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The Adenovirus E3-10.4K/14.5K Complex Mediates Loss of Cell Surface Fas (CD95) and Resistance to Fas-Induced Apoptosis†

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Cytotoxic T cells use Fas (CD95), a member of the tumor necrosis factor (TNF) receptor superfamily, to eliminate virus-infected cells by activation of the apoptotic pathway for cell death. The adenovirus E3 region encodes several proteins that modify immune defenses, including TNF-dependent cell death, which may allow this virus to establish a persistent infection. Here we show that, as an early event during infection, the adenovirus E3-10.4K/14.5K complex selectively induces loss of Fas surface expression and blocks Fas-induced apoptosis of virus-infected cells. Loss of surface Fas occurs within the first 4 h postinfection and is not due to decreased production of Fas protein. The decrease in surface Fas is distinct from the 10.4K/14.5K-mediated loss of the epidermal growth factor receptor on the same cells, because intracellular stores of Fas are not affected. Further, 10.4K/14.5K, which was previously shown to protect against TNF cytolysis, does not induce a loss of TNF receptor, indicating that this complex mediates more than one function to block host defense mechanisms. These results suggest yet another mechanism by which adenovirus modulates host cytotoxic responses that may contribute to persistent infection by human adenoviruses.

Fas (APO-1 or CD95) was originally described as a cell surface molecule that mediates apoptotic death of transformed human cell lines (53, 62). Molecular cloning of Fas revealed it to be a member of the tumor necrosis factor (TNF)-nerve growth factor receptor superfamily of cell surface proteins (55). Fas is constitutively expressed on a variety of epithelial cells and tissues (39, 55). Fas is also expressed on mature T cells (53), and activation of these cells further up-regulates its expression (31). Fas-mediated signal transduction begins when Fas ligand (FasL) or anti-Fas antibody binds to Fas and induces the cross-linking of Fas molecules (14). FasL is a member of the TNF family of membrane and secreted proteins (reviewed in reference 11). In contrast to that of Fas, FasL expression is limited to activated T cells (54), natural killer (NK) cells, and tissues of the "immune-privileged sites," such as the testis (48) and the eye (22).

In vitro models, which trigger cell death with the addition of monoclonal anti-Fas antibody (1), have delineated some mechanisms of Fas-FasL interactions for the regulation of cells in the immune system (for a review, see reference 41). FasL expression is induced on cytotoxic T cells after recognition of a target cell and engagement of the T-cell receptor/CD3 complex (1, 54). FasL engages with Fas on the target cell, triggering an apoptotic pathway and subsequent death of the target cell (42). $CD4^+$ cytotoxic T lymphocytes (CTL) utilize the Fas system as a major mechanism of cytotoxicity, while $CD8^+$ CTL utilize both Fas- and perforin-based mechanisms (30). The Fas-FasL system is also thought to play an important role in peripheral tolerance, since abrogation by genetic mutations, such as the mouse lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*) defects, leads to an autoimmune-like disease in mice (for a review, see reference 42).

Like many viruses, human adenoviruses encode a variety of gene products whose function appears to be suppression of host immune and inflammatory reactions (17). For example, the E3-gp19K product prevents translocation of major histocompatibility complex class I proteins from the endoplasmic reticulum to the cell surface, preventing adenovirus-specific CTL from recognizing adenovirus-infected target cells (44). Analysis of mouse cells infected with E3 deletion mutant viruses revealed that adenovirus has two redundant systems for protecting against TNF cytolysis: E3-14.7K (19), which acts autonomously (27), and the E3-10.4K and -14.5K gene products, which form a complex and function together (20, 52). The 55- to 60-kDa TNF receptor (TNFRI or p55), also known as type I, through which TNF cytotoxicity is triggered, shares a cytoplasmic "death domain" with Fas (49). In addition, similar (33), but not identical (60), downstream events following ligand binding have been described for Fas and TNFRI, suggesting these adenovirus gene products may block Fas-mediated cytolysis.

Here we investigate the ability of human adenovirus to modulate apoptosis induced by anti-Fas in two human carcinoma cell lines. The results indicate that the E3-10.4K/14.5K complex controls apoptosis by inducing a loss of Fas expressed at the cell surface. Further, E3-10.4K/14.5K does not alter intracellular levels of Fas or de novo synthesis of Fas, suggesting that this complex does not affect the synthesis of Fas. These results identify an additional mechanism employed by DNA viruses to evade host effector responses and to establish virus persistence.

MATERIALS AND METHODS

Cells and viruses. The HT-29.14S cell line (a gift from Jeff Browning, Biogen, Inc., Cambridge, Mass.) is a subclone from the human adenocarcinoma HT-29 cell line (American Type Culture Collection [ATCC], Rockville, Md.) and is

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sensitive to TNF, anti-Fas, and lymphotoxin α1β2 (LTα1β2) (6). HT-29.14S was maintained in RPMI (Gibco BRL, Grand Island, N.Y.) with 5% glucose supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan Utah). The ME180 human cervical carcinoma cell line (ATCC) was maintained in McCoy's medium with 5% glucose supplemented with 10% FCS (Hyclone). CL15.5 is a TNF-sensitive clone of a C3H-derived LMTK cell transfected with the entire simian virus 40 early region and expressing wild-type T antigen (3, 45). The C127 cell line (ATCC) is a clone derived from an RIII mouse mammary tumor. Both mouse cell lines were maintained in Dulbecco modified Eagle medium with 5% glucose supplemented with 10% FCS (Hyclone).

*rec*700 is an adenovirus type 5 (Ad5)-Ad2-Ad5 recombinant whose genome consists of the Ad5 *Eco*RI-A fragment (map position 0 to 76), the Ad2 *Eco*RI-D fragment (76 to 83), and the Ad5 *Eco*RI-B fragment (83 to 100) (59). Thus, *rec*700 expresses the Ad2 E3-10.4K gene product and the Ad5 E3-14.5K and -14.7K gene products (59). *rec*700 is the parent virus of all deletion mutants used in this study. *dl*752 has a 15-nucleotide deletion (nucleotides 2229 to 2243) within the 10.4K open reading frame which deletes five N-terminal amino acids (5), resulting in low-level production of a 10.4K mutant protein (25). *dl*753 has an internal deletion of 209 nucleotides (nucleotides 2229 to 2436) which deletes the entire 10.4K gene (5). This mutant does not produce the 10.4K protein (25, 51) but does produce small amounts (compared to wild-type levels) of the 14.5K protein (51). *dl*759 (nucleotides 2248 to 2803 deleted) is a deletion construct that deletes most of 14.5K and fuses the C-terminal 29 residues of 14.5K to the N terminus of the 10.4K protein, resulting in a $10.4K\text{-}14.5K_{104-132}$ fusion protein (36). *dl*799 (nucleotides 2229 to 2803 deleted) was constructed by building the E3 deletion of *dl*753 into the construct of *dl*764 (nucleotides 2243 to 2803 deleted) (18). Thus, neither 10.4K nor 14.5K is synthesized from this mutant (20), but E3-14.7K is still produced (20). *dl*762 (nucleotides 2904 to 3251 deleted) lacks only the E3-14.7K gene (4) but does produce wild-type levels of 10.4K and 14.5K (20). *dl*797 (nucleotides 2229 to 2436 and 2804 to 3002 deleted) lacks both the E3-10.4K and the E3-14.7K genes but does produce wild-type levels of 14.5K gene product (20). All adenovirus mutants mentioned above were generously provided by William S. M. Wold, St. Louis University, St. Louis, Mo. *dl*309 is an Ad5 mutant that lacks the E3B region, including the genes for the 10.4K, 14.5K, and 14.7K proteins (29).

Cytotoxicity assay. The protocol measuring sensitivity to anti-Fas cytolysis was adapted from a previous protocol measuring sensitivity to TNF cytolysis (19). Briefly, cell lines were infected with 10 to 40 PFU of virus per cell and incubated overnight with 200 μCi of $Na^{51}CrO_4$ (1,000 Ci/g) (New England Nuclear/Du-Pont, Boston, Mass.). Infected cells were added to standard 96-well microtiter plates (10^4 cells/well) which contained either medium alone (to determine spontaneous release) or increasing concentrations of anti-human Fas antibody (CH-11; Kamiya Biomedical Company, Tukwila, Wash.) and incubated for 18 h at 37°C in 5% CO_2. Supernatants were harvested and measured for the presence of radioactivity. The percent specific ^{51}Cr release was determined by the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The spontaneous release for all experiments was typically around 25% and no more than 40%. All determinations were done in triplicate and are presented as means ± standard errors of the means.

Infectivity was monitored by incubating fixed cells with the anti-E1A antibody M73 (46) (Oncogene Research Products, Cambridge, Mass.) and staining with the LSAB colorimetric assay (DAKO Corporation, Carpinteria, Calif.) according to directions given by the manufacturer.

FACS analysis. Cells were infected with 10 to 40 PFU of adenovirus, and at 12 to 15 h postinfection, cells were detached from the plate with 20 mM EDTA in phosphate-buffered saline (PBS), rinsed in binding buffer (PBS, 3% fetal bovine serum, 0.1% sodium azide), centrifuged, and resuspended to 10^7 cells/ml in binding buffer at 4°C. Cells were incubated with 10 μg of anti-human Fas antibody (UB2; Kamiya Biomedical Company), anti-LTβ receptor (anti-LTβR) antibody (BDA8; a gift from J. Browning, Biogen), or anti-epidermal growth factor receptor (anti-EGFR) antibody (Ab-1; Oncogene Sciences, Uniondale, N.Y.) per ml for 30 to 60 min at 4°C, rinsed in binding buffer, and then stained with 5 μg of phycoerythrin-labeled goat anti-murine immunoglobulin G (IgG) (Southern Biotechnology Associates, Inc., Birmingham, Ala.) per ml for 30 to 60 min at 4°C. Cells were washed in PBS and fixed in 1% paraformaldehyde. For measurement of TNFRI in mouse cell lines, cells were stained with a biotin-human recombinant TNF (biotin-hrTNF) conjugate, followed by avidin-fluorescein isothiocyanate (avidin-FITC), according to the instructions of the manufacturer (R&D Systems, Minneapolis, Minn.). Fluorescence-activated cell sorter (FACS) analysis was performed on a FACScan (Becton Dickinson, San Jose, Calif.) and analyzed with the LYSYS II (see Fig. 2) and CellQuest programs (Becton Dickinson).

Confocal microscopy. Cells were infected with 10 to 40 PFU of *rec*700 or *dl*799. At 12 h postinfection, intracellular Fas was detected by fixing cells in 4% paraformaldehyde for 10 min, washing in PBS, and then incubating in methanol for 6 min at −20°C. Fixed cells were incubated with 20% horse serum for 30 min, rinsed in PBS, incubated with the UB2 anti-human Fas antibody (10 μg/ml) for 2 h, washed in PBS, and incubated with goat anti-mouse IgG–FITC (Sigma, St. Louis, Mo.) for 1 h. Samples were washed with PBS and incubated with propidium iodide (Sigma) as a counterstain. To detect surface Fas, cells were incubated with anti-Fas (10 μg/ml) in PBS for 2 h, washed in PBS, fixed with 4% paraformaldehyde for 10 min, washed in PBS, and incubated with anti-mouse IgG–FITC for 1 h. Samples were washed with PBS and then incubated with propidium iodide (Sigma). All incubations were carried out at 4°C unless otherwise noted. As a negative control, cells were incubated with murine IgG (Sigma) instead of anti-Fas antibody. Samples were then analyzed by confocal microscopy (Zeiss model LSM 410 confocal microscope).

Immunoprecipitation. ME180 cells (10^7 cells per plate in 100-mm-diameter tissue culture dishes) were infected with 30 PFU of adenovirus (either *rec*700 or *dl*799) per cell at 37°C for 2 h. At 6 h postinfection, cells were labeled with 100 μCi of L-[^{35}S]cysteine (>1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) in cysteine-free medium at 37°C for 12 h. Cell lysates were prepared with 0.5 ml of cold single-detergent lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 100 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of aprotinin per ml, 1% Nonidet P-40) at 18 h postinfection. Immunoprecipitations were carried out by incubating the cell lysates with rabbit anti-human Fas antibody (APO-1; Calbiochem, Cambridge, Mass.) (2 μg/sample) at 4°C for 1 h. Protein A-Plus agarose (Calbiochem) was then added to the lysates and incubated at 4°C overnight. Proteins which had bound to the Protein A-Plus agarose were solubilized with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Equal amounts of radioactivity from each sample were separated by SDS–100% polyacrylamide gel electrophoresis. The gel was dried and exposed to Kodak X-OMAT X-ray film.

RESULTS

Adenovirus E3 proteins block anti-Fas cytolysis. The HT-29.14S cell line is killed following incubation with the monoclonal anti-Fas antibody CH-11 (62) (Fig. 1A). In contrast, HT-29.14S cells infected with wild-type adenovirus (*rec*700) are resistant to killing by anti-Fas antibody (Fig. 1A). Infection of this cell line with *dl*799, a double-deletion mutant which produces neither 10.4K nor 14.5K (20), abrogates resistance to anti-Fas killing; thus, infected cells die. Cells infected with *dl*759 (36), a mutant that lacks most of 14.5K (36), are also susceptible to anti-Fas cytolysis. The expression of this mutant protein may account for the partial protection from apoptosis. *dl*752 is a mutant virus that lacks five N-terminal amino acids of 10.4K and produces a small amount of the mutant protein (51). Cells infected with this virus are also susceptible to Fas-mediated cytolysis, albeit at lower levels than cells infected with *dl*799. Cells infected with a mutant lacking E3-14.7K (*dl*762) remain resistant. Thus, optimal protection from Fas-mediated apoptosis requires the expression of both 10.4K and 14.5K proteins but shows no requirement for 14.7K.

Another human cell line, ME180, is also killed by anti-Fas, although it is less sensitive than HT-29.14S (Fig. 1B). Infection of these cells with wild-type virus also protects them from anti-Fas cytolysis (Fig. 1B). In contrast to the HT-29.14S cells, ME180 cells infected with the *dl*799 double mutant become even more susceptible to anti-Fas lysis than uninfected cells (Fig. 1B). ME180 cells infected with an adenovirus mutant lacking both E3 and E1A do not exhibit this increased sensitivity to anti-Fas cytolysis (data not shown). Thus, it is likely that expression of adenovirus E1A increases susceptibility to Fas-induced apoptosis, similar to its ability to increase sensitivity to TNF (15). Cells infected with *dl*753, a mutant that lacks 10.4K and produces very low levels of 14.5K (51) (Fig. 1B), and cells infected with *dl*759, the mutant that does not produce 14.5K, are also sensitive to anti-Fas. ME180 cells infected with *dl*762 (14.7K deletion) are resistant to anti-Fas induced cytolysis. In this cell line as well, both 10.4K and 14.5K are required to prevent killing by anti-Fas antibody, while 14.7K expression has no effect.

E3-10.4K/14.5K induces loss of surface Fas from adenovirus-infected cells. Earlier studies indicated that the E3-10.4K/14.5K complex induces loss of EGFR from the cell surface (52). Deletion of either 10.4K or 14.5K abrogates EGFR down-regulation, suggesting that these two adenovirus proteins act as a complex (52). Because resistance to anti-Fas cytotoxicity maps to the E3-10.4K/14.5K complex, it was asked if these

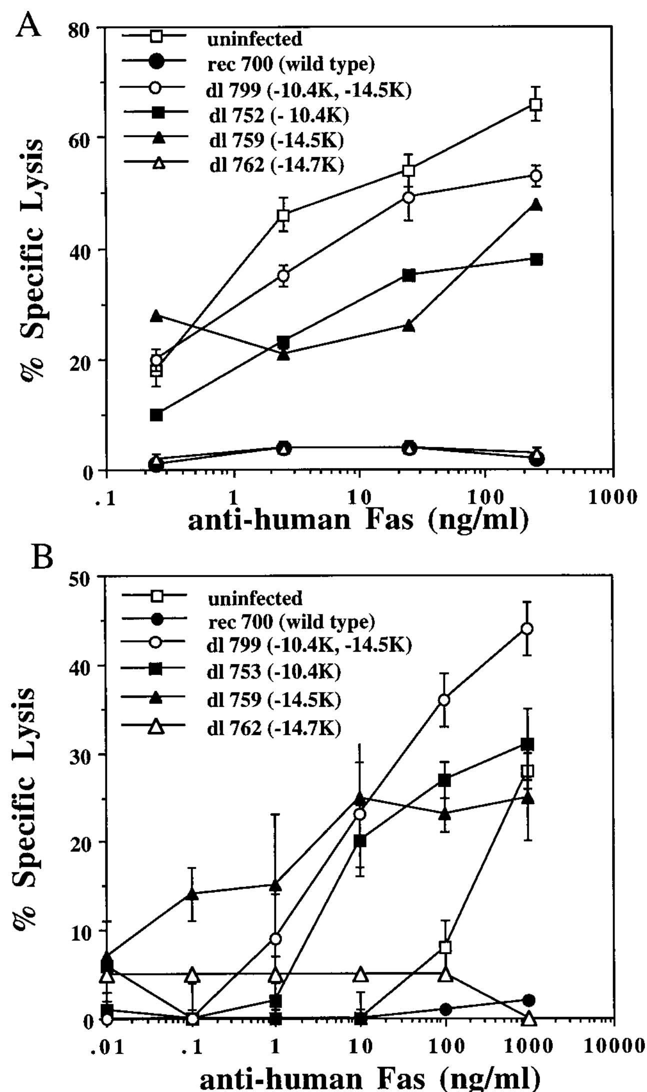

FIG. 1. The adenovirus E3-10.4K/14.5K heterodimer protects against anti-Fas induced cytolysis in the HT-29.14S (A) and the ME180 (B) human cell lines. Both cell lines were infected with adenovirus E3 mutants as indicated, and anti-Fas cytotoxicity was measured by a ^{51}Cr release assay as described in Materials and Methods.

viral proteins modulate cell surface levels of Fas. HT-29.14S cells were infected with wild-type or E3 mutant viruses as described above, and the infected cells were analyzed by flow cytometry for surface expression of Fas (Fig. 2B). Uninfected cells show the specific presence of Fas antigen on the cell surface, whereas Fas is undetectable on cells infected with wild-type adenovirus or with the 14.7K deletion mutant, *dl*762 (Fig. 2B). Cells infected with *dl*752, a mutant that lacks the five N-terminal residues of the 10.4K protein, also express lower levels of surface Fas, although not as low as cells infected with *rec*700 or *dl*762 (Fig. 2B). Compared to that in uninfected cells, Fas expression is not down-regulated in cells infected with *dl*759 (mutant E3-10.4K/14.5K fusion protein) or with *dl*799 (lacks 10.4K and 14.5K). Thus, optimal down-regulation of Fas requires expression of both 10.4K and 14.5K, with 14.7K expression not affecting Fas expression.

ME180 cells also express high levels of Fas antigen (Fig. 3). Infection of these cells with *rec*700 reduces the level of surface Fas expression to almost that of the negative control. Cells infected with *dl*799, which lacks both E3-10.4K and −14.5K, express surface Fas at the same levels as uninfected cells. Infection of cells with mutants *dl*753 and *dl*759, which lack E3-10.4K and E3-14.5K, respectively, also does not alter surface expression of Fas from that in uninfected cells. Infection of cells with *dl*762, a mutant that lacks E3-14.7K but expresses E3-10.4K/14.5K, also down-regulates Fas expression. In this cell line as well, both 10.4K and 14.5K are required to down-regulate surface Fas expression, while 14.7K has no effect on Fas.

The E3-10.4K/14.5K complex down-regulates EGFR but not TNFRI or LTβR. Virus-infected HT-29.14S cells were also stained for cell surface expression of EGFR (Fig. 2C). EGFR expression is decreased when cells are infected with wild-type adenovirus (*rec*700) or with the E3-14.7K deletion mutant *dl*762; however, loss of expression of EGFR is not as complete as it is for Fas. In contrast to that of Fas, EGFR expression is not down-regulated in cells infected with *dl*752 (having a small deletion in the 10.4K gene). Cells infected with *dl*759 (mutant E3-10.4K/14.5K fusion protein) or *dl*799 (lacks 10.4K and 14.5K) also do not show a down-regulation of EGFR expression. These results confirm the findings of Tollefson et al. (52) that both E3 proteins are required to down-regulate surface EGFR expression.

The ability of 10.4K/14.5K to induce loss of two other members of the TNF receptor superfamily, TNFRI and LTβR, was tested. Surface expression of LTβR is not affected when HT29-14.S cells are infected with wild-type or E3 mutant viruses (Fig. 2D). As mentioned above, the 10.4K/14.5K complex also protects infected mouse cells from lysis by TNF (20). To measure TNFRI levels in mouse cells, cells were incubated with a biotin-hrTNF conjugate that binds only to the murine TNFRI (21, 40). However, Fig. 4A shows that deletion of this complex does not alter TNFRI expression from that in wild-type-infected or uninfected CL15.5 mouse cells. TNFRI expression is also not altered in CL15.5 cells infected with *dl*762 (lacks E3-14.7K) or *dl*797 (lacks 10.4K and 14.7K). The C127 mouse cell line expresses very low levels of TNFRI, with TNFRI levels increasing after infection with either wild-type adenovirus (*rec*700) or *dl*797 (lacks 10.4K and 14.7K) (Fig. 4B). These results show that neither the 10.4K/14.5K complex nor 14.7K alters the TNFRI level as a mechanism for protection against TNF.

E3-10.4K/14.5K affects only surface-exposed Fas. There are several possible mechanisms by which E3-10.4K/14.5K expression leads to loss of surface Fas. E3-10.4K/14.5K may inhibit the production of Fas by blocking transcription or translation, it may prevent translocation of Fas to the cell surface, or it may remove Fas already present on the cell surface. Figure 5 shows that most Fas disappears from the surfaces of ME180 cells within 4 h of initial exposure to wild-type virus (*rec*700), with complete disappearance of surface Fas by 10 h postinfection. Infected ME180 cells were further analyzed for the presence of surface and intracellular Fas by confocal microscopy (Fig. 6). Corresponding with data from FACS analysis, uninfected cells express surface Fas (Fig. 6A), as do cells infected with *dl*799 (lacks 10.4K and 14.5K) (Fig. 6C), while *rec*700-infected cells lose surface Fas (Fig. 6B). In contrast, intracellular levels of Fas are not visibly decreased by infection with adenovirus with or without 10.4K/14.5K (Fig. 6D, E, and F). Thus, E3-10.4K/14.5K does not induce the loss of cytoplasmic Fas.

In addition, synthesis of Fas continues after infection with adenovirus. ME180 cells were infected, and newly synthesized proteins were labeled with [^{35}S]cysteine beginning at 6 h postinfection, at which point most Fas has already been lost

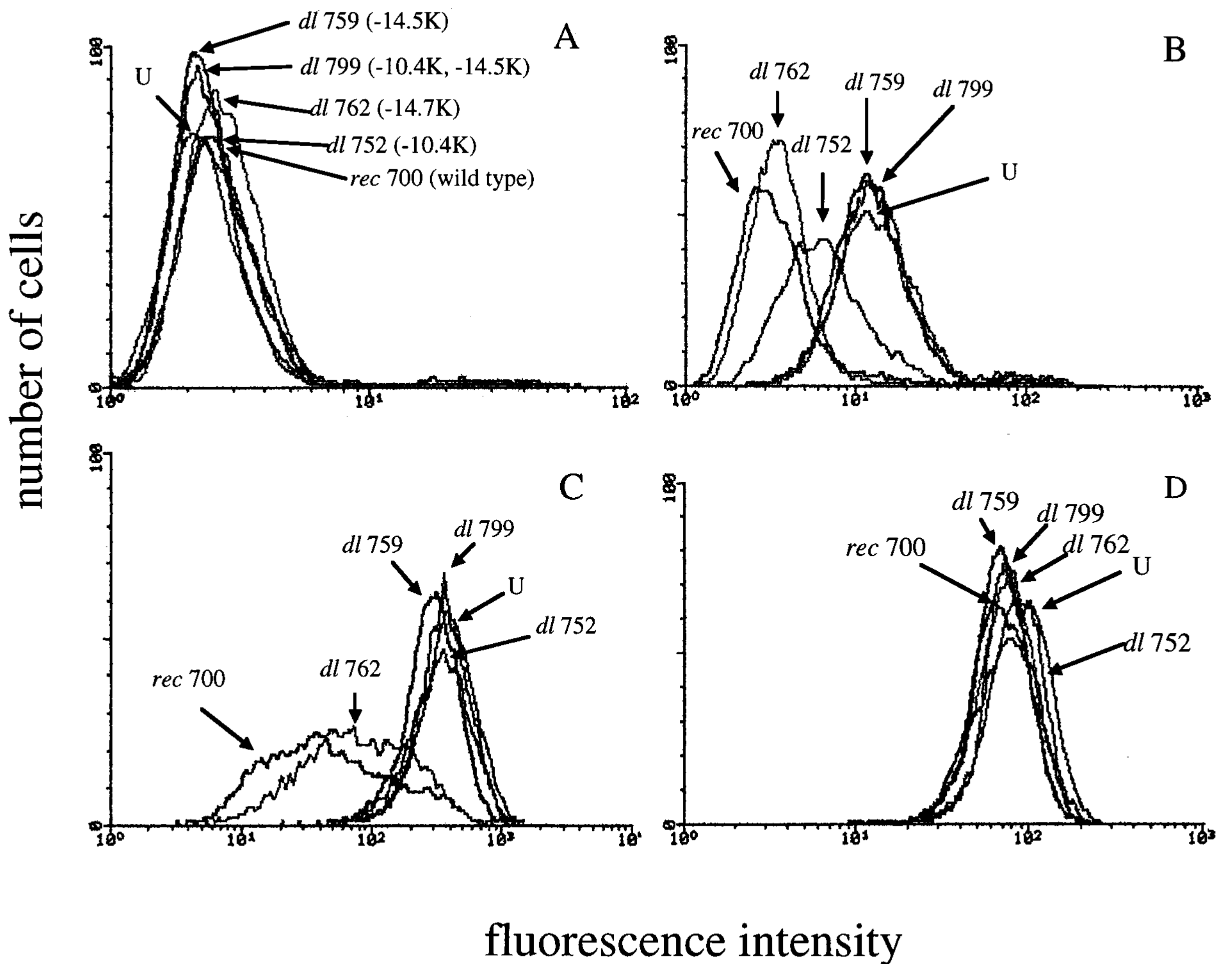

FIG. 2. Down-regulation of Fas by the adenovirus E3-10.4K/14.5K complex. HT-29.14S cells were infected with different adenovirus E3 mutants for 14 h and then stained with mouse monoclonal antibodies to Fas (B), EGFR (C), or LTβR (D). Nonspecific staining of cells infected with each mutant virus was assessed with normal mouse IgG (A). U, uninfected.

from the cell surface (Fig. 5). By using an anti-Fas antibody, newly synthesized Fas was immunoprecipitated from lysates of uninfected and infected cells. Cells infected with wild-type (*rec*700) virus continue to synthesize Fas, as is demonstrated by the presence of the 46-kDa band characteristic of Fas, at levels similar to those in uninfected cells (Fig. 7). These results confirm that adenovirus infection, and E3-10.4K/14.5K expression in particular, does not halt Fas synthesis. Cells infected with the 10.4K/14.5K deletion virus (*dl*799) also synthesize Fas (Fig. 7). While this particular experiment shows an apparent decrease in the amount of Fas in cells infected with *dl*799 compared to that in uninfected cells, other immunoprecipitations show that levels of Fas are the same in cells infected with wild-type and 10.4K/14.5K-deleted viruses (data not shown). Western blot analysis also confirms that steady-state levels of Fas are the same in cells infected with wild-type and 10.4K/14.5K-deleted viruses (data not shown).

DISCUSSION

The adenovirus E3-10.4K/14.5K complex protects against Fas-mediated cytolysis and alters Fas expression in two human carcinoma cell lines. The complete blockade of apoptosis and loss of surface Fas requires expression of both the 10.4K and

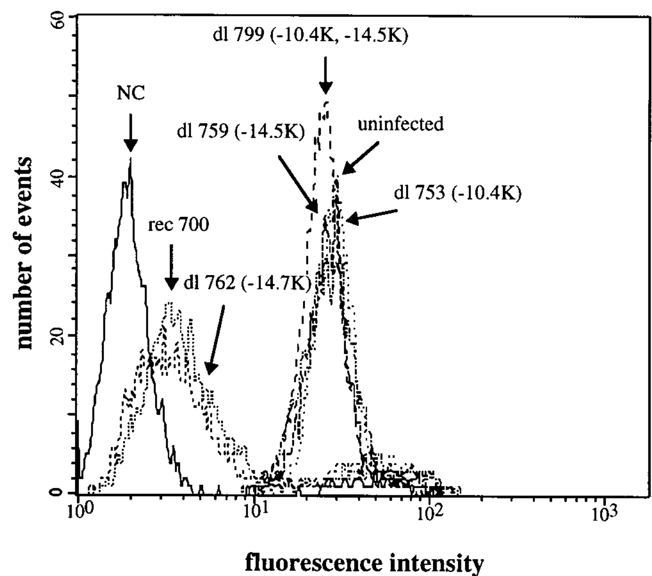

FIG. 3. Cell surface expression of Fas on the ME180 human cell line infected with a panel of adenovirus E3 mutants. Infected cells were stained with murine anti-human Fas or mouse IgG. NC, isotype-matched negative control.

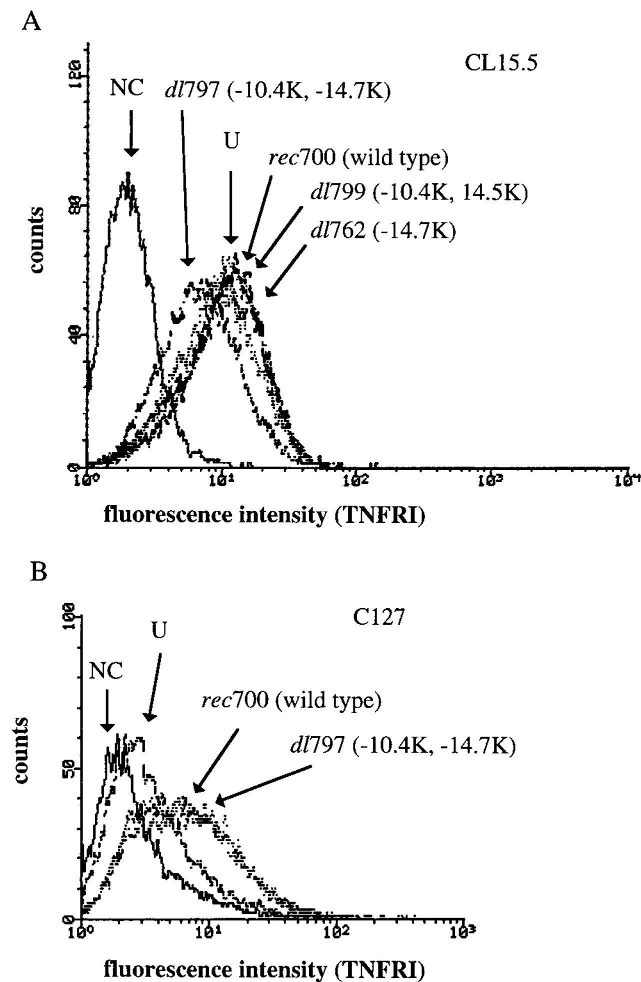

FIG. 4. Mouse TNFRI levels are not affected by expression of the E3-10.4K/14.5K complex. Infected CL15.5 cells (A) and C127 cells (B) were stained at 12 h postinfection with hrTNF-biotin, followed by FITC-avidin. NC, isotype-matched negative control; U, uninfected.

14.5K gene products as defined by a series of adenovirus mutants. In all adenovirus E3 mutants tested here, deletion or mutation of either the 10.4K or 14.5K gene dramatically affects both functions, indicating that a loss of receptor expression is associated with resistance to anti-Fas. Substantial biochemical evidence indicates that the 10.4K and 14.5K proteins function as a complex (20, 52) consisting of one 14.5K and two 10.4K molecules that localize in the plasma membrane (47). The 14.5K product orients as a type I transmembrane protein with O glycosylation of the extracellular domain and serine phosphorylation of the cytoplasmic domain (36, 37). The 10.4K protein forms a disulfide-linked dimer in which one subunit undergoes proteolysis at Ala 23 (34). The full complex consists of three nonidentical subunits with four membrane-spanning domains (47). The importance of the E3-10.4K and -14.5K gene products to virus survival is suggested by the high level of conservation of proteins in all major adenovirus groups (47).

Data presented here reveal that 10.4K/14.5K does not alter surface expression of two other members of the TNF receptor superfamily, TNFRI and LTβR. Fas, TNFRI, and LTβR have homology in their extracellular domains, but only Fas and TNFRI possess a death domain in their intracellular regions (6, 41). As previously shown, E3-10.4K/14.5K blocks TNF-mediated apoptosis activated by TNFRI in mouse cell lines (20). Thus, this complex's ability to also protect against Fas cytolysis is not unexpected, as Fas and TNFRI use common signaling proteins, i.e., FADD/MORT-1, to activate similar but not identical pathways for apoptosis (8, 60). However, E3-10.4K/14.5K does not cause loss of surface expression of TNFRI on murine cells, indicating a potential divergence in the mechanism(s) for protection against Fas-mediated cytolysis versus TNF-mediated cytolysis. TNFRI expression was analyzed with mouse cell lines, because in human cells TNF sensitivity is also inhibited by another viral protein, E1B-19K (18, 58). Thus a role for E3-10.4K/14.5K in inhibition of TNF cytolysis in human cells has not yet been definitively established.

E3-10.4K/14.5K has previously been shown to down-regulate EGFR (52), as well as other cellular receptors (38), from the surfaces of adenovirus-infected cells. However, down-regulation of receptors is selective, since the levels of other surface proteins, like LTβR (this report) and transferrin receptor (38), are not affected by adenovirus infection. For EGFR, 10.4K/14.5K induces a rapid loss of EGFR from both the surface and intracellular compartments (7, 24). E3-10.4K/14.5K causes constitutively internalized recycling EGFR molecules to accumulate in a prelysosomal compartment, where EGFR is degraded (24). In contrast, our results indicate that the intracellular levels of Fas are unaltered by 10.4K/14.5K at 12 h postinfection, at which point no detectable EGFR remains in either the cytoplasm or the plasma membrane (24, 25). Hence, E3-10.4K/14.5K possesses different mechanisms for altering Fas and EGFR expression. FACS data from HT-

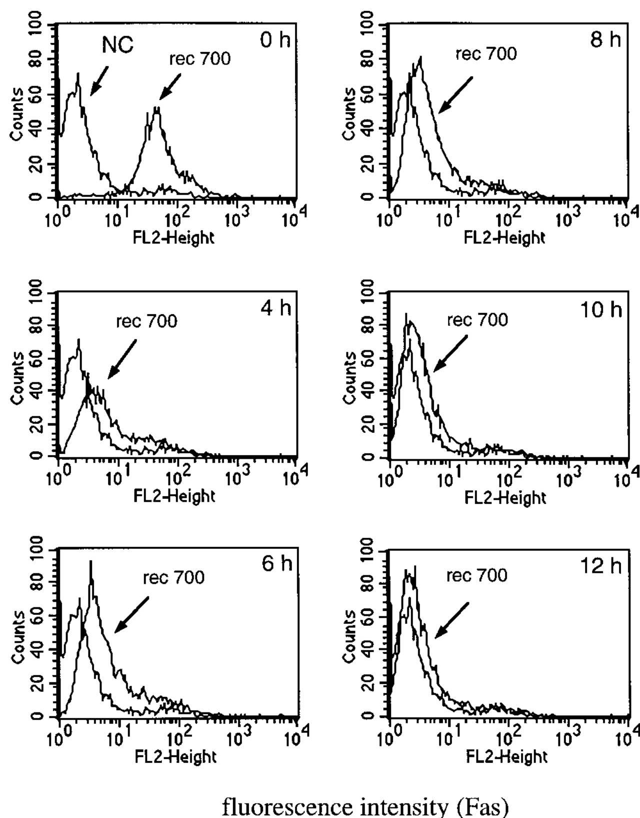

FIG. 5. Surface expression of Fas is down-regulated early during infection with adenovirus. ME180 cells were infected with wild-type adenovirus (*rec*700) and stained at the indicated times postinfection with either anti-Fas antibody or mouse IgG. NC, isotype-matched negative control.

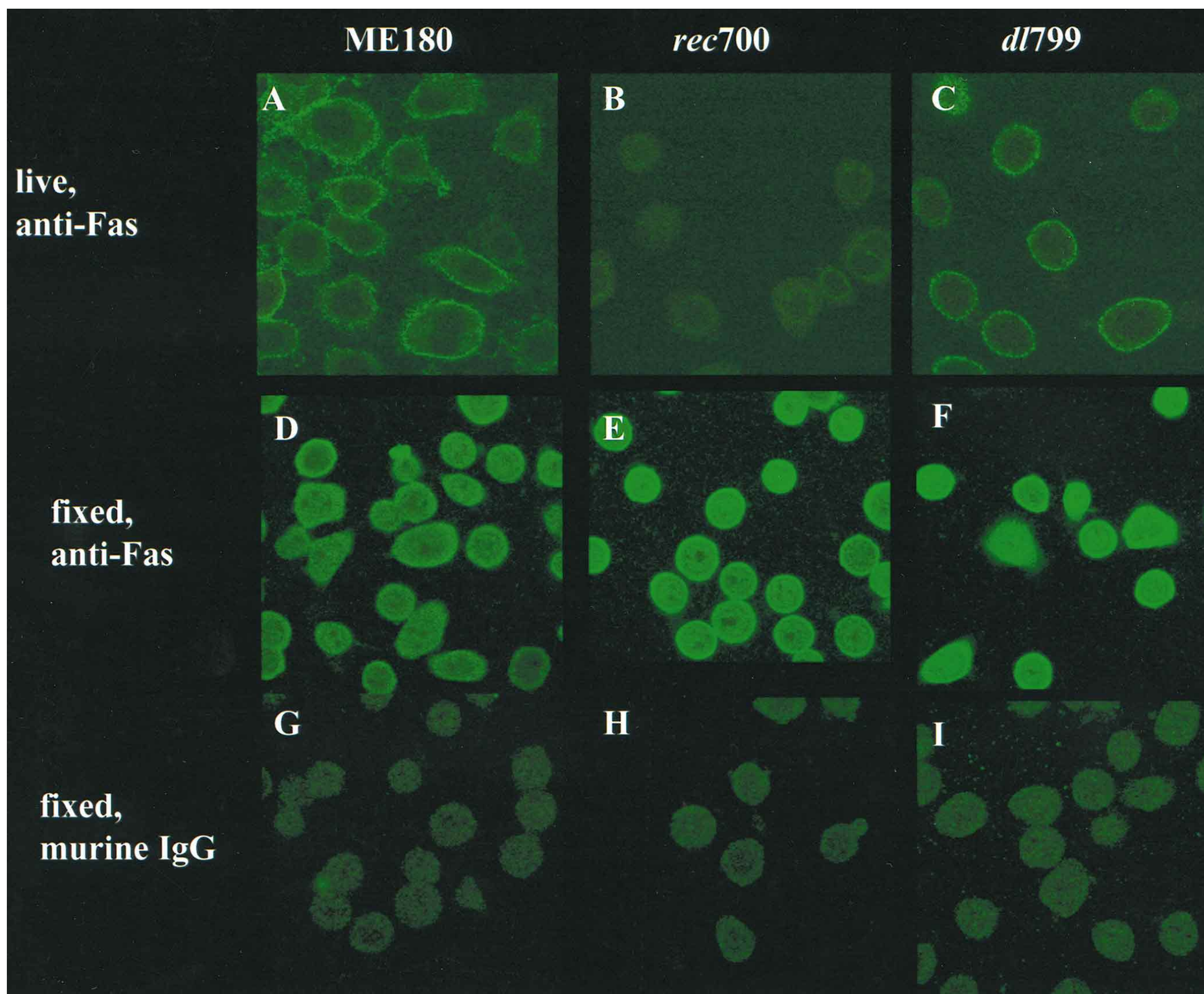

FIG. 6. Confocal microscopy of surface and intracellular Fas, showing that E3-10.4K/14.5K affects only surface Fas. ME180 cells were either uninfected (A, D, and G) or infected with wild-type (*rec*700) (B, E, and H) or E3-10.4K/14.5K mutant (*dl*799) (C, F, and I) adenovirus. At 12 h postinfection, cells were stained for surface Fas (A, B, and C) or cytoplasmic Fas (D, E, and F) as described in Materials and Methods. Fixed cells were stained with murine IgG as a negative control (G, H, and I).

29.14S cells infected with *dl*752 indicate that the mutant 10.4K-containing complex produced by this virus has no effect on EGFR expression but does produce partial down-regulation of Fas (Fig. 2B and C). This apparent inefficiency of EGFR down-regulation in comparison to that of Fas by *dl*752 may be due to a dosage effect, as EGFR expression is much greater than that of Fas (data shown here), or it may be due to distinct mechanisms regulating these two receptors.

Studies using E1A- or E1B-transfected human KB cells (an epithelial cell-like tumor cell line) have indicated that Ad2 E1B-19K prevents anti-Fas-mediated cytolysis (23). Clearly, in our studies E1B-19K is not preventing Fas lysis in HT-29.14S or ME180 cells, because E1B-19K-expressing *dl*799-infected cells are sensitive to anti-Fas killing. In addition to the HT-29.14S and ME180 cell lines described here, we have tested five other human cell lines and found that E1B-19K blocked Fas-mediated apoptosis in only two of those cell lines (one of which is the KB cell line) (data not shown). Thus, a total of two of seven human cell lines tested are protected from Fas-mediated killing by E1B-19K. The remaining five cell lines exhibit the same phenotype as HT-29.14S and ME180 cells; they are resistant only when both 10.4K and 14.5K are expressed. Virus mutants are not available to determine whether, in cell lines where E1B-19K prevents Fas-triggered cell death, 10.4K/14.5K is also protective.

E1B-19K is a member of the Bcl-2 family, whose members interact to regulate apoptosis (56). Similar to Bcl-2, E1B-19K binds to and neutralizes Bax and Bak function to block p53-induced, Bax-dependent apoptosis (9, 10). A second set of proteins, the ICE-related cysteine proteases, also regulate apoptosis, and it seems that Bcl-2 can block IL-1-converting enzyme (ICE)-like proteases by preventing the release of cytochrome *c* from mitochondria (32, 43, 61). Studies in the field of apoptosis indicate that there are myriad distinct signaling cascades that induce apoptosis. The cowpox virus protein CrmA and baculovirus protein p35 can each directly inhibit some members of the ICE-like protease family to block apoptosis induced by TNF (2, 50). However, only p35 can protect against

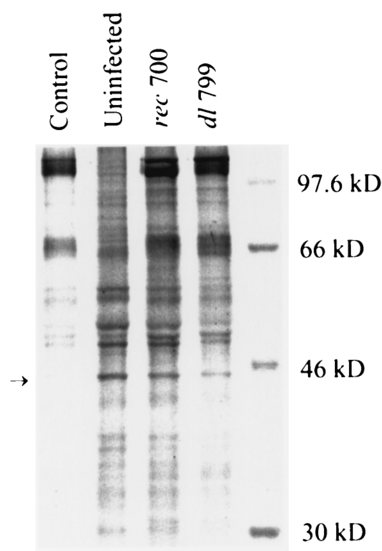

FIG. 7. E3-10.4K/14.5K does not inhibit Fas synthesis. [^{35}S]cysteine-labeled Fas was immunoprecipitated with anti-Fas antibody (APO-1) in cells that were uninfected or infected with wild-type (*rec*700) or E3-10.4K/14.5K deletion (*dl*799) virus. Fas is not immunoprecipitated in the lysates of *dl*799-infected cells precleared with murine IgM (control). Arrow, location of Fas band.

ionizing radiation, suggesting unique signaling cascades for radiation-versus TNF-induced cell death (13). Given that the cell possesses multiple pathways for inducing apoptosis, it is not surprising that adenovirus possesses products that target more than one of these pathways. E3-10.4K/14.5K, which localizes to the plasma membrane (34, 35), probably protects against Fas in a manner distinct from that of E1B-19K, which is found in the nuclear envelope (57). Further, while E3-10.4K/14.5K and E3-14.7K can each protect against TNF cytolysis (19, 20), only E3-10.4K/14.5K protects against Fas. These examples demonstrate the multiple mechanisms utilized by adenovirus to protect against a wide range of signaling cascades. Thus, it is beneficial for adenovirus to possess several distinct proteins that act on different apoptotic cascades to protect itself from cell death.

The results reported here raise the question of what benefit it is to this virus to alter Fas expression and signaling. Epithelial cells (39) and activated T and B cells (53), cell populations which adenovirus infects (26), express Fas. $CD4^+$ and $CD8^+$ CTL express FasL after recognition of specific antigen (30). Thus, removal of Fas from the host cell surface diminishes or eliminates the FasL-based mechanism that cytotoxic T cells normally employ to lyse virus-infected cells (42). It is possible that adenovirus-infected cells down-regulate Fas to avoid cell death triggered by cytotoxic cells and, hence, enhance virus survival. It seems likely that the effect of the E3-10.4K/14.5K complex on the CTL effector arm acts as an additional barrier along with the E3-gp19K protein, which binds MHC molecules to block antigen recognition by CTL (44).

It is tempting to speculate that the E3-10.4K/14.5K complex may also influence immune regulation. Mice bearing the *lpr* mutation, resulting from disruption of the Fas gene, phenotypically show a lymphoproliferative disorder, due to the inability of the mouse to eliminate self-reactive lymphocytes (42). Persistent infection of autoreactive T cells with adenovirus could alter their capacity to be eliminated via Fas. Thus, the continued presence of Fas-negative T lymphocytes which react inappropriately to self could in turn cause or contribute to the development of autoimmune disorders. This concept is supported by evidence showing that adenovirus type C viral DNA is found persistently in lymphoid tissues (28), although virus DNA is completely cleared from respiratory tissues (16) following the characteristic acute respiratory tract infections associated with adenovirus (26). To date, data linking adenovirus infection with the development of autoimmune diseases have been largely correlative. One such study found that patients with multiple sclerosis have antiadenovirus antibody titers higher than those of normal patients, suggesting a correlation between the presence of adenovirus and development of this disease (12).

This is the first report describing a specific viral gene product which alters surface Fas expression to prevent host cell death. These findings provide a plausible mechanism of how adenovirus, as well as other viruses, may persist in the host, which may eventually lead to late disease processes.

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