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The Amino-Terminal Portion of CD1 of the Adenovirus E1A Proteins Is Required To Induce Susceptibility to Tumor Necrosis Factor Cytolysis in Adenovirus-Infected Mouse Cells

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Previous work by our laboratory and others has shown that mouse cells normally resistant to tumor necrosis factor can be made sensitive to the cytokine by the expression of adenovirus E1A. The E1A gene can be introduced by either infection or transfection, and either of the two major E1A proteins, 289R or 243R, can induce this sensitivity. The E1A proteins are multifunctional and modular, with specific domains associated with specific functions. Here, we report that the CD1 domain of E1A is required to induce susceptibility to tumor necrosis factor cytolysis in adenovirus-infected mouse C3HA fibroblasts. Amino acids C terminal to residue 60 and N terminal to residue 36 are not necessary for this function. This conclusion is based on ^{51}Cr-release assays for cytolysis in cells infected with adenovirus mutants with deletions in various portions of E1A. These E1A mutants are all in an H5*dl*309 background and therefore they lack the tumor necrosis factor protection function provided by the 14.7-kilodalton (14.7K) protein encoded by region E3. Western blot (immunoblot) analysis indicated that most of the mutant E1A proteins were stable in infected C3HA cells, although with certain large deletions the E1A proteins were unstable. The region between residues 36 and 60 is included within but does not precisely correlate with domains in E1A that have been implicated in nuclear localization, enhancer repression, cellular immortalization, cell transformation in cooperation with *ras*, induction of cellular DNA synthesis and proliferation, induction of DNA degradation, and binding to the 300K protein and the 105K retinoblastoma protein.

The interactions between adenovirus and its host are complex. Persistent infections are common (16), suggesting that the virus has developed means to avoid elimination by the host immune response (reviewed in references 20, 52, and 76). For example, host cytotoxic T lymphocytes can recognize and destroy virus-infected cells. However, the adenovirus gp19K protein encoded by the E3 transcription unit can bind to host major histocompatibility complex class I and retain it in the endoplasmic reticulum, thus decreasing the amount of class I appearing on the cell surface (reviewed in references 20, 52, and 76). This interferes, in some cases, with cytotoxic T-lymphocyte recognition and destruction of virus-infected target cells (56, 67).

A second host system involved in the elimination of infected cells is the production of the cytokine tumor necrosis factor (TNF). TNF is known for its antitumor activity, inflammatory and immune responses, and antiviral activity (reviewed in references 3 and 51). It is directly lytic for cells infected by several viruses (7, 53, 79). Also, it inhibits the replication of a variety of RNA and DNA viruses, including adenovirus (42, 79).

Infection with adenovirus mutants can induce sensitivity to TNF cytolysis in cells normally resistant to the cytokine, such as the mouse fibroblast lines C3HA and NIH 3T3 (11, 19). To counter this sensitivity, adenovirus encodes another protein, 14.7K, also within E3, that protects virus-infected cells from cytolysis by TNF. Hence, although uninfected cells as well as cells infected with wild-type adenovirus are resistant to cytolysis by TNF, cells infected with virus mutants lacking the 14.7-kilodalton (14.7K) protein are sensitive to TNF lysis (19, 24). Sensitivity of virus-infected cells to TNF was subsequently shown to be a function of the E1A region in several (5, 11), although not all (70), cell lines. Using mutants that lack the 14.7K E3 protein, we found that viruses which produce either of the two major E1A proteins, 289R (289 amino acid residues) or 243R (243 amino acid residues), render infected cells sensitive to TNF cytolysis, while viruses that lack these proteins do not (11).

The 289R and the 243R proteins are products of alternatively spliced 13S and 12S mRNAs, respectively. They are identical except that the 289R contains a 46-amino-acid sequence lacking in the 243R product (Fig. 1). Both 289R and 243R proteins are multifunctional and have different domains required for a variety of functions (see Fig. 2), including nuclear localization, transcriptional activation, enhancer repression, cell immortalization, transformation in cooperation with *ras*, induction of cellular DNA synthesis and proliferation, and binding to various cellular proteins. The regions labeled CD1, CD2, and CD3 in Fig. 1 and 2 represent domains which are conserved among different adenovirus serotypes (30, 39, 46, and references therein), and the functions appear to map largely to one or more of these conserved domains.

The exact molecular mechanism by which TNF kills cells is unknown. However, certain aspects have been defined. TNF binds to specific receptors on the cell surface and is internalized and degraded (1, 32, 33, 40, 49, 58, 66, 68). This occurs in both sensitive and resistant cells. When normally resistant cells are treated with cyclohexamide or other inhibitors of protein synthesis, these cells become sensitive to TNF, implying that cellular proteins are essential for the

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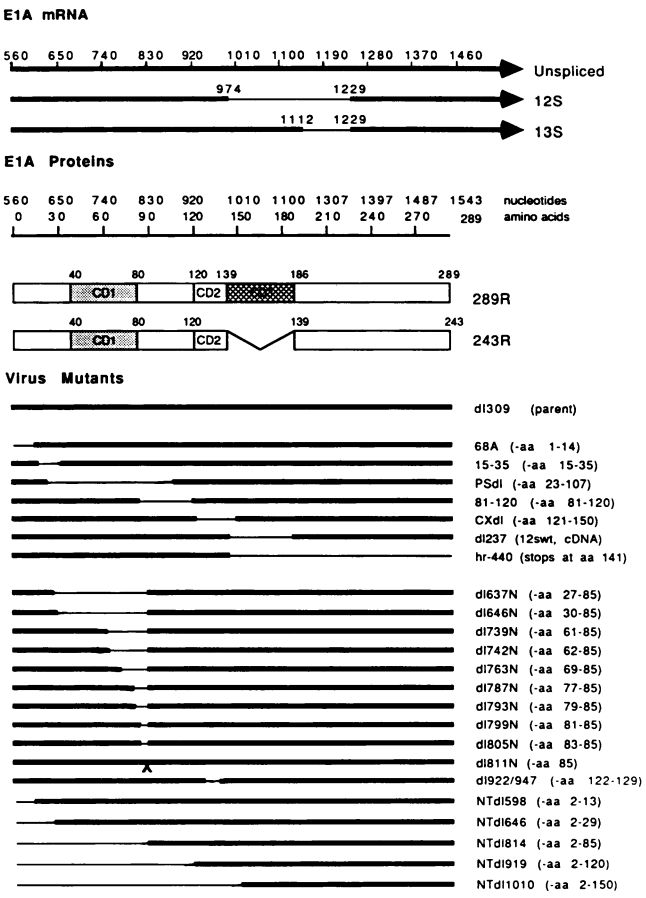

FIG. 1. Schematic map of adenovirus E1A 12S and 13S mRNAs, the 289R and 243R proteins, and the virus mutants used. The thin lines represent sequences deleted from the mutants.

resistant phenotype (31, 57, 72). Pretreatment of cells with TNF can protect them from subsequent killing by TNF plus cyclohexamide, suggesting that TNF can induce the synthesis of cellular protective proteins (21, 72) in cells that are TNF resistant. One of these induced proteins is manganous superoxide dismutase (77, 79), implying that the generation of reactive oxygen intermediates may be a mechanism of kill. Kill, as measured by the release of chromium from the cytoplasm, occurs over a period of 12 to 48 h (57) and, depending on the cell type studied, can occur by apoptosis (programmed cell death) or by necrosis (37).

Chen et al. (5) found that, of several *onc* genes tested, only E1A induced TNF sensitivity. This suggested that some feature unique to E1A was responsible. Since many functions of E1A have been localized by deletion mapping, mapping of TNF sensitivity to a region of E1A could (if it corresponded to a functional domain) shed light on a mechanism by which E1A induces TNF sensitivity and, in turn, tell us more about how TNF kills cells. To do this, we utilized a panel of virus mutants lacking region E3, including the 14.7K gene, and also deleting various parts of the E1A region. Cells infected with mutants lacking the sensitizing region should be resistant to TNF, while cells infected with mutants carrying this region should be sensitive. Our results show that only mutants carrying the CD1 region are capable of inducing TNF sensitivity and that only a part of this region is essential for induction.

MATERIALS AND METHODS

Materials. Human recombinant TNF (rTNF) was a generous gift from the Cetus Corp., Emeryville, Calif.; $Na^{51}CrO_4$ was obtained from New England Nuclear, Boston, Mass.; and fluorescein-conjugated anti-rabbit 7S globulins produced in goats were obtained from Meloy, Springfield, Va.

Cells and viruses. C3HA cells are an immortalized but not fully transformed 3T3-like cell line derived from mouse embryo fibroblasts (18). Cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Intergen, Purchase, N.Y.).

The adenovirus mutants H5*dl*309 (*dl*309) (28) and H5*sub*304 (*sub*304) (27) were obtained from T. Shenk, Princeton University, Princeton, N.J. *dl*309 lacks the E3B region, including the 14.7K gene, but has a wild-type E1A region. *sub*304 also lacks the E3B region, including the gene coding for the 14.7K protein, and thus is similar to *dl*309. Mutants 68A, 15-35, PS*dl*, 81-120, CX*dl*, and *hr*-440 (47, 62, 82) were obtained from E. Moran, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Mutants *dl*637N, *dl*646N, *dl*739N, *dl*742N, *dl*763N, *dl*787N, *dl*793N, *dl*799N, *dl*805N, *dl*811N, *dl*922/947, NT*dl*1010, NT*dl*919, NT*dl*814, NT*dl*646, and NT*dl*598 (75) were obtained from E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. The above mutants are all adenovirus type 5 constructs and were grown in monolayers of human 293 cells as described before (11). Nearly confluent monolayers in 150-cm^3 flasks were infected with 1.0 ml of virus for 1 h in a 37°C CO_2 incubator. After 2 days, when extensive cytopathic effect was apparent, cells were scraped into the medium, collected by centrifugation, resuspended in 1 ml of medium plus 1.5% fetal calf serum per flask, freeze-thawed three times, sonicated, and centrifuged at 2,000 rpm for 10 min in a centrifuge (International, Needham Heights, Mass.). The supernatant was quick-frozen and stored at −70°C.

Cytotoxicity assays. Cells were plated at a density of 8×10^5/60-mm plate. Some 5 to 6 h later, the cells were infected with up to 200 PFU of virus per cell. Cells were labeled by adding 200 μCi of $Na^{51}CrO_4$ to the media at least 3 h prior to harvest. Twenty-two hours after viral infection, cells were harvested for use in experiments. However, in designated experiments, cells were harvested 46 h postinfection. Cells were trypsinized, washed, and added to wells of 96-well microtiter plates (10^4 cells per well in a total volume of 200 μl) containing either medium alone (to determine spontaneous release) or the designated concentration of TNF. After an 18-h incubation at 37°C in 8% CO_2, 1 N HCl was added to some triplicate wells to determine the maximum releasable counts, and the plates were centrifuged at 180 × *g* for 10 min. A 100-μl portion of supernatant was removed from each well, and the radioactivity was determined. The percent specific ^{51}Cr release was determined based on the following formula: [(experimental − spontaneous release)/(maximum − spontaneous release)] × 100. All determinations were done in triplicate. Spontaneous release of ^{51}Cr by cells incubated in medium alone ranged from 25 to 39%. In all experiments, indirect immunofluorescence was done to determine whether all cells were infected. Infected cells were plated onto spot plates (Shandon Southern Instruments, Inc., Sewickley, Pa.), fixed, and incubated with adenovirus type 5 virion antiserum (from American Type Culture Collection). Cells were then washed and treated with fluorescein-conjugated anti-rabbit 7S globulins (produced in goats)

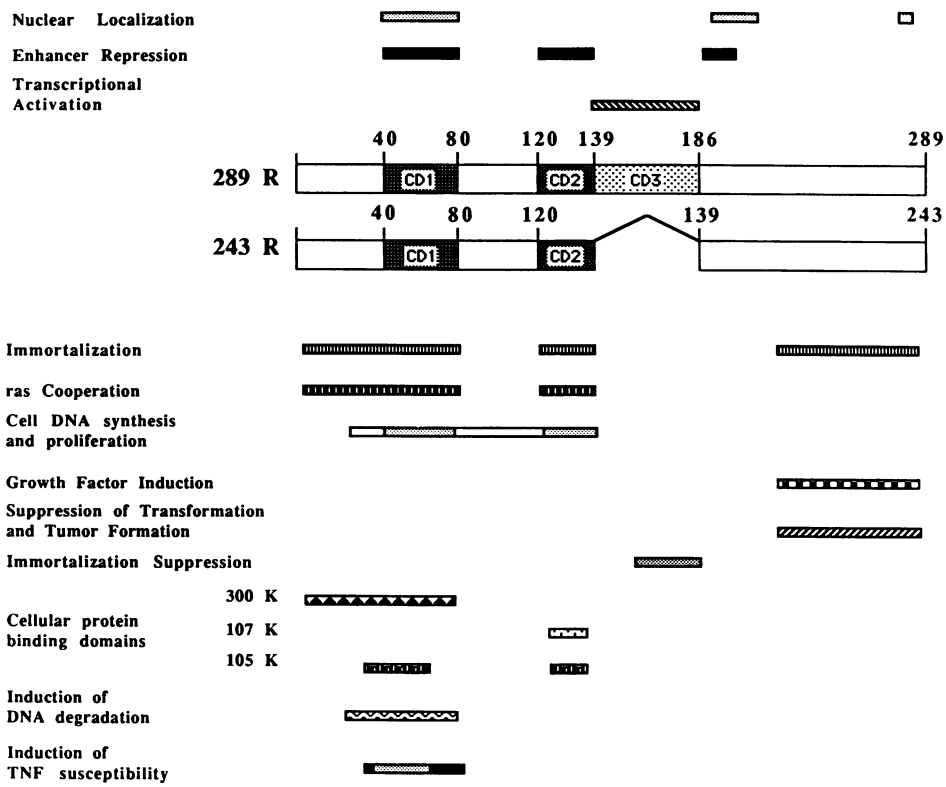

FIG. 2. Schematic representation showing the two major E1A proteins and indicating the functions mapped to the various domains. This schematic is based on data from a number of laboratories (2, 15, 25, 26, 34–36, 38, 39, 43, 46–48, 50, 55, 59, 60, 62, 64, 65, 69, 71, 73–75, 81, 82 and references therein). The region involved in inducing DNA degradation was mapped by E. White (72b). Conserved domains 1 and 2, but not conserved domain 3, have also been reported to be required to induce brain creatinine kinase in HeLa cells (29).

before determining infection rates. In all experiments reported here, cells were >80% infected.

Western blots (immunoblots). Cells were infected with the various virus mutants as described above. After 22 h, infected cells were removed from the plates with a virus scraper and transferred to tubes containing phosphate-buffered saline. The cells were centrifuged and the phosphate-buffered saline was removed. A 200-μl amount of lysis buffer (1% Nonidet P-40, 20 mM Tris chloride [pH 8.0], 150 mM NaCl, 1% aprotinin) was added, and the cells were vortexed and allowed to stand for 20 min on ice. The lysates were then microfuged for 10 min and the supernatant was removed. Supernatants were stored at −70°C until used.

Protein concentrations were determined for each lysate by the BCA (bicinchoninic acid) method (61), and equal amounts (18 μg) of protein were loaded onto lanes of a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose with a semidry blotter (Bio-Rad Laboratories, Richmond, Calif.). The nitrocellulose was blocked overnight in NET gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris base, 0.02% NaN_3, 0.05% Nonidet P-40, 0.25% gelatin, 5% nonfat dry milk, 10 U of heparin per ml), washed three times for 10 min each in TTBS (20 mM Tris chloride [pH 7.5], 500 mM NaCl, 0.5 ml of Tween 20 per liter), and then blotted in three steps. Initially, anti-E1A (a pool of equal volumes of supernatants from cells producing antibodies M1, M2, M37, M58, and M73 [22, 63]) dissolved in antibody buffer (1% gelatin dissolved into TTBS; 200 μl of antibody per 12 ml of buffer) was added to the nitrocellulose in a Seal-A-Meal bag, and the nitrocellulose was incubated for 2 h at room temperature with rocking. The nitrocellulose was removed, washed three times for 5 min each in TTBS, and then incubated with rabbit anti-mouse immunoglobulin (Cappel, Cochranville, Pa.) in antibody buffer (20 μl/12 ml) for 2 h with rocking. The nitrocellulose was again removed, washed three times for 5 min each with TTBS, and incubated for 2 h with rocking in ^{125}I-labeled protein A (5 μCi; ICN, Costa Mesa, Calif.) dissolved in SPA buffer (20 mM Tris chloride [pH 7.5], 150 mM NaCl, 25 mg of bovine serum albumin per ml, 0.02% NaN_3) (2 μl/12 ml). The nitrocellulose was then removed, washed four times for 20 min each in TTBS and twice in TBS (20 mM Tris chloride [pH 7.5], 500 mM NaCl) for 5 min each and rinsed in distilled water. The nitrocellulose was then air dried and exposed to X-ray film. The film was developed 2 days later.

RESULTS

Most but not all of the mutant E1A proteins are stable in mouse C3HA cells. Figure 1 depicts the E1A deletions in the adenovirus mutants that we have analyzed in mapping the region in E1A responsible for inducing susceptibility to TNF cytolysis in mouse C3HA cells. Figure 2 summarizes the functions that have been ascribed to the E1A 289R and 243R proteins as well as the domains that have been determined through the analysis of mutants to encode these functions. For many of the mutants in Fig. 1, the synthesis of the E1A proteins has been determined in human HeLa cells by pulse-labeling with [^{35}S]Met followed by immunoprecipita-

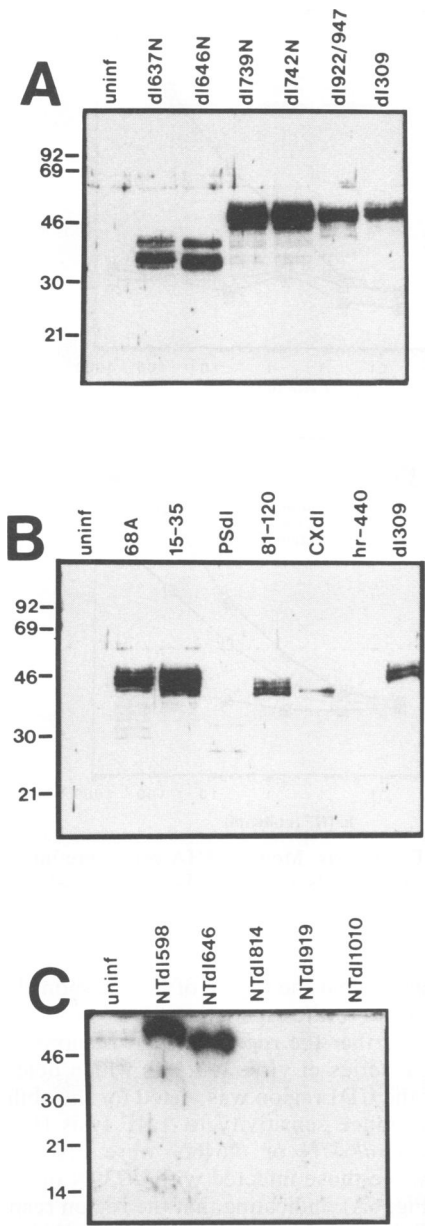

FIG. 3. Western blot analysis of E1A proteins produced in cells infected with virus mutants. Lysates were prepared from infected cells as described in Materials and Methods, separated by SDS-PAGE, transferred to nitrocellulose, blotted with anti-E1A, incubated with ^{125}I-labeled protein A, and exposed to X-ray film. Molecular weight markers (10^3) are indicated.

tion and SDS-polyacrylamide gel electrophoresis (PAGE) (44, 62, 75). However, since we were analyzing mouse C3HA cells, we examined whether the mutant E1A proteins were stable in these cells by using Western blot analysis, which measures the steady-state level of the E1A proteins. Western blotting was carried out with a pool of the monoclonal antibodies M1, M2, M37, M58, and M73.

The E1A proteins from most of the mutants appear to be at least as abundant as the E1A proteins from *dl*309 (Fig. 3). However, lesser amounts of the E1A proteins were obtained from mutant CX*dl*, less from PS*dl*, and even less from *hr*-440 (Fig. 3B). The proteins from *hr*-440 cannot be seen in this exposure, but are visible upon overexposure of the gel. After very long exposures, a trace of E1A protein was detected from NT*dl*814, but no E1A protein was detected from NT*dl*919 or NT*dl*1010 (Fig. 3C). In general, the E1A proteins from mutants with the larger deletions tended to be present at lower steady-state levels; this probably indicates that these E1A proteins were unstable, although it could mean that they were transcribed or translated in low amounts. Given that the level of E1A protein may correlate with the degree of susceptibility to TNF cytolysis in transfected cells (7, 70), it is important to consider the levels of E1A proteins from the mutants when evaluating the cytolysis data that follow.

The TNF-sensitizing region of E1A includes the N-terminal portion of CD1. *dl*309 is the parent virus from which all E1A deletion mutants used were derived. *dl*309 lacks the E3B region, which includes the 14.7K gene but contains a complete, wild-type E1A region. Hence, mouse C3HA cells infected with *dl*309 are sensitive to TNF (11). The virus mutants 68A, 15-35, PS*dl*, 81-120, CX*dl*, and *hr*-440 delete various large regions covering the entire E1A region (Fig. 1). C3HA cells were infected with each of these mutants and tested for TNF sensitivity. The results are shown in Fig. 4A to D. Cells infected with mutants 68A and 15-35 were sensitive to TNF (panel A), showing that the sensitizing region does not lie in the region N terminal to CD1. However, cells fully infected with mutant PS*dl* were resistant to TNF (panel B), even when the infection was allowed to proceed for 46 h before exposure to TNF (data not shown). This suggests that the CD1 region deleted in PS*dl* is necessary for TNF sensitization. The relatively low level in E1A from PS*dl* could account for the lack of TNF sensitivity, but other data (shown below) indicate that CD1 is important in TNF sensitization. Also, PS*dl* made nearly as much E1A protein as CX*dl* and more than *hr*-440, and the latter two mutants did induce TNF sensitivity (see below). Cells infected with mutant 81-120, deleting the region between CD1 and CD2, were sensitive to TNF (panel C), as were cells infected with the CD2 deletion mutant CX*dl* (panel D). In this experiment, cells infected with CX*dl* appear somewhat more resistant than cells infected with *dl*309, but this was not a consistent finding. Therefore, neither of these regions is required to produce TNF sensitivity.

To confirm our observation that the CD2 region is not necessary to produce TNF sensitivity, we tested mutant *dl*922/947, which also lacks the CD2 region (see Fig. 6C). Similar to the results with CX*dl*, cells infected with *dl*922/947 were sensitive to TNF cytolysis, verifying that CD2 is not necessary for the TNF-sensitive phenotype.

Mutant *hr*-440 deletes most of the C-terminal half of E1A (Fig. 1). When cells infected with *hr*-440 were tested for TNF sensitivity 22 h postinfection (as was done with the other mutants), the cells were largely resistant to TNF, although some kill was observed (Fig. 5). This mutant deletes the CD3 region as well as the regions C terminal to CD3. The CD3 region is required to transactivate adenovirus early genes, including E1A itself. Therefore, a TNF-resistant phenotype might simply be due to reduced E1A expression caused by the CD3 deletion. To test this, infections were allowed to proceed for an additional 24 h (for a total of 46 h), allowing additional E1A expression before exposure to TNF. Cells treated in this manner were fully sensitive to TNF (Fig. 5), showing that neither the CD3 region nor the sequences C terminal to it are required to produce TNF sensitivity. These

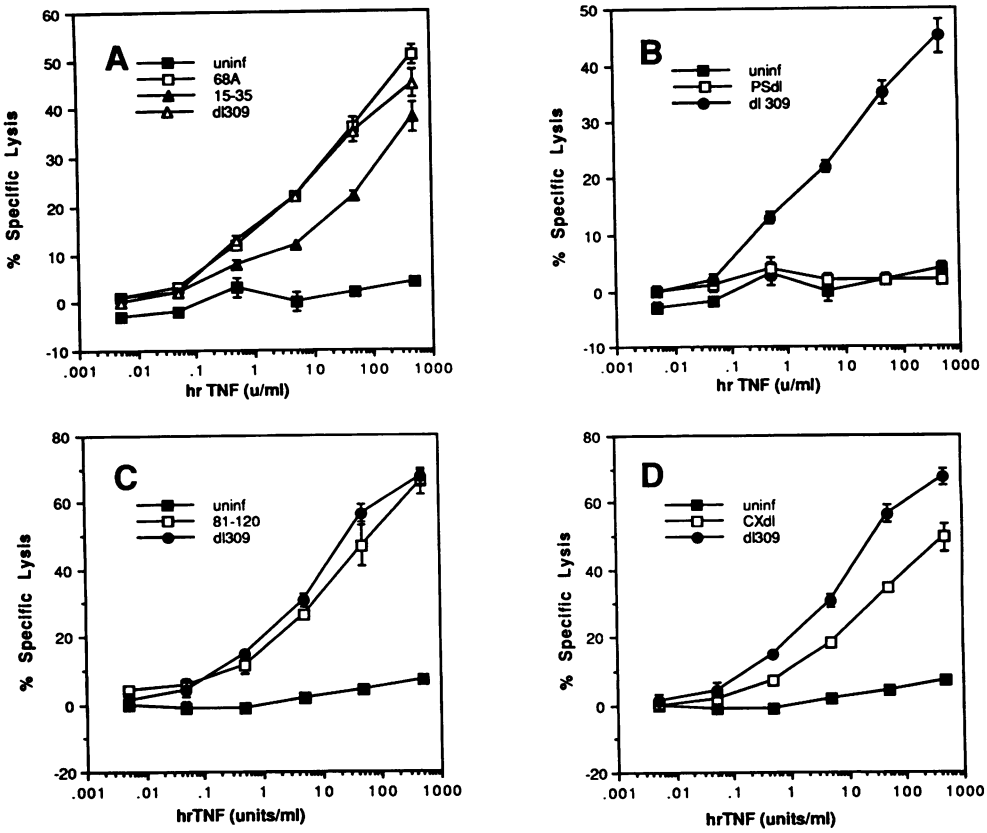

FIG. 4. Evidence that the CD1 region is required to induce susceptibility to TNF cytolysis. Mouse C3HA cells were infected with the mutants as shown and tested for TNF sensitivity in the cytolysis assay as described in Materials and Methods. Bars indicate standard error of the mean.

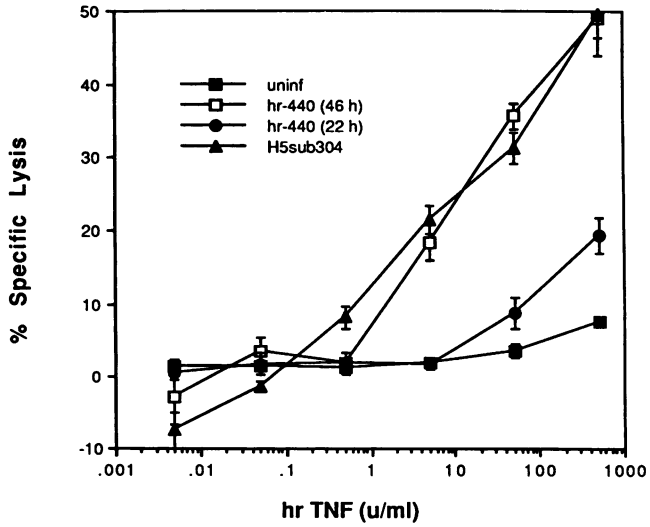

FIG. 5. Evidence that neither the CD3 region nor sequences C terminal to it are required for TNF cytolysis. Cells were infected with *hr*-440 for either 22 or 46 h, or with H5*sub*304 for 22 h, prior to harvest and assay for TNF sensitivity.

mutants suggest that the region of E1A responsible for TNF sensitivity maps to the vicinity of CD1.

To define further the region of E1A responsible for TNF sensitivity, a series of virus mutants which deleted various portions of the CD1 region was tested for the ability of these mutants to induce sensitivity to TNF lysis (Fig. 1). Cells infected with *dl*637N or *dl*646N were resistant to TNF cytolysis, while those infected with *dl*739N or *dl*742N were sensitive (Fig. 6A), indicating that the region responsible for inducing TNF sensitivity is missing in *dl*637N and *dl*646N but present in *dl*739N and *dl*742N. This implicates the N-terminal end of CD1. Consistent with this, cells infected with *dl*763N, *dl*787N, *dl*793N, and *dl*799N (Fig. 6B), as well as *dl*805N and *dl*811N (Fig. 6C), were all sensitive to TNF cytolysis. Importantly, the abundance of E1A proteins from *dl*637N and *dl*646N was roughly equivalent to that from *dl*309 as well as to that from the other E1A mutants in this series (Fig. 3A) and were much more abundant than in cells infected with CX*dl* and *hr*-440 (Fig. 3B). Thus, the lack of TNF cytolysis by *dl*637N and *dl*646N was not due to a lack of E1A proteins.

Five additional E1A mutants which delete increasing amounts of the gene from the opposite direction of the previous series were also tested (Fig. 1). Cells infected with NT*dl*598 and NT*dl*646 were sensitive to TNF cytolysis, while those infected with NT*dl*814, NT*dl*919, and NT*dl*1010 were resistant (Fig. 7). These data are consistent with our previous results which show that mutants which contain the N-terminal end of the E1A gene can induce TNF sensitivity,

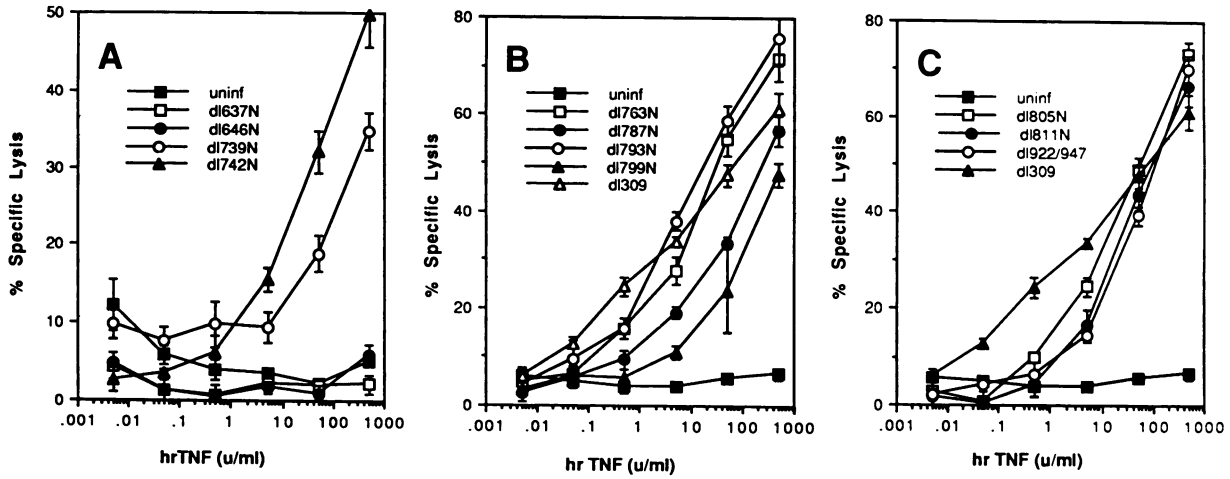

FIG. 6. Evidence that residues C terminal to amino acid 60 of E1A are not required for TNF sensitivity. Cells were infected with the mutants as shown and tested for TNF cytolysis.

whereas those that delete the N-terminal portion of CD1 cannot. However, with NT*dl*814, NT*dl*919, and NT*dl*1010 the lack of TNF sensitivity could also be due to the paucity of E1A proteins (Fig. 3C).

DISCUSSION

These results indicate that the region of E1A responsible for inducing TNF susceptibility in virus-infected C3HA cells resides in the N-terminal portion of CD1. Amino acids to the left of residue 36 or to the right of residue 60 are not necessary for the induction of TNF sensitivity. Three mutants which delete in the CD1 region confirm the involvement of CD1: PS*dl*, *dl*637N, and *dl*646N. Cells infected with any of these mutants produced E1A proteins, were TNF resistant, and, unlike the results with *hr*-440, allowing the infection to proceed further before the addition of TNF, did not induce sensitivity. Nearly all cells were actively infected with PS*dl*, *dl*637N, or *dl*646N, as determined by immunofluorescence with adenovirus type 5 antisera, indicating that the lack of TNF cytolysis was not due to poor infection. Also, although NT*dl*814, NT*dl*919, and NT*dl*1010 produced barely detectable or undetectable steady-state levels of E1A proteins, enough E1A presumably was made within the time of the standard assay conditions to transactivate the adenovirus gene products that we detected by immunofluorescence. Therefore, the lack of TNF sensitivity observed with these mutants could also be due to functional defective E1A proteins and not simply to a lack of E1A proteins.

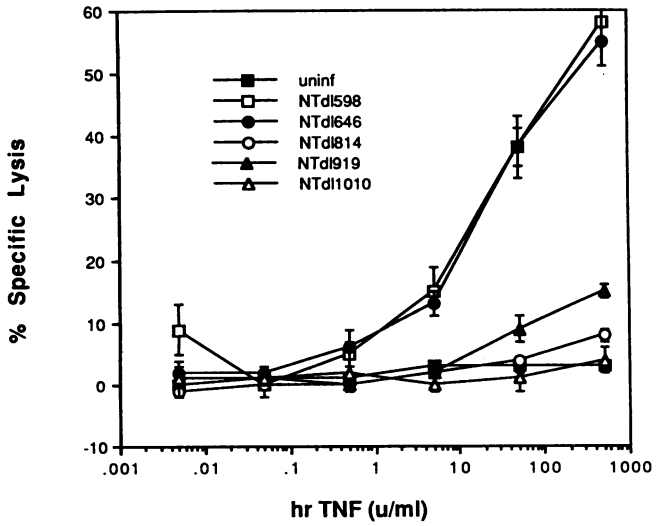

FIG. 7. C3HA cells were infected with the E1A N-terminal deletion mutants as shown and examined for TNF cytolysis.

The lack of a requirement for CD2 is confirmed with two separately derived deletion mutants, CX*dl* and *dl*922/947, both of which display the TNF-sensitive phenotype. Our results with mutants 68A, 15-35, 81-120 demonstrate that regions N terminal to CD1 (i.e., residues 1 to 35) and between CD1 and CD2 (residues 81 to 120) are also unnecessary for the TNF-sensitive phenotype. Our previous results and those of others indicated that both the 289R and the 243R proteins are capable of inducing TNF sensitivity (5, 7, 11). Since 243R lacks CD3, this region is not essential. These results were confirmed by our data with *hr*-440. Cells infected with this mutant displayed little TNF sensitivity in our conventional 22-h ^{51}Cr-release assay, but when the infection was allowed to proceed for an additional 24 h before the addition of TNF, the cells became fully sensitive. This indicates that both the C-terminal region of E1A and CD3 are unnecessary for the induction of TNF sensitivity.

Figure 2 shows a schematic map of the E1A proteins and indicates the regions which have been associated with various functions. The region mapped in this study for the induction of TNF susceptibility is also indicated. It is clear that this region, near the N-terminal end of CD1, does not correlate exactly with any of the other mapped functions. Several of the functions with which it does seem to colocalize, such as nuclear localization, enhancer repression, immortalization, cooperation with *ras*, induction of cellular DNA synthesis and proliferation, and binding to the 105K (retinoblastoma) and 300K proteins, all require regions in addition to that in CD1. Our study shows that these additional regions are not necessary for the induction of TNF sensitivity. Therefore, it is possible that our study has defined a new functional domain in E1A. However, since the mapped functions represent phenomena which may be complex and involve more than one system, it is possible that the colocalization of TNF susceptibility with these other func-

tions may indeed signify a common mechanism. Species differences may be responsible for some variation, since our studies were done in mouse cells and the studies by others were done in human or rat cells.

At present we are unable to analyze E1A function in human cells because of interference caused by E1B-19K. E1B-19K in the infecting virus suppresses TNF-mediated killing of human but not mouse cells (18a). However, triple mutants lacking E3, E1B-19K, and relevant regions of E1A will be tested to confirm these findings in human cells as soon as the viruses become available.

It should also be noted that the mutants used here have not been assayed for all of the functions shown in Fig. 2 and that in some cases there is even disagreement as to the properties of the mutants. These considerations make it difficult to determine definitively whether this TNF sensitivity-inducing region is indeed a new functional domain or whether it colocalizes with previously mapped domains.

A correlation between E1A transforming regions with binding to E1A-associated proteins has been noted (15, 62). It is interesting that the TNF sensitization region correlates with a region involved in the binding of the 105K retinoblastoma protein. Mutations in two E1A regions interfere with the ability of E1A to bind the human retinoblastoma protein. One region lies between amino acids 30 and 60, and the second lies between amino acids 121 and 127 (14, 15, 73, 75). The retinoblastoma protein appears to be involved in cell cycle regulation, utilizing a phosphorylation/dephosphorylation regulatory mechanism (4, 6, 8, 10, 41). The ability of E1A to bind to the retinoblastoma gene product may signify a mechanism for E1A's ability to immortalize cells, cooperate with *ras*, and/or induce cellular DNA synthesis and proliferation. If a region responsible for E1A binding to the retinoblastoma protein is also involved in inducing TNF sensitivity, this may shed light on immune surveillance and suggest a mechanism for recognition and destruction of transformed cells.

In human cells, binding of the retinoblastoma protein to E1A requires two regions, one of which is not required for the induction of TNF sensitivity in C3HA cells. While mouse cells do produce retinoblastoma protein (12), it is unclear whether mouse and human retinoblastoma proteins display the same binding behavior.

In addition to binding to the 105K retinoblastoma protein, the E1A proteins bind to a number of additional cellular products, including proteins of approximately 300K, 130K, 107K, 90K, 80K, 68K, 65K, 60K, 50K, 40K, and 28K in molecular mass (13, 23, 75, 80). The functions of these cellular proteins have yet to be determined, although binding to the 300-kD protein may play a role in E1A-mediated transformation (62). Binding to cyclin A, a 60-kD *cdc2*-associated polypeptide has also been reported (17, 54). The regions of E1A required for binding to these proteins are mostly unknown, but the 107K binding has been mapped to amino acids 121 to 127 and the 300K binding domain overlaps that required for induction of TNF sensitivity (15, 75). It may be that association between E1A and one or more of these proteins is part of the molecular mechanism mediating TNF sensitivity or resistance. In the case of the 300K protein, E1A proteins from *dl*637N, *dl*646N, *dl*739N, *dl*742N, and *dl*763N do not bind to the 300K in human cells, whereas only *dl*637N and *dl*646N are defective in TNF sensitization in mouse cells. Thus, as with the retinoblastoma protein, there is no strict correlation. But again, perhaps the putative 300K protein from mouse cells has different binding requirements to E1A than does the protein from human cells.

It is intriguing that the region required for TNF sensitivity lies within the region that has been shown by E. White (72a) to be required to induce a state that leads to DNA degradation in cells. It would not be surprising if induction of TNF sensitivity and induction of DNA degradation were not related phenomena associated with a program(s) leading to cell death. Indeed, this may be a response that evolved in the host to eliminate adenovirus-infected cells.

That only a small portion of the E1A molecule appears to be necessary to induce TNF sensitivity suggests that a specific mechanism, perhaps involving interaction of a single specific molecule with E1A, may be responsible for determining a cellular susceptibility to TNF. Alternatively, it is possible that this CD1 sequence is essential for stabilizing two or more other functional domains of the protein, when each of these domains must be deleted to eliminate the function. Understanding this mechanism will be important in understanding TNF cytolysis and perhaps in the eventual development of therapeutic approaches to both infection and cancer.

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