^{35}S-labeled proteins were immunoprecipitated with antisera to the 14.7K (A), 10.4K (B), or 14.5K (C) protein. In panel C, cells were either singly infected or doubly infected, as indicated. (D) C127 cells were infected with *in*724 or *dl*764, and immunoprecipitations were carried out with antisera to the 10.4K (lanes a and b) or 14.5K (lanes c and d) protein. The large arrows indicate the two 10.4K protein bands, and the small arrows indicate three 14.5K protein bands. Proteins were labeled with [^{35}S]Cys in panels A, B, and D and with [^{35}S]Met in panel C. The molecular weight markers of 18K, 14K, and 6K proteins are in lane MW.

proteins are required to prevent TNF cytolysis in the absence of the 14.7K protein.

*dl*797 is a double mutant that has the 10.4K protein gene mutation of *dl*753 (most of the 10.4K protein gene is deleted) and the 14.7K protein gene deletion of *dl*762. For reasons that are unclear, *dl*753 synthesizes very small amounts of the 14.5K protein in KB cells (38). *dl*797 appears to have the same phenotype in KB cells (not shown). *dl*797 also synthesizes only a minute amount of 14.5K protein in C127 and C3HA cells (Fig. 6C; a trace of 14.5K protein was seen after longer exposures of this autoradiogram). *dl*797 does not synthesize the 14.7K protein (Fig. 6A) or the 10.4K protein (Fig. 6B). As expected, therefore, the coinfection of C127 cells by *dl*797 and *dl*763 provided only slight protection, if any, against TNF cytolysis (Fig. 5C).

We conclude from all these experiments that both the 10.4K and 14.5K proteins are required to protect Ad-in-

fected C127 cells against lysis by TNF in the absence of the 14.7K protein.

E3 mutants used in TNF cytolysis experiments synthesize the expected E3 proteins in infected C3HA and C127 cells. C3HA and C127 cells infected with the key E3 mutants that distinguish the C3HA and C127 phenotypes were analyzed to ensure that the mutants synthesize or do not synthesize the 14.7K, 14.5K, and 10.4K proteins. Virus mutant-infected cells were metabolically labeled with [^{35}S]Cys (Fig. 6A, B, and D) or [^{35}S]Met (Fig. 6C), and the E3 proteins were immunoprecipitated and analyzed by SDS-PAGE. As expected, 14.7K protein was obtained from C3HA and C127 cells infected with *rec*700 or *dl*801 but not with *dl*762, *dl*763, *dl*796, or *dl*797 (Fig. 6A). The 10.4K protein was obtained with *rec*700, *dl*762, and *dl*763 but not with *dl*801, *dl*796, or *dl*797 (Fig. 6B). The 10.4K protein was also obtained with *dl*764 (Fig. 6D). The two 10.4K protein bands are characteristic of the 10.4K protein (39). The 14.5K protein was obtained from *dl*748 and *dl*796 but not from *dl*763 (Fig. 6C) or *dl*764 (Fig. 6D). The multiple bands seen are typical of the 14.5K protein (38), and they result because the 14.5K protein is both phosphorylated and glycosylated (30a). Although not apparent in Fig. 6C, a trace of the 14.5K protein was also obtained from *dl*797, which was visible after longer exposures of the autoradiogram. The 14.5K protein was also obtained from the coinfection with *dl*748 plus *dl*763 and *dl*796 plus *dl*763 (Fig. 6C). Note that the pattern of 14.5K protein bands differs in the coinfections and in the single infections; in particular, the top band is more abundant in the coinfections.

We have pointed out previously that the 14.5K protein band pattern is different in mutants that retain or lack 10.4K protein (38). This is because the 10.4K protein apparently forms a complex with the 14.5K protein (see below) (40), and it facilitates the posttranslational modification of 14.5K protein (30a). Thus, the 14.5K protein band patterns in Fig. 6C indicate that the 10.4K protein is interacting with the 14.5K protein in the coinfections, as is implied by the TNF cytolysis data. The results with these mutants are all in accord with results obtained from infected human KB cells (19, 38–41).

We have reported that in [^{35}S]Cys-labeled extracts of human KB cells, the 10.4K protein coimmunoprecipitates with the 14.5K protein with antipeptide antisera directed against the N and C termini of the 14.5K protein as well as an antiserum against a TrpE-14.5K protein fusion protein (40). We consider this to be strong evidence that the 10.4K and 14.5K proteins exist as a complex in vivo. As shown in Fig. 6D (lane c), the 10.4K protein was efficiently coimmunoprecipitated with the 14.5K protein from *in*724-infected C127 cells. Similar results were obtained with *in*724-infected C3HA cells (not shown). Coimmunoprecipitation of the 10.4K protein with the 14.5K protein is not apparent in Fig. 6C because the proteins were labeled with [^{35}S]Met and the 10.4K protein does not contain Met except for the initiator. As was the case with KB cells (40), antipeptide antiserum to 10.4K protein did not coimmunoprecipitate the 14.5K protein (Fig. 6D, lane a), presumably because the epitope for the antiserum was masked by the 14.5K protein in the complex.

We conclude that the mutants analyzed synthesize the expected proteins in C3HA and C127 cells and that the 10.4K and 14.5K proteins interact and probably form a complex in these cells.

The E3 10.4K and E3 14.5K proteins protect many Ad-infected mouse cell lines against cytolysis by TNF in the absence of the E3 14.7K protein. The 14.7K protein protects many different Ad-infected mouse cell lines against lysis by TNF, i.e., of 15 lines examined, only 2 were not protected, and these are cell lines that are spontaneously susceptible to TNF cytolysis (20; unpublished results). Although the 10.4K and 14.5K proteins cannot protect Ad-infected C3HA cells against TNF cytolysis, it appears that they can protect many or most mouse cells against cytolysis, i.e., of 15 lines tested, only 4 were not protected, and 2 of these also were not protected by 14.7K protein. Representative data are shown in Fig. 7. In panels A through E, neither uninfected nor wild-type Ad-infected cells were lysed by TNF. The cells in panel F were spontaneously sensitive to TNF cytolysis (uninfected cells were lysed by TNF), but infection with Ad5 prevented TNF lysis. When any of the cells in Fig. 7 were infected with *dl*327 (deletes all of E3 except the putative 12.5K protein gene), *dl*7001 (deletes all of E3), *dl*309 (deletes the 10.4K, 14.5K, and 14.7K protein genes), *dl*797 (deletes the 10.4K and 14.7K protein genes and synthesizes traces of the 14.5K protein), or *dl*763 (deletes the 14.5K and 14.7K protein genes), TNF lysis was observed. These are the expected results. Cells infected with *dl*801 (deletes all of E3 except the 14.7K and putative 12.5K protein genes), *dl*7000 (deletes all of E3 except the 14.7K protein gene), or *dl*799 (deletes the 10.4K and 14.5K protein genes) were not lysed, indicating that the 14.7K protein can prevent TNF cytolysis in the absence of other E3 proteins. Cells infected with *dl*758 or *dl*762 (deletes the 14.7K protein gene only) were not lysed, indicating that an E3 protein other than the 14.7K protein, presumably the 10.4K plus 14.5K proteins, can prevent TNF cytolysis in the absence of the 14.7K protein. Thus, it appears that, as was the case with C127 cells, either the 14.7K or 10.4K plus the 14.5K protein is required to prevent TNF cytolysis when these cells are infected with Ad.

DISCUSSION

We have previously reported that when mouse C3HA fibroblasts are infected with Ad wild type or Ad mutants, the E3 14.7K protein is the only Ad protein which is required to protect the cells from lysis by TNF (19). We have also observed that when human HEL-299 or ME-180 cells as well as four other human cell lines are infected with Ad mutants that have the E3 deletion in H5*dl*309, the E1B 19K protein is required to protect the cells from lysis by TNF (18). In the absence of the E1B 19K protein, one or more of the E3 proteins lacking in *dl*309 (i.e., the 10.4K, 14.5K, 14.7K, and putative 7.5K proteins) can also protect these human cells against TNF lysis, but we do not know which of these E3 proteins provides this protection (18). We have now shown here that the majority of mouse cells (11 of 15), typified by the C127 cell line, have yet a different TNF phenotype. In common with C3HA, the 14.7K protein expressed in the absence of other E3 proteins (i.e., the deletion in *dl*801) can protect cells of the C127 group against TNF lysis. However, in contrast to C3HA, with cells of the C127 type the 10.4K plus 14.5K proteins are required for protection against TNF in the absence of the 14.7K protein. This conclusion is based on the analysis of mutants that lack the 14.7K protein as well as either the 10.4K or 14.5K protein. Presumably all the C127-type cells are sensitized to TNF by expression of E1A, as are C3HA cells and NIH 3T3 cells (15), which have the C127 TNF phenotype (Fig. 7A). Although our results establish that the 10.4K and 14.5K proteins are necessary for protection of Ad-infected C127-type cells against TNF in the absence of the 14.7K protein, they do not establish that the 10.4K and 14.5K proteins are sufficient for this protection; this will require the isolation and analysis of transfected cells expressing the 10.4K and 14.5K proteins.

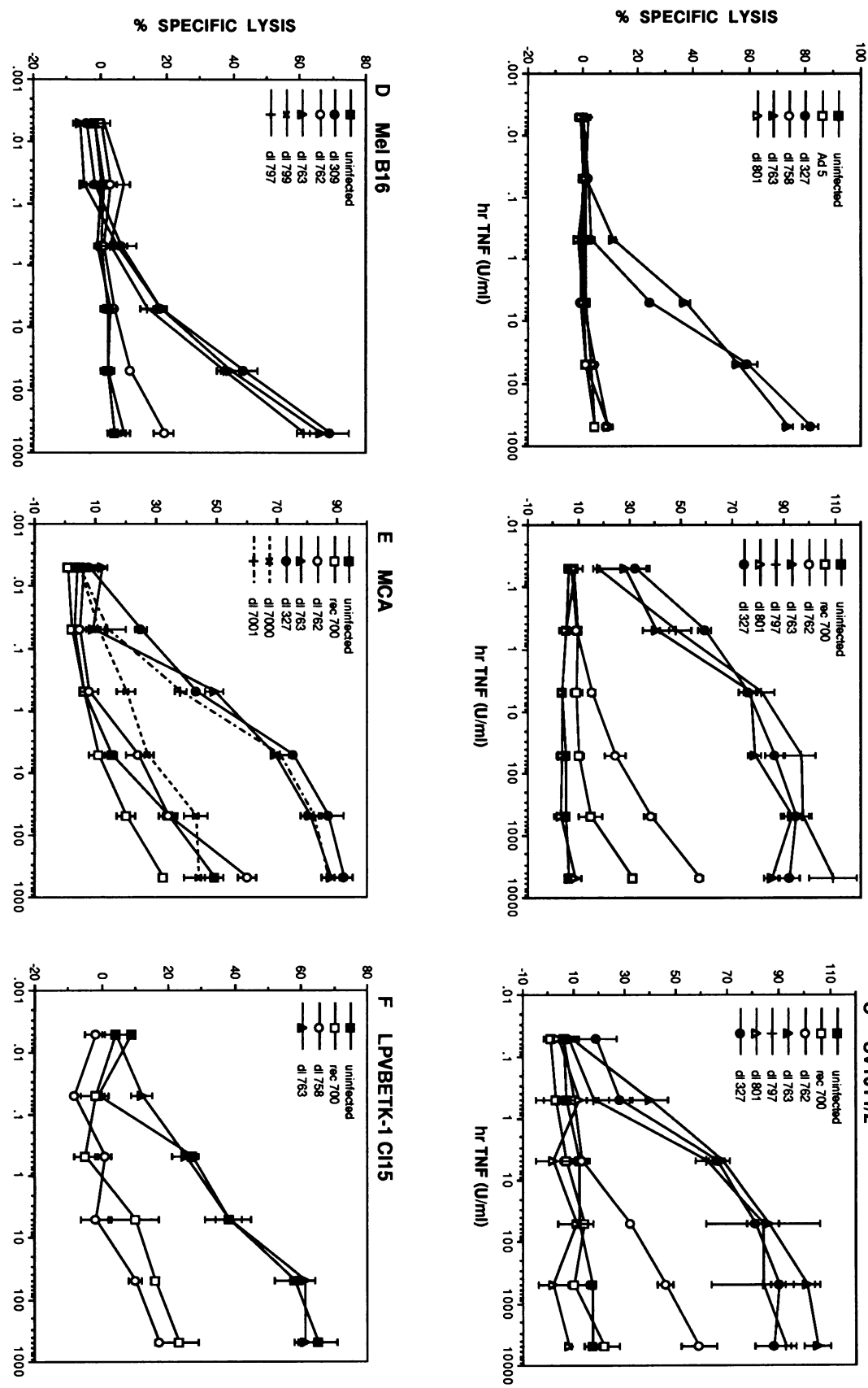

FIG. 7. The 10.4K plus 14.5K or the 14.7K protein can protect many mouse cell lines against TNF cytolysis. The cells were infected with Ad mutants, and TNF cytolysis was measured by ^{51}Cr release.

There are now four Ad proteins, or three "sets" of proteins, that have been implicated in protection against TNF lysis. The E3 14.7K protein protects nearly all mouse cells against TNF lysis, regardless of whether the cells are sensitized to TNF by expression of E1A, by treatment with CHI (which inhibits protein synthesis), or by treatment with cytochalasin E (which disrupts monofilaments) or whether the cells are spontaneously sensitive to TNF (19, 20, 24, 25). The E1B 19K protein protects Ad-infected human cells but not mouse cells against TNF, and it also prevents lysis when cells are sensitized to TNF by treatment with CHI (18). The E3 10.4K and 14.5K proteins protect many mouse cells, including C127, against TNF lysis; they do not protect C3HA cells against TNF, and we do not know whether they (or the 14.7K protein) protect human cells against TNF. The E3 10.4K and 14.5K proteins also block TNF cytolysis when cells are sensitized to TNF by treatment with CHI.

We will probably not understand why the E3 10.4K and 14.5K proteins do not protect C3HA cells and why the E1B 19K protein does not protect mouse cells until we know more about these proteins as well as the mechanism of TNF cytolysis. Perhaps the E1B 19K protein, being a human Ad protein, simply cannot interact with mouse proteins. In any event, it seems very unlikely that these proteins function in the same manner. The E1B 19K protein is primarily localized on the nuclear membrane, and it binds to and disrupts intermediate filaments and the nuclear lamina (44). The 14.7K protein is a hydrophilic protein localized in the cytosol and the nuclei (37a), and the 10.4K and 14.5K proteins are cytoplasmic membrane proteins (30a, 39). Considering this, these proteins probably act at different points in the pathway or pathways of TNF cytolysis. Given that at least three sets of proteins have evolved in Ad to prevent TNF cytolysis, it appears that TNF must be a major antiviral agent in the infected host. Perhaps three sets of proteins are required in Ad because some of the proteins can function in some tissues of the infected host but not in others.

We have noted previously (10) that Ad transcription units tend to encode genes with related functions, i.e., all three genes in region E2 function in DNA replication, region 1 genes function in gene regulation and cell transformation, and the genes in the major late transcription unit encode virus structural proteins. Our results here tend to support this notion. Four of the putative nine E3 proteins have now been implicated in allowing Ad to evade the host's antiviral defenses: gp19K prevents cytolysis by CTL, and the 14.7K and the 10.4K and 14.5K proteins are required to prevent cytolysis by TNF.

We have shown that in human cells, the 10.4K protein is coimmunoprecipitated with the 14.5K protein by an antipeptide antiserum to the C-terminal 15 amino acids of the 14.5K protein, by an antipeptide antiserum to residues 19 to 34 in the 14.5K protein, and by an antiserum against a TrpE-14.5K protein fusion protein (40). As shown here, the complex is also able to form in protein extracts from mouse cells. When obtained from human cells, the complex is stable to a solution containing 0.5 M NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS (40). These coimmunoprecipitation data strongly suggest that the 10.4K and 14.5K proteins exist as a complex in vivo, which is consistent with our observation that both 10.4K and 14.5K proteins are required to protect C127 cells from TNF lysis in the absence of the 14.7K protein.

There is also other evidence linking the 10.4K and 14.5K proteins. First, they are both translated from E3 mRNA *f* (38, 39), which suggests an evolutionary relationship between the proteins. Second, both the 10.4K (40) and 14.5K (30a) proteins are membrane proteins, probably integral membrane proteins with N-terminal signals, as judged by their sequence. Third, the pattern of 14.5K protein bands is different in mutants that lack the 10.4K protein than in viruses that express the 10.4K protein (Fig. 6) (38), suggesting that the 10.4K protein affects the posttranslational processing of the 14.5K protein. The fourth point relates to the fact that the 10.4K protein is required to stimulate endosome-mediated internalization and degradation of EGF-R in Ad-infected human and mouse cells (7). Although the 10.4K protein can function alone in this respect when expressed from a retrovirus vector (23), both the 10.4K and 14.5K proteins are required for efficient downregulation of EGF-R in Ad-infected cells (40).

The observation that the putative 10.4K-14.5K protein complex functions both in TNF protection and in downregulation of EGF-R raises the possibility that these phenomena are related. It will be of great interest to determine whether this is indeed the case. It will also be instructive to learn why the 10.4K and 14.5K proteins cannot protect C3HA cells against TNF lysis.

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