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Poliovirus Protein 3A Inhibits Tumor Necrosis Factor (TNF)-Induced Apoptosis by Eliminating the TNF Receptor from the Cell Surface

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Viral infections often trigger host defensive reactions by activating intrinsic (intracellular) and extrinsic (receptor-mediated) apoptotic pathways. Poliovirus is known to encode an antiapoptotic function(s) suppressing the intrinsic pathway. Here, the effect of poliovirus nonstructural proteins on cell sensitivity to tumor necrosis factor (TNF)-induced (i.e., receptor-mediated) apoptosis was studied. This sensitivity is dramatically enhanced by the viral proteinase 2A, due, most likely, to inhibition of cellular translation. On the other hand, cells expressing poliovirus noncapsid proteins 3A and 2B exhibit strong TNF resistance. Expression of 3A neutralizes the proapoptotic activity of 2A and results in a specific suppression of TNF signaling, including the lack of activation of NF-κB, due to elimination of the TNF receptor from the cell surface. In agreement with this, poliovirus infection results in a dramatic decrease in TNF receptor abundance on the surfaces of infected cells as early as 4 h postinfection. Poliovirus proteins that confer resistance to TNF interfere with endoplasmic reticulum-Golgi protein trafficking, and their effect on TNF signaling can be imitated by brefeldin A, suggesting that the mechanism of poliovirus-mediated resistance to TNF is a result of aberrant TNF receptor trafficking.

Viral infections often trigger a defensive apoptotic response, which may interfere with productive infection (44). Two major classes of apoptotic pathways, intrinsic (caused by metabolic disturbances) and extrinsic (receptor mediated), may be activated by the infection. Therefore, many viruses have developed a variety of mechanisms of apoptosis suppression by expressing proteins that inhibit different types of programmed cell death (44, 58, 64). Identification of novel viral antiapoptotic genes and elucidation of the mechanism of their activity are likely to lead to the discovery of new critical points in cell death regulation. On the other hand, understanding of viral counterdefensive tactics should help in designing new tools for the control of viral infections.

Most of our knowledge about viral antiapoptotic genes is based on analysis of DNA-containing viruses with relatively long life cycles, many of which contain more than one gene with apoptosis suppressor functions (adenoviruses, papovaviruses, herpesviruses, and baculoviruses) (12, 24, 44, 58, 64). Whether apoptosis suppressor genes exist in the genomes of small RNA-containing viruses with a fast replication cycle remains obscure. Among RNA viruses, an antiapoptotic protein has been identified only in hepatitis C virus (38, 42). It is unclear to what extent small RNA viruses with short replication cycles really depend on apoptosis suppression.

Poliovirus infection induces an apoptotic response only in some cells or under certain conditions (1, 2, 4, 37, 56). This response is a reaction to the damaging effects of viral proteinases 2A (29) and 3C (7) and possibly some other virus-encoded proteins. Activation of this intrinsic apoptotic pathway may, however, be prevented or interrupted by expression of a not-yet-identified viral antiapoptotic function(s) (1, 56). This viral function also suppresses apoptosis induced by such genotoxic agents as cycloheximide (CHI) and actinomycin D (56).

Here we address the possible role of the receptor-mediated apoptotic pathway in determining the fate of poliovirus-infected cells. One of the early events in poliovirus replication, as well as in the replication of many other picornaviruses, is severe suppression of host cap-dependent translation (25, 40, 50) caused by the cleavage of eukaryotic initiation factor 4GI (eIF4-GI) and eIF4-GII by viral proteinases (in the case of poliovirus, by protein 2A). Inhibition of translation, besides directly triggering rapid apoptosis in some cell types, is known to sensitize cells to tumor necrosis factor (TNF), a major inflammatory cytokine presented and secreted primarily by activated macrophages and T lymphocytes (27, 61). TNF is thought to suppress infections by a variety of microorganisms (57). In cell culture, TNF inhibits replication of various DNA or RNA viruses (17, 31, 41). The antiviral activity of TNF often correlates with its ability to induce apoptosis initiated by a signal from the death domain of TNF receptors (52, 65).

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Most cells are resistant to TNF under normal growth conditions. The mechanism of resistance may involve TNF-mediated activation of NF-κB translocation to the nucleus and transactivation of a set of NF-κB-responsive genes determining an antiapoptotic effect (8). Suppression of the NF-κB response by inhibitors of transcription or translation sensitizes many cell types to TNF. Inhibition of host translation by poliovirus 2A proteinase might be expected to enhance the sensitivity of the infected cells to TNF, which could potentially interfere with the viral infection. As shown here, this is indeed the case. However, we also demonstrate that poliovirus possesses a mechanism which suppresses cell sensitivity to TNF, ensuring efficient viral replication. We found that poliovirus noncapsid proteins 3A and 2B inhibit TNF-mediated apoptosis and that, at least in the case of 3A, this is accomplished by eliminating TNF receptors from the plasma membrane, presumably by affecting its intracellular trafficking. This represents a new mechanism of RNA virus-mediated suppression of apoptosis.

MATERIALS AND METHODS

Cell culture, DNA transfection, and viral infection. NIH 3T3, HeLa, 293, and Ecopack (Clontech) cell lines were cultured in Dulbecco modified Eagle medium (Gibco BRL) supplemented with 10% fetal calf serum (Life Technologies Inc.), 2 mM L-glutamine, and penicillin-streptomycin (100 U/ml; Gibco BRL). Transfections of NIH 3T3, HeLa, and Ecopack cells were conducted with the calcium phosphate transfection kit (Gibco BRL) and the Lipofectamine Plus reagent (Gibco BRL) according to the provider's protocols. The efficiency of transfection was estimated by cotransfection with the green fluorescent protein (GFP) expression vector and ranged between 10 and 20%. The efficiency of infection was calculated by coinfection with the GFP-expressing retroviral vector and by resistance of infected cells to G418. The efficiency of retrovirus infection was between 50 and 70%. Polybrene (4 μg/ml) (Sigma) was added to the medium during retrovirus infection. Poliovirus infection of HeLa cells was carried out as previously described (56).

Plasmids and vectors. For expression of poliovirus proteins 2A, 2B, 2BC, 2C, 3A, 3AB, and 3C, the retroviral expression vector LXSN (G418 resistant) was used. All the DNA segments encoding poliovirus proteins were prepared by PCR using a full-length type 1 poliovirus cDNA as the template. The identity of all PCR products with the original poliovirus genomic fragments and the absence of mutations were determined by sequencing. Each coding segment was provided with a Kozak sequence (CCACCATGG) at the 5′ end and a termination codon (TAG and TGA) at the 3′ end (lowercase letters). The following sense (s) and antisense (a) primers were used for the amplification of poliovirus genome fragments by PCR: 2A(s) (5′-ccaccatgGGATTCGGACACCAA-3′) and 2A(a) (5′-tcactaTTGTTCCATGGCTTC-3′); 2B(s) (5′-ccaccatgGGCATCACCAATTAC-3′) and 2B(a) (5′-tcactaTTGCTTGATGACATA-3′); 2BC(s) (5′-ccaccatgGGCATCACCAATTAC-3′) and 2BC(a) (5′-tcactaTTGAAAGAAAGCCTC-3′); 2C(s) (5′-ccaccatgGGTGACAGTTGGTTG-3′) and 2C(a) (5′-tcactaTTGAAAGAAAGCCTC-3′); 3A(s) (5′-ccaccatgGGCCCACTCCAGTAT-3′) and 3A(a) (5′-tcactaCTGGTGTCCAGCAAA-3′); 3AB(s) (5′-ccaccatgGGCCCACTCCAGTAT-3′) and 3AB(a) (5′-tcactaTTGTACCTTTGCTGT-3′); and 3C(s) (5′-ccaccatgGGACCAGGGTTCGAT-3′) and 3C(a) (5′-tcactaTTGACTCTGAGTGAA-3′). At the 5′ end, each primer was supplemented with the corresponding restriction sites for cloning in the vector. We used the pcDNA3HA expression vector, representing a modification of the pcDNA3 vector (Invitrogen) with the coding sequence of the standard hemagglutinin (HA) TAG epitope. This vector was used for expression of proteins 2B, 2BC, 2C, and 3A as fusion proteins with the HA TAGs at their N ends. The sense primers for cloning in the pcDNA3HA vector did not have the Kozak sequence or the ATG codons. Expression of the 2BHA, 2BCHA, 2CHA, and 3AHA proteins was tested by anti-HA TAG antibodies (Santa Cruz Biotechnology) in extracts of transfected cells. To estimate the efficiency of transfection and to check the induction of apoptosis, we used the LXSN vector with the coding sequence of the GFP gene or the pcDNA3-LacZ plasmid expressing bacterial β-galactosidase. β-Galactosidase activity was determined by the manufacturer's protocol (Promega). The retroviral vector LXIG with the GFP gene under the control of an internal ribosomal entry sequence (IRES) regulatory element has been used to coexpress proteins 2A and GFP in the same cells without the suppression of GFP mRNA translation. To analyze the status of the Golgi apparatus, we used the pEYFP-Golgi vector (Clontech). The enhanced yellow fluorescent protein (EYFP) was fused to the membrane-anchoring signal peptide of UDP-galactose-D-*N*-acetylglucosaminide β-1,4-galactosyltransferase. The fusion protein localizes to the Golgi apparatus (36).

Apoptosis analysis. To stimulate apoptosis, cells were treated with TNF (0.1 ng/ml; R&D Systems) and CHI (1 or 5 μg/ml in NIH 3T3 or HeLa cells, respectively). In the absence of TNF, these concentrations of CHI were not toxic. As additional apoptosis inducers, a 1:3,000 dilution of anti-Fas antibodies (R&D Systems) and 10 nM staurosporine were used. Brefeldin A was used at a concentration of 1 μg/ml. Cell resistance to TNF-, Fas- and staurosporine-stimulated apoptosis was estimated by 4′,6′-diamidino-2-phenylindole (DAPI) staining, counting of GFP-expressing cells by microscopy, and staining of surviving cells with methylene blue. The dye was extracted from cells resistant to apoptosis by using 0.1 N HCl, and its absorbance was measured at 560 nm.

NF-κB gel shift assay. Five million NIH 3T3 or NIH 3T3 3A cells were treated for 1 h with 0.1 ng of TNF/ml with or without pretreatment with 1 μg of brefeldin A (Sigma)/ml. Cells were collected, and the pellet was resuspended in 300 μl of the lysis buffer, containing 10 mM KCl, 0.1 mM EGTA, 10 mM HEPES (pH 7.9), 1.0 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim). Samples were then transferred to 1.5-ml microcentrifuge tubes, incubated for 15 min on ice, and subjected to mechanical disruption by 7 passages through a 25-gauge needle. Lysates were centrifuged at 600 × *g* for 5 min at 4°C. Nuclear pellets were resuspended in 40 μl of the nuclear extract buffer (400 mM NaCl, 3 mM EGTA, 20 mM HEPES [pH 7.9], 1 mM DTT, 20% [vol/vol] glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) and incubated for 15 min on ice. Samples were microcentrifuged at 4°C (12,400 × *g*), and the supernatants containing the nuclear protein extracts were collected. An aliquot of each nuclear extract was quantified by a Coomassie Plus protein assay (Bio-Rad).

A double-stranded oligonucleotide probe comprising an NF-κB-binding region of the mouse B-cell light chain enhancer (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega) was end-labeled with [γ-^{32}P]ATP according to the manufacturer's protocol. The labeled probe was purified using a Nuctrap column (Stratagene), and the activity of the purified probe was determined on a Beckman LS 6500 scintillation counter. A probe (200,000 cpm; approximately 1.0 μl) was combined with 10 μg of nuclear protein extract, poly(dI-dC) (0.01 μg per 1.0 μg of nuclear protein), and electromobility shift assay buffer (50 mM NaCl, 0.5 mM EDTA, 10 mM Tris · HCl [pH 7.5], 1 mM $MgCl_2$, 4% glycerol, 0.5 mM DTT) to a final volume of 25 μl. Reaction mixtures were incubated for 20 min at room temperature, and 20 μl of each reaction mixturewas loaded on a 5% acrylamide–0.5× TBE (45 mM Tris, 44 mM boric acid, 11 mM EDTA, pH 8.3) gel and run at 10 V/cm. The gel was removed after the probe had run three-quarters of the length of the gel, dried on Whatman paper, and exposed to X-ray film overnight at −70°C.

Flow cytometry. For determination of the surface expression of Fas and TNF receptor 1 (TNFR1), cells were collected using phosphate-buffered saline with 0.5 mM EDTA. Live cells were incubated in phosphate-buffered saline with 10 mg of bovine serum albumin/ml at 4°C, first with a primary anti-human TNFR1 or anti-human Fas monoclonal antibody (R&D Systems) and then with a secondary fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody (Serotec). Staining with antibodies was followed by incubation with 50 μg of propidium iodide (Sigma)/ml. Dead cells were excluded from the analysis by gating in FL3. The DNA fluorescence of cells was measured with a FACScan flow cytometer (Becton Dickinson, Bedford, Mass.), and percentages of TNFR1- and Fas-positive cells were analyzed using FACScan software programs.

Western blot analysis. Total protein extracts from 10^7 HeLa cells expressing 2BHA, 2BCHA, 2CHA, and 3AHA proteins were obtained by lysis in radioimmunoprecipitation assay solution (150 mM NaCl, 1% sodium dodecyl sulfate, 10 mM Tris [pH 8.0], 1% sodium deoxycholate, 1% NP-40) with a protease inhibitor cocktail (Sigma). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond P nylon membranes (Amersham). After incubation with primary anti-HA TAG antibodies (Santa Cruz Biotechnology) or anti-TNFR1 antibodies (R&D Systems), immune complexes were visualized by enhanced chemiluminescence (Amersham).

RESULTS

In infected cells, poliovirus RNA is translated into a 247-kDa polyprotein precursor. Its proteolytic processing begins cotranslationally and eventually generates several polypeptides with different functions. A map of poliovirus genomic RNA is shown in Fig. 1. VP1, VP2, VP3, and VP4 are the structural

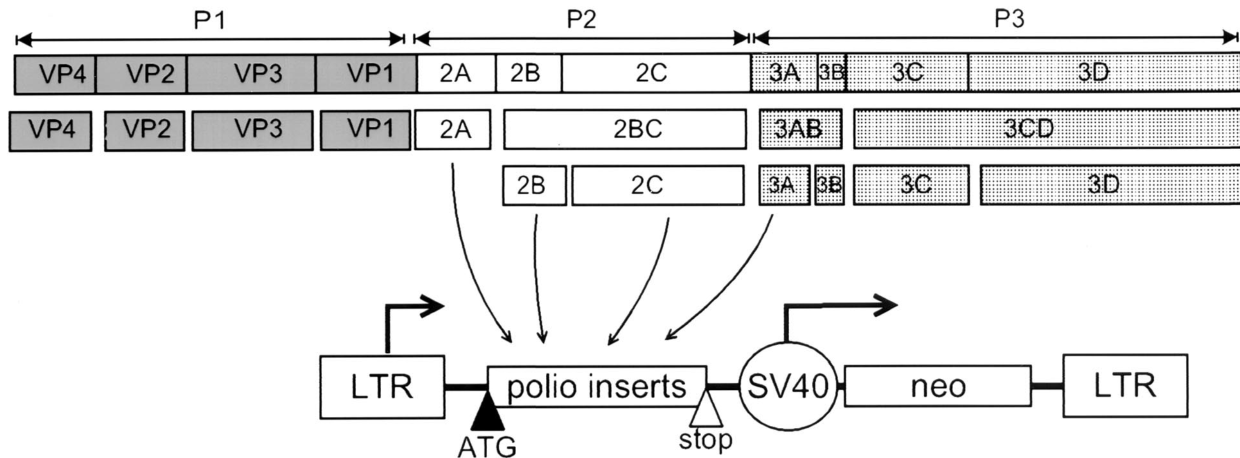

FIG. 1. Scheme of the organization of the poliovirus genome, processing of the polyprotein, and cloning of fragments encoding individual peptides into the retroviral vector. SV40, simian virus 40.

proteins involved in formation of the viral capsid. 2A, 3C, and 3CD are proteinases involved in viral polypeptide processing. In addition, 2A is responsible for the turnoff of cellular cap-dependent translation by cleavage of eIF-4G, an essential subunit of the translation initiation complex eIF-4F (40). 3D is viral RNA polymerase. 3AB and 3B (VPg) are involved in the initiation of RNA synthesis. The functions of the 3A, 2B, 2BC, and 2C proteins have not been clearly defined, although they have been shown to be involved in membrane proliferation, alterations in membrane permeability, inhibition of cellular protein secretion, and viral RNA replication (5, 6, 10, 14, 22, 23, 53).

To determine which of the virus-encoded polypeptides affect the host apoptotic system, we first expressed them individually in cells and then tested cells for sensitivity to apoptosis. DNA fragments corresponding to the poliovirus nonstructural proteins 2A, 2B, 2BC, 2C, 3A, 3AB, 3C, and 3CD were synthesized by PCR using full-length poliovirus cDNA as a template. Each fragment was supplied with a starting ATG codon in the Kozak consensus context and with a termination codon to encode proteins exactly corresponding to the viral processed polypeptides (with the exception of one additional N-terminal methionine). These fragments were cloned into the LXSN retrovirus expression vector, conferring resistance to G418. In this vector, poliovirus inserts were under the control of the Moloney murine leukemia virus long terminal repeat (LTR) promoter (Fig. 1).

The whole panel of retroviral constructs expressing individual poliovirus proteins was tested for their effects on cell sensitivity to TNF. Constructs were delivered to the target NIH 3T3 cells, highly sensitive to TNF, by massive infection allowing virus transduction in at least 50% of the cells. Mouse cells, when provided with the human poliovirus receptor, are fully permissive for poliovirus replication (66), suggesting that all the required viral functions are adequately expressed in mouse cells.

Poliovirus 2A protein sensitizes cells to TNF. Generally, cell sensitivity to TNF is greatly enhanced by inhibition of translation. Therefore, induction of apoptosis by TNF usually requires additional treatment with CHI (or another protein synthesis inhibitor), which inhibits TNF-dependent activation of NF-κB (59, 60). We expected that suppression of cap-dependent translation by poliovirus protein 2A would have a similar effect on cell sensitivity to TNF. This hypothesis was tested on NIH 3T3 cells, which are sensitive to TNF in the presence of CHI (Fig. 2).

Since protein 2A is toxic if highly expressed and can induce apoptosis by itself (29), its effect on TNF-induced apoptosis could be analyzed only under conditions of transient expression. NIH 3T3 cells were transfected with the vector expressing 2A from the LTR promoter, making (cap-dependent) translation of the resulting mRNA sensitive to 2A. Such a feedback regulation, by limiting the expression level of 2A, did not allow it to induce apoptosis . To monitor the cellular effects of the poliovirus protein, cells were cotransfected with a plasmid expressing GFP under the control of an IRES from encephalomyocarditis virus, making GFP expression insensitive to 2A. Transfected cells were treated with TNF, and proportions of apoptotic cells were quantified by fluorescence microscopy at varying times after transfection. The results (Fig. 2b and d) showed that although moderate expression of protein 2A by itself did not induce apoptosis in transfected cells, it made them sensitive to TNF even in the absence of CHI.

Poliovirus proteins 2B and 3A suppress cell sensitivity to TNF. Vector-infected cells were treated with TNF in the presence of a low concentration of CHI, resulting in induction of apoptosis in the majority of control cells infected by the insert-free vector (in these experiments CHI was used at concentrations that were not toxic and not apoptogenic in the absence of TNF). Cells were treated shortly (24 to 48 h) after the last infection in order to avoid potential complications that might be caused by the effects of expression of poliovirus proteins on cell growth. The proportion of surviving cells was estimated for each cell population. The results of a representative experiment are shown in Fig. 3. To monitor the expression of poliovirus proteins, we tagged them at their N termini with the HA epitope, which did not affect their abilities to confer TNF resistance (data not shown). The proteins expressed were of the expected sizes (Fig. 3a). Poliovirus protein 3A acted as a potent inhibitor of TNF-induced apoptosis, and 2B showed a

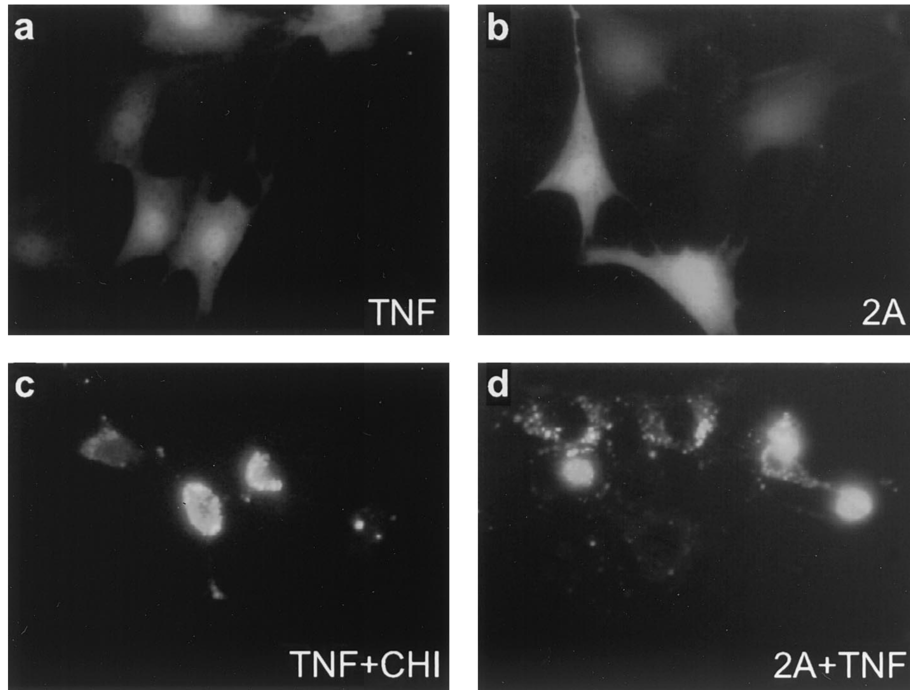

FIG. 2. Expression of 2A makes cells more sensitive to TNF-induced apoptosis. NIH 3T3 cells were transfected with the insert-free LXSN vector or with vectors encoding protein 2A and GFP. At 24 h posttransfection, cells were incubated for 10 h either with TNF alone (a and d) or with a combination of TNF and CHI (c). Note that neither expression of 2A by itself (b) nor incubation of NIH 3T3 cells with TNF alone (a) induced apoptosis under these conditions (less than 1% of GFP-expressing cells showed apoptotic morphology). However, TNF efficiently induced apoptosis in cells transfected with 2A (d) or treated with CHI (c) (>80% of cells showed apoptotic morphology after 10 h of TNF treatment).

rather strong and reproducible effect, but the others did not appreciably protect cells from TNF (Fig. 3b). Similarly, expression of 3A could neutralize 2A-mediated sensitization of cells to TNF (Fig. 3c).

Effects of 3A and 2B on protein trafficking. Alterations in the plasma membrane and profound modification in the vesicular systems result from poliovirus infection (14, 53). During the first 2 to 3 h of infection the *cis*-Golgi network is disrupted

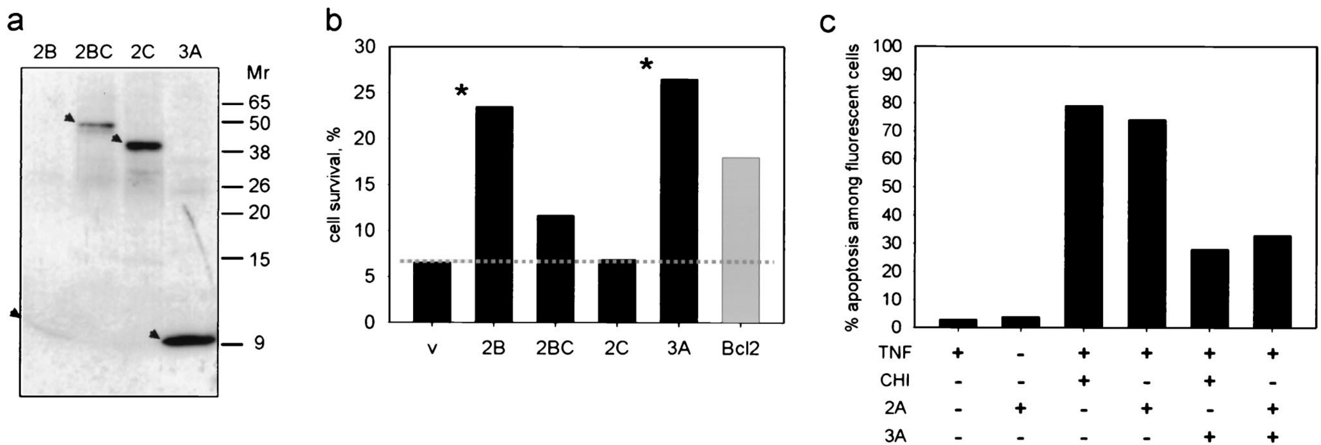

FIG. 3. Expression of poliovirus proteins 2B and 3A protects NIH 3T3 cells from TNF-induced apoptosis. (a) Western blot analysis of expression of HA-tagged 2B, 2BC, 2C, and 3A proteins. Portions (30 μg) of total protein extracts from Ecopack cells transfected with the indicated retroviral vectors were probed with antibodies against HA TAG (Santa Cruz Biotechnology). (b) Quantitation of the results of a representative experiment with NIH 3T3 cells infected with empty retrovirus vector (V) or with a retrovirus expressing poliovirus protein 2B, 2BC, 2C, 3A, or Bcl2. Cells were incubated for 10 h with 0.1 ng of TNF/ml and 1 μg of CHI/ml and then were fixed with methanol, and cell survival was estimated by methylene blue assay. Values were normalized relative to control untreated cells. It should be noted that only a fraction (not less than 50%) of the cell population was infected with the retroviruses. (c) Protein 3A protects NIH 3T3 cells from apoptosis induced by a combination of TNF and protein 2A expression. NIH 3T3 cells were cotransfected with a GFP-expressing vector and either the LXSN vector (first and third bars), a 2A coding vector (second and fourth bars), a combination of LXSN and 3A coding vectors (fifth bar), or a combination of 2A and 3A coding vectors (sixth bar). Cells were treated with TNF alone (first, fourth, and sixth bars) or with TNF and CHI (third and fifth bars).

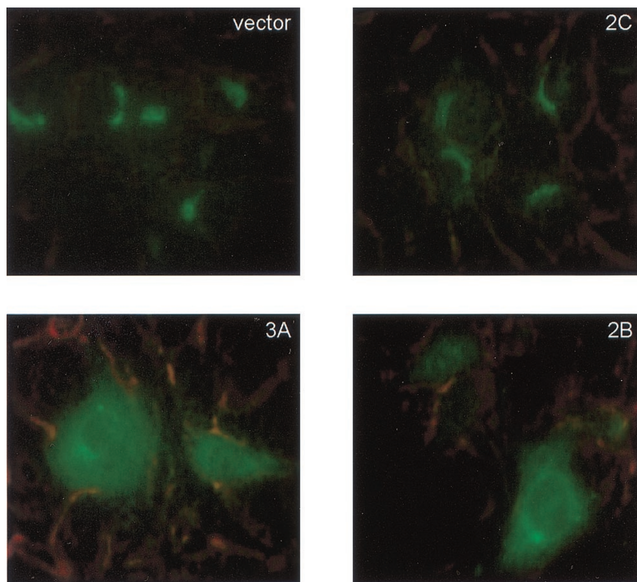

FIG. 4. Effects of expression of various poliovirus proteins on ER-Golgi protein trafficking, monitored by using a Golgi-specific marker protein (EYFP-Golgi). NIH 3T3 cells were transiently transfected with the pEYFP-Golgi vector in combination with a plasmid expressing poliovirus protein 2B, 2C, or 3A. Fluorescence consistent with the normal morphology of the Golgi apparatus was observed in >90% of cells transfected with the control insert-free vector. When cells were cotransfected with the vector coding for 3A, the EYFP-Golgi protein showed diffuse cytoplasmic distribution in more than 80% of transfected cells. Expression of 2B also prevented EYFP-Golgi protein from localizing in a typical Golgi pattern, with efficiency close to that of protein 3A expression. Expression of protein 2C had no effect on EYFP-Golgi protein localization. The ratio of each poliovirus protein-encoding vector to the EYFP-Golgi coding vector was 10:1.

into fragments scattered throughout the cytoplasm (3). Transient expression of the viral protein 2B in COS-7 cells also causes disassembly of the Golgi complex (45). Expression of the proteins 3A and 2B (22, 45) blocked protein trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus as well as protein secretion. We supposed that the mechanism of the antiapoptotic activities of 3A and 2B could be linked to their abilities to affect protein trafficking.

To monitor the effects of poliovirus proteins on the Golgi complex in our systems, we cotransfected 3A-, 2B-, and 2C-expressing plasmids together with the vector expressing modified GFP fused to a Golgi localization signal (pEYFP-Golgi vector). Upon microscopic examination, the EYFP revealed typical Golgi-like structures if it was expressed alone in NIH 3T3 cells (Fig. 4). However, both 3A and 2B dramatically changed the pattern of fluorescence, which, in these cases, was distributed evenly throughout the cytoplasm (Fig. 4). These changes in the fluorescence pattern apparently reflect the block in protein trafficking from the ER to the Golgi apparatus, possibly causing a disruption of the Golgi complex, as has been described previously for cells expressing proteins 2B and 3A (22, 45). Expression of 2C, which did not protect cells from TNF, did not alter the location of the EYFP-Golgi protein (Fig. 4).

We attempted to establish cell populations with stable expression of the selected constructs. Retrovirus-driven expression of 2B had a detrimental effect on cell growth, resulting in selection of cells with undetectable levels of protein expression. However, expression of 3A did not markedly change cell growth properties (data not shown). Therefore, we chose 3A for a detailed characterization using polyclonal populations of freshly infected or transfected mouse (NIH 3T3) and human (HeLa) cells stably expressing HA-tagged 3A (Fig. 5a).

To further explore the possibility of a functional link between impairment of the Golgi function and TNF resistance, we used brefeldin A, which can rapidly destroy the Golgi complex (21). Indeed, treatment of NIH 3T3 cells with brefeldin A for 30 min caused a change in the intracellular distribution of EYFP-Golgi similar to that caused by expression of 3A and 2B (data not shown). Consistently, pretreatment of HeLa and NIH 3T3 cells with brefeldin A for 1.5 h made them resistant to TNF. Longer pretreatment made this effect more profound (see below). Neither 3A expression nor pretreatment with brefeldin A protected cells from Fas- or staurosporine-induced apoptosis (Fig. 5). These observations indicate that inhibition of ER-Golgi protein trafficking can make cells specifically resistant to TNF and that poliovirus protein 3A likely acts through this mechanism.

Protein 3A and brefeldin A suppress activation of NF-κB by TNF. A common mechanism of cellular resistance to TNF consists in the TNF-induced activation of NF-κB and its transport to the nucleus (8, 59). We investigated whether NF-κB activation took place in TNF-treated NIH 3T3 cells that either expressed 3A or were pretreated with brefeldin A (Fig. 6). No activation was detected by the gel shift assay in either case. Expression of 3A and pretreatment with brefeldin A each prevented TNF-induced translocation of NF-κB to the nucleus (Fig. 6a). Cells expressing 3A or treated with brefeldin A lost the ability to activate NF-κB only in response to TNF, not in response to other inducing agents. Thus, the combination of phorbol myristate acetate (PMA) and ionomycin activated NF-κB to the same level in 3A-expressing, brefeldin A-treated, and control NIH 3T3 cells (Fig. 6b). Thus, 3A and brefeldin A appeared to protect specifically against TNF-stimulated apoptosis at a step upstream of NF-κB activation.

Protein 3A and brefeldin A decrease abundance of TNFRs on the cell surface. TNF activity is initiated by its binding to cellular receptors. Fluorescence-activated cell sorter (FACS) analysis was used to quantify the amounts of TNFR1 on the surfaces of control HeLa cells, HeLa cells expressing protein 3A (after a short selection with G418), and HeLa cells treated with brefeldin A for 3 h, using antibodies against human TNFR1 (R&D Systems). Similar experiments were performed with 293 cells transiently transfected with the 3A-expressing plasmid. The results of these experiments are presented in Fig. 7. Treatment of HeLa cells and 293 cells with brefeldin A decreased levels of TNFR1 on cell surfaces approximately 10-fold. The amounts of TNFR1 on the surfaces of 3A-expressing cells were also decreased. A significant proportion of these cells showed levels of the receptor as low as those on negative-control cells or cells treated with brefeldin A. The 3A-expressing cells had greater heterogeneity in the pattern of TNFR1 expression, possibly due to variations in 3A expression within the mixed population. Similar effects of 3A were observed with the less-abundant TNFR2 in HeLa cells and with hu-

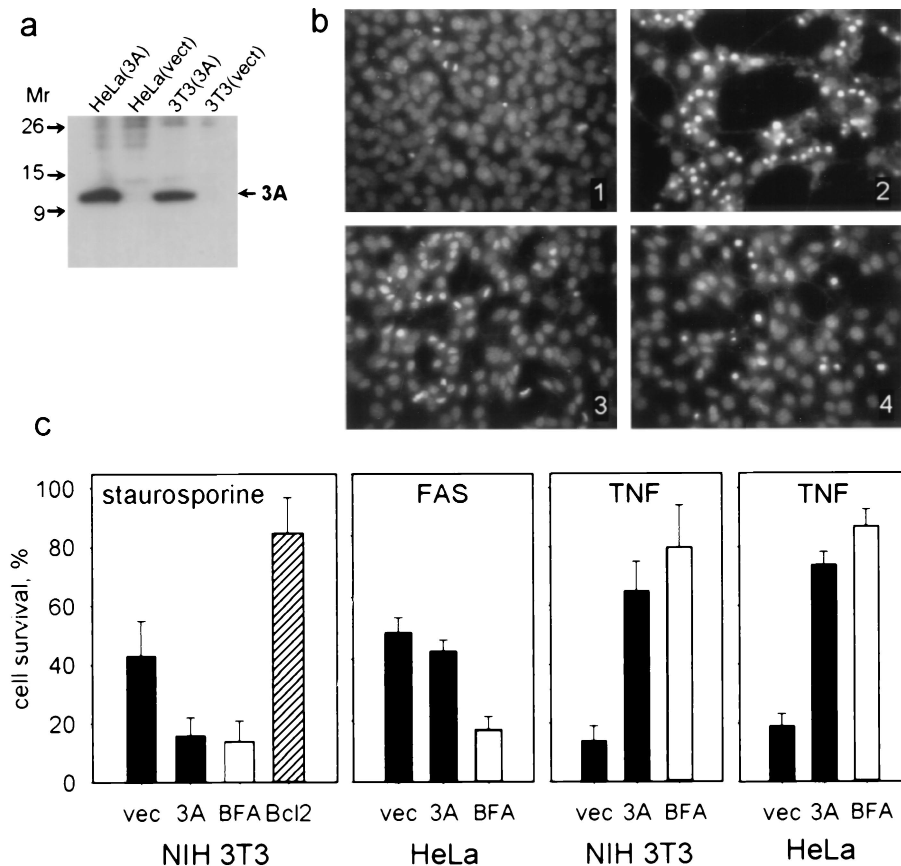

FIG. 5. Poliovirus protein 3A and brefeldin A protect cells against TNF-induced apoptosis but not against apoptosis induced by anti-Fas antibodies or staurosporine. (a) Western blot analysis of expression of HA-tagged 3A protein. Portions (30 μg) of total protein extracts from HeLa or NIH 3T3 cells transfected with an empty vector (vect) or a retroviral vector expressing protein 3A-HA were analyzed with antibodies against HA TAG (Santa Cruz Biotechnology). (b) DAPI staining of NIH 3T3 cells transduced either with the insert-free retroviral vector (panels 1, 2, and 3) or with a similar vector (LXSN) expressing protein 3A (panel 4) and treated with TNF and CHI either without pretreatment (panels 2 and 4) or with a 3-h brefeldin A pretreatment (panel 3). Bright spots correspond to the condensed chromatin in apoptotic cells. (c) Protein 3A and brefeldin A do not protect HeLa cells from Fas-induced apoptosis or NIH 3T3 cells from staurosporine-induced apoptosis. Results are shown for representative experiments with NIH 3T3 and HeLa cells transduced with an empty vector (vec), a 3A-HA-expressing vector (3A), or a vector expressing human Bcl2 (Bcl2) and for cells pretreated for 3 h with 1 μg of brefeldin A/ml (BFA). (Left panels) NIH 3T3 cells were treated with staurosporine (10 nM), and HeLa cells were treated with antibodies against Fas (R&D Systems). (Right panels) Cells were treated with 0.1 ng of TNF/ml in combination with CHI (1 μg/ml for NIH 3T3 cells and 5 μg/ml for HeLa cells), and cell survival was estimated by methylene blue assay. All results were normalized to the amount of dye in control untreated cells.

man TNFR1 ectopically expressed in NIH 3T3 cells (data not shown).

Protein 3A and brefeldin A caused a decrease in the amount of TNFR1 only on the cell surface, not affecting the total amount of TNFR1 in the HeLa cell, as judged by the results of Western analysis (Fig. 7c). This observation indicated that TNFR1 molecules were either trapped or delayed inside the cell on their way to the surface, presumably due to the alterations in protein trafficking from ER to the Golgi. It may be noted parenthetically that the apparent absence of Golgi-specific O-linked glycosylation in brefeldin A-treated and 3A-expressing cells did not appreciably affect the molecular mass of TNFR1 molecules (slightly above 55 kDa) (Fig. 7c). This could be explained by the fact that more than 95% of TNFR1 molecules have only N-linked oligosaccharides, which are added in the ER, and less than 5% of TNFR1 species have Golgi-derived long O-linked oligosaccharide chains (15).

Expression of 3A or treatment with brefeldin A did not protect HeLa cells from Fas-induced apoptosis. Consistent with this observation, both 3A and brefeldin A had only minor effects on the abundance of endogenous Fas (also known as CD95) receptor on the surfaces of HeLa and 293 cells (Fig. 7a, b, and d). The difference between the responses of two death receptors to the inhibitors of protein trafficking may be due to differences in stability. Unlike Fas, TNFR1 is relatively unstable, with a half-life of about 2 h (63). This correlates well with the time course of brefeldin A-mediated protection of cells from TNF-induced apoptosis (Fig. 8) and the ability of brefeldin A to suppress the activation of NF-κB (Fig. 6a). If the mechanism of protection from TNF by 3A and brefeldin A was based on a low stability of TNF receptors, inhibitors of translation could be expected to produce a similar effect. In fact, CHI has been reported to rapidly reduce the amount of TNFR1 on the cell surface (63). We therefore decided to test whether CHI could reduce cell sensitivity to TNF if it was added before the cytokine (to stimulate apoptosis, CHI is usu-

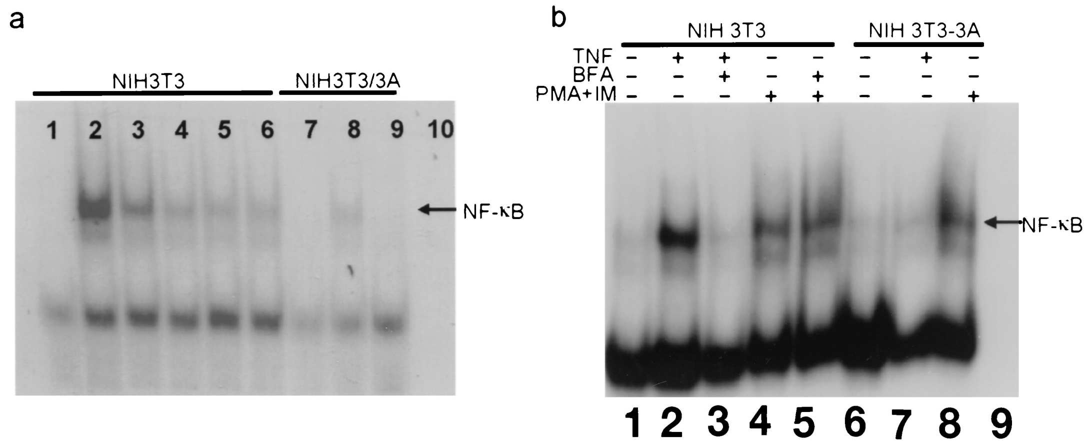

FIG. 6. Preincubation with brefeldin A and expression of protein 3A suppress activation of NF-κB by TNF (a) but not by a combination of PMA with ionomycin (IM) (b). A gel shift assay was carried out by incubating nuclear protein extracts (10 μg) from control NIH 3T3 cells or from NIH 3T3 cells transfected with a 3A-expressing vector (NIH 3T3/3A) with a ^{32}P-labeled oligonucleotide probe specific for NF-κB protein and then subjected to electrophoresis. The arrow marks the position of the NF-κB-specific complex. (a) Lanes 1 and 7, cells without induction; lanes 2 and 8, cells treated with TNF for 1 h; lanes 3 and 9, preincubation with brefeldin A for 1 h, followed by 1 h of TNF; lane 4, preincubation with brefeldin A for 2 h, followed by 1 h of TNF; lane 5, preincubation with brefeldin A for 3 h, followed by 1 h of TNF; lane 6, preincubation with brefeldin A for 4 h, followed by TNF for 1 h; lane 10, no protein added. (b) Lanes 1 and 6, cells without induction; lanes 2 and 7, cells treated with TNF for 1 h; lane 3, preincubation with brefeldin A for 3 h, followed by 1 h with TNF; lanes 4 and 8, incubation with PMA (0.1 μM) and IM (10 ng/ml) for 1 h; lane 5, preincubation with brefeldin A for 3 h, followed by 1 h with PMA and IM; lane 9, no protein added.

ally added simultaneously with TNF). As shown in Fig. 8a, NIH 3T3 cells pretreated with 5 μg of CHI/ml for 3 h were indeed more resistant to TNF-triggered apoptosis than control cells. However, the antiapoptotic effect of CHI was not as strong and as fast as that of brefeldin A (Fig. 8b). These differences correlated with the results of FACS analysis of TNFR1 abundance on the surfaces of cells treated with either of the inhibitors (Fig. 8c). Importantly, neither brefeldin A (within 3 h of treatment) nor 3A affected cell protein synthesis, as judged by [^{35}S]methionine incorporation (data not shown).

Thus, inhibition of trafficking through the Golgi apparatus (by brefeldin A) is likely to be a more efficient and rapid way to suppress TNF-dependent apoptosis than inhibition of translation (by CHI).

TNFR1 rapidly disappears from the cell surface during poliovirus infection. Depletion of TNFR1 not only resulted from the isolated expression of 3A but was also observed in the course of infection with intact poliovirus. FACS analysis was used to examine the stabilities of TNFR1 and Fas on the surfaces of virus-infected HeLa cells (Fig. 9). The amount of TNFR1 strongly decreased after 2 h postinfection (p.i.), resulting in its almost-complete disappearance by 4 h p.i. Only a minor reduction in the amounts of Fas receptor was observed, with an approximately twofold decrease by 8 h p.i.

DISCUSSION

One of the major fields where the "struggle" between viruses and cells takes place is the host apoptotic system. Some metabolic and structural changes induced by viral infection are "sensed" by the host as a signal to turn on the defensive, infection-limiting death program. Viral genes responsible for these alterations are considered "apoptotic," even though their primary role is usually just to ensure efficient virus reproduction. On the other hand, many viruses possess a variety of counterdefensive, antiapoptotic tools. In some cases, interference with the host apoptotic system is a major, or even the sole, job of a viral protein, whereas in other cases, the antiapoptotic function is merely a by-product of an activity required for viral reproduction. It is quite common for a virus to express both apoptotic and antiapoptotic proteins.

The host apoptotic system may be activated by viral infection in a variety of ways, which schematically can be classified as intrinsic and extrinsic, although some of the components involved in the relevant pathways may overlap. The present study is focused primarily on the extrinsic pathway, which starts from the interaction of cellular receptors with a class of "immunity molecules" represented by soluble or cell-associated ligands, in our case TNF, an inflammatory cytokine widely used by the host to combat bacterial and viral infections (31, 41, 61). Viral antiapoptotic proteins targeting the receptor-mediated defensive pathway operate at several levels (44). Poxviruses encode soluble forms of TNFR-like molecules (viroceptors), which serve as scavengers preventing TNF interaction with the bona fide cellular TNF receptors (39, 47, 49). Several DNA viruses can affect presentation of a variety of proteins on the plasma membrane. Thus, adenovirus E3 proteins stimulate internalization and subsequent degradation of Fas (26, 48, 55) and interfere with the TNF-triggered generation of arachidonic acid through inhibition of the cytoplasm-to-membrane relocation of phospholipase 2A (20, 34). Herpesviruses have developed diverse mechanisms to inhibit major histocompatibility complex (MHC) class I-restricted antigen presentation (16, 43, 51). The activation of caspase-8 by the adapter proteins bound to the cytoplasmic domain of TNFR and related receptors can be suppressed by death effector domain-containing proteins encoded by several herpesviruses and poxviruses (9, 32, 33, 54, 62). Farther-downstream steps of the receptor-mediated apoptotic pathway can also be suppressed by variously acting proteins of numerous viruses (11, 13, 30, 35, 44, 61, 64). It is noteworthy that all the above data were obtained with

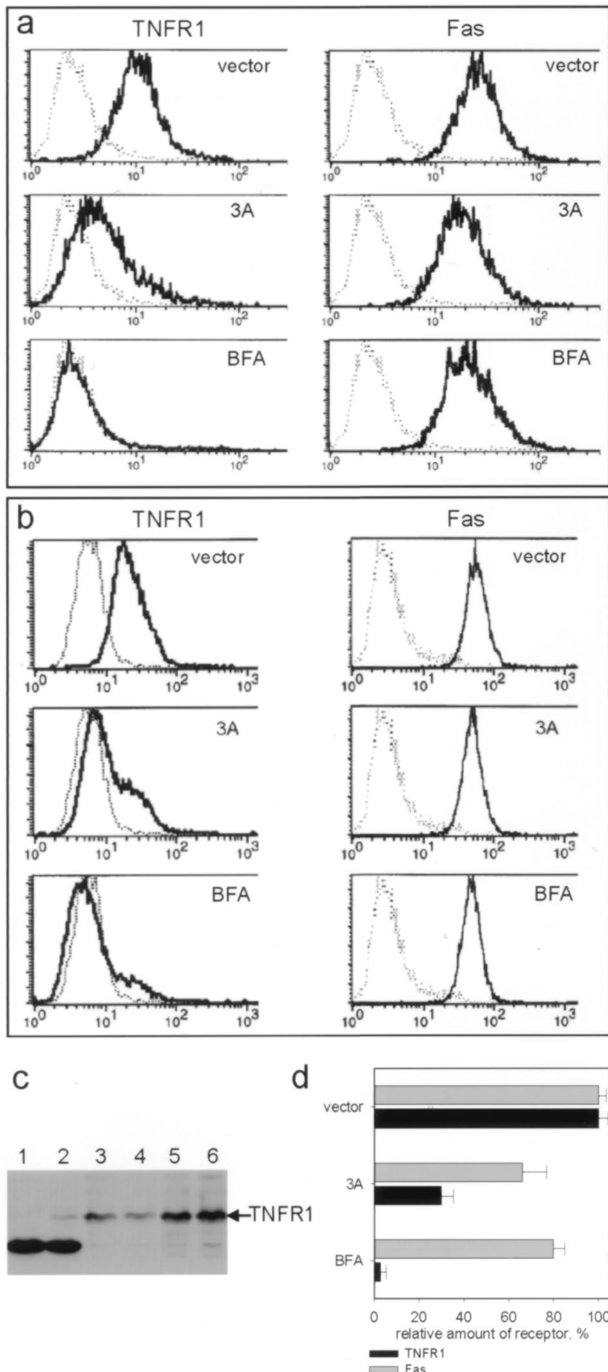

FIG. 7. Treatment with brefeldin A and expression of 3A deplete TNFR1 from the cell surface but have much less effect on Fas surface presentation. (a) Flow-cytometric analysis of live 293 cells stained by antibodies against human TNFR1 or Fas and secondary FITC-labeled antibodies (solid lines) or by secondary antibodies alone (dotted lines). *x* axis, intensity of fluorescence; *y* axis, cell numbers. The proportion of cells with a high surface presentation of TNFR1 was decreased in the population transiently transfected with the vector expressing 3A (transfection efficiency was higher than 80%, as judged by the proportion of fluorescent cells expressing GFP from a cotransfected plasmid on a parallel plate) and, even more dramatically, in the cells treated for 3 h with brefeldin A (BFA). Expression of 3A and pretreatment with brefeldin A for 3 h have only minor effects on the surface amount of Fas. (b) Flow-cytometric analysis with antibodies against human TNFR1 or Fas and secondary FITC-labeled antibodies (solid lines) or with secondary antibodies alone (dotted lines) of live HeLa cells stably transfected with the 3A-expressing plasmid. (c) Detection of TNFR1 in NIH 3T3 and HeLa cells by Western immunoblotting with anti-human TNFR1 antibodies (R&D Systems). Lanes: 1, NIH 3T3 cells; 2, NIH 3T3 cells transfected with a human TNFR1-expressing vector; 3, control HeLa cells; 4, HeLa cells treated for 3 h with 1 μg of BFA/ml; 5, control HeLa cells; 6, HeLa 3A-expressing cells. Fifty micrograms of protein from total lysates was loaded. The position of TNFR1 is slightly above 55 kDa. The nonspecific band of about 50 kDa was detected in NIH 3T3 protein extracts. (d) Quantitation of the results of the flow-cytometric analyses of Fas and TNFR1 on cell surfaces shown in panel a. Data are averages from two experiments with transiently transfected 293 cells.

DNA viruses, predominantly with those that possess large genomes.

The present study was carried out with poliovirus, a small RNA-containing virus. It is demonstrated that viral proteins 3A and 2B suppress cell sensitivity to the apoptotic effect of TNF. Decreased sensitivity to TNF is accompanied, and most likely is caused, by the depletion of the TNFR species from the surfaces of cells expressing the viral proteins. Relative tolerance to TNF is also exhibited by cells that express 3A together with another poliovirus protein 2A, which by itself sensitizes cells to the cytokine in the absence of CHI. These and other data reported here are consistent with the following hypothetical scheme. TNFR depletion appears to be largely achieved through the 3A-mediated inhibition of protein trafficking from the ER to the Golgi apparatus, which normally ensures fast replenishment of the short-lived receptor. Indeed, the effects of 3A on TNF sensitivity and on TNFR abundance are mimicked by brefeldin A, a known inhibitor of ER-Golgi transport (21). On the other hand, the most likely mechanism of the 2A-induced enhancement of sensitivity to TNF involves inhibition of host protein synthesis. This sensitization to TNF is likely due to interference with the antiapoptotic functions of NF-κB, usually activated by TNF. However, depletion of TNFR from the cell surface interrupts signal transduction from TNF to NF-κB (no NF-κB activation by TNF was detected in 3A-expressing cells), and that is why the anti-TNF effect of 3A dominates over the pro-TNF effect of 2A. This scheme is significantly buttressed also by important observations made in other laboratories. Thus, poliovirus 3A and 2B have been shown to disrupt the Golgi complex and interfere with both ER-to-Golgi traffic and protein secretion in other systems as well (22, 23, 45). While this paper was in preparation, Deitz et al. (18) reported on the ability of 3A to inhibit MHC class I-dependent antigen presentation.

It is noteworthy that the decrease in TNF sensitivity of 3A-expressing cells is receptor specific and is not accompanied by a comparable decrease in sensitivity to the Fas ligand. This difference can be readily explained by the much shorter half-life of TNFR than of Fas (63). The protein 3A- and probably protein 2B-mediated deregulation of protein trafficking may affect other membrane or secreted cellular proteins that can also contribute to host antiviral defense mechanisms, such as MHC proteins, interferon, TRAIL, transforming growth factor β, and interleukin receptors. In agreement with this hypothesis, it was recently shown that the amounts of highly abundant and stable MHC class I molecules on the cell surface were not

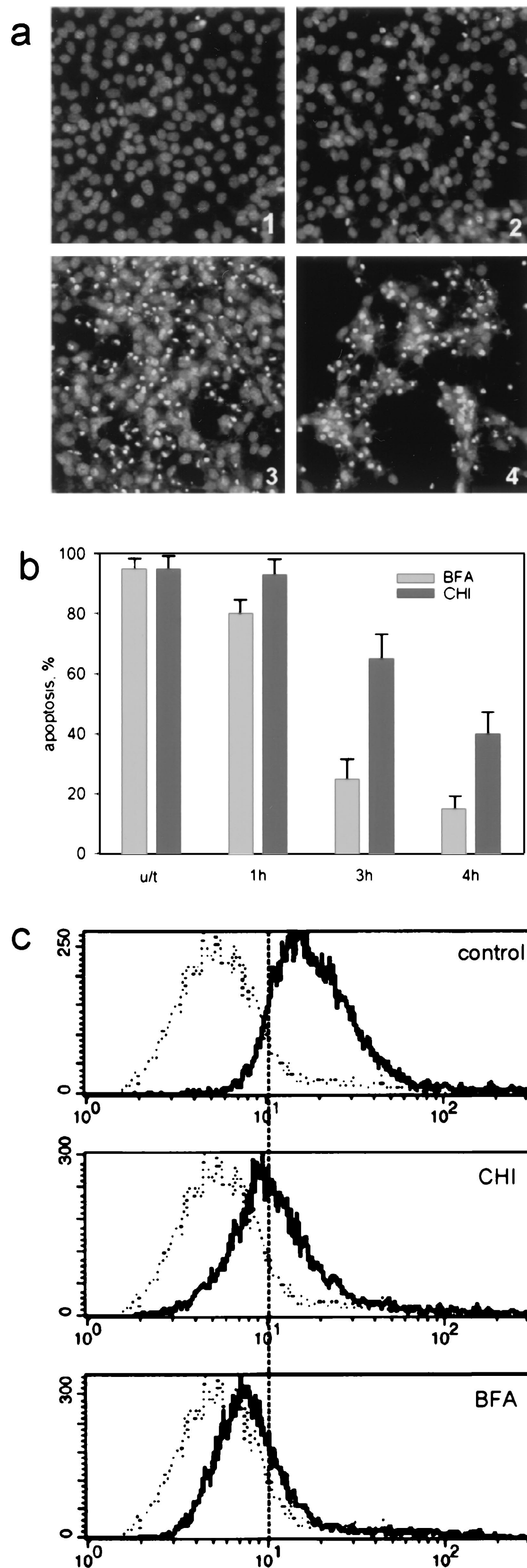

FIG. 8. Brefeldin A protects cells from TNF-stimulated apoptosis faster than pretreatment with CHI. (a) DAPI staining of untreated control NIH 3T3 cells (panel 1), cells pretreated for 3 h with brefeldin A (panel 2) or CHI (panel 3) before treatment with TNF and CHI, and cells treated with TNF and CHI without pretreatment (panel 4). Light spots of condensed chromatin indicate apoptotic cells. (b) Quantitative presentation of average results from two experiments. NIH 3T3 cells were either treated with TNF and 1 μg of CHI/ml for 7 h (u/t) or were pretreated with 1 μg of brefeldin A (BFA)/ml or 5 μg of CHI/ml for 1, 3, or 4 h before addition of TNF with the lower concentration of CHI. After selection, cells were fixed with formaldehyde and treated for DAPI staining. The number of apoptotic cells was estimated by microscopic analysis of 400 to 500 cells from each plate. Note that pretreatment with BFA protected cells from apoptosis faster and more efficiently than pretreatment with CHI. (c) Flow-cytometric analysis of live HeLa cells with antibodies against human TNFR1 and secondary FITC-labeled antibodies (solid lines) or with secondary antibodies alone (dotted lines). Note that a 3-h pretreatment of HeLa cells with CHI does not decrease the abundance of TNFR1 on the cell surface as dramatically as a 3-h treatment with BFA.

affected by poliovirus infection, but transport of newly synthesized viral antigens was suppressed, and as a result, infected cells were not sensitive to the virus-specific cytotoxic T cells (18). We anticipate that not only TNFR1, but all other short-lived surface proteins, should disappear from the cell surface during poliovirus infection (starting between 2 and 4 h p.i.) and that this process may contribute to the efficiency of poliovirus infection and its protection against the cellular antiviral response. These experiments are now in progress, and our preliminary results support this hypothesis.

Depletion of TNFR from the cell surface also takes place in the course of productive poliovirus infection. Therefore it is interesting to consider temporal relationships between the opposing effects of 2A (sensitization to TNF) and 3A (desensitization) during the relatively short cycle of poliovirus reproduction (6 to 8 h in HeLa cells). In this context it is important that 2A-mediated inhibition of host translation occurs rather early, e.g., 2 to 3 h p.i., and that TNF-induced apoptosis is very fast: the activated form of caspase 8 can be detected within 30 min after TNF application (46). On the other hand, the arrest of protein trafficking during poliovirus infection occurs before the suppression of cellular protein synthesis (22), and as shown here, the reduction in TNFR1 levels on cell surfaces after treatment with agents that destroy Golgi functions also occurs very rapidly, even faster than it occurs after inhibition of protein synthesis. Thus, inhibition of ER-Golgi protein trafficking may efficiently serve to neutralize, at least in part, the proapoptotic activity of 2A. The ability of poliovirus to suppress TNF-induced apoptosis may therefore represent a biologically important viral countermeasure against host defense.

This study did not address the mechanism of TNFR1 down-modulation during poliovirus infection. At least two mechanisms for TNFR1 turnover from the cell surface have been described. One involves an endocytic pathway and usually is stimulated by ligand binding to the receptor molecules (63). The clathrin-mediated endocytic pathway was reported to be totally suppressed by 5 h of poliovirus infection in HeLa cells (19). Because, as shown here, the majority of TNFR1 molecules disappear from the cell surface between 2 and 4 h p.i., a contribution of endocytosis to TNFR1 down-modulation cannot be ruled out. The other mechanism of turnover of TNFR1 consists in its proteolytic shedding from the cell surface (28). This also can contribute to the fast disappearance of TNFR1 from the surfaces of poliovirus-infected cells.

It seems warranted to briefly comment on the fact that cells expressing a level of 3A that interfered with ER-Golgi protein

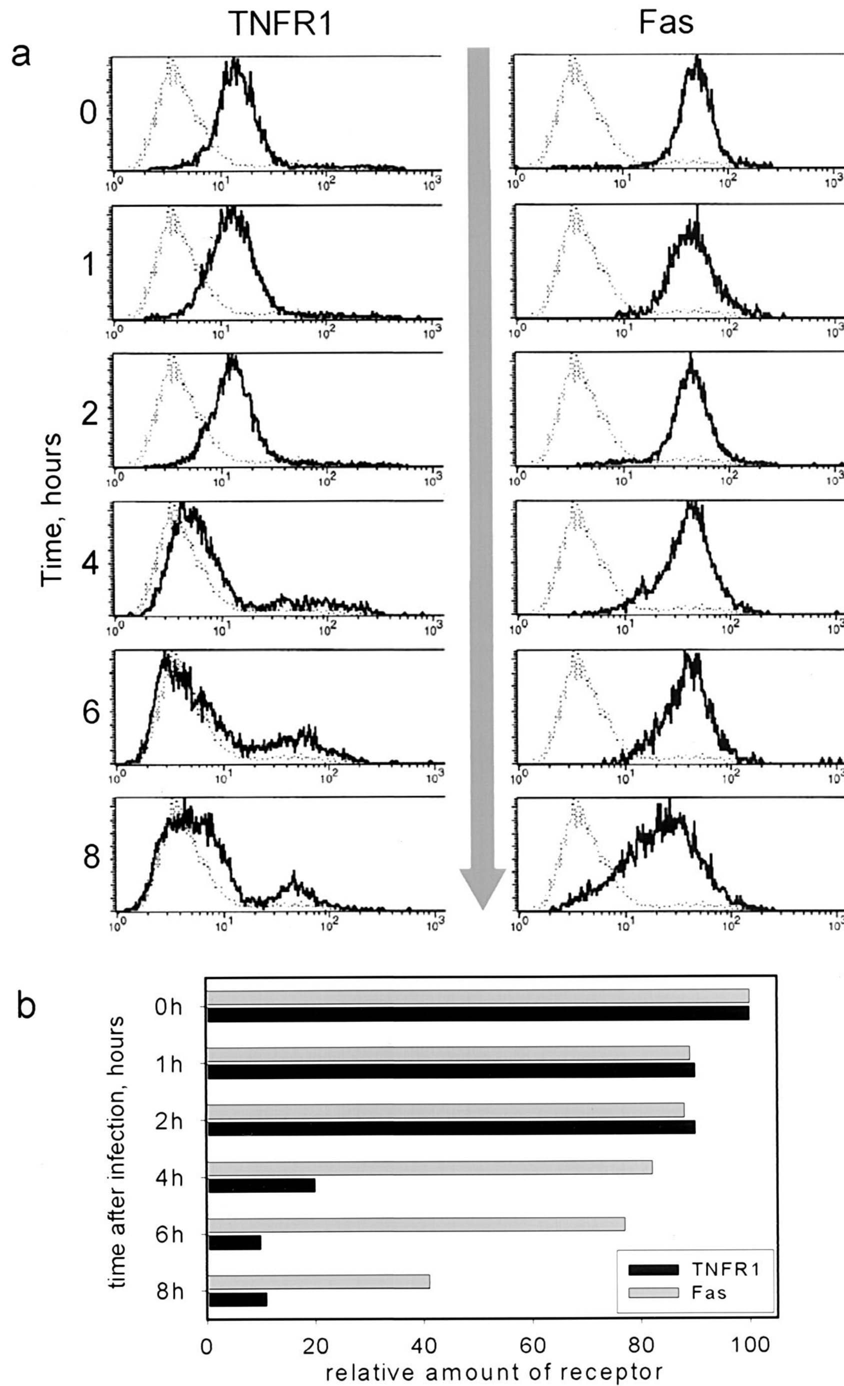

FIG. 9. TNFR1 disappears rapidly from the surfaces of poliovirus-infected cells. (a) Flow-cytometric analysis with antibodies against TNFR1 or against Fas of live HeLa cells infected with poliovirus (solid lines). Results of staining with secondary antibodies alone are shown by dotted lines. Detection of TNFR1 molecules on poliovirus-infected cells was carried out at 1, 2, 4, 6, and 8 h p.i. Note the fast and profound decrease in the amounts of TNFR1 between 2 and 4 h p.i.; in contrast, there was only a slow decline in Fas levels. (b) Results of poliovirus infection experiments presented as percentages of TNFR1 and Fas molecules on infected cell surfaces at different times p.i. The amount of receptor molecules on the surfaces of uninfected HeLa cells was taken as 100%.

trafficking remained viable. It is noteworthy that the severe inhibition of protein trafficking by 3A shown in Fig. 4 was observed only under the condition of transient transfection, when expression levels were significantly higher than in the stable cell lines constitutively expressing the protein. The Golgi morphology of the majority of HeLa and NIH 3T3 cells stably expressing 3A was close to normal (data not shown). Moreover, a direct comparison of the effects of brefeldin A and 3A on protein secretion showed that the 3A-expressing cells were still capable of secretion, although at a lower rate (data not shown). The reduced rate of protein secretion by 3A-expressing cells has also been demonstrated recently by Deitz et al. (18).

Poliovirus infection is known not only to affect receptor-mediated interaction with external apoptotic stimuli but also to dramatically interfere with the intracellular apoptotic system. An intrinsic apoptotic pathway(s) is activated early after infection (1) as a response to the damaging effects of viral proteinases 2A (29) and 3C (7), and possibly some other proteins. Implementation of this suicide program may, however, be prevented or interrupted by the expression of viral antiapototic functions (1, 56). These functions certainly include mechanisms other than interference with the Golgi-mediated traffic. Thus, poliovirus is equipped to withstand both extrinsic and intrinsic defensive apoptotic responses.

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