

Three Adenovirus E3 Proteins Cooperate to Evade Apoptosis by Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor-1 and -2*

Received for publication, September 8, 2000
Published, JBC Papers in Press, October 24, 2000, DOI 10.1074/jbc.M008218200

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Adenovirus encodes multiple gene products that regulate proapoptotic cellular responses to viral infection mediated by both the innate and adaptive immune systems. The E3-10.4K and 14.5K gene products are known to modulate the death receptor Fas. In this study, we demonstrate that an additional viral E3 protein, 6.7K, functions in the specific modulation of the two death receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The 6.7K protein is expressed on the cell surface and forms a complex with the 10.4K and 14.5K proteins, and this complex is sufficient to induce down-modulation of TRAIL receptor-1 and -2 from the cell surface and reverse the sensitivity of infected cells to TRAIL-mediated apoptosis. Down-modulation of TRAIL-R2 by the E3 complex is dependent on the cytoplasmic tail of the receptor, but the death domain alone is not sufficient. These results identify a mechanism for viral modulation of TRAIL receptor-mediated apoptosis and suggest the E3 protein complex has evolved to regulate the signaling of selected cytokine receptors.

Adenoviruses, as well as other DNA viruses, have developed distinct strategies to counteract host immune defenses and cellular responses to viral infection. These strategies include blocking cellular apoptosis at critical junctions in the death-signaling cascade, suppressing the interferon response, and inhibiting presentation of viral antigens (reviewed in Refs. 1 and 2). The E3 region of the adenovirus genome contains seven expressed open reading frames, most of which encode proteins with immunomodulatory functions, and viral deletion mutants lacking E3 genes induce stronger pro-inflammatory responses in animal models (3). Although the E3 region is dispensable for viral replication in tissue culture, emerging data reveal that several E3 genes are involved in the evasion of host immune

defenses. Uncovering the mechanism by which E3 genes temper host immune responses could lead to the development of novel anti-inflammatory therapeutic strategies.

The E3-10.4K and 14.5K open reading frames encode type 1 transmembrane glycoproteins that form a heteromeric complex (4–6). Both viral 10.4K and 14.5K proteins are required to down-regulate epidermal growth factor receptor (EGF-R)¹ and some related tyrosine kinase receptors from the surface of infected cells (5, 7). More recently, the E3-10.4K/14.5K complex has been shown to mediate the down-regulation of cell surface Fas (8–10), a proapoptotic member of the tumor necrosis factor receptor (TNFR) superfamily. Other members of the TNFR family, such as the LT β R and TNFR1 were not modulated by the 10.4K/14.5K complex. Loss of cell surface Fas results in the desensitization of virus-infected cells to apoptosis induced by Fas signaling, thus counteracting a key defense pathway of cytotoxic T cells and NK cells. It is not clear whether the targeting of both EGF-R and Fas, which show no primary sequence homology, reflects independent or linked functions of the E3-10.4K/14.5K complex. This issue of receptor specificity exhibited by the E3 protein complex prompted us to address whether additional death domain-containing receptors in the TNFR family are targeted by adenovirus.

Cell surface receptors for the TNF-related apoptosis-inducing ligand (TRAIL) are members of the TNFR superfamily, and currently four membrane-anchored TRAIL receptors have been described (reviewed in Refs. 11 and 12). TRAIL-R1 (DR4) and TRAIL-R2 (DR5) both contain a cytoplasmic death domain that when ligated or overexpressed recruits the adaptor FADD, allowing direct activation of the caspase cascade and apoptosis (13–15). In contrast, neither TRAIL-R3 (DcR1) nor TRAIL-R4 (DcR2) induce cell death because they lack a death domain. TRAIL-R3 is linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor (15–17), whereas ligation of TRAIL-R4, which is unable to recruit FADD, can initiate anti-apoptotic signals through the activation of the transcription factor

* This work was supported in part by United States Public Health Service Grants AI03368 and PO1CA69381 from the National Institutes of Health (to C. F. W.), Swiss National Science Foundation Grant 31-47279.96 (to J. T.), National Institutes of Health Training Grant T32AG00252 (to C. A. B.), and NRSA Fellowship Grant 1F2AI10414-01AI (to P. S. N.). This is manuscript 378 from the La Jolla Institute for Allergy and Immunology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: EGF-R, epidermal growth factor receptor; Ad2, adenovirus type 2; Ara-C, arabinofuranosylcytosine; CTL, cytotoxic T lymphocytes; FADD, Fas-associated death domain-containing protein; GPI, glycosylphosphatidylinositol; LAMP-1, lysosome-associated membrane protein-1; NF- κ B, nuclear factor- κ B; NK cells, natural killer cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SAEC, small airway epithelial cells; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; VSV, vesicular stomatitis virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; m.o.i., multiplicity of infection; FACS, fluorescence-activated cell sorter; DD, death domain.

NF- κ B (18). However, TRAIL-R3 and TRAIL-R4 expression correlates poorly with the sensitivity of tumor cell lines to TRAIL-mediated death (19). These findings, and the fact that TRAIL-R1 and TRAIL-R2 can activate NF- κ B in addition to inducing apoptosis (16, 20), suggest a complex hierarchy in the regulation of TRAIL signaling. Additionally, TRAIL has been implicated in both CTL and NK cell killing of target cells (21–23), suggesting a possible role for this cytokine in the innate response to viral pathogens.

Here we report that adenovirus infection results in the down-regulation of TRAIL-R1 and TRAIL-R2 from the cell surface causing desensitization of infected cells to TRAIL-mediated apoptosis. The E3-6.7K protein (24, 25) is required in addition to the E3-10.4K/14.5K proteins for TRAIL receptor down-regulation. These results identify a strategy for viral modulation of TRAIL receptor-mediated apoptosis and suggest the E3 trimolecular complex has evolved to regulate the signaling of selected cytokine receptors.

EXPERIMENTAL PROCEDURES

Cell Lines, Viruses, and Reagents

HT29.14S cells are a human colon adenocarcinoma line that are sensitive to the death inducing activities of TNF-related ligands (26). 293T-Fas cells were generated by transfecting a Fas expression vector into 293T cells (from ATCC), and drug selecting cells that stably expressed a “noncytotoxic” level of Fas on their surface (provided by P. Schneider and J. Tschopp). HeLa cells were obtained from the ATCC. Primary small airway epithelial and normal human bronchial epithelial cell lines were acquired from Clonetics (San Diego, CA) and were propagated in the company’s recommended medium. All other cell lines were propagated in Dulbecco’s modified Eagles medium (Life Technologies, Inc.) supplemented with 10 mM glutamine and 10% fetal calf serum (HyClone, Logan, UT).

All viruses used in these experiments have been described in detail previously (27, 28) and were kind gifts of W. Wold (St. Louis University, St. Louis, MO). Briefly, rec700 is a recombinant Ad5 subtype virus containing the Ad2 10.4K protein (*EcoRI*-D fragment, map position 73–86) and the Ad5 14.5K and 14.7K proteins. All viral deletion mutants were generated from this parent recombinant “wild-type” virus. dl752 lacks the first 5 amino acids of the 10.4K protein; dl759 deletes the N-terminal 103 amino acids of 14.5K resulting in a nonfunctional 10.4K/14.5K fusion protein; dl762 is deleted for 14.7K but expresses wild-type levels of 10.4K/14.5K, and dl799 is deleted for both 10.4K and 14.5K.

For production of anti-TRAIL-R1, -R2, and -R3 antibodies, a custom antibody production service was utilized (Eurogentec, Seraing, Belgium). Rabbits were immunized with TRAIL-R1:Fc, TRAIL-R2:Fc, and TRAIL-R3:Fc (Alexis Biochemicals, San Diego, CA). For antibody purification, the various TRAIL receptors were coupled to HighTrap NHS-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Fc-specific antibodies were first depleted by repeated passage over human IgG1-agarose (Sigma). TRAIL-R1, -R2, and -R3 specific antibodies were then purified on TRAIL-R1(R2 and R3)-Fc-Sepharose, eluted in 50 mM Tris-HCl, pH 2.7, neutralized with citrate NaOH, pH 9, and dialyzed against PBS. The anti-10.4K polyclonal antiserum was generated by immunizing rabbits with keyhole limpet hemocyanin-coupled peptide corresponding to amino acids 60–91 of Ad2-10.4K. Total IgG was purified from crude rabbit serum using protein G-Sepharose, eluted in 50 mM glycine, pH 3.0, neutralized with Tris, pH 8.0, and dialyzed against PBS. The anti-TRAIL-R4 goat polyclonal antibody was obtained from R & D Systems (Minneapolis, MN). FLAG-tagged FasL and TRAIL were from Alexis Biochemicals (San Diego, CA); the anti-EGF-R antibody (clone Ab-1) was from Calbiochem (Cambridge, MA); the anti-Fas antibody (clone DX2) was obtained from PharMingen (San Diego, CA), and the anti-FLAG and anti-VSV antibody were obtained from Sigma.

Plasmids

Plasmids Used in 293T Transfections—The pBluescriptII-Ad2E3 region plasmid (Ad2-E3) has been described previously (9) and was a generous gift from Hans-Gerhard Burgert (Max Von Pettenkofer-Institut, Munich, Germany). The E3-10.4K expression plasmid was generated by excising the Ad2 10.4K coding sequence from pMAM-10.4K (gift of W. Wold) with *SalI* and ligating the fragment into pcDNA3.1(–)

(Invitrogen, Carlsbad, CA) cut with *XhoI*. The E3-14.5K expression plasmid was generated by amplifying the 14.5K coding sequence from isolated Ad5 genomic DNA using *Pfu* polymerase (Stratagene, San Diego, CA) (primers, 5′-ggactatagctatctctc-3′ and 5′-cgggatccatccatcttagctatctag-3′), digesting with *BamHI* and *EcoRI*, and ligating the fragment into pcDNA3.1(–). FLAG-14.5K was generated by amplification of the mature 14.5K coding sequence from E3-14.5K (primers, 5′-acgtgaattcccgactccaagcctcaa-3′ and 5′-acgtgaattccatcagtcattctctctc-3′) with *Pfu* polymerase. The amplified PCR product was cut with *EcoRI* and ligated into PS497 (provided by F. Martinon and J. Tschopp), an engineered PCRIII (Invitrogen)-based vector containing the signal peptide from human IgG fused to an N-terminal FLAG epitope tag. VSV-6.7K was constructed by amplifying the Ad2 E3-6.7K coding sequence from isolated adenoviral genomic DNA (primers, 5′-acgtgaattcagcaatccaagtaactctacaagc-3′ and 5′-acgtgaattcttatctcttgatgtt-gcctcag-3′) using *Pfu* polymerase. The amplified PCR product was digested with *EcoRI* and ligated into PL507, an engineered PCRIII-based vector containing an N-terminal start codon fused to the VSV epitope tag. FLAG-6.7K was generated in exactly the same manner as VSV-6.7K; however, the *EcoRI*-digested PCR product was ligated into PL508, an engineered PCRIII-based vector containing an N-terminal start codon fused to the FLAG epitope tag.

The FLAG-tagged full-length TRAIL-R2 expression construct was generated by PCR amplification of the mature coding sequence of TRAIL-R2 (amino acids 52–440) from a PCRIII-TRAIL-R2 plasmid (16) with the addition of a flanking 5′ *BamHI*-FLAG sequence and a 3′ *PstI* site allowing subsequent ligation into a PCRIII-derived vector (Invitrogen, San Diego, CA) containing a signal peptide from the heavy chain of human IgG (PS089). The TRAIL-R2 Δ C16 construct, lacking the 16 C-terminal amino acids, was derived from the full-length FLAG-TRAIL-R2 construct by PCR amplification using the primers 5′-ctg-cagctagctcaacaagtggtc-3′ and T7. The resulting product was subcloned as a *BamHI*-*PstI* fragment in PS089. The TRAIL-R2 Δ DD deletion mutant (ALeu³⁴⁸-Ser⁴²⁴) was generated using a dual stage PCR approach. In the first PCR round, two overlapping fragments corresponding to the 5′ end and 3′ end of TRAIL-R2 devoid of the DD sequence were amplified using the primers 5′-caccaaattgtctctcagccc-3′ and T7 for the 5′ fragment and 5′-tttcagactctggaagttcag-3′ and sp6 for the 3′ end fragment. The two PCR products obtained were allowed to anneal and were re-amplified using sp6 and T7 to generate FLAG-TRAIL-R2 Δ DD, which was subcloned as a *BamHI*-*PstI* fragment in PS089. TRAIL-R2: GPI was obtained by sub-cloning the sequence of the 5 TAPE tandem repeats of human TRAIL-R3 (amino acids 157–259) as a *SalI*-*NotI* fragment in replacement of the human IgG1 Fc cassette of a TRAIL-R2:Fc construct (described in Ref. 16).

Retroviral Vector Plasmids—pBMN-10.4K/14.5K was constructed by amplification of the E3-10.4K/14.5K coding sequence from Ad2-E3 using *Pfu* polymerase (Stratagene) and the following primers: 5′-agacg-gatccgcatgattctctcagttcttata-3′ and 5′-tcgtaagcttcagtcattccactgt-caa-3′. The amplified product was digested with *BamHI* and *HindIII* and ligated into pBMN-LacZ (derived from pBABE series vectors, gift of Garry Nolan, Stanford University), and the resulting retroviral vector expresses both E3 proteins. The pBABE-6.7K plasmid was generated by amplification of the Ad2 E3-6.7K-coding sequence from isolated adenoviral genomic DNA using the following primers: 5′-atgagcaatccaagta-actc-3′ and 5′-tcactcttgatgttgc-3. The amplified product was ligated into the PCRII-Topo vector (Invitrogen). The E3-6.7K-coding sequence was then excised from PCRII-Topo with *EcoRI* and ligated into pBABE-puro (29) to generate pBABE-6.7K. pBABE-FLAG-6.7K was generated by digesting FLAG-6.7K with *SmaI* and *EcoRV* to excise the N-terminal FLAG-tagged 6.7K gene, and this fragment was ligated into *SnaBI*-digested pBABE-puro. The sequences of all constructs were verified unambiguously using an ABI Prism 310 genetic analyzer automatic sequencer (PerkinElmer Life Sciences).

Cell Death Assays

Cell viability was determined using an MTT-based assay as described previously (30) with the following modifications. For adenovirus infection, cells were infected at a m.o.i. of ~30 in 100- μ l volume; virus was allowed to preadsorb for 60 min, and then 100 μ l of media containing cytokine (FLAG-tagged FasL or TRAIL), 80 units/ml human interferon- γ , and 20 μ g/ml cytosine 1- β -D-arabinofuranosylcytosine (Ara-C) (Sigma) were added (13). One μ g/ml anti-FLAG M2 antibody was added to wells containing FasL (2 μ g/ml for TRAIL-containing wells) to enhance the activity of the FLAG-tagged cytokines. Fresh Ara-C was added to wells every 12–18 h, and cell viability was determined with MTT 48 h after infection. All cytokine concentrations were performed in

triplicate, and error bars represent standard deviations. Retrovirally transduced cells (5×10^3) were assayed for viability with MTT 72 h after addition of cytokines. Fold differences in sensitivity to TRAIL and FasL mediated killing were determined using IC_{50} concentrations of cytokine calculated from cell viability plots.

Retrovirus Production and Cell Transduction

Retroviral vectors were generated as described previously (31) based on the method of Soneoka *et al.* (32) and were pseudotyped with the vesicular stomatitis virus G protein (VSV-G). HT29.14S cells (1.5×10^5) were transduced multiple times in vector supernatant containing 8 μ g/ml Polybrene. The volume of vector supernatant was kept constant for all wells by the addition of growth medium. Retroviral vector titers were $\sim 1\text{--}2 \times 10^7$ colony-forming units/ml when determined on HT29.14S cells, and transduction efficiency was $>99\%$ as gauged by resistance to puromycin (pBABE-6.7K) or staining with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) (pBMN-LacZ virus made in parallel). Cells were harvested for analysis by FACS or plating into 96-well dishes for killing assays ~ 48 h after transduction.

Detection of Receptor Surface Levels by Flow Cytometry

HT29.14S and SAEC were infected with adenovirus at a m.o.i. ~ 30 , and cells were detached from plastic with 5 mM EDTA in PBS 18 h after infection. Cells were resuspended in FACS binding buffer (PBS + 2% fetal bovine serum + 0.02% sodium azide), and 1×10^5 cells were used for each staining. Cells were incubated in 50 μ l of FACS buffer plus appropriate antibodies (all antibodies used at 10 μ g/ml) for 1 h, at which time cells were washed and incubated in either goat anti-rabbit F(ab')₂ (for detection of TRAIL-R1, R2 or R3), goat anti-mouse F(ab')₂ (for detection of Fas and EGF-R), or biotinylated rabbit anti-goat IgG followed by streptavidin (for TRAIL-R4). All secondary detection reagents were conjugated to R-phycoerythrin and were from Southern Biotechnology Associates (Birmingham, AL). Cells were analyzed on a FACS-Calibur (Becton Dickinson, Mountain View, CA), and each histogram represents 5×10^3 cells gated on forward and side-angle light scatter. HT29.14S cells transduced with retroviral vectors were analyzed by the same methods. In all presented figures, dotted histograms represent either isotype control antibody (Fas and EGF-R) or goat anti-rabbit F(ab')₂ (TRAIL receptors) staining as a negative control.

293T-Fas and 293T cells were transfected by the CaPO₄ precipitation method as described previously (33). Two μ g of E3-10.4K and E3-14.5K expression plasmid (4 μ g of Ad2 E3 plasmid) were used to examine the down-regulation of endogenously expressed receptor in 293T-Fas cells (Fig. 4). For analysis of E3-6.7K surface expression (Fig. 6), 3 μ g of VSV-6.7K (or FLAG-6.7K), 1 μ g of E3-10.4K, and 1 μ g of E3-14.5K or 0.5 μ g of FLAG-14.5K were used. For analysis of TRAIL-R2 mutants (Fig. 7), 0.1 μ g of wild-type or mutant receptor plasmid was used plus or minus 3 μ g of Ad2-E3 plasmid. Total DNA concentration was always kept equivalent by addition of empty vector. Cells were detached from plastic 36 h after transfection for analysis by FACS as described above.

Confocal Microscopy

The subcellular distribution of down-modulated receptors was analyzed by confocal imaging of immunofluorescently labeled HeLa cells. HeLa cells (3×10^5) were infected with virus at a m.o.i. of 100 in permanox chamber slides (Nalge Nunc, Naperville, IL). Twelve hours after infection, the cells were washed with PBS and fixed in 4% formaldehyde. After washing with 0.2 M glycine, the cells were permeabilized with 0.1% saponin. The cells were then incubated in 10% donkey serum for 20 min prior to the addition of the primary antibodies. Fas was detected with murine anti-Fas (DX2) followed by incubation with goat anti-mouse Fab (Jackson ImmunoResearch) and donkey anti-goat fluorescein isothiocyanate-conjugated antibody (Jackson ImmunoResearch). TRAIL-R2 was detected with rabbit anti-TRAIL-R2 polyclonal antibody followed by donkey anti-rabbit fluorescein isothiocyanate (Jackson ImmunoResearch). The biotinylated anti-LAMP1 antibody (PharMingen, San Diego, CA) was used at a 1:8 dilution according to the manufacturer's instructions followed by detection with streptavidin-Texas Red. All primary antibodies were used at 20–25 μ g/ml and secondary detection reagents were used at 2 μ g/ml. The immunofluorescently labeled cells were analyzed with a Bio-Rad MRC 1000 (Emeryville, CA) confocal microscope using the 60 \times objective. Two-color Z-series were collected in the simultaneous mode, and the overlap of green and red fluorescence was depicted by a yellow signal. The Z-sections were then projected as stacks using the Lasersnap image processing software.

Co-immunoprecipitation of 10.4K, 14.5K, and 6.7K

293T cells were transfected in 10-cm dishes as described above using E3-10.4K (10 μ g/dish), FLAG-14.5K (5 μ g/dish), and VSV-6.7K (10 μ g/dish). Cells were lysed 36 h after transfection (lysis buffer: 1% Nonidet P-40, 50 mM HEPES, 150 mM NaCl, 20 mM EDTA, 500 μ M phenylmethylsulfonyl fluoride, and 0.018 units of aprotinin), and the lysates were pre-cleared with mouse IgG (5 μ g/ml) and protein G-Sepharose beads for 3 h at 4 $^{\circ}$ C. Pre-cleared lysates were then incubated with anti-FLAG (M2, Sigma, 5 μ g/ml) or anti-VSV (for analysis of total transfected VSV-6.7K) antibody and protein G beads overnight at 4 $^{\circ}$ C. Samples were then analyzed by SDS-PAGE on 18% Tris glycine gels and transferred to polyvinylidene difluoride membrane. Membranes were incubated with anti-VSV antibody (1:5000) followed by rabbit anti-mouse horseradish peroxidase-conjugated antibody. Protein (VSV-6.7K) was then visualized by enhanced chemiluminescence using the Super-Signal detection kit (Pierce). The membranes were then stripped and re-probed with anti-FLAG or rabbit polyclonal anti-10.4K antibody to visualize FLAG-14.5K and 10.4K, respectively.

RESULTS

Adenovirus Infection Results in Down-regulation of Cell Surface TRAIL-R1 and TRAIL-R2 via an E3-10.4K/14.5K-dependent Mechanism—HT29.14S cells, which have been shown previously to down-regulate cell surface Fas and EGF-R upon infection with adenovirus (8), were infected with either wild-type adenovirus (rec700) or viral deletion mutants lacking functional E3 region proteins as follows: 10.4K (dl752), 14.5K (dl759), 14.7K (dl762), or both 10.4K/14.5K (dl799). After infection, cells were analyzed by FACS for surface levels of TRAIL-R1, -R2, -R3, and -R4 as well as for Fas and EGF-R (Fig. 1a). Infection with wild-type virus resulted in significant down-regulation of TRAIL-R1, TRAIL-R2, Fas, and EGF-R, but surface levels of TRAIL-R3 and TRAIL-R4 were not down-regulated. TRAIL-R1 and TRAIL-R2 surface levels decreased 66 and 60%, respectively, after viral infection (based on peak mean fluorescence), as compared with a 96% decrease in the levels of Fas and 88% for EGF-R. Three viral deletion mutants (dl752, dl759, and dl799) were incapable of down-regulating any receptors analyzed, indicating that the E3-10.4K/14.5K complex was necessary for TRAIL receptor down-regulation as has been shown previously for both Fas and EGF-R. By contrast, infection with a virus deleted for E3-14.7K (14.7K is encoded by various polycistronic E3-transcripts which also encode 10.4K/14.5K (34)) was similar to the effect of wild-type virus, indicating the E3-14.7K protein is not required for TRAIL receptor down-regulation.

Although various studies have analyzed E3-mediated down-regulation of cell surface receptors, all these experiments have been performed using transformed human cell lines. To determine whether cell surface levels of TRAIL receptors decrease in cells that represent the normal target tissue for adenoviral infection *in vivo*, primary small airway epithelial cells (SAEC) were infected with wild-type virus or dl799 (Fig. 1b). Infection with wild-type virus resulted in TRAIL-R1 and TRAIL-R2 down-regulation, as well as Fas and EGF-R, from the cell surface. Notably, TRAIL receptor down-regulation in SAEC was significantly more pronounced than in HT29.14S cells. TRAIL-R1 was undetectable, and TRAILR2 was reduced by 93% of normal levels (Fig. 1b). TRAIL-R3 and TRAIL-R4 levels were unaffected by wild-type virus, and infection with dl799 caused no down-regulation of the receptors examined. These data indicate that modulation of TRAIL-R1 and TRAIL-R2 by adenovirus occurs in primary cells that are targeted during host infection. Similar results were seen in infected primary normal human bronchial epithelial cells, HeLa, 293T, and 293-HEK cells (data not shown).

Subcellular Localization of Fas and TRAIL Receptors in Infected Cells—The steady-state levels of TRAIL-R1 and TRAIL-R2 on the plasma membrane were reduced within 4 h

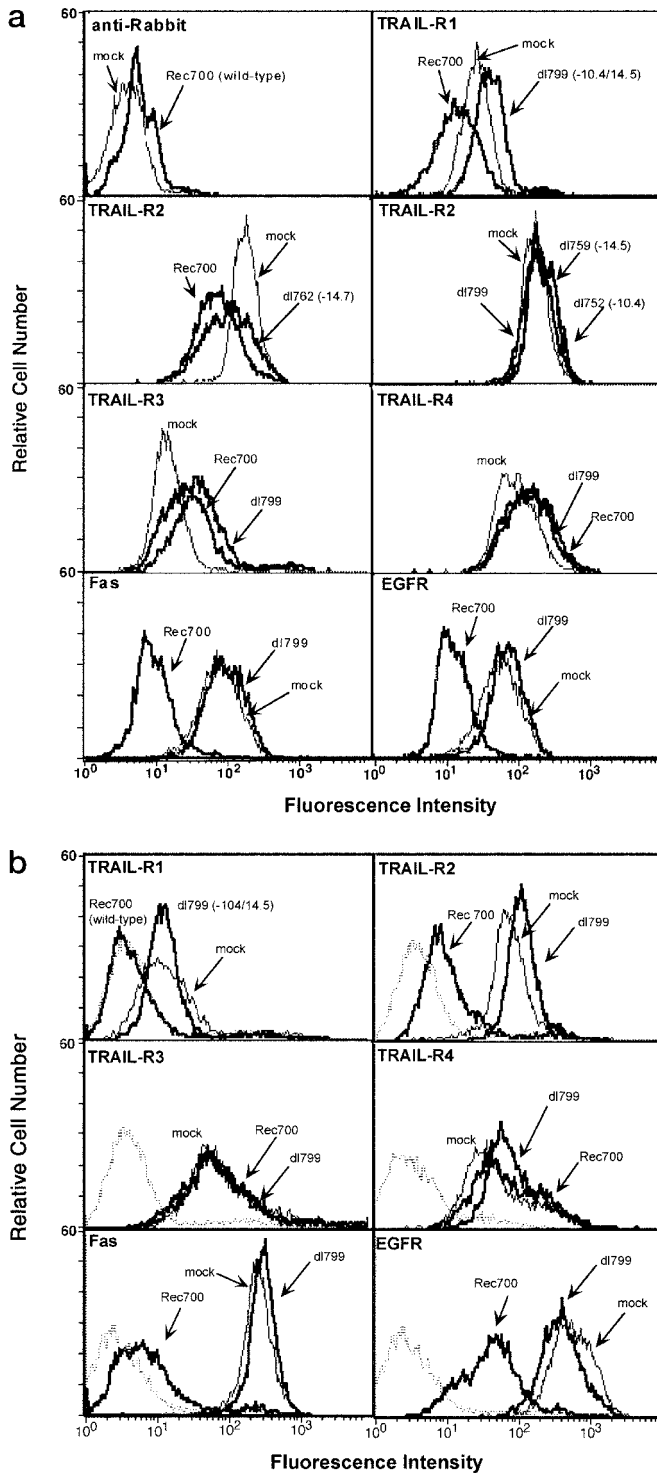


FIG. 1. Down-regulation of TRAIL-R1 and TRAIL-R2 by adenovirus requires E3-10.4K/14.5K. *a*, HT29.14S cells were infected with wild-type (rec700) adenovirus or viral mutants deleted for E3-14.7K (dl762), E3-10.4K (dl752), E3-14.5K (dl759), or 10.4K/14.5K (dl799). *b*, primary SAEC were infected with wild-type virus or dl799. Infected cells were analyzed for cell surface expression of TRAIL receptors, Fas and EGF-R, by flow cytometry.

after viral infection (data not shown), suggesting that the adenovirus E3 proteins stimulate TRAIL receptor internalization rather than interfering with their biosynthesis, similar to what has been shown for Fas (8, 10). The intracellular distribution of Fas and TRAIL-R2 was analyzed in HeLa cells infected with either wild-type virus or dl799. The infected cells were double-labeled with antibodies directed against Fas or TRAIL-R2 and

lysosome-associated membrane protein-1 (LAMP-1), a marker of late endosomes and lysosomes (35). Confocal imaging (Fig. 2) of cells infected with wild-type virus revealed that both Fas and TRAIL-R2 were abundant in perinuclear vesicles (Fig. 2, *a* and *g*). In contrast, both Fas and TRAIL-R2 were absent from these compartments in cells infected with dl799 (Fig. 2, *d* and *j*), and both Fas and TRAIL-R2 co-localized with LAMP-1 (Fig. 2, *c* and *i*). However, not all internalized Fas and TRAIL-R2 were present in LAMP-1-positive vesicles. Consistent with the presence of Fas and TRAIL-R2 in late endocytic compartments, these two proteins extensively co-localized in cells infected with wild-type virus (Fig. 2*o*) suggesting both receptors have a similar endocytic pathway.

The E3-10.4K/14.5K Proteins Are Not Sufficient for Down-regulation of TRAIL Receptors—To assess whether the E3-10.4K/14.5K proteins alone were sufficient for down-regulation of TRAIL-R1 and TRAIL-R2 from the cell surface, expression vectors were transfected into 293T cells that modestly overexpress Fas (293T-Fas), and receptor surface levels were examined by FACS (Fig. 3*a*). As expected co-transfection of E3-10.4K plus E3-14.5K expression vectors resulted in down-regulation of both Fas and EGF-R from the surface of 293T-Fas cells (Fig. 3*a*), whereas transfection of either vector alone did not affect Fas or EGF-R levels (data not shown). Surprisingly, transfection with both E3-10.4K and 14.5K did not alter the cell surface levels of TRAIL-R1 and TRAIL-R2 (Fig. 3*a*). This result indicated that an additional viral protein(s) acts in concert with the E3-10.4K/14.5K complex to down-regulate TRAIL receptors.

Treatment of adenovirus-infected HT29.14S cells with cytosine arabinoside (AraC), an inhibitor of viral DNA replication, did not inhibit down-regulation of TRAIL-R1 and TRAIL-R2 (data not shown), also suggesting that the additional viral protein(s) needed for TRAIL receptor down-modulation was expressed as an early gene. To test whether this gene was contained within the E3 region, a plasmid containing the entire Ad2 E3 locus (*EcoRV* C fragment) (9) was transfected into 293T-Fas cells, and the down-regulation of surface receptors was analyzed by FACS (Fig. 3*b*). Decreased levels of both TRAIL-R1 and TRAIL-R2, as well as Fas, were detected on the surface of cells transfected with the Ad2-E3 plasmid (Fig. 3*b*), indicating that the adenoviral protein(s) needed for down-regulation of TRAIL receptors, in addition to E3-10.4K/14.5K, was included in the E3 region.

The E3-6.7K Protein Is Required to Down-regulate TRAIL Receptors from the Cell Surface—The E3-6.7K protein was identified as a potential candidate for assisting in TRAIL receptor down-regulation by its structure as an integral membrane protein (24, 25); however, no function for the E3-6.7K protein has been previously identified. An E3-6.7K-expressing retroviral vector (pBABE-6.7K) was generated to test whether this protein might function in concert with E3-10.4K/14.5K in the down-regulation of TRAIL-R1 and TRAIL-R2. HT29.14S cells were transduced with either control retroviral vector expressing LacZ (Vector), vectors expressing E3-10.4K/14.5K (pBMN-10.4K/14.5K) and/or E3-6.7K (pBABE-6.7K). Cells transduced with pBMN-10.4K/14.5K or pBABE-6.7K were then compared with cells transduced with both vectors for surface receptor levels by flow cytometry (Fig. 4). Transduction of HT29.14S with pBMN-10.4K/14.5K resulted in Fas down-regulation as expected, and co-transduction with pBABE-6.7K did not show any additional effects on Fas surface levels (Fig. 4). The identical results were seen for EGF-R as well (data not shown). TRAIL-R1 was modestly down-regulated by transduction with pBMN-10.4K/14.5K alone (51% of normal levels), which was not seen in 293T cells, but co-transduction with pBABE-6.7K significantly increased TRAIL-R1 down-regula-

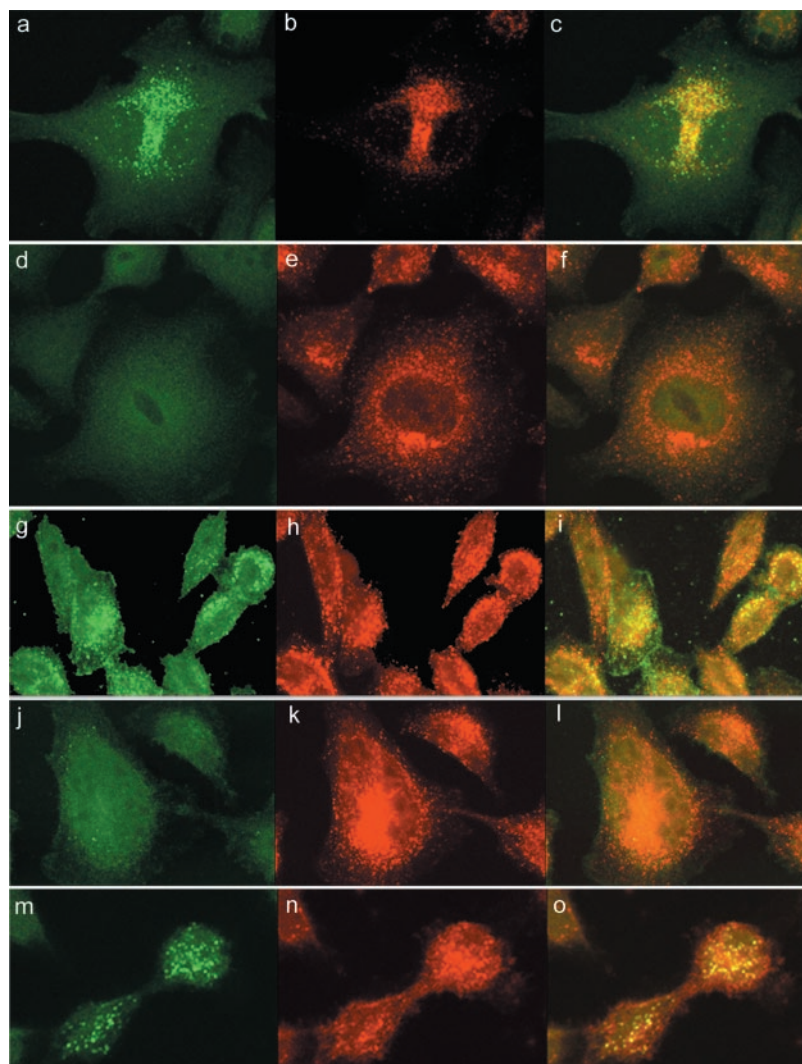


FIG. 2. Fas and TRAIL-R2 co-localize in intracellular vesicles after adenovirus infection. HeLa cells were infected with wild-type (rec700) or dl799(-10.4K/14.5K) virus and stained with antibodies against Fas, TRAIL-R2, and LAMP-1. *a–c*, *g–i*, and *m–o* represent cells infected with wild-type virus, and *d–f* and *j–l* represent cells infected with dl799. The images as follows: *a–f*, anti-Fas (green) and anti-LAMP-1 (red); *g–l*, anti-TRAIL-R2 (green) and anti-LAMP-1 (red); and *m–o*: anti-Fas (green) and anti-TRAIL-R2 (red). The last panel in each row is a merged image of the previous two panels where yellow indicates the co-localization of the two analyzed proteins.

tion (28% of normal levels). Significant TRAIL-R2 down-regulation was only observed when HT29.14S was co-transduced with pBMN-10.4K/14.5K and pBABE-6.7K (44% of normal levels). Neither TRAIL-R1 nor TRAIL-R2 surface levels were affected by transduction with pBABE-6.7K alone (Fig. 4). The surface levels of TRAIL-R3 and TRAIL-R4 were not affected by transduction with any combination of E3 protein-expressing vectors. These data indicate that the E3-6.7K protein is required for the down-modulation of the death domain-containing TRAIL receptors seen in adenovirus infection.

E3-6.7K Is Detectable on the Cell Surface and Forms a Complex with E3-10.4K/14.5K—The E3-10.4K and 14.5K proteins have previously been shown to exist as a heteromeric complex on the cell surface composed of one 14.5K monomer and two alternatively processed, disulfide-bridged forms of 10.4K (36, 37). E3-6.7K has been reported to reside in the endoplasmic reticulum, based upon the sensitivity of this protein to digestion by endoglycosaminidase H (25). Our functional analysis of E3-6.7K led us to hypothesize that the E3-6.7K protein might associate with the E3-10.4K/14.5K complex. Epitope-tagged versions of E3-14.5K (FLAG-14.5K) and E3-6.7K (FLAG-6.7K and VSV-6.7K) were generated to facilitate biochemical analysis of protein-protein interactions and cell surface expression. FLAG-14.5K and FLAG/VSV-6.7K were capable of down-regulating Fas and TRAIL-R equivalent to the untagged proteins when co-expressed with E3-10.4K, indicating that epitope tagging of these two E3 proteins did not disrupt their function (data not shown). 293T cells were transfected with various

combinations of the E3 protein expression vectors and were analyzed for protein cell surface expression by flow cytometry (Fig. 5, upper panel), or cell lysates were subjected to immunoprecipitation for analysis of protein complex formation (Fig. 5, lower panel). Flow cytometry analysis revealed that the E3-6.7K protein is expressed on the surface of transfected cells (Fig. 5, *a* and *b*) and that co-expression of E3-10.4K/14.5K does not affect the surface levels of E3-6.7K. Additionally, E3-6.7K cannot supplant the function of E3-10.4K in helping to transport E3-14.5K to the cell surface (see Fig. 5*d*). Western blot analysis of immunoprecipitated cell lysates revealed that E3-6.7K co-immunoprecipitates with E3-10.4K/14.5K. E3-6.7K also co-precipitated with E3-14.5K in the absence of E3-10.4K, suggesting a direct interaction between these two proteins. E3-6.7K did not immunoprecipitate with E3-10.4K in the absence of E3-14.5K (data not shown).

E3 Protein-mediated Down-regulation of TRAIL-R2 Is Dependent upon Sequences in the Cytoplasmic Tail of the Receptor—In an attempt to elucidate which sub-domains of TRAIL-R2 are required for down-regulation by E3-10.4K/14.5K/6.7K, we generated various mutants of the cytoplasmic domain of TRAIL-R2 that lack either the C-terminal 16 amino acids of the cytoplasmic tail (TRAIL-R2 Δ C16), the entire death domain (TRAIL-R2 Δ DD), or a chimeric TRAIL-R2/R3 receptor encoding the extracellular domain of TRAIL-R2 fused to the domain of TRAIL-R3 coding for the addition of the GPI link to the cell surface (TRAIL-R2:GPI). The mutant TRAIL-R2 expression plasmids were transfected into 293T cells either with

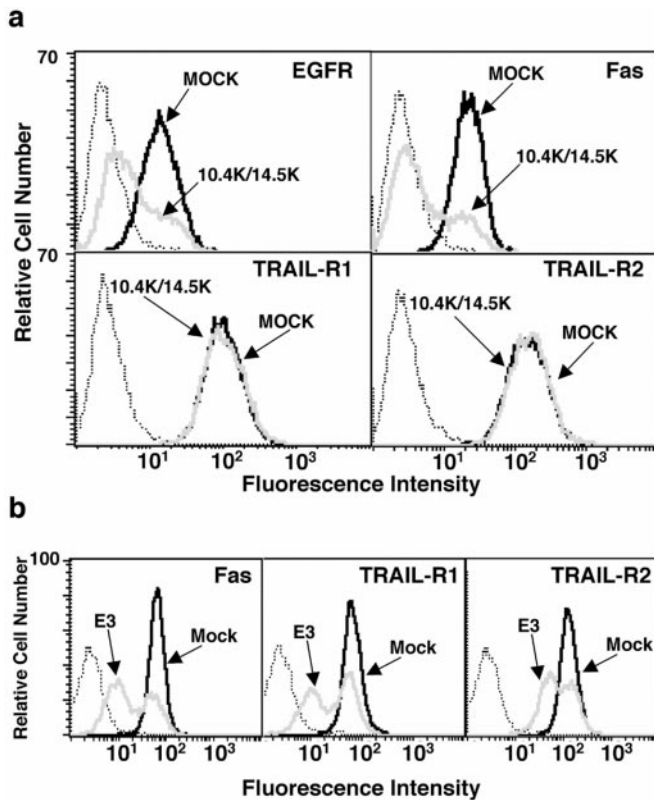


FIG. 3. E3-10.4K/14.5K is not sufficient for down-regulation of TRAIL-R1 and TRAIL-R2. *a*, 293T-Fas cells were co-transfected with vectors expressing E3-10.4K and E3-14.5K or were mock-transfected, and cell surface levels of TRAIL-R1, TRAIL-R2, Fas, and EGF-R were analyzed by flow cytometry. *b*, 293T-Fas cells were transfected with a vector containing the entire adenovirus E3 coding sequence (pBlue-script-Ad2E3) or were mock-transfected and subjected to flow cytometric analysis to determine receptor surface levels.

or without the Ad2-E3 plasmid, and cell surface receptor levels were analyzed by FACS (Fig. 6). Full-length TRAIL-R2 was included as a positive control for Ad2-E3 down-regulation. Interestingly, the cell surface levels of the TRAIL-R2 mutants were unaffected by co-expression of Ad2-E3 (Fig. 6), indicating that the cytoplasmic tail of TRAIL-R2 plays a critical role in the susceptibility of the receptor to down-regulation by E3 proteins. More specifically, the results seen with the TRAIL-R2 Δ C16 mutant indicate that the C-terminal 16 amino acids are required for sensitivity of TRAIL-R2 to down-regulation, and the determinant of specificity does not reside in the death domain.

Adenovirus E3 Proteins Are Required to Reduce the Sensitivity of Infected Cells to Apoptosis by TRAIL and FasL—To test whether the down-modulation of death receptors from the cell surface by E3-6.7K/10.4K/14.5K results in the desensitization to ligand-induced apoptosis, HT29.14S cells transduced with E3 protein-expressing retroviral vectors (Fig. 7*a*) or infected with wild-type and deletion mutant adenovirus (Fig. 7*b*) were treated with TRAIL and FasL. Cells transduced with pBMN-10.4K/14.5K plus pBABE-6.7K showed significant resistance to TRAIL killing when compared with cells transduced with vector alone (~8-fold) (Fig. 7*a*). Transduction with pBMN-10.4K/14.5K showed some protection against TRAIL killing (~3-fold) and is probably due to the limited down-regulation of TRAIL-R1 in HT29.14S cells expressing these two E3 proteins (see Fig. 4). Transduction with pBABE-6.7K alone showed no protection against TRAIL-mediated apoptosis. For comparison, transduced cells were also tested for their sensitivity to FasL. Consistent with the virtual complete down-regulation of Fas in

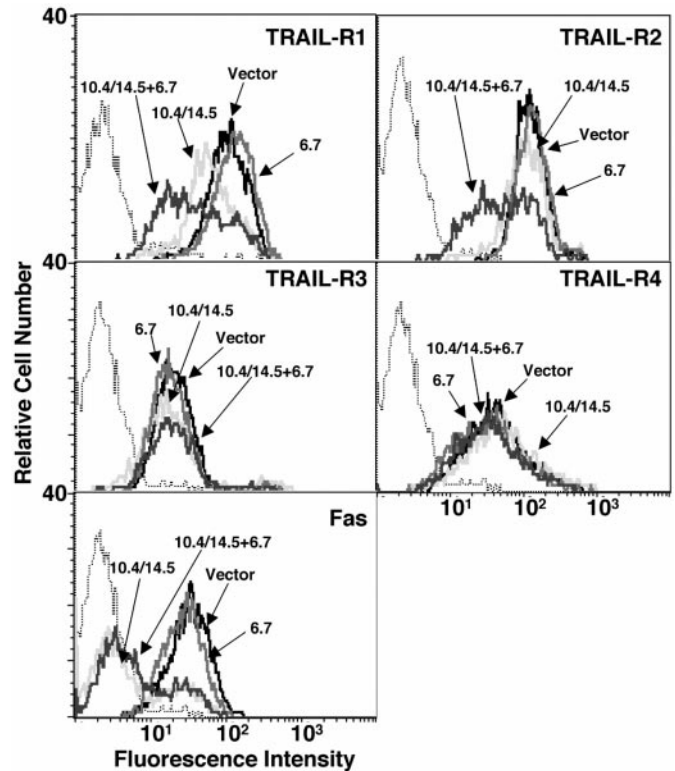


FIG. 4. E3-6.7K functions together with E3-10.4K/14.5K to down-regulate TRAIL-R1 and TRAIL-R2. HT29.14S cells were transduced with recombinant retroviral vectors expressing β -galactosidase (pBMN-LacZ, serves as "vector" negative control), E3-10.4K/14.5K (pBMN-10.4K/14.5K), and/or E3-6.7K (pBABE-6.7K) and were analyzed by flow cytometry to determine receptor cell surface levels.

HT29.14S (see Fig. 4), transduction with either pBMN-10.4K/14.5K or pBMN-10.4K/14.5K plus pBABE-6.7K rendered cells almost entirely resistant to killing by FasL.

Next, adenovirus-infected cells were tested for sensitivity to TRAIL and FasL-mediated apoptosis. HT29.14S cells infected with viral mutants lacking E3-10.4K/14.5K showed an ~6-fold increased sensitivity to killing by TRAIL when compared with cells infected with wild-type virus, virus deleted for 14.7K (dl762), or mock-infected cells (Fig. 7*b*). Similar data were seen when infected cells were analyzed for their sensitivity to FasL-mediated killing, although, analogous to the retroviral transduction experiments, desensitization to FasL (~20-fold) was more dramatic than TRAIL. As was observed for TRAIL, infection with deletion mutant viruses also increased the sensitivity of HT29.14S cells to FasL (~2–3-fold). Together, these data suggest that the E3 protein complex acts to counteract the increased sensitivity of cells to TRAIL and FasL that occurs during viral infection.

DISCUSSION

This report identifies the specific targeting of the proapoptotic receptors for TRAIL by adenovirus and is the first example of a viral defense strategy directed toward this group of death receptors. The E3-6.7K protein is required for the specific down-regulation of TRAIL receptors seen during adenoviral infection, providing an additional mechanism that potentially blocks the host innate and immune responses to viral infection. The adenoviral genome contains several distinct proteins that regulate cellular apoptosis including E1b-19K, E1b-55K, E3-14.7K, and E3-10.4K/14.5K. Each viral protein has evolved a different mechanism to modulate apoptotic signaling pathways. E1b-19K, a functional homologue of the human BCL-2 protein, is thought to interfere with apoptosis at the level of the

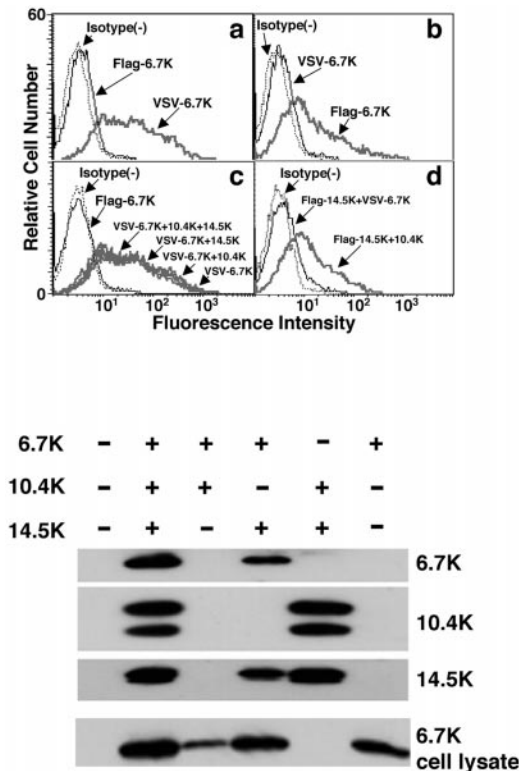


FIG. 5. E3-10.4K/14.5K/6.7K are expressed on the cell surface and form a trimolecular complex. Upper panel, 293T cells were transfected with E3 protein expression vectors (VSV-6.7K, FLAG-6.7K, E3-10.4K, E3-14.5K, and FLAG-14.5K) and were subjected to analysis by flow cytometry. Histograms *a* and *c* were samples incubated with anti-VSV antibody to detect the N-terminal epitope-tagged VSV-6.7K protein. *b* and *d* were samples incubated with anti-FLAG antibody to detect either FLAG-6.7K or FLAG-14.5K, respectively. Lower panel, 293T cells were transfected with various combinations of E3 protein expression vectors (FLAG-14.5K, VSV-6.7K, and E3-10.4K). +/- indicates the presence or absence of the designated expression vectors in the various transfections. Cell lysates were precipitated using anti-FLAG antibody followed by SDS-PAGE and Western blot. For Western blots of immunoprecipitated proteins, anti-FLAG antibody was used to detect FLAG-14.5K, anti-10.4K polyclonal antiserum for detection of the two isoforms of 10.4K, and anti-VSV for detection of VSV-6.7K. The bottom panel indicates the relative levels of VSV-6.7K protein present in the various transfections and was determined by immunoprecipitation of cell lysates (approximately half of that used for analysis of E3 complexes) with anti-VSV antibody followed by Western blot using anti-VSV.

mitochondria (reviewed in Ref. 38), whereas E1b-55K suppresses p53-mediated apoptosis (39). Both of these E1b proteins function to block cell death mediated through the intrinsic cellular apoptotic pathway (reviewed in Ref. 40). Contrary to the E1b proteins, the E3-encoded proteins appear to act on components of the extrinsic apoptotic pathways, which are initiated by signaling through the death domain-containing receptors of the TNFR superfamily. E3-14.7K is a cytosolic protein that has been shown to inhibit TNF-mediated apoptosis (41) by a still unknown mechanism, although recently 14.7K has been implicated in modulating the transcription factor NF- κ B through interactions with IKK γ /NEMO (42), which may induce protection against TNF-mediated death. The commitment of adenoviral genes dedicated to blocking apoptosis signaling by death receptors indicates an indispensable role for this pathway in immune control of adenovirus infection *in vivo*.

The E3-6.7K protein has the predicted structure of a type III integral membrane protein, a family of transmembrane proteins that encode no cleavable signal peptide but contain a signal anchor sequence for targeting and insertion into the membrane of the endoplasmic reticulum, a compartment that

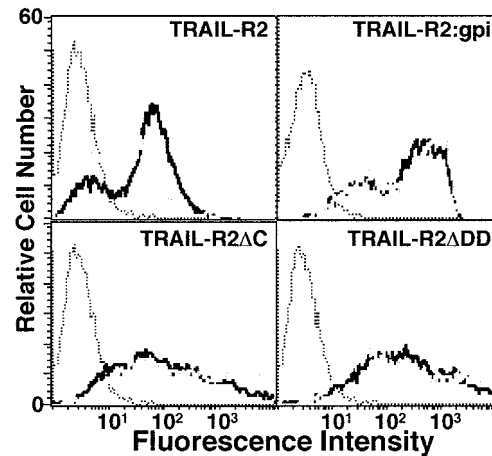
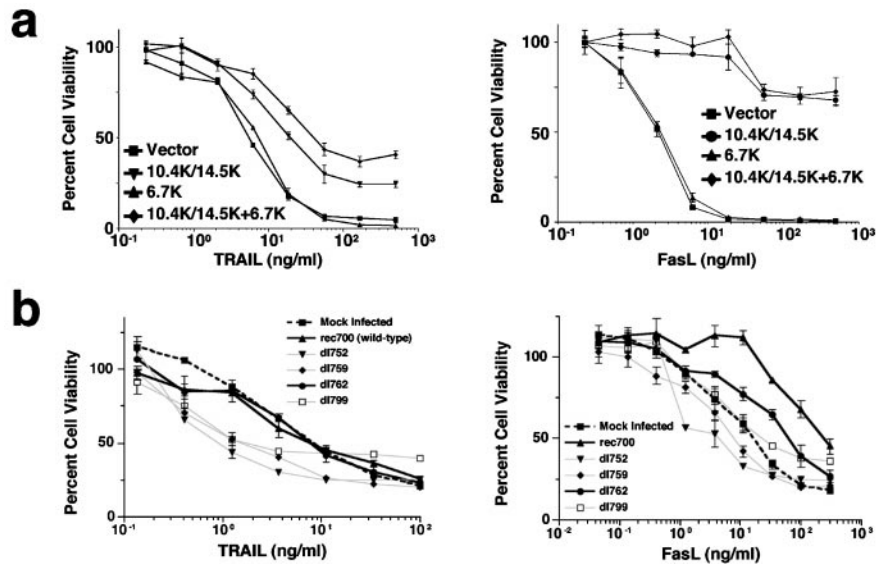


FIG. 6. TRAIL-R2 down-regulation by E3 proteins is dependent upon the cytoplasmic tail of the receptors. 293T cells were transfected with either wild-type TRAIL-R2 or various TRAIL-R2 mutants in the presence (gray histogram) or absence (black histogram) of the Ad2-E3 plasmid. All receptor constructs were detected using anti-FLAG antibody, except TRAIL-R2:GPI where anti-TRAIL-R2 antibody was used.

E3-6.7K has previously been reported to reside (25). In contrast, the E3-10.4K/14.5K proteins are type I membrane proteins that are thought to localize primarily to the plasma membrane (4), raising the question of where this trimolecular protein complex resides in the infected cell. Our data prove that the E3-6.7K protein can be expressed on the cell surface and interacts directly with the E3-10.4K/14.5K complex (see Fig. 6), suggesting that the trimolecular complex exists on the plasma membrane. E3-6.7K is highly expressed on the cell surface in the absence of E3-10.4K/14.5K, indicating there is no requirement for additional viral proteins to assist in its transport to the plasma membrane as is the case for E3-10.4K/14.5K (4). However, there are detectable levels of E3-10.4K and E3-14.5K in both the endoplasmic reticulum (where E3-6.7K has been described to be localized previously) and Golgi of adenovirus-infected cells (4), raising the possibility that the E3 complex may reside in multiple cellular compartments. We are currently examining the subcellular localization of the E3-10.4K/14.5K/6.7K complex to understand better the mechanism for TRAIL receptor down-regulation.

Although the E3-10.4K/14.5K complex was known to down-regulate both Fas and EGF-R prior to this study, little was known about the mechanism of action for these proteins. Various deletion mutants were generated in the cytoplasmic tail of TRAIL-R2 to analyze the role of this receptor domain in E3 protein-mediated down-regulation, and none of the mutants were susceptible to down-regulation. Notably, even TRAIL-R2 Δ C16, which deletes the last 16 amino acids of the receptor cytoplasmic tail, but still encodes the entire death domain (DD), was unable to be down-regulated by adenovirus. These data delineate that the DD, which is highly conserved between Fas and TRAIL receptors, is not the exclusive target of the E3 complex. Additionally, the E3-10.4K/14.5K/6.7K complex is unable to down-regulate the DD encoding receptors DR3/TRAMP and TNFR-1 from the surface of HT29.14S cells (data not shown), further highlighting the specificity of E3 proteins for Fas and TRAIL-R. The inability of the TRAIL-R2 Δ DD mutant to be down-regulated by the E3 complex suggests a positional requirement for the C-terminal 16 amino acids of TRAIL-R2 in determining sensitivity to E3-mediated down-regulation. Interestingly, all of the TRAIL-R2 mutants are expressed on the cell surface of transfected 293T cells at significantly higher levels than wild-type TRAIL-R2 (see Fig. 7). Since the total

FIG. 7. E3 proteins reduce the sensitivity of cells to FasL and TRAIL apoptosis. *a*, HT29.14S cells were infected with wild-type adenovirus (Rec700) or various E3 deletion mutants, and cells were tested for their sensitivity to TRAIL and FasL-mediated apoptosis using a MTT-based assay. Adenovirus mutants lacking E3-10.4K, 14.5K, or both proteins are indicated by the *gray lines*. *b*, HT29.14S cells were transduced with various retroviral vectors expressing E3 proteins and tested for sensitivity to FasL and TRAIL using an MTT assay.



protein levels of the TRAIL-R2 mutants were equivalent to that of wild-type receptor in 293T cells (data not shown), this result indicates that the overall steady-state distribution of the mutants is altered. A significant fraction of TRAIL-R2 is normally present in intracellular compartments (43), and our data suggest that manipulation of the TRAIL-R2 cytoplasmic tail can result in a redistribution of TRAIL-R2 to the cell surface, similar to what has been shown for the $LT\beta R$ (44). Our continuing efforts to dissect the role trafficking and subcellular localization of TRAIL-R1 and R2 in E3-mediated down-regulation should help to elucidate further the mechanism of action of these viral proteins.

Although the trimeric E3 protein complex clearly reduces the sensitivity of cells to TRAIL killing when expressed in *trans* (see Fig. 7*a*), the role of these proteins in desensitizing cells to TRAIL in the context of a viral infection appears a bit more complicated. As has been shown previously for FasL, infection of cells with viral mutants lacking E3-10.4K and 14.5K dramatically sensitizes them to TRAIL-induced apoptosis. Down-regulation of TRAIL-R1 and -R2 by the E3 complex counteracts this sensitivity, reducing it to the level of mock-infected cells. The desensitization (when compared with uninfected cells) of adenovirus-infected HT29.14S cells to FasL killing is likely to be due to the more pronounced down-regulation of Fas in this cell line when compared with TRAIL-R1 and -R2. Quite interestingly, down-regulation of TRAIL-R1 and -R2 is most dramatic in primary small airway epithelial cells (see Fig. 1*b*), suggesting that these cells would be largely resistant to TRAIL-mediated apoptosis if infected *in vivo*. However, because most primary cells are not killed by TRAIL in tissue culture models, we have been prohibited from assaying for TRAIL sensitivity in this cell line.

Based on our studies in HT29.14S cells, it appears that E3-6.7K is absolutely required for down-regulation of TRAIL-R2, but that E3-10.4K/14.5K can function alone to down-regulate TRAIL-R1, albeit at significantly reduced efficiency when compared with the trimeric E3 complex. If TRAIL is important in mediating innate immune responses against pathogens *in vivo*, then perhaps the TRAIL receptor family has co-evolved to counteract viral strategies directed at quelling this response, and this is a possible explanation for differences seen in E3-induced down-regulation of the two proapoptotic receptors for TRAIL. However, cell-specific issues regarding the extent of receptor down-regulation are likely to play a role as well, and this notion is supported by the observation that E3-10.4K/

14.5K complex alone is insufficient to down-regulate TRAIL-R1 in 293T cells in the absence of E3-6.7K (Fig. 3*a*).

TRAIL has been implicated in the killing of target cells by both CTL and NK cells (21–23). Cell lines that were insensitive to killing by FasL and TNF were susceptible to death mediated by TRAIL (23), suggesting that the TRAIL signaling pathway is independent of Fas and TNF. Thus, TRAIL may serve as an additional armament of CTL or NK cells in the initial defense mounted against viral infection (reviewed in Ref. 45). The E1A gene product of adenovirus sensitizes infected cells to NK cell killing (46, 47), further supporting the hypothesis discussed above that a role for the E3 complex may be to compensate for the increased sensitivity of infected cells to apoptosis induced by other viral genes. Taken together, these data indicate that the inhibition of TRAIL killing by E3-10.4K/14.5K/6.7K may be critical for adenovirus to evade several host cytotoxic effector mechanisms *in vivo*. The apoptotic strategies, together with the E3-gp19K gene that alters antigen presentation by blocking major histocompatibility complex transport to the cell surface, may underlie the success of adenovirus to maintain a persistent infection.

Acknowledgments—We thank Cheryl McLaughlin and Danielle Franco for assistance with the manuscript; Pascal Schneider and Patrizia Vinciguerra for providing TRAIL-R2 expression constructs; Hans-Gerhard Burgert for providing the Ad2-E3 vector; Davide Mauri for providing reagents; and Vincent Piguet and Doug Green for extremely helpful discussions about the manuscript.

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