Inhibition of Tumor Necrosis Factor (TNF) Signal Transduction by the Adenovirus Group C RID Complex Involves Downregulation of Surface Levels of TNF Receptor 1

Shawn P. Fessler, † Y. Rebecca Chin, and Marshall S. Horwitz*

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York

Received 12 May 2004/Accepted 29 June 2004

Adenoviruses employ multiple genes to inhibit the host antiviral responses. There is increasing evidence that these immunoregulatory genes may function either during lytic or latent infection. Adenovirus early transcription region 3 (E3) encodes at least seven proteins, five of which block the acquired or innate immune response. Previous findings from this laboratory demonstrated that the E3 proteins 10.4K and 14.5K, which form a complex in the plasma membrane, inhibit tumor necrosis factor (TNF)-induced activation of NF-κB and the synthesis of chemokines. To determine the mechanism of inhibition of these pathways by the adenovirus E3 10.4K/14.5K proteins, we have examined the effects of this viral complex on the inhibition of AP-1 and NF-κB activation by TNF and found a reduction in assembly of the TNF receptor 1 (TNFR1) signaling complex at the plasma membrane accompanied by downregulation of surface levels of TNFRI.

Adenoviruses (Ad) contain a number of genes involved in regulation of host immune and inflammatory responses, presumably necessary to prevent elimination during lytic or latent infection. Of interest to our laboratory is early transcription region 3 (E3), which encodes proteins involved in the inhibition of major histocompatibility complex class I processes (Ad E3-gp19K), and inhibition of cytolysis by tumor necrosis factor (TNF) receptor (TNFR) family members, including TNF, Fas, and TRAIL (Ad E3-10.4K, Ad E3-14.5K, and Ad E3-14.7K) (13, 24, 51). The only E3 protein with a known function not involved in immunomodulation is Ad E3-11.6K, also known as the adenovirus death protein, a proapoptotic protein involved in release of progeny virus from infected cells (45, 46). The function of the Ad E3 region in protection against the host immune response has been demonstrated by in vivo murine studies, which showed that deletion of E3 genes resulted in an increased inflammatory response in the cotton rat (Sigmodon hispidus) pulmonary model of viral infection (16), as well as in a murine model of arterial gene transfer (50). Other work done in our laboratory with the E3 region expressed as transgenes in several mouse models has also demonstrated its efficacy in the prevention of autoimmune diabetes (11, 25, 38, 49) and in facilitating allogeneic transplantation (10).

Studies in our laboratory investigated the mechanism of adenovirus E3’s inhibition of signaling through TNFR1, a proinflammatory receptor that activates both NF-κB and AP-1 as diagrammed in Fig. 1. Proapoptotic or proinflammatory signaling poses a significant threat to the survival of many viral types, which have evolved mechanisms to block or modulate signaling through the TNF receptor. For example, cytomegalovirus (1) and poliovirus (37) encode proteins that eliminate TNFRI from the cell surface. Protein E6 from human papillomavirus 16 inhibits TNFRI signaling based on its ability to interact with the receptor (14), and VP4 from rotavirus mod-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2083. Fax: (718) 430-8702. E-mail: horwitz@aecom.yu.edu.
† Present address: Department of Pathology, Tufts University, Sackler School of Biomedical Sciences, Boston, Mass.

FIG. 1. TNF activation of NF-κB and AP-1 through TNFRI. Upon ligand binding, TNFRI trimerizes, leading to signalosome formation and activation of both NF-κB and AP-1 transcription factors in parallel. Assembly of TRADD, TRAF-2, and RIP at the receptor is required for recruitment and activation of the IKK complex, which in turn phosphorylates IκB-α, signaling its polyubiquitination and proteasomal degradation. The p65/p50 heterodimer NF-κB is then able to translocate into the nucleus and bind its nuclear targets (2,6). For AP-1 activation, the assembly of TRADD and TRAF-2 at the receptor upon ligand-induced trimerization results in the recruitment and activation of the mitogen-activated protein kinases MEKK1, MKK4 or MKK7, and JNK, which phosphorylates and activates nuclear c-Jun of the Jun/Fos heterodimer comprising AP-1. This simplified diagram omits a number of interacting molecules that can affect the activities of the pathways shown.
ulates TNF signaling by modulating TRAF-2 activity, which results in the activation of NF-κB signaling and inhibition of AP-1 signaling to promote viral survival (31). Similarly, inhibitory effects of Ad-E3 proteins on the TNF pathways have also been described (reviewed in (13), implicating both Ad E3-14.7K (18, 22, 23, 54) and the Ad E3-10.4K/14.5K protein complex (9,17) in the inhibition of apoptosis (17, 18, 22, 23), or arachidonic acid release (9,54) in response to TNF. The antiapoptotic effects of Ad E3-14.7K may be based on its ability to interact with caspase-8 (7,29). The anti-TNF mechanism of Ad E3-10.4K/14.5K has not been previously demonstrated, but an initial report failed to detect the internalization of the TNF receptor in murine C15.5 and C127 cells (42).

The Ad E3-10.4K and E3-14.5K proteins exist as a heterotrimeric complex spanning the plasma membrane, consisting of two molecules of adenovirus E3 10.4K, joined by a disulfide bridge, and bound to a single copy of adenovirus E3 14.5K, as described previously (43). The role of these proteins in downregulation of surface levels of epidural growth factor receptor was determined prior to the discovery by several groups of their ability to downmodulate cell surface levels of Fas (12, 42, 44) as well as TRAIL-R1 and TRAIL-R2 by endosomal internalization and lysosomal degradation (3, 47). This resulted in the renaming of these proteins as RID (receptor internalization and degradation), and the subunits as RID-α (Ad E3-10.4K) and RID-β (Ad E3-14.5K). Further work has demonstrated the necessity of a tyrosine sorting motif on RID-β (21, 35) and a dileucine sorting motif on RID-α (21, 53) in the downregulation of Fas, TRAIL, and epidermal growth factor receptors.

Results of experiments presented in this manuscript show that the RID complex is both necessary and sufficient for modulation of signal transduction through the TNF receptor, and that RID’s inhibitory effect extends to the AP-1 pathway in addition to the NF-κB pathway. The successful use of plasmids expressing RID-α and RID-β proteins in the inhibition of NF-κB activation further supports the idea that RID can inhibit TNF signal transduction in the absence of other viral proteins. Investigation of the recruitment of NF-κB signal transduction factors to the receptor in response to TNF also demonstrated RID’s ability to block the association of members of the IKK complex as well as the protein kinase RIP with the TNFR1. Downregulation of surface TNFR1 by RID accompanied this inhibition, demonstrating the mechanism of inhibition of TNFR1-induced signal transduction and chemokine synthesis. The downmodulation of TNFR1 and Fas occurred simultaneously and required similar amounts of virus for the effects on each of these receptors. Inhibition of TNF-induced NF-κB activation coincided with receptor downregulation, indicating that RID’s inhibition of signal transduction through the TNF receptor is by elimination of TNFR1 from the cell surface. RID’s downregulation of surface TNFR1 and Fas was inhibited by hypotonic medium, indicating that clathrin-mediated endocytosis is probably involved in the mechanism of this effect.

### MATERIALS AND METHODS

#### Cell culture
293, HeLa, and A549 cultures were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen or Cellgro) with 10% fetal bovine serum (Gemini Bio-Products) and penicillin/streptomycin (Invitrogen or Cellgro).

#### Viruses
Ad/RID and Ad/null are E1- and E3-deleted adenoviral vectors, described in Table 1, and were kind gifts of William S. M. Wold (St. Louis University School of Medicine, St. Louis, Mo.). Ad/RID expresses the RID-α and RID-β open reading frames from the adenovirus type 2 (Ad2)-Ad5 chimera rec700 by alternative splicing of a transcript derived from the cytomegalovirus promoter inserted in the E1 region, and Ad/null is the corresponding negative control, which contains the cytomegalovirus promoter in the E1 region but no open reading frames. Ad-CMV-GFP was constructed in our laboratory, and d309 was a gift from Thomas Shenk (Princeton University, Princeton, N.J.) (Table 1). All infections were done in Dulbecco’s modified Eagle’s medium with fetal bovine serum (2 to 10%).

#### Plaque assays
Plaque assay titrations were performed in 60-mm dishes of confluent 293 cells by adsorbing monolayers in 0.2 ml of serial dilutions of virus, followed by overlaying cells with 0.9% granulated agar (Fisher) in Dulbecco’s modified Eagle’s medium with penicillin, streptomycin, and 2% fetal bovine serum. Cells were fed with this medium-agar mixture 7 days postinfection, and live cells were stained overnight 14 days postinfection with medium-agar mixture with neutral red solution (Invitrogen) prior to counting plaques for titer calculation. One PFU is equivalent to approximately 20 virus particles.

#### Cytokine treatment
Cells were treated with recombinant human TNF (R&D Systems) for indicated amounts of time by adding it directly to medium at final concentrations of 10 or 20 ng/ml, as indicated in the figure legend for each experiment.

#### Antibodies
Antibodies used in Western blot analysis were as follows: rabbit anti-IκB-α and β-tubulin, mouse monoclonal phospho-ε-Jun (Ser 63), ε-Jun, and mouse monoclonal TNFR1 were purchased from Santa Cruz Biotechnologies; monoclonal IKK-α antibody was purchased from BD PharMingen; monoclonal IKK-β and RIP antibodies were purchased from BD Transduction Laboratories; rabbit anti-RID-β antibody was kindly provided by Hans-Gerhard Burgert (University of Warwick, Coventry, United Kingdom).

Goat anti-TNFRI antibody was purchased from R&D Systems for use in immunoprecipitation. For use in flow cytometry experiments, biotinylated mouse immunoglobulin G2A anti-TNFRI, its corresponding biotinylated isotype control antibody, phycoerythrin (PE)-conjugated mouse immunoglobulin G1 anti-Fas antibody, and its corresponding PE-conjugated isotype control were purchased from BD Pharmingen.

#### Western blot analysis
Cell pellets were frozen in microcentrifuge tubes immediately after cytokine treatment and resuspended in 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue), by vortexing. Samples were boiled for 5 min on a heating block, and briefly centrifuged before resolution by SDS-PAGE on a 10% acrylamide–bisacrylamide gel (Bio-Rad) at 130 V for 1 h. Proteins were electrothermally transferred to Hybond C (Amersham) membrane at 50 V for 30 min, and blocked for 5 min in 5% nonfat dry milk in phosphate-buffered saline (PBS). All primary and secondary antibodies were used at a titer of 1:1,000, diluted in blocking solution overnight at 4°C in 5% nonfat dry milk in PBS. Membranes were washed three times in PBS and incubated with secondary antibody for 30 min at room temperature. Membranes were washed again and developed with Western Lightning reagent (Perkin Elmer) or Supersignal West Femto Maximum Sensitivity Sub-

### TABLE 1. Viruses used in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Expression from CMV promoter in E1 region</th>
<th>E1/E3 genotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad/RID</td>
<td>RID-α is from the Ad2 region of the Ad2-Ad5 recombinant rec700, and RID-β is from the Ad5 region of rec700</td>
<td>E1/E3 deleted</td>
<td>48</td>
</tr>
<tr>
<td>Ad/null</td>
<td>No ORFs in E1</td>
<td>E1/E3 deleted</td>
<td>48</td>
</tr>
<tr>
<td>d309</td>
<td>Wild-type E1</td>
<td>RID-α, 14.7K deleted</td>
<td>4, 26, 27</td>
</tr>
<tr>
<td>Ad-CMV-GFP</td>
<td>GFP</td>
<td>E1 deleted, E3 region from d309</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(RID-α, 14.7K deleted)</td>
<td></td>
</tr>
</tbody>
</table>
strate (Pierce). Blots were stripped with 0.2 N NaOH or Restore Western blot Stripping Buffer (Pierce) prior to reprobing with c-Jun or /H9252-tubulin antibodies.

Plasmids. Constructs expressing RID-/H9251 (pMT2-RID-/H9251), RID-/H9252 (pMT2-RID-/H9252), and the corresponding empty vector pMT2 were kindly provided by William S. Wold (St. Louis University School of Medicine). The NF-/H9260-dependent re-reporter plasmid pIg-/H9260-Luc was kindly provided by Bruce Horwitz (Whitehead Institute at the Massachusetts Institute of Technology, Cambridge), and the thymidine kinase promoter-driven Renilla luciferase reporter control plasmid pRL-Tk was from Promega.

Transfection and luciferase reporter assay. 293 cells (2x1 05) were seeded on six-well plates. Cells were transfected the following day with 100 ng of NF-/H9260-dependent luciferase reporter construct (pIg-/H9260-Luc), 10 ng of Renilla luciferase reporter construct (pRL-Tk), 400 ng of pMT2-RID-/H9260 plus 400 ng of pMT2-RID-/H9252-tubulin, 10 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Six hours prior to harvest, cells were treated with TNF-/H9260 (20 ng/ml) or left untreated. Twelve hours posttransfection, cells were harvested and luciferase reporter assays were performed with a luciferase assay kit (Promega) following the manufacturer’s protocols, using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Luciferase activities were normalized on the basis of Renilla luciferase activity.

Flow cytometry. Cells were harvested by scraping into medium and pelleted by centrifugation. Pellets were washed three times in ice-cold 0.5% bovine serum albumin in PBS, and incubated in FB (2% fetal bovine serum in PBS) for 5 min on ice. Cells were pelleted and resuspended in anti-Fas or anti-TNFR1 antibodies or isotype controls at 0.5 g/ml, and incubated on ice for 30 min. Cells were washed twice in FB. Cells stained for Fas receptor or its control were fixed in 2% paraformaldehyde (Tousimis) in PBS. Cells stained for TNFR1 were incubated in streptavidin-PE (Molecular Probes) at a final concentration of 1 g/ml for 30 min on ice, washed twice in FB, and fixed in 2% paraformaldehyde (Tousimis) in PBS. Fluorescence-activated cell sorting (FACS) analysis was performed with either a FACScan or FACSCalibur (Becton-Dickinson), and CellQuest software (BD Biosciences).

Immunoprecipitation of cell lysates. HeLa cells (8 x 106) were infected for 18 h with 1,000 particles/cell of Ad/RID or Ad/null. Prior to harvest, cells were treated with TNF-/H9260 (20 ng/ml) for 10 min or left untreated. Cell pellets were lysed in 1 ml of lysis buffer (0.1% NP-40, 1% Triton X-100, 150 mM NaCl, 1 mM
RESULTS

RID is both necessary and sufficient for the inhibition of NF-κB and AP-1 activation by TNF. Inhibition of the NF-κB pathway by RID was previously demonstrated in our laboratory by examining changes at the level of the IKK complex as well as downstream of this step including electrophoretic mobility shift assays on the nuclear components of the NF-κB transcription factors. However, our previous mapping studies utilized selected deletion mutants of the adenovirus E3 region and there were always other E3 proteins present during these experiments. The following studies used recently constructed adenoviruses expressing only RID-α/β to the exclusion of the other E3 proteins.

In order to determine the upstream target for the inhibition of NF-κB as well as extend the observations of RID’s effects to the AP-1 pathway, other steps on both of these signal transduction pathways were assayed. 293 cells were infected for 16 h with the E1- and E3-deleted adenovirus vector reconstituted to express RID from the cytomegalovirus promoter in place of the deleted E1A genes (Ad/RID) or a similar adenovirus vector which did not express any E1 or E3 genes (Ad/null) (Table 1). After treatment with TNF for 15 and 30 min, IκB-α was undetectable in Ad/null-infected cells; however, there was no degradation of IκB-α in Ad/RID-infected cells (Fig. 2A). Degradation of IκB-α, which tethers NF-κB in an inactive state in the cytoplasm, correlates with the activation of NF-κB. Because these infections were done in 293 cells that complement the E1 defect in viral vectors, adenovirus proteins other than those from the deleted E3 region could be expressed to normal levels and potentially could act together with RID-α and -β.

However, similar experiments were done in noncomplementing HeLa cells and yielded similar results. (Fig. 2B) Twenty minutes posttreatment with TNF, IκB-α was degraded in mock-infected or Ad-CMV-GFP-infected cells (E1 and RID deleted), but not in Ad/RID-infected cells. Thus, RID-α and -β are the only viral proteins necessary for the inhibition of TNF-induced IκB-α degradation. RID was able to inhibit TNF-induced IκB-α degradation also in A549 cells, but this effect was dependent upon a higher dose of virus (data not shown).

RID’s effects on TNF activation of the AP-1 pathway were also examined to determine not only the scope of RID’s inhibitory capacity, but also to assist in finding the target of RID’s block on the TNFR1 signaling pathway. Any inhibition of AP-1 signaling by RID would indicate either a separate target or a common upstream molecule necessary for both NF-κB and AP-1 inhibition. Molecules involved in both pathways, such as TRADD, TRAF-2, or RIP, or the receptor would be candidates for such investigations. Examination of the same extracts in Fig. 2B with an antibody that detects activated c-Jun showed that RID is also sufficient to block the phosphorylation of c-Jun on Ser 63 and subsequent TNF activation of AP-1 (Fig. 2C). These results are consistent with the target of RID’s block being at a molecule common to both pathways.

Further data to support this hypothesis were obtained by stimulating the AP-1 pathway independently of NF-κB. For example, the addition of adenovirus E1 genes, which were previously shown to stimulate AP-1 activity through the activation of MEKK1 (41), resulted in the phosphorylation of c-Jun independently of TNF and was not inhibited by RID (Fig. 2D). The experiment was performed in HeLa cells infected with the adenovirus mutant dl309, which is deficient in both RID proteins and 14.7K, but expresses all of the E1 proteins. IκB-α was degraded in response to TNF, and as expected for a virus containing the E1A region, c-Jun was phosphorylated independently of TNF. Coinfection of cells with equivalent amounts of dl309 and Ad/RID resulted in an inhibition of IκB-α degradation by TNF, but no reduction in the TNF-independent phosphorylation of c-Jun. This is consistent with the hypothesis that RID blocks TNFR1 signaling at or above MEKK1 (Fig. 1), most likely at one of the molecules common to the NF-κB and AP-1 pathways.
RID's inhibitory effects on NF-κB-dependent transcription were also demonstrated by luciferase assays with the pIg/H9260-Luc reporter (Fig. 3). Infection of 293 cells with Ad/RID blocked TNF induction of pIg/H9260-Luc, in contrast to cells infected with Ad/null (Fig. 3A). The RID plasmids pMT2-RID-α and pMT2-RID-β were able to inhibit TNF induction of pIgK-Luc, unequivocally proving that RID does not require any other viral proteins to block NF-κB-dependent transcription. (Fig. 3B).

RID inhibits signalosome formation in response to TNF. The effect of RID α/β on TNF-induced assembly of the signalosome at the receptor was measured by immunoprecipitation of TNFR1 followed by Western blotting for proteins bound to the receptor. The rapid, sequential assembly of TRADD, TRAF-2, RIP, and the IKK complex at the receptor has been well documented (2, 6). In HeLa cells infected with Ad/RID for 18 h, there was a decrease in the recruitment of IKK-α, IKK-β, and RIP to TNFR1 in comparison to the Ad/null control (Fig. 4). These results explain our previous findings of a RID-dependent decrease in TNF-induced IKK activity (15). A similar outcome occurred for the recruitment of TRAF-2 to the receptor (data not shown), indicating that RID’s block was above TRAF-2, probably at TRADD or the receptor itself.

Cell Surface Levels of TNFR1 are reduced in the presence of RID. As our results suggested that RID’s block in TNF-α signaling was located at or close to the TNF receptor, the amount of TNFR1 in the plasma membrane of the cells used in these experiments was quantified by FACS analysis. In suspension HeLa cells (15), we showed only an approximately 10% reduction of TNFR1. In the murine cell lines CL15.5 and C127, no downregulation of TNFR1 from the cell surface in the presence of RID was observed (42), and this issue was readdressed with the cell lines in which NF-κB signaling by TNF was reduced by RID and a new anti-TNFR1 antibody which detected TNFR1 with a higher sensitivity.

In 293 cells infected with either Ad/RID or Ad/null for 16 h, FACS analysis of cell surface levels of TNFR was performed (Fig. 5A), and demonstrated extensive reduction in cell surface expression of TNFR1 in Ad/RID-infected cells. Ad/RID-infected cells had an average sixfold reduction in geometric mean fluorescence of surface TNFR1, compared to Ad/null-infected cells. Eighty percent of the Ad/RID-infected cells were contained within region M1 (compared to 90% for isotype control), which was considered negative for surface TNFR1 (Fig. 5A). These results corresponded to the inhibition of IκB-α degradation shown in Fig. 2 as well as in assays done on aliquots of cells at the time of FACS analysis. These data provide compelling evidence for the reduction of TNFR1 as the target for RID’s inhibition of TNF activation of NF-κB and AP-1. Identical results were also demonstrated with monolayer HeLa cells (Fig. 5B).

The kinetics and amount of adenovirus required for the RID-induced downregulation of surface TNFR1 were measured and compared to the downregulation of Fas. At 8 h postinfection, a small downregulation of TNFR1 (Fig. 6A), and an intermediate downregulation of surface Fas (Fig. 6B) was observed in 293 cells. Concordant with this minor reduction in surface TNFR1, there was no inhibition of IκB-α degradation by TNF (Fig. 6C). Extensive downregulation of TNFR1 (Fig. 6D) and Fas (Fig. 6E) was observed by 12 h postinfection. Consistent with the downregulation of surface TNFR1, Ad/RID-infected cells were also resistant to TNF-induced IκB-α degradation (Fig. 6F). These results indicate that the inhibition of NF-κB activation by RID is dependent upon the downregu-
lation of the receptor, and that downregulation of TNFR1 and Fas from the surface is concurrent.

The extent of downregulation of surface TNFR1 and Fas is dependent upon the amount of Ad/RID virus added during infection, and the extent of downregulation of each receptor is nearly equivalent for each amount of input multiplicity of Ad/RID (Fig. 7). Similarly, dose-dependent degradation of total amounts of TNFR1 and Fas receptor by Ad/RID in 293 cells was also observed, although complete degradation of TNFR1 was not observed (Fig. 8). Failure of complete degradation of TNFR1 may be due to the fact that a large amount of TNFR1 resides in the Golgi (28), and therefore, this sequestered population is not able to be targeted by RID, unlike that on the cell surface.

TNFR1 downregulation by RID is inhibited by hypertonic medium. Hypertonic sucrose has been shown to be a selective inhibitor of clathrin-mediated endocytosis (19,20). To test the inhibitory capacity of hypertonic sucrose on the elimination of TNFR1 from the cell surface by RID, FACS analysis of TNFR1 and Fas was performed on cells infected with Ad/RID or Ad/null. Other laboratories have shown that RID internalizes Fas, epidermal growth factor receptor and TRAIL-R via clathrin-mediated endocytosis. Consistent with a clathrin-mediated mechanism of RID’s downregulation of surface TNFR1 and Fas, treatment of Ad/RID-infected cells with medium containing hypertonic sucrose not only reversed RID’s downregulation of Fas (Fig. 9A), but also resulted in an increase of surface Fas when compared to untreated Ad/null-infected cells. Ad/RID-infected cells treated with hypertonic medium also had a greater amount of surface TNFR1 than untreated Ad/RID infected cells, but less than surface levels of untreated Ad/null-infected cells (Fig. 9B). The differences in amounts of each of these receptors compared to control infection with Ad/null suggest that the molecular mechanism of RID down-

![Fig. 6. Kinetics of downregulation of receptors and inhibition of TNF-induced degradation of IκB-α. 293 cells were infected with 4,000 particles/cell of Ad/RID or Ad/null virus. At 8 or 12 h postinfection, surface levels of TNFR1 (A, D) or Fas (B, E) were measured by flow cytometry as in Fig. 5. Cells infected in parallel with Ad/RID or Ad/null were treated for 30 min with TNF (10 ng/ml) or left untreated. Cells were harvested at 8 h (C) or 12 h (F) postinfection and analyzed for IκB-α levels by Western blot.](http://jvi.asm.org/)

![Fig. 7. Multiplicity of infection requirements for RID’s downregulation of receptors. 293 cells were infected with various amounts of Ad/RID (3, 10, 30, or 100 PFU/cell) or 30 PFU/cell of Ad/null. Cells were analyzed for surface levels of TNFR1 or Fas, and the resulting geometric mean fluorescence intensities were plotted as percentages of geometric mean fluorescence obtained from Ad/null-infected cells (Ad/null fluorescence = 100, isotype control fluorescence = 0).](http://jvi.asm.org/)
regulation of Fas and TNFR1 may not be identical, but both involve a clathrin-mediated process.

**DISCUSSION**

Because we observed that RID inhibited the NF-κB and AP-1 pathway and a previous report indicated that TNFR1 was not downregulated at the surface of other cell lines, such as C15.5 and C127 murine cells (42), we began a systematic search for the step on these signal transduction pathways that might be affected by the Ad RID complex. Data presented in this manuscript demonstrate that the adenovirus type 5 RID complex is sufficient for inhibition of TNFR1 signaling in two human cell lines, as shown by RID’s ability to block key processes in NF-κB/H9260B and AP-1 activation, such as the degradation of IκB/H9260B and the phosphorylation of c-Jun, respectively.

Consistent with the inhibition of the NF-κB pathway by RID at the IKK step, results of luciferase assays showed that this block also affected NF-κB activation at the promoter level. Also, RID’s ability to inhibit the assembly of the TNFR1 signalosome at the receptor was demonstrated. We reexamined TNFR1 levels in 293 and monolayer HeLa cells. Similar to data presented for Fas and epidermal growth factor receptor, RID also was shown to be sufficient for the downregulation of TNFR1 from the cell surface, as shown by RID’s ability to block key processes in NF-κB and AP-1 activation, such as the degradation of IκB-α and the phosphorylation of c-Jun, respectively.

Kinetic studies showed that reduction of surface TNFR1 and Fas by RID occurs concurrently, and that RID’s inhibition of NF-κB activation by TNF coincides with an extensive downregulation of TNFR1. Varying the multiplicity of infection of Ad/RID demonstrated dose-dependent reductions of surface and total TNFR1 and Fas. At each multiplicity, the amount of reduction of surface TNFR1 or Fas was nearly identical. However, the dose-dependent degradation of total amounts of TNFR1 was incomplete, unlike the reduction of Fas to undetectable levels. This may be the result of RID’s inability to affect the TNFR1 population sequestered in the Golgi (28). Elimination of Fas and TNFR1 from the cell surface by RID was blocked in the presence of hypertonic medium, a known inhibitor of clathrin-mediated endocytosis (19, 20), indicating that RID probably internalizes TNFR1 through a clathrin-mediated mechanism similar to that of Fas, epidermal growth factor, and TRAIL receptors (21, 35). However, there are differences between the restoration of TNFR1 and Fas on the cell surface in the presence of sucrose, which suggest that there may be an additional mechanism of decreasing TNFR1 on the plasma membrane. In addition, it has also been reported that hypertonic sucrose can inhibit clathrin- and caveolin-independent endocytosis (39); thus, further dissection of cellular pathways involved in the internalization of TNFR1 by RID must be done to determine exactly which cellular processes are involved in RID-dependent endocytosis of TNFR1.

Uncovering the mechanism of action of individual E3 proteins is an important step in the development and use of E3 proteins as tissue-specific therapeutic agents in the inhibition of TNFR1.
of the host immune response in tissue transplants or autoimmune conditions (10, 11, 49; reviewed in 25). Understanding the immunomodulatory mechanisms employed by adenoviruses might also be helpful in developing antiviral therapies to combat fatal adenovirus infections in immunosuppressed patients, such as those following organ transplants (30). To these ends, interactions of other Ad E3 proteins, such as the antiapoptotic Ad E3–14.7K, with cellular proteins have been examined (reviewed in 13). For example, in our laboratory, four Ad E3–14.7K-interacting proteins (14.7-interacting proteins, FIP 1 to 4) that were novel at the time of their isolation were identified. FIP-3 (34) was shown to be identical to the third subunit of the IKK complex, IKK-γ/NEMO/IKKAP (36, 40, 52). Although expression of 14.7K was shown to reverse cell death induced by FIP-3 overexpression, this interaction did not result in the inhibition of NF-κB activation by TNF (15) or the inhibition of chemokine synthesis induced by TNF or lipopolysaccharide (32). The significance of the IKK-γ–Ad E3–14.7K interaction, although potentially fascinating, has yet to be determined.

Identification of cellular proteins that interact with RID-α/β have thus far identified complex formation between not only epidermal growth factor receptor and RID-α (8) but also an association between RID proteins and the adaptor proteins involved in clathrin-mediated endocytosis (21). These data complement the mutagenesis data demonstrating the requirement of the dileucine sorting motif on RID-α and the tyrosine sorting motif on RID-β for the reduction of cell surface Fas (21, 35), as well as data demonstrating the use of lysosomal trogic agents in the inhibition of Fas degradation (12, 44). Similar studies are in progress in the study of RID’s effects on TNFR1.

REFERENCES


