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Adenovirus E3-6.7K Protein Is Required in Conjunction with the E3-RID Protein Complex for the Internalization and Degradation of TRAIL Receptor 2

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Adenoviruses (Ads) encode several proteins within the early region 3 (E3) transcription unit that help protect infected cells from elimination by the immune system. Among these immunomodulatory proteins, the receptor internalization and degradation (RID) protein complex, which is composed of the RIDα (formerly E3-10.4K) and RIDβ (formerly E3-14.5K) subunits, stimulates the internalization and degradation of certain members of the tumor necrosis factor (TNF) receptor superfamily, thus blocking apoptosis initiated by Fas and TNF-related apoptosis-inducing ligand (TRAIL). The experiments reported here show that TRAIL receptor 2 (TR2) is cleared from the cell surface in Ad-infected cells. Virus mutants containing deletions that span E3 were used to show that the RID and E3-6.7K proteins are both necessary for the internalization and degradation of TR2, whereas only the RID protein is required for TRAIL receptor 1 downregulation. In addition, replication-defective Ad vectors that express individual E3 proteins were used to establish that the RID and E3-6.7K proteins are sufficient to clear TR2. These data demonstrate that E3-6.7K is an important component of the antiapoptosis arsenal encoded by the E3 transcription unit of subgroup C Ads.

To replicate successfully, many viruses must protect infected cells from destruction by apoptosis, a principal mechanism by which the host eliminates infected cells. Apoptosis can be induced via intrinsic or extrinsic pathways, with the latter being mediated through receptors in the tumor necrosis factor (TNF) receptor superfamily. The ligands for these receptors are TNF, Fas, and TNF-related apoptosis-inducing ligand (TRAIL).

TRAIL (Apo-2L), a member of the TNF superfamily, induces apoptosis by binding to TRAIL receptor 1 (TR1/DR4) or TRAIL receptor 2 (TR2/DR5). Three other members of the TNF receptor superfamily also bind to TRAIL (TR3/DcR1, TR4/DcR2, and osteoprotegerin) but are unable to induce apoptosis. TRAIL is produced by a large number of cell types and exists as a membrane protein and in a proteolytically cleaved soluble form (29, 44). Although the normal physiological function of TRAIL is an active area of investigation, it is known that T lymphocytes and NK cells use TRAIL to induce apoptosis in virus-infected and tumor cells (33). Many viruses are known to modulate the TRAIL pathway by altering the expression of TRAIL and/or TRAIL receptors or by synthesizing proteins that interfere with the normal signaling pathway, suggesting that the TRAIL pathway plays an important role in combating virus infections (2).

Human adenoviruses (Ads) use several strategies to prevent apoptosis. Ads block apoptosis induced extrinsically by TNF, Fas, and TRAIL and prevent apoptosis initiated from within the cell (16, 24, 25, 47, 48). In particular, five Ad-encoded polypeptides contribute to the blocking of TRAIL-induced apoptosis, namely, E1B-19K, E3-14.7K, receptor internalization and degradation (RID) protein complex subunits RIDα and RIDβ, and E3-6.7K (1, 41).

The RID complex (formerly E3-10.4K/E3-14.5K), which is composed of the RIDα and RIDβ subunits (13, 40), stimulates the destruction of specific death receptors, such as Fas (10, 32, 35), TR1 (1, 41), and TR2 (1). The RID complex also mediates the degradation of other cell surface receptors, such as the epidermal growth factor receptor (EGFR) (6, 40). Through a mechanism that requires a tyrosine sorting motif present in the cytoplasmic tail of RIDβ (14, 23) and a dileucine sorting motif in RIDα (14), the RID complex mediates the internalization of target receptors from the cell surface into the endocytic pathway, where the receptors are ultimately degraded in lysosomes.

The RID complex has functions in addition to mediating the degradation of cell surface receptors. The RID complex blocks the TNF-mediated translocation of cytoplasmic phospholipase A_2 from the cytoplasm to membranes (8), thus preventing the release of arachidonic acid (18), a potent mediator of inflammation. The RID complex also interferes with activation of the NF-κB pathway in response to treatment with TNF alpha or interleukin 1 by preventing a critical phosphorylation step (11).

Both RID protein subunits are encoded within the early region 3 (E3) transcription unit and are integral membrane proteins (19, 20), with RIDβ being O glycosylated (21) and phosphorylated on serine residue 116 (22, 23). RIDα and RIDβ are localized to the plasma membrane when coexpressed (15, 23, 34, 35) but are localized predominantly in the Golgi apparatus (RIDα) (34, 35) or the endoplasmic reticulum (ER) and the Golgi apparatus (RIDβ) (23, 34, 35) when expressed individually. These data and the fact that neither sub-

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TABLE 1. Viruses and vectors used in this study[a]

Virus or vector	Expression of:							Reference or source
	E3-12.5K	E3-6.7K	E3-gp19K	ADP	RIDα	RIDβ	E3-14.7K	
*rec*700[b]	+	+	+	+	+	+	+	51
*dl*731	−	+	+	+	+	+	+	5
*dl*701	+	−	+	+	+	+	+	4
*dl*740	+	−	+	+	+	+	+	This report
*dl*754[c]	+	−	−	+	+	+	+	12
*dl*704	+	+	−	+	+	+	+	4
*pm*734.1	+	+	+	−	+	+	+	39
*dl*799	+	+	+	+	−	−	+	13
*dl*762	+	+	+	+	+	+	−	5
*dl*309[d]	+	−	+	+	−	−	−	17
Ad/null	−	−	−	−	−	−	−	43
Ad/E3	+	+	+	−	+	+	+	43
Ad/RID/14.7K	−	−	−	−	+	+	+	43
Ad/RID	−	−	−	−	+	+	−	43
Ad/14.7K	−	−	−	−	−	−	+	43
Ad/6.7K	−	+	−	−	−	−	−	This report

[a] Replication-competent viruses used in this study were *rec*700, *dl*731, *dl*701, *dl*740, *dl*754, *dl*704, *pm*734.1, *dl*799, *dl*762, and *dl*309. RD (EIA-negative) vectors used in this study were Ad/null, Ad/E3, Ad/RID/14.7K, Ad/RID, Ad/14.7K, and Ad/6.7K. The expression cassette, which consists of the cytomegalovirus promoter driving the expression of the indicated protein(s), is placed into the deleted E1A region. The natural E3 region is deleted in these vectors. −, no expression; +, expression.

[b] The virus *rec*700 is an Ad5-Ad2-Ad5 recombinant and is considered to be the wt parent of virus mutants with "700" designations.

[c] The mutant *dl*754 contains a deletion that removes the C-terminal coding sequence of E3-6.7K and the N-terminal coding sequence of gp19K.

[d] The mutant *dl*309 is a derivative of Ad5. Sequence analysis showed that the E3-6.7K gene in *dl*309 contains a mutation (3) that renders the E3-6.7K protein nonfunctional (A. E. Tollefson, unpublished observations).

unit by itself promotes the degradation of target receptors support the hypothesis that the RID protein functions primarily on receptors that are located in the plasma membrane (35).

Another E3-encoded protein, E3-6.7K, was shown to have a role in blocking apoptosis. E3-6.7K is a small integral membrane protein (45) that is encoded by subgroup C Ads and that exists in various topological conformations within membranes (26, 45). E3-6.7K contains three potential N-glycosylation sites, only one of which is modified with high-mannose oligosaccharides, suggesting that the protein is localized to the ER (46). Analysis by immunofluorescence microscopy confirmed that E3-6.7K is localized primarily in the ER, but a small amount of E3-6.7K reaches the plasma membrane (1, 26, 46). Cells stably transfected with E3-6.7K showed reduced levels of TNF alpha-induced release of arachidonic acid and apoptosis (27). The stable transfectants also exhibited reduced levels of apoptosis and Ca^{2+} efflux after treatment with thapsigargin (a compound that induces apoptosis by mimicking sustained Ca^{2+} flux) (27). Based on these data, E3-6.7K was proposed to function in the ER as a general repressor of apoptosis by maintaining cytosolic Ca^{2+} homeostasis (27). In addition, E3-6.7K associates with the RID complex and assists with some of its antiapoptotic functions. E3-6.7K was shown to be required for the RID protein-mediated degradation of TR2 (1) but is not generally required for the function of the RID protein, since the RID protein alone mediates the degradation of EGFR and Fas (35, 40). With regard to TR1, one group showed that the degradation of this receptor is entirely independent of E3-6.7K (41), whereas another group showed that E3-6.7K is necessary for the optimal degradation of TR1 (1).

In this report, we have investigated the effect of Ad on TR2 in more detail. We confirm that Ad infection induces the downregulation of TR2 from the cell surface. Using Ad mutants and recombinant Ad vectors that express individual E3 proteins, we have demonstrated that a combination of E3-6.7K and the RID protein is both necessary and sufficient for RID protein-mediated internalization and degradation of TR2. In contrast, only the RID protein by itself is necessary and sufficient for the downregulation of TR1.

MATERIALS AND METHODS

Cells, viruses, and vectors. Human A549 lung carcinoma cells (American Type Culture Collection [ATCC]), 293 cells, and human HeLa cervical carcinoma cells were grown in Dulbecco's modified essential medium. HT29.14S cells (obtained from Jeff Browning, Biogen, Inc., Cambridge, Mass.) are a subclone derived from the HT29 colon carcinoma cell line (ATCC) and were grown in McCoy's medium. All media were supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Virus stocks were prepared in suspension cultures of human KB cells or 293 cells (only for replication-defective [RD] Ad vectors) and then purified by banding in CsCl; titers were determined on A549 cells or 293 cells (for RD vectors) as described previously (36). The viruses and viral vectors used in this study are described in Table 1.

An E1A-negative, E3-negative RD Ad vector named Ad/6.7K was constructed for this study. The Ad2 E3-6.7K gene was amplified by PCR from plasmid pED, which contains the Ad2 EcoRI D fragment, with the following primers: 5′-GGATAAGAATTC**ACC**ATG**G**GCAATTCAAGTAACTC-3′ and 5′-GGATTAAGAATTCTCATCTTGGATGTGGC-3′; an EcoRI site (underlined type) was introduced at each end of the PCR product, and the nucleotide context surrounding the start codon was changed (boldface type) to improve mRNA translation efficiency. The mutation in the coding sequence changed the first amino acid after the initiator methionine from serine to glycine. The corresponding residue in the Ad5 E3-6.7K protein is asparagine, indicating that there is heterogeneity at this position (7). The PCR product was digested with EcoRI and cloned into EcoRI-digested pMT2 vector DNA (35) to create plasmid pMT2-6.7K. The E3-6.7K protein was expressed abundantly from pMT2-6.7K (data not shown). A PCR product containing a PacI site at the 5′ end and a SwaI site at the 3′ end was generated with pMT2-6.7K as a template and the following primers: 5′-GGGATTTTTTAATTAAGACCATGGGC-3′ (PacI site is underlined) and 5′-AGCTTCTGATTTAAATGTAACATTGC-3′ (SwaI site is underlined). This PCR product was digested with PacI and SwaI and then used to create plasmid p231-6.7K(KOZ) by replacement of the PacI-SwaI fragment of plasmid p231. Plasmid p231 was generated by cloning the ClaI-MfeI fragment containing all of E3 of p181 (43) into ClaI-EcoRI-digested pΔE1SP1A (Microbix, Toronto, Ontario, Canada). In order to delete all of the E3 genes downstream of the E3-6.7K gene, plasmid p231-6.7K(KOZ) was digested with SwaI and HpaI and then religated to create plasmid p231-6.7K(KOZ)ΔSH. Ad/6.7K was generated by

cotransfecting 293 cells with p231-6.7K(KOZ)ΔSH and pBGH10 (Microbix). The resulting Ad vector was plaque purified three times, expanded in 293 cells, and purified by banding in CsCl; titers were determined on 293 cells. Although the E3-12.5K protein might be expected to be expressed from this vector, the protein was not detected by Western blot analysis of lysates of Ad/6.7K-infected 293 cells (data not shown).

The Ad mutant *dl*740 was constructed in a manner similar to that for mutant *dl*701 (4). The deletion in *dl*740 extends from nucleotide 878 to nucleotide 1075 (the Ad2 E3 transcription initiation site is considered +1). The coding sequence for the wild-type (wt) Ad type 2 (Ad2) E3-6.7K gene extends from nucleotide 1021 to nucleotide 1207.

Antibodies. Mouse monoclonal antibodies (MAbs) specific for TR1 (M271) and TR2 (M413) were obtained from Immunex Corp. (Seattle, Wash.) and used for fluorescence-activated cell sorting (FACS) and immunofluorescence studies. MAbs against Fas (M38) and transferrin receptor (TfnR) (OKT9) were prepared from hybridoma cell lines obtained from ATCC. The anti-EGFR MAb (528) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit polyclonal antibodies against RIDα (38), RIDβ (37), E3-6.7K (45), and E3-14.7K (42) were described previously. Fluorochrome- and horseradish peroxidase-conjugated secondary antibodies were purchased from Cappel/ICN (Costa Mesa, Calif.).

Immunological assays. For flow cytometry, cells were infected with 20 to 300 PFU of virus or Ad vector, and staining was begun at various times. Staining was performed as previously described (41). MAb M413 was used at a concentration of 5 μg/ml. Indirect immunofluorescence of cells infected at 100 PFU/cell was performed as previously described (34). For Western blot and immunoprecipitation analyses, HeLa cells and KB cells, respectively, were infected at 100 PFU/cell. The cycloheximide (CHX) block procedure was used for metabolic labeling of proteins with [^{35}S]cysteine (Perkin-Elmer Life Sciences, Boston, Mass.) (49, 50). Preparation of cell lysates, Western blot analysis, and immunoprecipitation were carried out as described by Lichtenstein et al. (23). For Western blot analysis, antibodies against RIDβ and E3-14.7K were used at a dilution of 1:400. For immunoprecipitation, 5 μl of anti-RIDα or anti-E3-6.7K antiserum was used for each sample.

Apoptosis assay. The apoptosis assay was conducted at 2 days postinfection (p.i.) so that it detected only apoptosis and not virus-induced cytotoxicity, which occurs much later. HT29.14S cells were infected at 150 PFU/cell. At 4 to 5 h p.i., cells were trypsinized, diluted, and replated on 96-well plates so that each infection or condition was assayed in triplicate. At 24 h p.i., the cell culture medium was replaced with fresh medium containing 25 μg of CHX/ml and either 0, 0.5, 5.0, or 50.0 ng of TRAIL/ml. CHX was used because it sensitizes cells to TRAIL-induced apoptosis (41). After 24 h of TRAIL treatment, the culture medium was removed and assayed for lactate dehydrogenase (LDH) release by using a CytoTox96 assay (Promega, Madison, Wis.). Percent specific lysis was calculated as follows: [(absorbance with TRAIL − absorbance with CHX)/(maximum absorbance − absorbance with CHX)] × 100.

RESULTS

E3-6.7K and RID proteins are necessary for downregulation of TR2 from the cell surface. A panel of mutant viruses, each of which is deficient in the expression of a different E3 protein or proteins, was used recently to show that the RID protein is both necessary and sufficient for the downregulation of TR1 (41). This same panel of mutant viruses was used to determine which E3 protein(s) is required for the clearance of TR2 from the cell surface (Table 1). Cell surface expression of EGFR was also monitored because the RID protein is known to downregulate this receptor (6, 40). FACS analysis of cells stained for TR2 showed that it was absent from the surface of cells infected with wt Ad (*rec*700) as well as cells infected with mutant viruses lacking genes for the following proteins: E3-gp19K (*dl*704), E3-12.5K (*dl*731), ADP (*pm*734.1), and E3-14.7K (*dl*762) (Fig. 1A). In contrast, cells infected with mutant viruses lacking genes for E3-6.7K only (*dl*701) or RIDα plus RIDβ (*dl*799) showed the same level of cell surface staining for TR2 as mock-infected cells (Fig. 1A). The only mutant virus that did not clear EGFR from the cell surface was *dl*799 (Fig. 1B). In cells infected with the remaining virus mutants, EGFR was removed from the cell surface to the degree seen in *rec*700-infected cells (Fig. 1B). These data strongly suggest that both E3-6.7K and RID proteins were required for the downregulation of TR2 and confirm that only the RID protein was needed for EGFR clearance.

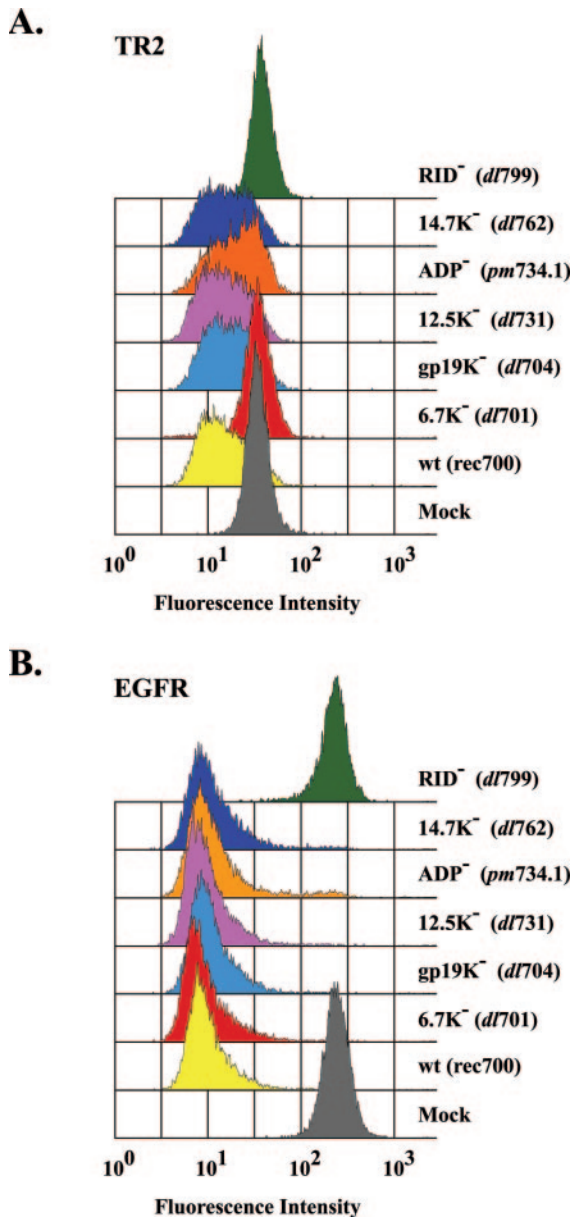

FIG. 1. Downregulation of TR2 in Ad-infected cells requires the RID and E3-6.7K proteins. HeLa cells were mock infected or infected with wt (*rec*700) or mutant viruses at a multiplicity of infection of 150 PFU/cell. At 23 h p.i., cells were detached from their dishes, stained with anti-TR2 (A) or anti-EGFR (B) antibody, and analyzed by flow cytometry. The virus used for infection (in parentheses) and the protein(s) deleted or mutated in that virus are shown to the right of each FACS profile.

Mutations within the E3 genes often alter the pattern of expression of the E3 proteins because these mutations may also affect the complex splicing scheme required for the ap-

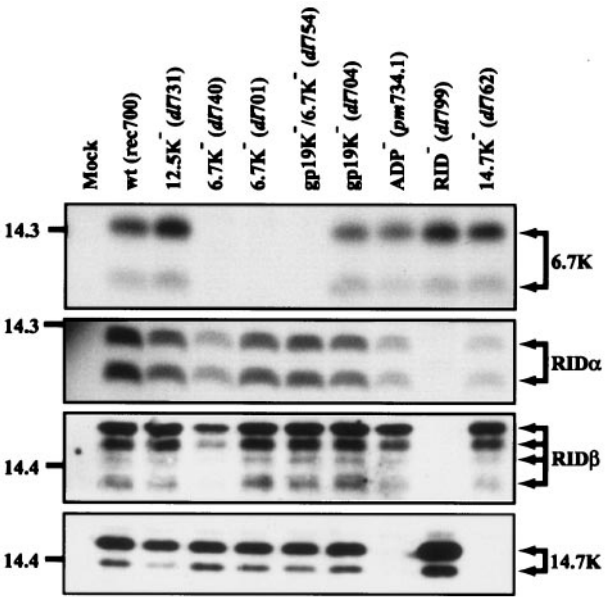

FIG. 2. Mutant Ads generally express equivalent amounts of E3 proteins. KB cells were mock infected or infected with wt (*rec*700) or mutant viruses at 100 PFU/cell. Proteins metabolically labeled with [^{35}S]cysteine were immunoprecipitated from cell lysates with anti-E3-6.7K or anti-RIDα antiserum. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography of the dried gel (top two panels). Both E3-6.7K (45) and RIDα (38) migrated as two bands when analyzed by SDS-PAGE. For Western blot analyses, HeLa cells were infected as described above. Proteins from cell lysates prepared at 24 h p.i. were separated by SDS-PAGE and then subjected to Western blot analysis with anti-RIDβ or anti-E3-14.7K antiserum (bottom two panels). RIDβ migrated as multiple bands (37) and E3-14.7K migrated as a doublet or a triplet (42) when analyzed by SDS-PAGE. The virus used for infection (in parentheses) and the protein(s) deleted or mutated in that virus are shown above each lane. The sizes of molecular weight standards (in thousands) are shown to the left of each gel or blot. The migration positions of the proteins detected are shown to the right of each gel or blot.

propriate expression of the E3 genes (5, 31). The levels of expression of various E3 proteins known to be important in regulating the sensitivity of infected cells to TRAIL were analyzed by immunoprecipitation or Western blotting of lysates of cells infected with various mutant viruses (41). As shown in Fig. 2, E3-6.7K was not detected in cells infected with mutant viruses from which the gene was deleted but was expressed at normal levels (compared to the level seen with wt virus *rec*700) in cells infected with the remaining mutant viruses. With two exceptions, the remaining E3 proteins examined were all expressed at levels similar to those seen in *rec*700-infected cells (Fig. 2). The two exceptions were that *dl*740-infected cells expressed a slightly smaller amount of RIDβ and *dl*799-infected cells expressed a much larger amount of E3-14.7K (Fig. 2). These data indicate that the results obtained by FACS analysis of TR2 were specifically the result of a lack of E3-6.7K or RID protein expression and not a generally altered pattern of E3 protein expression.

E3-6.7K expression is required for internalization and degradation of TR2 but not TR1. The RID protein normally acts on its target receptors by stimulating their entry into the endocytic pathway and their subsequent degradation in lysosomes (1, 10, 35). Internalization of TR2 as well as TR1 and EGFR was examined by indirect immunofluorescence of cells that were mock infected or infected with wt or mutant viruses. Mock-infected cells showed faint cell surface expression of TR1 and TR2 and strong expression of EGFR (Fig. 3A). Cell surface expression of all three receptors was absent after infection with wt Ad, and instead the receptors were present in intracellular vesicles (Fig. 3B). All three receptors were still present at the cell surface after infection with a mutant that did not express the RID protein (*dl*799), although the pattern of TR2 localization was altered from an even to a punctate distribution over the cell surface (Fig. 3C). With two different mutant viruses lacking E3-6.7K expression (*dl*701 and *dl*754), TR1 and EGFR were internalized in a manner similar to that seen in wt Ad-infected cells, but TR2 showed the punctate surface staining pattern seen with *dl*799 (Fig. 3D and E). These data, which confirm those obtained by FACS analysis, indicate that the E3-6.7K and RID proteins were needed for the internalization of TR2 but that only the RID protein was required for the internalization of EGFR and TR1 (41).

As described previously, the intracellular punctate pattern seen for Fas in wt Ad-infected cells is indicative of lysosomes, the site of RID protein-mediated degradation of Fas (10, 35). We investigated whether TR2 and TR1 are also degraded. Immunoprecipitates prepared from lysates of infected cells with anti-TR2 or anti-TR1 MAbs were subjected to Western blot analysis with TR2- or TR1-specific rabbit antisera, respectively. As controls, nonimmunoprecipitated lysates were also analyzed by Western blotting for Fas, TfnR, Ad late proteins, and ERp72 (a cellular chaperone). As shown in Fig. 4, TR2 was degraded (i.e., could not be detected) in cells infected with any virus that expressed both E3-6.7K and RID proteins but was not degraded when either E3-6.7K or RID protein expression was lacking. The situation for TR1 was identical to that for Fas: these receptors were degraded in all virus-infected cells except for those infected with *dl*799, which lacks RID protein expression (Fig. 4). As anticipated, TfnR was not degraded in any of the virus-infected cells (Fig. 4). Analysis of Ad late protein expression showed that all infections were equivalent (Fig. 4). The same amounts of protein were loaded in all of the wells, as demonstrated by the uniform signal obtained for ERp72 (Fig. 4). These data show that TR2 and TR1 were degraded, that the E3-6.7K and RID proteins were necessary for the degradation of TR2, and that only the RID protein was required for the degradation of TR1.

Complementation of virus mutants restores downregulation of TR2. Although TR2 was not downregulated in cells infected with individual virus mutants deficient in the expression of the E3-6.7K or RID protein, the receptor should be cleared following coinfection with these mutant viruses because the E3-6.7K or RID protein should be provided by the complementing virus. Mock- or virus-infected cells were stained for various cell surface receptors and then analyzed by FACS. As expected, cell surface expression of TR2 was eliminated by infection with wt Ad but was not affected in uninfected cells or by infection with mutant viruses lacking the expression of the E3-6.7K (*dl*701) or RID (*dl*799) protein (Fig. 5A). Coinfection of cells with *dl*701 and *dl*799 resulted in the removal of TR2 from the cell surface in a manner similar to that seen with wt Ad infection (Fig. 5A). As reported previously and as shown in Fig. 5B

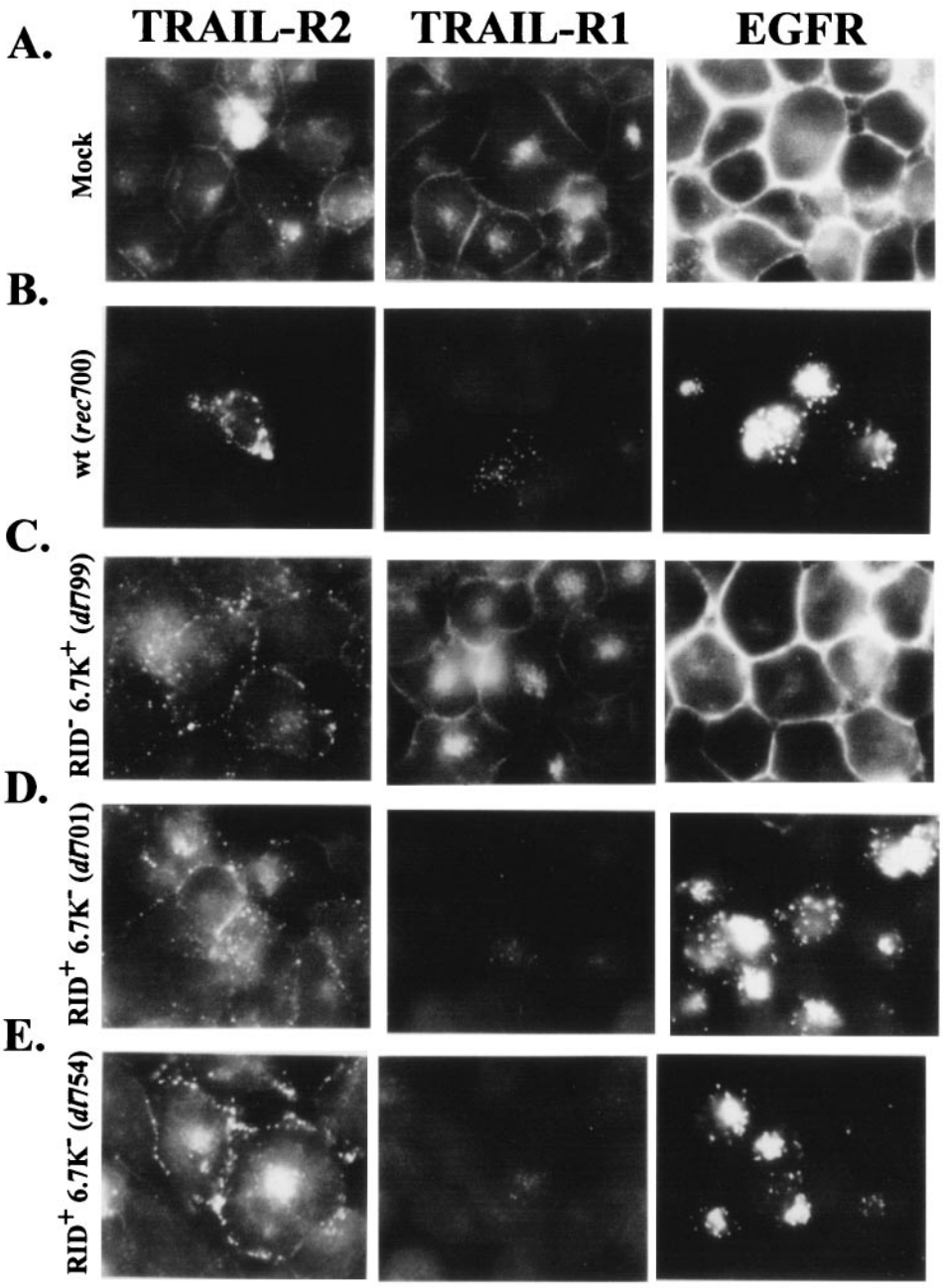

FIG. 3. Internalization from the cell surface of TR2, but not TR1 or EGFR, requires both RID and E3-6.7K protein expression. HeLa cells were mock infected or infected with wt (*rec*700) or mutant viruses at 100 PFU/cell. At 17 h p.i., cells were fixed and then immunostained for TR2, TR1, or EGFR. The phenotype with respect to RID and E3-6.7K protein expression along with the name of the virus used for infection are shown.

and C, the downregulation of TR1 and Fas requires the expression of the RID protein but not that of the E3-6.7K protein. Cell surface expression of TfnR was unaffected by infection with any virus (Fig. 5D). These data confirm that both E3-6.7K and RID proteins were necessary for the removal of TR2 from the cell surface and also show that mutant viruses that were unable to express either the E3-6.7K or the RID protein could complement one another.

E3-6.7K and RID proteins are required to fully protect Ad-infected cells from TRAIL-induced apoptosis. TRAIL initiates apoptosis by binding to and causing the trimerization of TR1 and/or TR2. Considering the data described above regarding the internalization and degradation of TR2 and TR1, we examined the ability of various E3 mutant Ads to block TRAIL-induced apoptosis. HT29.14S cells were mock infected or infected with wt or mutant Ads and then treated with TRAIL at various concentrations at 24 h p.i. HT29.14S cells were used for this experiment because neither the E1B-19K protein, which is expressed by all of the mutant viruses analyzed, nor the E3-14.7K protein inhibits TRAIL-induced apoptosis in these cells

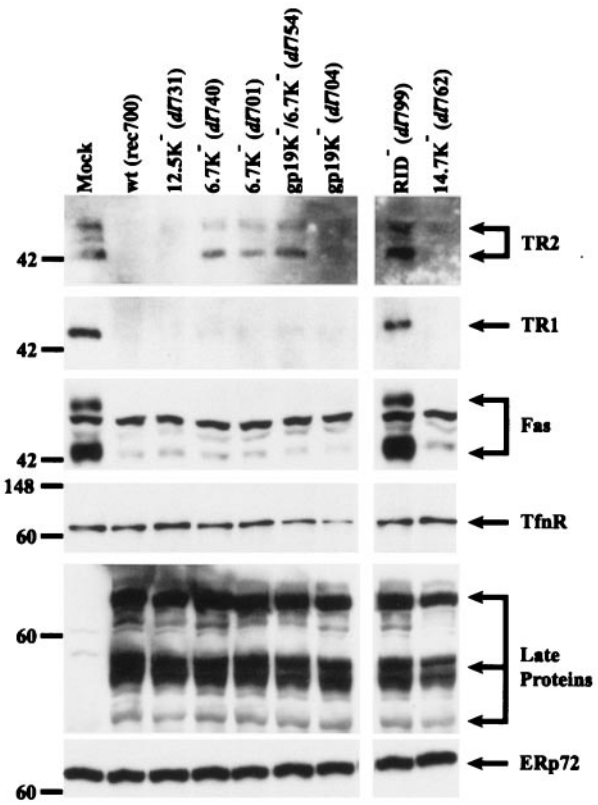

FIG. 4. TR1 and TR2 are degraded in Ad-infected cells. HeLa cells were mock infected or infected with wt or mutant viruses at 50 PFU/cell. Lysates were prepared at 26 h p.i., and a portion of each lysate was subjected to Western blot analysis to detect Fas, TfnR, Ad late proteins, and ERp72 (bottom four panels). In addition, a portion of each lysate was immunoprecipitated with anti-TR1 or anti-TR2 antibody prior to being subjected to Western blot analysis with anti-TR1 or anti-TR2 rabbit antiserum (top two panels). See the legend to Fig. 2 for additional explanations of designations.

(41). After 24 h of TRAIL treatment, cell viability was assessed by using an LDH release assay. Under the conditions used, about 80% of mock-infected cells were killed at the highest dose of TRAIL (Fig. 6). Cells infected with wt Ad (*rec*700) or mutant Ads (*dl*704, *dl*762, *pm*734.1, and *dl*731) that expressed both E3-6.7K and RID proteins were substantially protected from TRAIL-induced apoptosis (30 to 50% killing). Cells infected with virus mutants lacking RID protein expression (*dl*309 and *dl*799) were not protected from killing by TRAIL, since nearly 100% of the cells were lysed. An intermediate level of killing (60 to 70%) was observed in cells infected with E3-6.7K-deficient virus mutants (*dl*740 and *dl*754), indicating that these cells were still somewhat protected from TRAIL-induced apoptosis. This partial protection likely was a consequence of the fact that the RID protein, which was still expressed by these two virus mutants, was able to downregulate TR1 but not TR2. These data demonstrate that E3-6.7K was necessary for maximal protection from TRAIL-induced apoptosis. In addition, the results show that the RID protein alone could provide substantial protection from killing by TRAIL in the time frame tested and with the concentrations of TRAIL that were examined.

The combination of the E3-6.7K and RID proteins is sufficient to downregulate TR2. Because the E3-6.7K and RID proteins are required for the downregulation of TR2, we sought to determine whether these two viral proteins are sufficient for this activity. To this end, we used E1-negative, E3-negative RD Ad vectors (43) that expressed all of E3 (Ad/E3), only the RID protein (Ad/RID), only E3-14.7K (Ad/14.7K), or the RID protein plus E3-14.7K (Ad/RID/14.7K). These vectors were used previously to demonstrate that the RID protein alone was sufficient to downregulate TR1 (41). Cells were mock infected or infected with wt Ad or the Ad vectors, stained for TR2, and analyzed by FACS. TR2 was cleared from the cell surface when the E3-6.7K and RID proteins were expressed (*rec*700 and Ad/E3) but was not cleared when the vectors lacked E3-6.7K (Ad/RID/14.7K and Ad/RID) or both E3-6.7K and RID proteins (mock, Ad/null, and Ad/14.7K) (Fig. 7A).

To test whether the expression of the E3-6.7K and RID proteins was sufficient for TR2 clearance, an RD Ad vector (Ad/6.7K) that expressed only E3-6.7K was constructed. Ad/6.7K-infected cells expressed E3-6.7K to a level similar to that seen in Ad/E3-infected cells (Fig. 7B). FACS analysis was used to examine the cell surface levels of TR2, TR1, EGFR, and TfnR in cells infected with various Ad vectors. As expected, TR2 was cleared from the cell surface in Ad/E3-infected cells (Fig. 7C). TR2 was not downregulated by infection of cells with only Ad/6.7K or Ad/RID, but cell surface expression of TR2 was substantially reduced after coinfection with Ad/6.7K and Ad/RID (Fig. 7C). In agreement with previous work, the expression of the RID protein was sufficient to cause the clearance of TR1 and EGFR (Fig. 7C). In addition, TfnR was not downregulated by the expression of the E3-6.7K or RID protein, either alone or in combination (Fig. 7C). These data demonstrate that the E3-6.7K and RID proteins were sufficient to downregulate TR2 and confirm that the RID protein by itself was sufficient to clear TR1.

DISCUSSION

We demonstrated that the E3-6.7K and RID proteins are jointly necessary and sufficient for the downregulation of TR2, in contrast to the situation for EGFR, Fas, and TR1 (see below), for which the RID protein alone is necessary and sufficient for downregulation (10, 32, 35, 40, 41). We showed that infection with mutant viruses lacking the expression of either the E3-6.7K (*dl*701) or the RID (*dl*799) protein prevented the clearance of TR2 from the cell surface (Fig. 1A and 3) but that TR2 downregulation was restored upon coinfection with these two mutant viruses (Fig. 5A). As with other RID protein-targeted receptors, both RIDα and RIDβ were required to clear TR2 from the cell surface (data not shown). Furthermore, the expression of either the E3-6.7K or the RID (RIDα plus RIDβ) protein alone from Ad expression vectors did not result in TR2 downregulation, but simultaneous expression of these proteins demonstrated that they are sufficient to mediate the clearance of TR2 from the cell surface (Fig. 7C).

Importantly, the clearance of TRAIL receptors from the cell surface reduced the ability of TRAIL to induce apoptosis. The

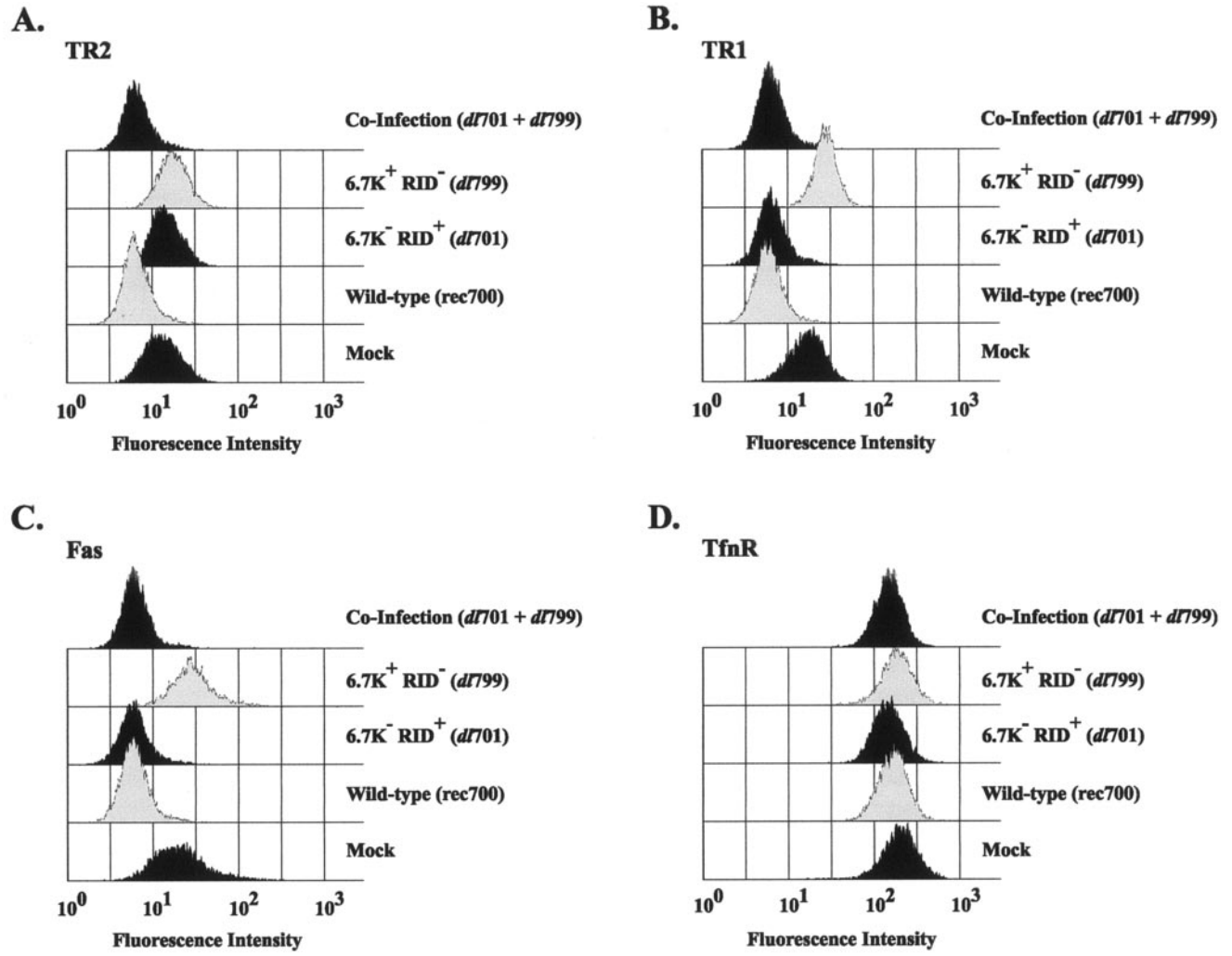

FIG. 5. Coinfection with E3-6.7K and RID protein-deficient viruses rescues the ability of Ads to downregulate TR2. A549 cells were mock infected or infected with wt (*rec*700) or mutant viruses at 150 PFU/cell. Note that each virus was used at 100 PFU/cell for coinfection (total of 200 PFU/cell). At 23 h p.i., cells were detached from their dishes, stained with a MAb against TR2 (A), TR1 (B), Fas (C), or TfnR (D), and analyzed by flow cytometry. The virus used for infection (in parentheses) and the protein(s) deleted or mutated in that virus are shown to the right of each FACS plot.

level of apoptosis corresponded to whether the infecting virus was able to clear one or both receptors. wt Ad provided good protection, whereas mutant viruses not expressing the RID protein did not provide any protection; in fact, cells infected with mutant viruses showed enhanced sensitivity to TRAIL, consistent with the observation that E1A enhances sensitivity to apoptosis induced by TRAIL (30, 41). Mutant viruses that did not express E3-6.7K provided an intermediate level of protection, consistent with the observation that TR1 but not TR2 was downregulated in cells infected with mutant viruses (Fig. 6).

Other receptors that are affected by the RID protein, such as EGFR, Fas, and TR1, are targeted for degradation in lysosomes (1, 10, 35, 41). We demonstrated that TR2 is also degraded, even though this process requires the E3-6.7K protein in addition to the RID protein (Fig. 4). Similar to results obtained for other RID protein-targeted receptors, degradation likely occurs in lysosomes because TR2 accumulates upon bafilomycin A_1 treatment of wt Ad-infected cells (data not shown).

These data suggest that TR2 is internalized and degraded by a mechanism that at the very least shares some features with that for the RID protein-mediated degradation of other receptors. It was recently shown that the cytoplasmic tails of RIDα and RIDβ each contain sorting motifs and that these motifs are important in the function of the RID protein (14, 23). RIDα contains a dileucine motif that appears to be important in preventing rapid turnover of the RID complex (14). It was suggested that this dileucine motif mediates recycling back to the cell surface of the RID protein that has been internalized, thereby preventing degradation of the RID protein in lysosomes (14). Mutation of the dileucine motif either prevents (EGFR and TR2) or significantly inhibits (Fas and TR1) RID protein-mediated receptor downregulation, possibly by reducing the half-life of the RID complex and thus decreasing the amount of the RID protein present at the cell surface (14).

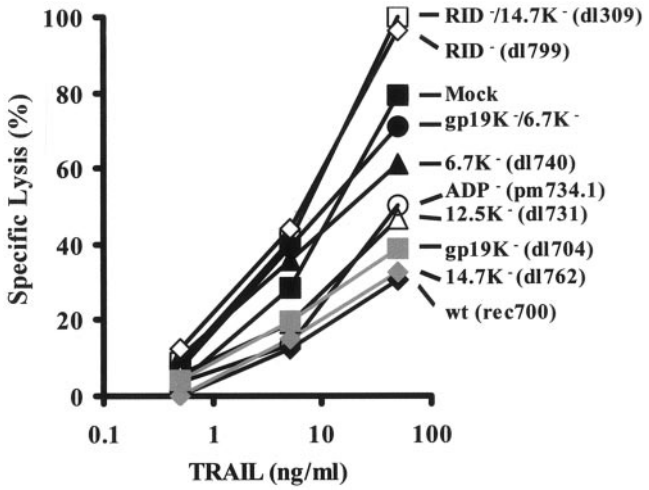

FIG. 6. Maximal protection against TRAIL-induced apoptosis requires the expression of the E3-6.7K and RID proteins. HT29.14S cells were mock infected or infected with the indicated virus at 150 PFU/cell. Starting at 24 h p.i., cells were treated with TRAIL at 0, 0.5, 5.0, or 50.0 ng/ml plus CHX at 25 μg/ml for 24 h. Cell viability was determined by assaying for LDH release into the culture medium. The percent specific lysis was calculated as described in Materials and Methods and then plotted against the TRAIL concentration. Filled black circle, *dl*754 (gp19K$^-$/6.7K$^-$).

RIDβ contains a YXXΦ motif, where Y is tyrosine, X is any amino acid, and Φ is a hydrophobic amino acid with a bulky side chain, near its C terminus; this motif is required for the internalization of Fas, EGFR, and both TRAIL receptors and for the protection of cells from Fas- and TRAIL-induced apoptosis. It was suggested that this motif is involved in the internalization of the RID protein from the cell surface (14, 23), a hypothesis that is supported by the observation that mutation of the tyrosine residue in this motif enhances the expression of the RID protein at the cell surface (14). These sorting motifs typically function by binding to a specific subunit of the heterotetrameric adaptor protein (AP) complex, of which four types have been identified (AP-1 through AP-4). In this regard, peptides corresponding to the C termini of RIDα and RIDβ were shown to bind to AP-1 and AP-2 (14). In addition, AP-2 (but not AP-1, AP-3, or AP-4) was identified as a binding partner for RIDβ in a yeast two-hybrid assay (9). These data strongly suggest that the RID protein stimulates the degradation of specific cell surface receptors by modulating the normal function of the cellular trafficking machinery, but this proposed mechanism does not address the question of how the RID protein achieves specificity.

A molecular mechanism that accounts for the fact that the RID protein requires the cooperation of E3-6.7K for the downregulation of TR2 but not for that of other receptors remains to be determined. Any proposed mechanism should explain the following observations. First, E3-6.7K interacts with RIDβ (1). Second, deletion of the C-terminal 16 amino acids of the cytoplasmic tail but not the death domain of TR2 inhibits its downregulation by the RID and E3-6.7K proteins (1). Third, degradation of TR2 is very slow, taking longer than that of EGFR and Fas, suggesting that TR2 downregulation is inefficient compared to that of other receptors (data not shown). Fourth, the pattern of TR2 staining on the cell surface changes from an even distribution in mock-infected cells to a punctate pattern in cells infected with mutant viruses not expressing either the RID or the E3-6.7K protein (Fig. 3C to E), suggesting that the Ad protein by itself can affect the localization of TR2 but is not able to complete the tasks of internalization and degradation. Based on these observations, one can speculate that in the absence of the E3-6.7K protein, the RID protein may deliver TR2 to the endocytic pathway, but because of an efficient recycling motif present in the cytoplasmic tail of TR2 (similar to that found in RIDα), the receptor is not shunted into late endosomes or lysosomes to be degraded but is instead delivered back to the cell surface. In this proposed mechanism, the E3-6.7K protein would bind to TR2, perhaps to mask its recycling motif, and to the RID protein, thereby allowing the RID protein to direct the degradation of TR2. Although no information regarding the rate of recycling of TR2 is available, it has been shown that TR2 turns over rapidly in brefeldin A-treated cells, with a half-life of 60 to 90 min (28). In addition, the observation that the cytoplasmic tail of TR2 contains a potential dileucine motif located 19 to 20 amino acids from the C terminus of the protein may have a bearing on this proposed mechanism, although there is no evidence regarding the functionality of this putative motif (D. L. Lichtenstein, unpublished observations). Confirmation or refutation of this highly speculative mechanism awaits experimental observations.

There is no evidence to indicate which region of E3-6.7K is important for its ability to mediate TR2 internalization and degradation. E3-6.7K is an integral membrane protein that, although predominantly localized to the ER, has been detected at the plasma membrane, a setting that is appropriate for its role in downregulating TR2 (1, 26, 46). E3-6.7K contains an internal signal sequence that directs its insertion into the membrane. It was recently shown that E3-6.7K could adopt three different topologies within microsomal membranes in a cell-free translation system, a form with a luminal N terminus and an intracellular C terminus (N^{lumen}/C^{cyt}), a form with an intracellular N terminus and a luminal C terminus (N^{cyt}/C^{lumen}), and a form in which both termini have a luminal localization (N^{lumen}/C^{lumen}) (26). In addition, transient transfection assays with various FLAG-tagged versions of E3-6.7K were used to demonstrate that the N^{lumen}/C^{cyt} and N^{cyt}/C^{lumen} forms of E3-6.7K are present at the cell surface; the authors could not rule out the possibility that the N^{lumen}/C^{lumen} form also localizes to the cell surface (26). It will be of interest to determine whether a specific form(s) of E3-6.7K is required for its function in downregulating TR2.

In this report, we confirmed and extended earlier observations that only the RID protein is necessary for the internalization of TR1 from the cell surface (41). We showed that the clearance of TR1 from the cell surface and TR1 degradation were efficient in cells infected with virus mutants not expressing E3-6.7K (Fig. 3D, 4, and 5B). We also used RD Ad vectors expressing various E3 proteins to show that the RID protein is sufficient for the complete and efficient internalization of TR1 (Fig. 7C). These data provided convincing evidence that E3-6.7K is not required for the downregulation of TR1. This finding contrasts that of another report indicating that E3-6.7K is needed along with the RID protein for the efficient down-

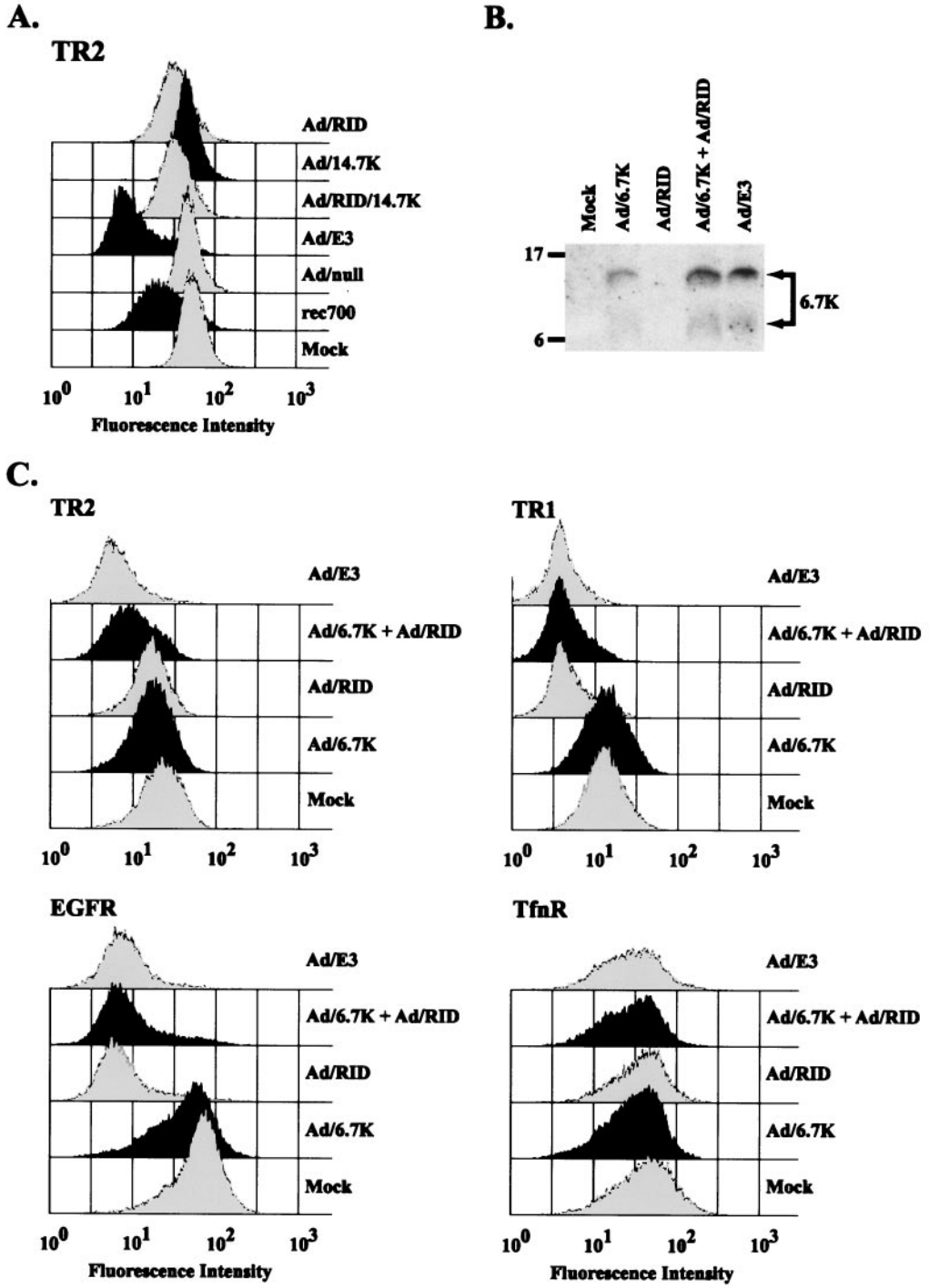

FIG. 7. Infection with RD Ad vectors show that the E3-6.7K and RID proteins are sufficient for the downregulation of TR2. (A) HeLa cells were mock infected or infected with wt Ad (*rec*700) or Ad vectors that express different E3 proteins (Table 1). At 23 h p.i., cells were detached from their dishes, stained with anti-TR2 antibody, and analyzed by flow cytometry. (B) 293 cells were mock infected or infected at 20 PFU/cell with the vectors shown above the lanes. Note that each vector was used at 20 PFU/cell for coinfection. Proteins from cell lysates prepared at 24 h p.i. were separated by SDS-PAGE and then subjected to Western blot analysis with anti-E3-6.7K antiserum. The sizes of molecular weight standards (in thousands) are shown to the left of the blot. The migration positions of the two bands corresponding to E3-6.7K are shown to the right of the blot. (C) HeLa cells were mock infected or infected with 200 (Ad/RID and Ad/E3) or 300 (Ad/6.7K) PFU/cell. Note that Ad/RID at 200 PFU/cell and Ad/6.7K at 300 PFU/cell were used for coinfection. Following infection, cells were maintained in medium containing 1-β-D-arabinofuranosylcytosine (araC) in order to block cell division and to ensure that the infection did not progress from the early to the late stage. At 12-h intervals, the medium was replaced with medium containing fresh araC. At 48 h p.i., cells were detached from their dishes, stained with MAb against TR2, TR1, EGFR, or TfnR, and analyzed by flow cytometry.

regulation of TR1 (1). This discrepancy may be accounted for by differences in the methods and/or the reagents used in these studies. For example, Benedict et al. (1) expressed the RID and E3-6.7K proteins from plasmid and retrovirus vectors, respectively, in cotransfection experiments; perhaps the abundance, stoichiometry, posttranslational processing, or intracellular localization of the RID and/or E3-6.7K proteins differed in their experiments and ours. In any case, continued progress in identifying the functions of the Ad E3 proteins should enable researchers to gain insight into the mechanism of action of these important viral proteins and to take advantage of their immunomodulatory activities in developing improved reagents for combating human disease.

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