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Induction of Sensitivity to the Cytotoxic Action of Tumor Necrosis Factor Alpha by Adenovirus E1A Is Independent of Transformation and Transcriptional Activation

ROBERT S. AMES,[1]* BEVERLY HOLSKIN,[1] MICHELLE MITCHO,[2] DAVID SHALLOWAY,[2] AND MANN-JY CHEN[1]

Department of Molecular Genetics, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-0939,[1] and Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802[2]

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We have previously shown that expression of the adenovirus E1A 12S or 13S products in NIH 3T3 fibroblasts induces susceptibility to the cytotoxic actions of tumor necrosis factor alpha (TNFα). A large number of studies have mapped the multiple biological functions of the 12S and 13S products to three highly conserved regions (CR) within the E1A sequence. Here we used plasmids coding for E1A deletion and point mutants in these regions to generate target cell lines for TNFα cytotoxicity assays to determine which regions and functions are necessary for the induction of TNFα sensitivity. Expression of CR1 was required for the induction of TNFα sensitivity. This finding did not reflect a requirement for transforming or transcriptional repression activity, since some mutants that were defective in both of these properties were able to induce TNFα sensitivity. CR2 transformation-defective point mutants, but not a CR2/3 region deletion mutant, were also able to induce sensitivity. In addition, NIH 3T3 cells expressing the retroviral transcription activators *tat* from human immunodeficiency virus type 1 and *tax* from human T-lymphotropic virus type I were not sensitive to TNFα. However, the possibility that E1A-mediated transcriptional activation can augment the induction of TNFα sensitivity is not excluded. Comparison of data from previous biological studies with the TNFα cytotoxicity assays presented here suggested that the mechanism by which E1A induces sensitivity to TNFα in NIH 3T3 cells is independent of many of the known E1A biological functions, including transformation in cooperation with *ras*, immortalization, induction of DNA synthesis in quiescent cells, and transcriptional repression. A novel E1A-mediated effect may be involved, although our data do not exclude the possibility that sensitization to TNFα is mediated through E1A binding to cellular proteins.

Tumor necrosis factor alpha (TNFα), a product primarily of activated macrophages, is cytostatic or cytotoxic for a variety of transformed cell lines. The sensitivity of target cells to TNFα does not correlate with the number or the affinity of the TNF receptors (53). The mechanism of cytotoxicity by TNFα has not yet been fully elucidated. It may involve inhibition of phospholipase A_2 activity (1, 52), ADP ribosylation (2), inhibition of rRNA processing (10), increased lysosomal activity (35), or increased hydroxyl radical production (67). Recent evidence has suggested that TNFα induces manganous superoxide dismutase and that this activity may be necessary for resistance to TNFα cytotoxicity (64, 66).

TNFα has antiviral as well as antineoplastic properties and is directly cytotoxic to herpes simplex virus type 1 (HSV-1)-infected cells (30). It also has a synergistic antiviral effect with gamma interferon on HSV-1 replication (18), as well as inhibitory activity on vesicular stomatitis virus, encephalomyocarditis virus, adenovirus type 2, and HSV-2 (39, 65). The role(s) of TNFα in human T-lymphotropic virus type I (HTLV-I) and immunodeficency virus (HIV) infections is less clear. TNFα expression is induced (57) in HTLV-I-infected T cells, and in some cell lines TNF even stimulates HIV replication (25).

Adenovirus early-gene products have been shown to effect sensitivity to TNF cytolysis. Expression of adenovirus E1A renders NIH 3T3 cells susceptible to TNFα killing (12, 16), whereas expression of a variety of other oncogenes, including v-*src*, c-*src*, Ha-*ras*, c-*myc*, and polyomavirus middle and large T, does not (12). Enhanced TNFα sensitivity may partially or fully account for the fact that E1A induces sensitivity to macrophage and natural killer cell-mediated cytotoxicity (14, 15, 48), and both of these factors may be involved in the reduced tumorigenicity of adenovirus-transformed cells in animals (34). In addition to E1A, a 14,700-molecular-weight protein encoded by the adenovirus E3 transcription unit alters sensitivity to TNF; cells expressing this protein are protected from lysis by TNF (22).

The relationship between the TNF sensitization and the other multiple biological activities has not been explored. E1A expression transcriptionally activates other adenovirus early genes and can repress or stimulate expression of other viral enhancers and cellular genes (8, 36, 37, 54, 55, 58, 59, 69; reviewed in reference 7). E1A can stimulate DNA synthesis in quiescent cells and immortalize primary cell cultures, although its expression alone is not sufficient for transformation. However, E1A can cooperate with adenovirus E1B or other oncogenes, including *ras* and c-*src*, in transformation of primary and immortalized cell cultures (49, 50).

Deletion and point mutations can separably inhibit E1A transcription-modulation, immortalization, and transformation-cooperation activities, indicating that these functions are physically and functionally distinct (31, 38, 43; reviewed in reference 41). E1A generates two overlapping mRNAs, designated 12S and 13S, encoding nuclear-localized phosphoproteins of 243 and 289 amino acids, respectively. These contain three regions that are highly conserved between

* Corresponding author.

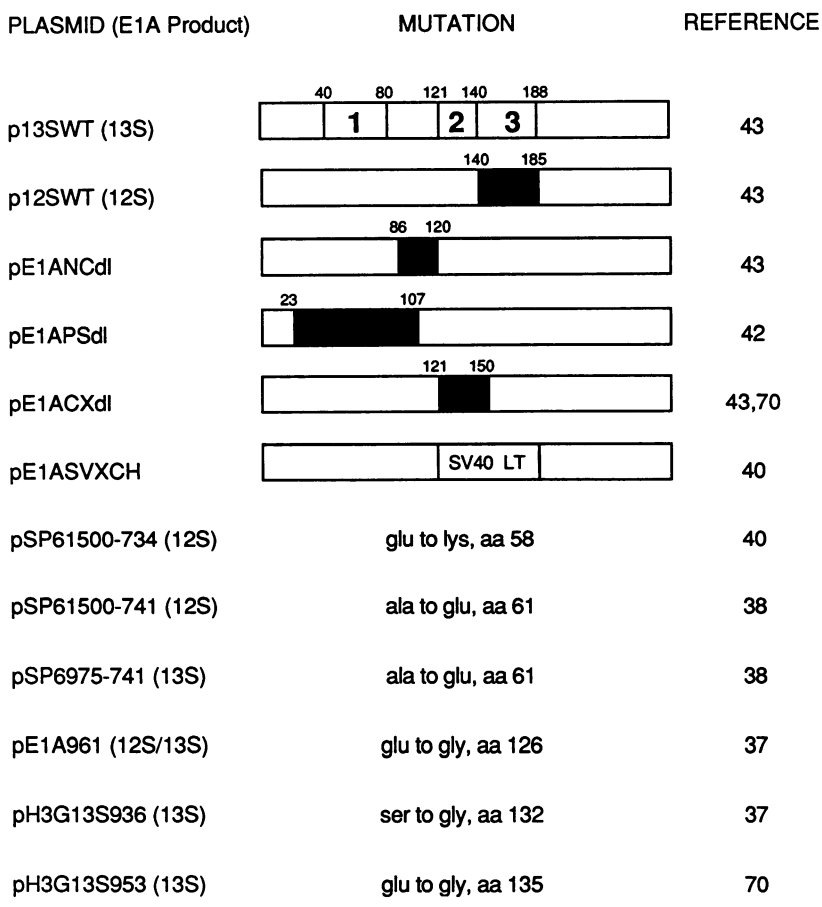

FIG. 1. Adenovirus E1A mutants analyzed for their ability to induce sensitivity in NIH 3T3 cells to the cytotoxic actions of TNF. The locations of the CR1 to -3 of E1A are indicated at the top. Black regions indicate sequences deleted; numbers refer to amino acid (aa) positions as they would occur in the 289-amino-acid 13S product. pE1ASVXCH is a chimeric gene in which coding sequences for SV40 T antigen substitute for E1A CR2. The plasmids are described in the indicated references.

different serotypes (29, 56). Conserved regions 1 and 2 (CR1 and CR2) are required for cooperation with *ras* in primary cell transformation; this activity can be blocked by deletion or point mutations (41). These regions are also thought to be necessary for the transcriptional repression of viral and cellular enhancers (26, 37, 38, 58). CR3, which is present only in the 13S E1A product, is necessary and sufficient (20, 38, 41, 47) for transcriptional activation.

Previous studies have shown that cell lines expressing only 12S E1A are generally somewhat less susceptible to TNFα killing and to macrophage and natural killer cell-mediated cytotoxicity than are cells expressing 13S E1A (12, 14), suggesting that transcriptional activation, associated with the presence of CR3 in 13S E1A, might be involved in the induction of TNF sensitivity. We found that results of experiments with functionally related retroviral transactivators *tat*-1 (from HIV type 1 [HIV-1]) and *tax*-1 (from HTLV-I) do not support this suggestion. To determine the regions of E1A that are required for the induction of TNFα sensitivity and to study the correlation between this and other E1A-mediated biological activities, we generated clonal cell lines that express a well-characterized series of E1A deletion and point mutants and examined their sensitivity to the cytotoxic actions of TNFα.

MATERIALS AND METHODS

TNF. Recombinant normal human TNFα was produced in *Escherichia coli* and purified to homogeneity (12). Its specific activity was 10^7 U mg^{-1} as determined by L929 cytotoxicity assay (in absence of metabolic inhibitor), as previously described (12).

Plasmids. Previously characterized wild-type and mutant E1A expression plasmids p13Swt, p12Swt, pE1ANCdl, pE1APSdl, pE1ACXdl, pE1ASVXCH, and pE1A961 (gifts from E. Moran) and pH3G13S936, pH3G13S953, pSP61500-741, pSP6975-741, and pSP61500-734 (gifts from J. Lillie) were used to generate the target cell lines used in the TNF cytotoxicity assays. All plasmids express either adenovirus type 5 serotype or chimeric adenovirus type 2-type 5 sequences. References describing all E1A plasmids are listed in Fig. 1. The eucaryotic expression plasmid RSVtatDB5 (gift from D. Brake) contains a full-length HIV-1 *tat* cDNA (3) inserted downstream of the Rous sarcoma virus long terminal repeat promoter and upstream of a bovine growth hormone polyadenylation signal sequence (45). RSVtatDB5 also contains the simian virus 40 (SV40) origin of replication for use in COS-1 cells (23). The plasmid HBBLOR (gift from C. Debouck) contains the HTLV-I *tax* gene inserted down-

stream from the Rous sarcoma virus promoter, followed by the bovine growth hormone polyadenylation signal sequence. This plasmid also contains the neomycin resistance gene transcribed from the mouse major β-globin promoter (13).

Cell lines. The cell lines used as target cells in the TNF cytotoxicity assays were generated by cotransfection of NIH 3T3 cells with plasmid pSV2neo (51) or β-globinNEO (13) and the test plasmid, using the calcium phosphate procedure. Coselected cell lines (except where indicated) were isolated from individual G418-resistant colonies by filter paper transfer as previously described (27, 50). The plasmids used to generate the cell lines are indicated in parentheses. All cell lines were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% calf serum, antibiotics, and 200 μg of G418 (GIBCO) per ml.

Immunofluorescence. Cells were grown overnight on chamber slides (Nunc, Inc., Naperville, Ill.) and washed with Dulbecco phosphate-buffered saline, pH 7.4 (PBS; GIBCO). Cells were fixed for 30 min with 3% formaldehyde in PBS, washed with PBS, permeabilized for 3 min with 0.05% Triton X-100 in PBS, washed with PBS, and then incubated for 1 h at 37°C in 5% CO_2 with a 1:1,000 dilution in PBS of rabbit antiserum raised against recombinant E1A (19; gift from J. Culp). Cells were washed with PBS, incubated with a 1:2,000 dilution of fluorescein-conjugated goat anti-rabbit antiserum for 1 h at 37°C in 5% CO_2, and rinsed with PBS; the slides were then examined by fluorescence microscopy.

Immunoblots. Cells were washed with PBS and suspended in 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (0.125 M Tris [pH 6.8], 4% SDS, 10% glycerol, 4% 2-mercaptoethanol) at a concentration of 3×10^7 cells ml^{-1}. Cell lysates were passed through a 27-gauge needle and heated to 100°C for 3 min; 10 to 20 μl of the lysate was resolved on a 10% SDS-polyacrylamide gel (32) and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Membranes were blocked for 1 h at 37°C with 0.5% gelatin in PBS, rinsed with Western buffer (PBS with 0.04% gelatin and 0.25% Triton X-100), incubated for 30 min with a 1:2,000 dilution of a rabbit antiserum to recombinant E1A (19), rinsed with Western buffer, and incubated for an additional 30 min at 37°C with 1 μCi of ^{125}I-labeled protein A (ICN Biomedicals, Inc., Costa Mesa, Calif.). Membranes were washed with Western buffer and autoradiographed with Kodak XAR-2 film at −70°C.

TNF cytotoxicity assays. Cells were plated at 3×10^4/0.1 ml of medium per well in 96-well plates. After 12 to 18 h, 0.1 ml of medium containing TNF (at 14 concentrations derived by twofold serial dilution starting from 2 μg/ml) without any metabolic inhibitor was added to each well. Each concentration was tested in duplicate. Plates were incubated 48 h at 37°C in 5% CO_2; the cells were then fixed and stained with 0.5% crystal violet in 20% methanol. Cells were solubilized in 33% acetic acid, and the optical density at 570 nm (OD_{570}) was determined on a BIOTEK spectrophotometer. Data are expressed as 50% inhibitory concentration (IC_{50}), the concentration of TNF resulting in 50% inhibition of the OD obtained with cells incubated in medium alone.

RESULTS

HIV and HTLV transactivators do not induce TNFα sensitivity. NIH 3T3 cells were cotransfected with a plasmid coding for the HIV-1 *tat* gene and G418 resistance plasmid pSV2neo or with a plasmid coding for both G418 resistance and the HTLV-1 *tax* gene and assayed for sensitivity to TNFα. Three cell lines isolated from individual G418-resistant colonies that expressed HIV-1 *tat* and a culture derived from a pool of G418-resistant colonies that expressed HTLV-1 *tax* were not growth inhibited or killed by concentrations of TNFα up to 1 μg/ml (Table 1). In contrast, as previously reported (12, 14), cell lines expressing 12S or 13S E1A were sensitive to TNFα, and the IC_{50} was approximately 2 to 8 ng/ml. NIH 3T3 cells transfected with only pSV2neo were not sensitive to TNFα. No inhibition was noted at the highest concentration of TNFα used, 1 μg/ml. Activity of the retroviral transcriptional activators in the NIH 3T3 cells was confirmed by their ability to transactivate a plasmid coding for chloramphenicol acetyltransferase under the control of the adenovirus E3 promoter (data not shown).

TABLE 1. TNFα sensitivity of NIH 3T3 cells expressing viral transcriptional activators

Cell line[a]	Gene expressed	IC50[b] (ng ml^{-1})	
		Expt 1	Expt 2
NIH(pSV2neo/RSVtatDB5/cos)3	HIV *tat*	>1,000	>1,000
NIH(pSV2neo/RSVtatDB5/cos)4	HIV *tat*	>1,000	>1,000
NIH(pSV2neo/RSVtatDB5/cos)5	HIV *tat*	>1,000	>1,000
NIH(HBBLOR)MC	HTLV-I *tax*	>1,000	>1,000
NIH(pSV2neo/p13Swt/cos)2	E1A 13S	4.0	2.0
NIH(β-globinNEO/p12Swt/cos)2	E1A 12S	8.0	8.0
NIH(pSV2neo)10		>1,000	>1,000

[a] Cell line name denotes the transfected plasmid(s); cos, coselected.
[b] The concentration of TNFα resulting in a 50% decrease in OD_{570} compared with OD_{570} obtained with cells incubated in the absence of TNF.

TNFα sensitivity of cells expressing E1A deletion mutants and an E1A-SV40 chimera. NIH 3T3 cells were cotransfected with pSV2neo and separately with plasmids coding for the E1A deletion and substitution mutants shown in Fig. 1, and individual G418-resistance cell lines were cloned. When G418-resistant cell lines were screened for anti-E1A immunofluorescence, only 20 to 40% were found to coexpress E1A. This percentage was lower than we and others (63) usually observed in cotransfections. Since E1A can repress transcription from the SV40 promoter (38, 59) used by pSV2neo, we performed identical experiments with β-globinNEO, a plasmid in which the neomycin resistance gene is transcribed from the mouse major β-globin promoter (13). However, a similar fraction of G418-resistant cells coexpressed E1A in these experiments as well. E1A-expressing lines that had been coselected by using either pSV2neo or β-globinNEO were used in subsequent experiments.

Expression of E1A polypeptides of the predicted molecular weights in these lines was confirmed by immunoblot analysis (Fig. 2), and nuclear localization of the E1A products was demonstrated by indirect immunofluorescence (data not shown). Although the CR1 deletion mutant pE1APSdl appeared not to be expressed as well as 12S or 13S (Fig. 2, lanes 8 and 9), this result could have been due to reduced affinity of the antiserum, which was raised against recombinant 13S E1A, with epitopes present on this mutant.

The cell lines were tested for TNF sensitivity (Table 2).

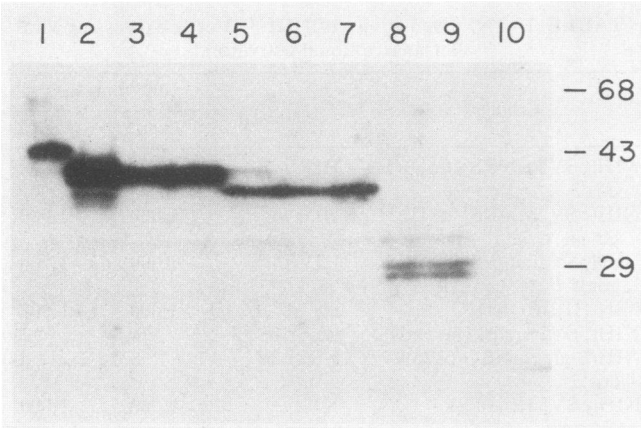

FIG. 2. Immunoblot analysis of E1A deletion mutant proteins in transfected NIH 3T3 cells. Cell lysates were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with a rabbit antiserum to E1A followed by ^{125}I-labeled protein A. Lanes: 1, NIH(pSV2neo/p13Swt/cos)2; 2, NIH(β-globinNEO/p12Swt/cos)2; 3 and 4, NIH(pSV2neo/pE1ASVXCH/cos)5 and -6; 5, NIH (β-globinNEO/pE1ANCdl/cos)F; 6 and 7, NIH (pSV2neo/pE1ACXdl/cos)5 and -6; 8 and 9, NIH(pSV2neo/pE1APSdl/cos)3 and -4; 10, NIH(β-globinNEO)A.

NIH 3T3 cells that expressed only β-globinNEO or pSV2neo were not killed by TNFα concentrations up to 1 μg/ml. Lines expressing wild-type 12S or 13S E1A or pE1ASVXCH, an E1A-SV40 large-T-antigen chimera in which CR3 is deleted and CR2 is replaced by T-antigen amino acids 101 to 118, were killed at low concentrations of about 1 to 62.5 ng/ml. In contrast, lines expressing the CR1 (pE1APSdl) or CR2/3 (pE1ACXdl) deletion mutants were not killed by TNFα. Deletion of the nonconserved sequences between CR1 and CR2 (amino acids 86 to 120) had no effect: cells that expressed mutant plasmid pE1ANCdl were as sensitive to TNFα as were those expressing p13Swt or p12Swt.

TNFα sensitivity of cells expressing CR1 and CR2 point mutants. Moran et al. (43) and Lillie et al. (37, 38) have created a large number of E1A CR1 and CR2 point mutants that are defective for specific E1A biological functions. To analyze the role of CR1 and CR2 in the induction of TNFα sensitivity in greater detail, multiple G418-coselected NIH 3T3 cell lines expressing selected partial functional-defect mutants were isolated and tested for sensitivity to TNF as described above (Table 3). E1A expression in all cell lines was demonstrated by indirect immunofluorescence and immunoblot analysis (Fig. 3 and data not shown).

Whereas all of the CR2 point mutants have previously been shown to be defective (or to have reduced activity) in *ras* cotransformation assays (37, 42, 43; Table 4), and some (pH3G13S936 and pH3G13S953) have been shown to be defective for transcriptional repression (37), all induced TNFα sensitivity. Of the CR1 region point mutants, which have also been shown to be defective for *ras* cooperation and transcriptional repression (38; Table 4), two (pSP6975-741 and pSP61500-734) were able and one (pSP61500-741) was not able to induce TNF sensitivity. The lack of induction by pSP61500-741, which expressed 12S E1A with a Ala-61→Glu substitution, was tested in four coselected cell lines (Table 3). Surprisingly, the same mutation in the 13S E1A protein (pSP6975-741) did not significantly impair the ability of E1A to induce TNF sensitivity. The difference in the activities of these two mutants was not the result of different levels of gene expression, since similar levels of E1A mutant protein were detected in cell lines expressing both mutants (Fig. 3, lanes 4 to 7 and 8 to 11).

The sensitivity to TNFα in cells expressing E1A was not due simply to growth inhibition during the 48-h assay but rather to cytotoxicity. In one experiment (Table 3, experiment 3), twice the number of cells was added per well (6×10^4) than was added in the standard assay, TNFα was added immediately after the cells were plated, and the assay was harvested 24 h later. After overnight incubation, the number of surviving cells in the TNFα-treated wells was reduced by 50% relative to the starting cell number by concentrations of TNFα similar to that resulting in an IC_{50} in the 48-assay.

DISCUSSION

We have used E1A deletion and point mutants to identify functions of E1A that are required for the induction of sensitivity in NIH 3T3 cells to the cytotoxic actions of TNFα. The previously characterized biological functions of the deletion, substitution, and point mutants, and their abilities to induce TNF sensitivity, are compared in Table 4.

We conclude that the ability of E1A to transform primary cells in cooperation with *ras* or to repress transcription is not required for the induction of TNFα sensitivity, since CR1 and CR2 region point mutants that are defective for these

TABLE 2. TNFα sensitivity of NIH 3T3 cells expressing E1A deletion mutants and an E1A-SV40 chimera

Cell line[a]	Deletion[b]	IC_{50}[c] (ng ml^{-1})			
		Expt 1	Expt 2	Expt 3	Expt 4
NIH(pSV2neo)10		>1,000	>1,000	—[d]	—
NIH(pSV2neo/pE1APSdl/cos)3	aa 22–107, CR1	>1,000	>1,000	—	—
NIH(pSV2neo/pE1APSdl/cos)4		>1,000	>1,000	>1,000	—
NIH(pSV2neo/pE1ACXdl/cos)1	aa 121–150, CR2/3	>1,000	>1,000	—	—
NIH(pSV2neo/pE1ACXdl/cos)2		>1,000	>1,000	>1,000	—
NIH(pSV2neo/pE1ASVXCH/cos)5	CR2, substitute aa 101–118 of SV40 large T antigen	15.5	—	62.5	31.5
NIH(pSV2neo/pE1ASVXCH/cos)6		31.5	—	62.5	62.5
NIH(β-globinNEO/pE1ANCdl/cos)F	aa 86–120, nonconserved sequences	31.5	8.0	—	—
NIH(β-globinNEO)A		>1,000	>1,000	—	—
NIH(β-globinNEO/p12Swt/cos)2		8.0	8.0	—	—
NIH(pSV2neo/13Swt/cos)2		—	—	2.0	1.0

[a] Cell line name denotes the transfected plasmid(s); cos, coselected.
[b] aa, Amino acids.
[c] The concentration of TNFα resulting in a 50% decrease in OD_{570} compared with OD_{570} obtained with cells incubated in the absence of TNF.
[d] —, Not tested.

TABLE 3. TNF sensitivity of NIH 3T3 cells expressing E1A CR1 and CR2 point mutants

Cell line[a]	Mutation (E1A product expressed)[b]	IC_{50}[c] (ng ml^{-1})			
		Expt 1	Expt 2[d]	Expt 3	Expt 4
NIH(β-globinNEO/pSP61500-734/cos)A	Glu to Lys; aa 58 (12S)	15.5	31.5	—[e]	—
NIH(β-globinNEO/pSP61500-734/cos)B		31.5	8.8	—	—
NIH(β-globinNEO/pSP61500-734/cos)C		8.0	—	—	—
NIH(β-globinNEO/pSP61500-734/cos)F		4.0	0.3	—	—
NIH(β-globinNEO/pSP61500-741/cos)A	Ala to Glu; aa 61 (12S)	>1,000	>1,000	—	—
NIH(β-globinNEO/pSP61500-741/cos)B		>1,000	>1,000	—	—
NIH(β-globinNEO/pSP61500-741/cos)C		>1,000	>1,000	—	
NIH(β-globinNEO/pSP61500-741/cos)F		500	>1,000	—	—
NIH(β-globinNEO/pSP6975-741/cos)1	Ala to Glu; aa 61 (13S)	—	15.5	—	—
NIH(β-globinNEO/pSP6975-741/cos)2		—	2.0	—	—
NIH(β-globinNEO/pSP6975-741/cos)3		—	250	—	—
NIH(β-globinNEO/pSP6975-741/cos)4		—	31.5	—	—
NIH(β-globinNEO/pH3G13S936/cos)A	Glu to Gly, aa 126 (13S)	—	—	31.5	—
NIH(β-globinNEO/pH3G13S936/cos)B		—	—	4.0	—
NIH(β-globinNEO/pH3G13S953/cos)A	Ser to Gly, aa 132 (13S)	—	—	8.0	—
NIH(β-globinNEO/pH3G13S953/cos)B		—	—	31.5	—
NIH(β-globinNEO/pE1A961/cos)A	Glu to Lys, aa 135 (12S/13S)	—	—	—	8.0
NIH(β-globinNEO/pE1A961/cos)B		—	—	—	15.5
NIH(β-globinNEO/pE1A961/cos)D		—	—	—	15.5
NIH(β-globinNEO/pE1A961/cos)F		—	—	—	31.5
NIH(β-globinNEO/p12Swt/cos)2	(12S)	4.0	4.0	8.0	2.0
NIH(pSV2neo/p13Swt/cos)2	(13S)	4.0	8.0	—	—
NIH(β-globinNEO)A		>1,000	—	>1,000	>1,000

[a] Cell line name denotes the transfected plasmid(s); cos, coselected.
[b] aa, Amino acid.
[c] The concentration of TNFα resulting in a 50% decrease in OD_{570} compared with OD_{570} obtained with cells incubated in the absence of TNF.
[d] 24-h assay; TNFα was added to wells immediately after cells were plated, and the assay was harvested 24 h later.
[e] —, Not tested.

functions confer TNFα sensitivity to the same extent as does wild-type E1A. In addition, the ability to induce TNFα sensitivity is not correlated with the ability to induce DNA synthesis in quiescent cells.

The small amount of available data describing the ability of the mutants to immortalize primary cell cultures precludes an evaluation from these experiments of the role of this factor in the induction of TNFα sensitivity. However, we have previously shown that TNFα sensitivity is not induced by expression of *myc*, the polyomavirus large-T-antigen gene, or Ha-*ras*, even though these genes are known to be capable of primary culture immortalization (28, 46). It therefore seems unlikely that this function is relevant.

E1A-mediated transcriptional activation was neither necessary nor sufficient for induction of TNF sensitivity. Both 12S and 13S E1A induced sensitivity to TNF (12, 15, 16; Tables 1 to 3), yet CR3, which is required for efficient transactivation, is not present in 12S. Furthermore, the pE1APSdl mutant, which expressed CR3 and has transcriptional activation activity (42) but which lacks amino acids 22 to 107 spanning CR1, did not induce TNFα sensitivity. The relationship of transcriptional activation and TNFα sensitivity was directly tested with cell lines that expressed the retroviral transactivators *tax* from HTLV-1 (which can activate transcription from E1A-dependent promoters and may activate the same cellular genes [11]) and *tat* from HIV-1. However, neither transactivator induced TNFα sensitivity. This conclusion is consistent with our previous finding that *myc* expression does not enhance sensitivity to TNFα (12), since *myc* possesses a transactivating activity that is mediated through the same DNA target sequences as are used by E1A (44).

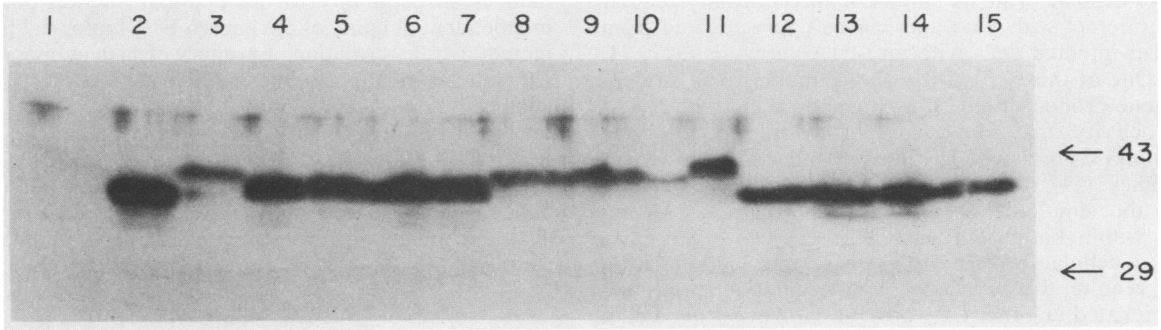

FIG. 3. Immunoblot analysis of E1A CR1 point mutant proteins in transfected NIH 3T3 cells. Cell lysates were immunoblotted with rabbit antiserum to E1A as described in the legend to Fig. 2. Lanes: 1, NIH(β-globinNEO)A; 2, NIH(β-globinNEO/p12Swt/cos)2; 3, NIH(pSV2neo/p13Swt/cos)2; 4 to 7, NIH(β-globinNEO/pSP61500-741/cos)A, -B, -C, and -F; 8 to 11, NIH(β-globinNEO/pSP6975-741/cos) 1 to -4: 12 to 15, NIH(β-globinNEO/pSP61500-734/cos)A, -B, -C, and -F.

TABLE 4. Summary of adenovirus E1A mutant plasmid induction of TNFα sensitivity and biological activities[a]

Plasmid	Induction of TNF sensitivity	Cooperation with *ras*	Transcriptional activation	Transcriptional repression[b]	Immortalization	Induction of DNA synthesis in quiescent cells
p13Swt	+	+ (43)	+ (43)			+ (70)
CR2 mutations						
pH3G13S936	+	− (37)	+ (37)	− (37)		
pH3G13S953	+	− (37)	+ (37)	− (37)		
pE1A961	+	− (43)	+ (43)		+ (43)[c]	
CR2/3 mutation						
pE1ACXdl	−	− (42, 70)	− (43)		−(43)	+ (43, 70)
CR1 mutations						
pE1APSdl	−	− (42)	+ (42)			− (42)
pSP6975-741	+	− (38)	+ (38)			
CR1/3 mutations						
pSP61500-734	+	− (38)		− (38)		− (38)
pSP61500-741	−	− (38)		− (38)		− (38)
CR3 mutation						
p12Swt	+	+ (43)	− (42)	+ (37, 38)	+ (43)	+ (70)
Nonconserved region mutation						
pE1ANCdl	+	+ (43, 70)	− (43)			+ (42, 70)[c]
SV40-E1A chimera						
pE1ASVXCH	+	+ (40)				+ (40)

[a] Data from previous investigations with the E1A mutants used in this study are summarized along with the TNFα sensitivity results presented in this report. +, Function was not significantly lower in the mutant plasmid than in a wild-type E1A expression plasmid; −, function was severely impaired although in some cases not completely eliminated. References to the primary data are given in parentheses. Because different cell types and experimental protocols were used in the various studies, the data may not be strictly comparable.

[b] Experiments were performed with plasmids that contained these mutations.

[c] Experiments were performed with a 12S expression plasmid having the same mutation.

However, our results do not exclude the possibility that E1A transcriptional activation, mediated by CR3, augments the induction of TNFα sensitivity. This effect could explain the observation that the negative effect of the CR1 Ala-61→Glu mutation was only apparent when the mutation was introduced into 12S E1A (pSP61500-741, which also lacked transcriptional activating activity) and not when introduced into 13S E1A (pSP6975-741). This augmentation could also explain why pE1ACXdl (defective both in CR2 and CR3) did not induce TNFα sensitivity, whereas CR2 region point mutants that contained the wild-type CR3 region did induce sensitivity. Alternatively, the difference between the activities of pE1ACXdl and the CR2 point mutants could be due to the total elimination of CR2 in pE1ACXdl.

The fact that deletion of amino acids 23 to 107 (pE1APSdl) but not deletion of amino acids 86 to 120 (pE1ANCdl) eliminated the ability to induce TNFα sensitivity indicated that CR1 is required for the induction of sensitivity, even though, as discussed above, some of the functions mediated by CR1 are not required. It is possible that CR1-mediated binding to cellular proteins may be involved. Many cellular proteins interact with E1A and can be coprecipitated from adenovirus-infected cells with anti-E1A antibodies (17, 24, 62, 68). One of these, p105-Rb, the retinoblastoma susceptibility gene product (61, 62), a phosphoprotein with DNA-binding activity, may play an important role in cell cycle progression (33; reviewed in reference 60). Mutagenic mapping studies (17, 62) have shown that p105-Rb binding requires the simultaneous presence of separate E1A sequences within both CR1 and CR2, whereas other E1A-associated cellular proteins (e.g., 107- and 300-kilodalton species) require the presence of different contiguous sequences located in either CR1 or CR2. However, since data describing the protein-binding properties of the mutants used in this study are not available, the relevance of cellular protein binding to the induction of TNFα sensitivity cannot yet be evaluated.

It is interesting that the chimeric E1A-SV40 protein expressed by pE1ASVXCH, in which CR2 and CR3 were replaced with homologous sequence from SV40 large T antigen, displayed wild-type TNFα sensitization activity even though deletion of a subset of this region (pE1ACXdl) did not. This expands the catalog of E1A functions that can be mediated by this E1A-SV40 chimera (40; Table 4) and underscores the high degree of functional similarity between these regions in the two proteins.

Fletcher et al. (21) have demonstrated one case in which resistance to TNFα cytolysis is correlated with the presence of gap junctions between target cells. E1A expression reduces gap junctional communication in NIH 3T3 cells (5), and it is possible that this activity plays a role in, and may be required for, its induction of TNFα sensitivity. However, this activity is clearly not sufficient, since TNFα sensitivity is not enhanced by expression of v-*src*, c-*src*, or polyomavirus middle T, agents which cause even more significant decreases in gap junctional communication (4, 6, 9).

In summary, the molecular mechanism by which E1A induces susceptibility to the cytotoxic action of TNFα is independent of many of the known E1A biological functions, including transformation of primary cells in cooperation with *ras*, immortalization, induction of DNA synthesis in quiescent cells, and transcriptional repression. Although E1A-mediated transcriptional activation may augment, it is not necessary or sufficient for induction of TNFα sensitivity. A novel E1A-mediated effect may be involved, although our data do not exclude the possibility that sensitization to TNFα is mediated through E1A binding to cellular targets such as p105-Rb.

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