Coordinate Inhibition of Cytokine-mediated Induction of Ferritin H, Manganese Superoxide Dismutase, and Interleukin-6 by the Adenovirus E1A Oncogene*

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Adenovirus E1A sensitizes cells to the cytotoxic action of tumor necrosis factor α (TNF-α). This effect has been attributed to direct blockade of NF-κB activation, as well as to increased activation of components of the apoptotic pathway and decreases in inhibitors of apoptosis. In this report we evaluated the mechanism by which E1A modulates the expression of the cytokine-inducible cytoprotective genes manganese superoxide dismutase (MnSOD), interleukin-6 (IL-6), and ferritin heavy chain (FH). We observed that E1A blocks induction of MnSOD, IL-6, and FH by TNF-α or IL-1α. Because NF-κB plays a role in cytokine-dependent induction of MnSOD, IL-6, and FH, we assessed the effect of E1A on NF-κB in cells treated with TNF. IκB, the inhibitor of NF-κB, was degraded similarly in the presence and absence of E1A. TNF induced a quantitatively and temporally equivalent activation of NF-κB in control and E1A-transfected cells. However, TNF-dependent acetylation of NF-κB was diminished in cells expressing E1A. E1A mutants unable to bind p400 or the Rb family proteins were still capable of repressing TNF-dependent induction of FH. However, mutants of E1A that abrogated binding of p300/CBP blocked the ability of E1A to repress TNF-dependent induction of FH. These results suggest that p300/CBP is a critical control point in NF-κB-dependent transcriptional regulation of cytoprotective genes by cytokines.

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine that serves as a potent immunomodulator and regulator of cell function (1, 2). Binding of TNF to its receptor TNFR1 triggers both survival and death pathways. TNF recruits the adapter molecules TRADD and FADD, initiating a signaling cascade that culminates in apoptosis. Conversely, TRADD can also recruit TRAF2 and RIP, which promote survival by triggering the activation of NF-κB (see Refs. 3 and 4 for review).

Signaling events involved in the activation of NF-κB include recruitment of IκB kinase (IKK), resulting in the phosphorylation of the NF-κB inhibitory protein, IκB. IκB is then targeted for proteasome-mediated degradation, freeing NF-κB to translocate to the nucleus, where it transcriptionally induces target genes.

The NF-κB family of transcription factors consists of 5 proteins that form homo- or heterodimers. The prototypical NF-κB complex is a heterodimer composed of RelA (p65) and p50. Recent work has revealed that the activity of NF-κB is modulated by post-translational modification as well as by subcellular localization. Thus phosphorylation and acetylation control the duration and amplitude of NF-κB signaling (5) (6). The activity of NF-κB is further modulated by association with histone acetylases and deacetylases. For example CREB-binding protein (CBP)/p300, a transcriptional coactivator and histone acetylase, interacts with the p65 subunit of NF-κB. Such an interaction is thought to increase the transcriptional activity of NF-κB both directly, through acetylation of NF-κB, and indirectly, through acetylation of histones.

Interleukin-1α (IL-1) shares many of the biological activities of TNF (reviewed in Ref. 7) although it is structurally unrelated and binds to different cell receptors (8) (9). Like TNF, IL-1 activates NF-κB. TNF and IL-1 induce expression of a common set of cellular genes including intracellular adhesion molecule 1 (ICAM1) in endothelial cells (10), collagenase in synovial cells (11), as well as plasminogen activator inhibitors (12), interleukin-6 (IL-6) (13), interleukin-8 (IL-8) (14), manganese superoxide dismutase (MnSOD) (15), and ferritin H (16) in fibroblasts.

Many genes induced by TNF via an NF-κB-dependent pathway serve a cytoprotective function. These include antiapoptotic genes such as IAP-1, IAP2, c-FLIP, and TRAF that inhibit the apoptotic process at the death-inducing signaling complex (DISC) or the mitochondrion (17). IL-6 is essential for certain antiviral responses and the synthesis of acute phase proteins (reviewed in Ref. 18). Other cytoprotective genes induced by NF-κB inhibit oxidative stress. Such genes include MnSOD (19) and ferritin H (20). MnSOD reduces levels of the superoxide radical produced as a byproduct of respiration. Ferritin, an iron storage protein, sequesters iron and prevents iron from participating in reactions that generate oxygen free radicals. Cytosolic ferritin is composed of two types of subunits, termed H and L that assemble to form a 24 subunit protein. The H subunit of ferritin possesses a ferroxidase activity that catalyzes the oxidation of iron into a ferrihydrite mineral core in the center of the protein. We have shown that activation of NF-κB by TNF leads to the transcriptional induction of ferritin H via an NF-κB-containing element in the ferritin H promoter, which we term FER2 (20). Induction of ferritin H blunts the sustained phase of JNK activation...
required for the accumulation of ROS in TNF-treated cells, and is a critical component of the cytoprotective response to TNF (21).

The balance between survival and apoptotic pathways induced by TNF can be modulated by the E1A oncogene. E1A is an immediate early gene of adenovirus with multiple effects, including sensitization of cells to TNF-induced apoptosis. Many of its effects are mediated by interaction and perturbations of important intracellular regulators, including the Rb tumor suppressor family (p107, p105Rb, and p130), the transcriptional coactivators and histone acetylases p300 and CBP, the cell cycle regulator p21(WAF1) (22), the tumor suppressor p53 (23), and a p40 chromatin remodeling complex (24).

In this article, we demonstrate that E1A inhibits the induction of the survival genes ferritin H, MnSOD, and IL-6 by TNF and IL-1. We show that E1A exerts its activity on ferritin H by targeting an NF-κB binding domain of the ferritin H promoter and inhibiting the induction of ferritin H by TNF. However, although E1A inhibits the activity of NF-κB, E1A does not prevent the accumulation of NF-κB, its translocation to the nucleus, or its nuclear stability. However, E1A blocks TNF-dependent acetylation of NF-κB. Mapping experiments reveal that the p300 binding domain of E1A is critical to its ability to inhibit TNF-dependent induction of ferritin H. These results indicate that in cytokine-treated cells, E1A regulates the activity of NF-κB at the ferritin H promoter through binding and inactivation of p300, preventing p300 from acetylating NF-κB. These results suggest that p300 functions as a nodal point for functional regulation of NF-κB by modulating its ability to activate transcription of cytoprotective genes in cells treated with cytokines such as TNF.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse fibroblast NIH3T3 parental cells as well as stable E1A and control vector NIH3T3 transfectants were cultured at 37 °C in a 5% CO2 atmosphere in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% bovine calf serum (Hyclone). Isolation and construction of stable NIH3T3 transfectants expressing E1A or the Δ23–150 E1A deletion mutant were as described (25). Stably transfected IP1 cells (derived from SKOV3 human ovarian cancer cells) expressing either type Ad5 E1A (IP1/E1A) or a dysfunctional frameshifted E1A mutant (IP1/EFS) were kindly provided by M.-C. Hung (University of Texas, Houston, TX).

**Plasmids**—All E1A mutants and wild-type E1A plasmids used in these experiments are shown in Fig. 1. All mutants and their binding properties have been described in detail elsewhere (26–28). The FER2 minimal ferritin H promoter linked to human growth hormone (HGH) reporter plasmid (FER2-min/HGH) was constructed from previously described constructs of 0.941 kb ferritin H promoter–HGH and −5.6 kb ferritin H promoter–HGH (20). The TNF responsive element of the ferritin H gene, FER2, was amplified from the −5.6 kb construct by PCR, adding restriction sites for Sphl and EcoRI. This element was then cloned into the Sphl/EcoRI sites of the −0.941 kb construct to create the FER2/−0.941 kb/HGH construct. This construct was used to generate the FER2 minimal promoter construct (FER2-min/HGH) by digesting with EcoRI/MscI to delete an internal sequence of 886 bp. This was followed by fill-in and blunt-end ligation, resulting in a construct containing FER2 immediately 5′ of the 55-bp proximal promoter. To construct 4×FER2–FH–Luc, −4.8kbA3.5 FH–Luc (29) was digested with Smal and MscI to create −0.059kb FH–Luc. Sense and antisense oligonucleotides corresponding to 2× FER2 were synthesized by the Wake Forest University School of Medicine Comprehensive Cancer Center DNA synthesis facility, PAGE-purified, phosphorylated using T4 polynucleotide kinase, annealed, and ligated into the bunt-ended Smal–MscI site of 0.059 kb FH–Luc. Clones expressing 4× FER2 were identified by restriction digestion. All constructs were confirmed by dideoxynucleotide sequencing.

**Transient Transfections**—Transient transfections were carried out using electroporation or Lipofectamine (Invitrogen). For electroporation, NIH3T3 cells were suspended in electroporation buffer described previously (20) at a density of ∼1 × 10^7 cells per 0.6 ml of buffer. 1 × 10^7 cells were then transferred to ice-cold electroporation cuvettes (Bio-Rad) in which DNA was already present. Electroporations were carried out at 300 mV and 960 mF on a Bio-Rad gene pulser. The contents of the cuvettes were then resuspended into serum-containing media and distributed into 100-mm tissue culture plates. Cells were allowed to recover for 24 h prior to treatment with 10 ng/ml recombinant human TNF-α (R&D Systems) for 24 h. Control plates were left untreated. In transfections of 12SE1A mutants with the FER2-min/HGH construct, 20 μg of the FER2-min/HGH DNA and 30 μg of wild-type 12S E1A DNA were utilized. DNA concentrations of E1A mutants were adjusted to give E1A protein expression equivalent to that of wild-type E1A as determined by Western blotting. Empty pHygSRv vector DNA was added to each electroporation so that all cuvettes received an equal concentration of total DNA. For transfection experiments in which 13SE1A or 13SE1AΔ23–150 were used, 15 μg of plasmid DNA was used for each construct as well as for the FER2-min/HGH reporter construct. Transfections with Lipofectamine were carried out essentially as described (29) using Renilla luciferase vector as a transfection control.

**RNA Isolation and Northern Blot Analysis**—Cells were treated with recombinant human TNF-α or with recombinant human IL-1α (a gift from Hoffman-La Roche Inc.) and collected at the indicated time points. RNA was isolated by the method of Chirgwin et al. (30). Briefly, cells were lysed in 4 M guanidium isothiocyanate, and total RNA was collected by ultracentrifugation through 5.7 M CsCl. Ferritin mRNA was analyzed by blotting using hybridization to cDNA from mouse ferritin H (31) and human ferritin L (32) as described previously (33). The cDNA for human MnSOD was obtained from ATCC and mouse IL-6
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from F. Lee (DNAX Research Inst., Palo Alto, CA). Gels were subjected to autoradiography and signal intensities quantified by scanning densitometry.

RNase Protection Assay—All RNA prepared for use in RNase protection assays was collected as described for Northern blot analysis. RNA probe was prepared by in vitro transcription of a ferritin H-HGH template using T7 RNA polymerase (Promega) and [α-32P]CTP (> 400Ci/mmol; Amersham Biosciences) essentially as described (34). The probe spans the junction between ferritin H and HGH and includes from nt −255 to +86 of ferritin H and the HindIII/DraIII fragment of HGH. Hybridization was carried out using 10 μg of total RNA and 1 × 106 cpm of RNA probe overnight at 56 °C in 80% formamide.

Single-stranded RNAs were then digested with RNases A and T1 (both from Sigma). Remaining RNA was precipitated, and the products were separated on a 6% denaturing urea-acrylamide gel. Results were quantitated by densitometry of phosphorimages on a Molecular Dynamics 445 SI phosphorimager.

Nuclear/Cytoplasmic Extractions—Protein extractions were carried out essentially as described (35). Briefly, cells were scraped from plates in phosphate-buffered saline/25 mM EDTA and pelleted at 4 °C by centrifugation. Cells were rinsed with ice-cold phosphate-buffered saline, repelleted, and then resuspended in cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl2, 1 mM phenylmethylsulfonfonyl fluoride) for 15 min on ice. Cells were then lysed by pulse vortexing with addition of ~6% v/v 10% Nonidet P-40 in high salt buffer (50 mM HEPES, 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Nuclear pellets were collected, resuspended in the above high salt buffer and rocked for 30 min to extract nuclear proteins. Protein concentrations were determined using the Bio-Rad Bradford protein assay system. Whole cell protein extracts for assessment of NF-kB acetylation were prepared by freeze-thaw lysis, as described (36).

Electrophoretic Mobility Shift Assay (EMSA)—25 ng of consensus NF-kB oligonucleotide (Promega) was used for end-labeling by [γ-32P]ATP (ICN) by T4 polynucleotide kinase (Promega). The assay was carried out as described (37) with modifications. Binding reactions were carried out on ice using a mixture of 10 μg of nuclear extract, 1 μg of poly(dIdC), and between 100,000–250,000 cpm of radiolabeled oligonucleotide in a final volume to 20 μl. Where indicated, unlabeled oligonucleotide at a 100-fold molar excess was used as competitor. SP1 oligonucleotide (Promega) was used as a nonspecific competitor. SP1 oligonucleotide (Promega) was used as a nonspecific competitor. In supershift experiments, antibodies against the p50 and p65 NF-kB family members (Santa Cruz Biotechnology) or against nonspecific antibody for p53 (Santa Cruz Biotechnology), were added where indicated and preincubated for 45 min prior to addition of competitors and probe. Samples were electrophoresed on a 5% native acrylamide gel containing 1.25% glycerol, fixed, and dried. Results were obtained by phosphorimaging and by radiography.

Western Blotting—For E1A expression, 20–40 μg of nuclear extracts were separated by electrophoresis on 12% SDS-PAGE gels. E1A was detected using anti-E1A clone M58 (PharMingen), followed by HRP-conjugated goat anti-mouse IgG (Calbiochem) and detection by enhanced chemiluminescence (ECL) (Amersham Biosciences). For IκB expression 60 μg of cytoplasmic protein were used. Primary antibody incubations were carried out using anti-IκB-α (Cell Signaling Technology) followed by HRP-conjugated goat anti-rabbit IgG (Bio-Rad) and detection with ECL Plus. Anti-acetylated K310 RelA(p65) antibody was a generous gift of Warner C. Greene (University of California, San Francisco), and anti-NF-kB p65 (C-20) was obtained from Santa Cruz Biotechnology. These antibodies were used following electrophoresis of 50 μg of total cell lysate. Secondary antibody was HRP-conjugated goat anti-rabbit IgG (Bio-Rad) and detection was by ECL.

 Luciferase Assays—Cells were lysed in reporter lysis buffer (Promega) and dual luciferase assays carried out using the Luciferase Assay kit (Promega) according to the manufacturer’s protocol.

RESULTS

E1A Inhibits TNF-dependent Induction of Ferritin H—To explore the influence of E1A on TNF-dependent gene expression, NIH3T3 cells were transfected with an E1A expression vector, pHygSR+E1A (NIH3T3/E1A), or with the empty vector pHygSR (NIH3T3/HYG) as a control, and stable transfectants were isolated. Stable E1A and hygromycin clones were either untreated or treated with TNF-α for 15 h, a time point that precedes cytotoxic effects of TNF in 3T3 cells (not shown). Both pooled and clonal transfectants were examined. As shown in Fig. 2, TNF treatment led to a marked induction of ferritin H mRNA in NIH3T3/HYG and NIH3T3/E1A stably transfected cells treated with 30 ng/ml IL-1α or not at time 0, and total RNA was isolated at intervals (0, 4, 8, 12, 16, and 24 h). Blotted gels were hybridized against cDNA probes for H ferritin, L ferritin, MnSOD, and IL-6.

E1A blocks TNF induction of ferritin H. Northern blot analysis for ferritin H (FH) and L (FL) in NIH3T3 cells transfected with either pHygSR+E1A (NIH3T3/E1A), a plasmid containing adenovirus 135E1A cDNA under the control of a hybrid SV40/human immunodeficiency virus promoter, or with pHygSR (NIH3T3/HYG) as a control vector. Stable transfectants were treated with 30 ng/ml TNF-α or not at time 0, and total RNA was isolated at 15 h. Control (3T3/HYG), an E1A clonal transfectant (3T3/E1Af2), and pooled transfectants (3T3/E1A) are shown for FL and FH.

E1A Blocks TNF-induced Gene Expression—To test whether E1A inhibition is specific to TNF, we used a second cytokine, IL-1. Treatment of control 3T3 cells with IL-1 led to an induction of ferritin H mRNA (Fig. 3), as we have previously reported for human fibroblasts (33). A robust induction was present at 4 h, with a gradual increase in mRNA levels up to 24 h. Ferritin L mRNA was not induced by IL-1, indicating that, like TNF, IL-1 exhibits a selective effect on gene expression. As we had observed in TNF-treated cells, E1A dramatically inhib-
E1A Inhibits Induction of Multiple Cytokine-inducible Genes—To test whether E1A-mediated inhibition of IL-1 inducible gene expression was specific to the ferritin H gene, we examined the cytokine-inducible genes MnSOD and IL-6. As seen in Fig. 3, MnSOD and IL-6 genes were dramatically induced in response to treatment with IL-1α. As observed with ferritin H, introduction of wild-type 13S E1A completely blocked IL-1α-dependent induction of MnSOD and IL-6 in NIH3T3 cells (NIH3T3/E1A) at all time points measured.

E1A Does Not Inhibit Nuclear Translocation of NF-κB—One potential mechanism by which E1A might repress cytokine-mediated induction of multiple genes is through interference with NF-κB, a stress-activated transcription factor that has been identified as mediating the induction of multiple genes in response to TNF or IL-1, including MnSOD (19), IL-6 (39), and ferritin H (20, 21). Because activation of NF-κB can be blocked by inhibiting the activation of IKK (IKκB-kinase), we first assessed effects of E1A on TNF-dependent IkB degradation in NIH3T3. As shown in Fig. 4, IkB was degraded following TNF treatment of NIH3T3 cells regardless of whether or not they expressed E1A. These results indicate that E1A does not inhibit IkB degradation in response to TNF in NIH3T3 cells.

Because the transcriptional activity of NF-κB also depends on its subunit composition and ability to reach the nucleus, we next tested whether E1A inhibited nuclear targeting of NF-κB. NIH3T3 cells stably transfected with the pHygSRα control vector (NIH3T3/Hyg) or the E1A expression vector, pHygSRα/E1A vector (NIH3T3/E1A) were treated with TNF and EMSA for NF-κB were performed at intervals ranging from 15 min to 24 h. As shown in Fig. 5, NF-κB was activated to similar levels in both control and E1A vector-transfected NIH3T3 cells at all time points. In three independent time course experiments, peak activation of NF-κB by TNF occurred at 30 min in both E1A transfectants and controls. Fold increase in NF-κB binding activity at this time was 24 ± 8 (mean ± S.E.) for controls and 27 ± 7 for E1A transfectants, indicating that E1A has no effect on the time course or magnitude of NF-κB activation. Further, as shown in Fig. 6, supershift analysis using anti-p50 and p65 NF-κB antibodies demonstrated the presence of both p50 and p65 NF-κB subunits in activated NF-κB, and indicated that E1A did not demonstrably affect this composition. In particular, levels of p50, which can homodimerize to inhibit transcription, were not increased in firefly luciferase activity in the presence of TNF; means and standard deviations of triplicate determinations are shown.

E1A Directly Inhibits the Activity of NF-κB—We have previously shown that TNF-dependent induction of ferritin H requires a cis-acting element of the ferritin H gene, which we termed FER2. FER2 consists of two tandem NF-κB consensus elements that both bind NF-κB (20). Consistent with these results, FER2 ligated to a minimal ferritin H promoter (FER2-FH) was induced following treatment of cells with TNF (Fig. 7). This indicates that the NF-κB binding domain of the ferritin promoter is both necessary and
sufficient for induction of ferritin H by TNF. To test whether E1A targets this region of the ferritin promoter, we cotransfected cells with FER2-FH and an expression plasmid for E1A. As shown in Fig. 7, TNF-dependent induction of this construct was effectively inhibited by E1A. Thus, despite the inability of E1A to directly inhibit nuclear translocation of NF-κB (Fig. 5), E1A functionally interfered with the activity of NF-κB at the ferritin H promoter.

Blockade of TNF Induction Requires Conserved Domains of E1A—To elucidate the mechanism by which E1A inhibits TNF inducible gene expression, we mapped the domain of E1A required for this effect. E1A is a complex protein with many activities, including transcriptional activation, transcriptional repression and cell immortalization (reviewed in Refs. 40–42). Key functional domains of E1A have been mapped to four highly conserved domains termed CR1, CR2, CR3, and CR4 (43). To determine whether repression of TNF induction required functional E1A, and to begin to map the domain of E1A that mediates this activity, cells were stably transfected with an E1A mutant in which the CR1 and CR2 functional domains have been deleted, and the response of these cells to TNF assessed. As shown in Fig. 8, deletion of conserved regions 1 and 2 (NIH3T3/Δ23–150E1A) blocked the ability of E1A to inhibit TNF induction of ferritin H, MnSOD, and IL-6. Thus, E1A exhibits a concerted ability to inhibit TNF inducible gene expression, and this effect is mediated by the CR1 and/or CR2 domains of E1A.

E1A Blocks TNF-α-inducible Expression of the Ferritin H Gene through Interaction with p300/CBP and Blockade of NF-κB Acetylation—CR1 and CR2 contain domains of E1A required for its interaction with key cellular proteins. These interactions represent an important mechanism by which E1A modulates cellular transcription (26, 44, 45, 46). To determine whether the ability of E1A to interact with p300, p400, or the Rb family proteins accounted for its ability to block cytokine-dependent induction of gene expression, we tested the effect of E1A and mutants of E1A selectively defective for binding to these proteins in cotransfection experiments with a TNF-responsive ferritin H construct.

Consistent with the repression of TNF-inducible expression seen for the endogenous gene, both 13 S and 12 S wild-type E1A constructs pHygSRα-12Swt and pHygSRα-13Swt led to a 2–3-fold repression of TNF-α-inducible expression of ferritin H in transient transfections (Fig. 9). Tole delineate the domains of E1A required for this effect, a series of E1A mutants was used (Fig. 1). As seen in Fig. 10, constructs deficient in Rb family binding (YH47, and YH47/928) were both capable of E1A-mediated repression, indicating that the Rb family of proteins is dispensable for repression of TNF-mediated induction of ferritin H. Similarly, the Δ26–35 mutant of E1A, which is deficient in binding to p400, retained its ability to block TNF induction of ferritin, suggesting that the ability to bind p400 is not critical to the E1A effect. In contrast, mutants that lost the ability to bind p300/CBP were inhibited in their ability to repress TNF-dependent induction of ferritin. These mutants included RG2, a point mutant deficient in its ability to bind p300/CBP; a double point mutant RG2/928, deficient in the ability to bind both pRb and p300/CBP; as well as the N-terminal deletion mutant Δ23–36, which is unable to bind p300 or p400. Thus, binding of p300/CBP is required for effective blockade of TNF-dependent induction of ferritin H by E1A.
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p300/CBP serves as a co-activator of NF-κB in multiple promoters (47–49). p300/CBP possesses intrinsic histone acetyltransferase activity, and can also acetylate NF-κB directly. Because acetylation has been implicated in activation of NF-κB, we tested the hypothesis that targeting of p300 by E1A might impede transcriptional activation of target genes by blocking NF-κB acetylation. Consistent with results shown in Fig. 6, expression of E1A did not affect total levels of the p65 subunit of NF-κB in cells treated with TNF (Fig. 11). However, acetylation of NF-κB p65 at lysine 310 was markedly reduced in cells expressing E1A (Fig. 11). Thus, E1A inhibits TNF-dependent acetylation of the p65 subunit of NF-κB.

DISCUSSION

The prosurvival activity of TNF is attributable at least in part to its ability to transcriptionally activate target genes that encode cytoprotective proteins. These include MnSOD, IL-6, and ferritin H, as well as antiapoptotic proteins that inhibit the apoptotic process at the DISC or the mitochondrion (17). Genes encoding some of these proteins, including ferritin H, IL-6, and MnSOD, are also induced by IL-1α. Induction of such cytoprotective proteins may contribute to the ability of pretreatment with IL-1α to protect cells from the cytotoxic effects of subsequent exposure to TNF (16) (50). Ferritin H, MnSOD, and IL-6 play different but important roles in cytoprotection. IL-6 is essential for certain antiviral responses and stimulates the synthesis of acute phase proteins (reviewed in Ref. 18). Both MnSOD and ferritin H are critical in buffering cells from oxidative stress. Dismutation of superoxide by MnSOD is an essential step in the detoxification of superoxide, an unavoidable byproduct of mitochondrial respiration. Ferritin is a protein with a pivotal role in iron storage and detoxification. By oxidizing and sequestering iron, ferritin prevents the participation of iron in deleterious reactions generating reactive oxygen species. Accordingly, ferritin over-expression protects against oxidative stress (51). Conversely, decreases in ferritin H are associated with increasing sensitivity to oxidative stress (52, 53). Thus, E1A, which represses basal levels of ferritin H, enhances cellular sensitivity to oxidants (54). We have shown that E1A exerts its effects on basal ferritin H expression by targeting FER1, a basal enhancer of ferritin H that binds SP1/3 and members of the Jun and Fos families (55).

Although ferritin H is critical in the protection from stress induced by both cytokines and oxidants, the induction of ferritin H by TNF occurs via an independent mechanism from that triggered by exposure to chemical oxidants or electrophiles. We have shown that induction of ferritin H by TNF does not require FER1, but rather requires FER2, a cis-acting element containing two tandem NF-κB sites. p65/p50 NF-κB subunits bind to this site and activate transcription of ferritin H in response to TNF challenge (20). Other laboratories have reported that NF-κB also mediates cytokine-dependent induction of IL-6 (56) and MnSOD (57).

Here we demonstrate that E1A inhibits induction of ferritin H, MnSOD, and IL-6 mRNA by both TNF (Figs. 2 and 8) and IL-1 (Fig. 3). Our studies of the mechanism by which E1A modulates the response to these cytokines focused on TNF. We first assessed whether E1A affected NF-κB, a transcription factor that mediates TNF-dependent induction of ferritin H (20, 21). We examined the initial step in NF-κB activation, IκB degradation. However, the degradation of IκB was unaffected by E1A in our cells (Fig. 4). This differs from results obtained in ovarian carcinoma cells, suggesting that the mechanism of NF-κB regulation is cell lineage specific. In human ovarian cancer cell lines, E1A has been shown to prevent IκB degradation (58), a result which we recapitulated (data not shown). In contrast, we observed that steps up to and including nuclear localization are unaffected by E1A in immortalized fibroblastic cells (Figs. 5 and 6). Thus mechanisms of NF-κB regulation differ in transformed epithelial and immortalized mesenchymal cells.

We next examined the transport of NF-κB to the nucleus, since inhibition of nuclear translocation would prevent NF-κB from functioning as a transcription factor. However, EMSA analysis of nuclear extracts demonstrated that NF-κB translocation was unaffected by E1A. Because alteration of NF-κB subunit composition can affect NF-κB function (for example the p50/p65 dimer is a transcriptional activator, whereas the p50/p50 dimer is a transcriptional repressor (6)), we then explored the effect of E1A expression on NF-κB subunit composition. Using supershift assays, we found that NF-κB composition was unaffected by E1A expression (Fig. 6). Finally, we tested whether E1A affected the dwell time of NF-κB in the nucleus, because NF-κB transcriptionally activates IκBα, a protein that eventually leads to chaperoned NF-κB nuclear egress. These experiments demonstrated that the kinetics of NF-κB nuclear accumulation was similar in E1A and control cells (Fig. 5). Thus, E1A impedes TNF-dependent activation of ferritin H without demonstrable effect on NF-κB nuclear accumulation.

Because we were unable to show an effect of E1A on NF-κB translocation, we considered that E1A might reduce the activity of NF-κB by affecting its interactions with other proteins required for NF-κB func-
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tion. To test this possibility, we first confirmed that the cis-acting element in the ferritin H gene that we had previously identified as the TNF regulatory sequence (FER2) was the region targeted by E1A. This was important to verify, because E1A can also target the basal enhancer of ferritin (FER1) (55). Transfection of the FER2 element ligated to a basal enhancer conferred full TNF-dependent induction on a luciferase reporter gene, and this induction was completely inhibited by E1A (Fig. 7). Thus, FER2 is the critical cis-acting sequence of the ferritin H promoter targeted during E1A-dependent inhibition of TNF induction. To identify E1A binding partners required for E1A-dependent inhibition of induction of ferritin H by TNF, we used a series of E1A mutants. These included mutants that alter binding of E1A to the Rb family members pRb, p107, and p130, as well as p400 and p300/CBP (Figs. 1 and 10). This analysis identified p300 as the protein targeted by E1A during E1A-mediated repression of cytokine gene induction.

p300/CBP has been shown to be a coactivator of NF-κB in multiple promoters including HIV (47), IFNβ (48), VCAM1, and E-selectin (49). Additionally, p300/CBP binds to the basal transcription factor TFIIB, which in turn contacts the TATA box-binding protein (TBP) of the basal transcription apparatus (65) (46). p300/CBP also binds to the histone acetylating enzyme PCAF, and possesses intrinsic histone acetyltransferase activity (66) giving p300/CBP the dual functionality of bridging enhancers to the basal transcription machinery and enabling chromatin remodeling through histone acetylation. In addition to histone acetylation, p300/CBP activates transcription through direct acetylation of transcription factors, including NF-κB (6). Our results demonstrate that E1A inhibits cytokine-dependent gene transcription by interfering with p300/CBP-dependent activation of NF-κB, at least in part by interfering with acetylation of NF-κB.

These results are consistent with studies demonstrating that E1A augments TNF cytotoxicity by disabling cytoprotective pathways normally triggered following TNF exposure (59–63). Although E1A enhances TNF cytotoxicity by multiple mechanisms, its effects have been attributed at least in part to its p300 binding domain (25, 64). By interfering with p300-dependent acetylation of NF-κB, E1A may facilitate TNF-induced apoptosis by blocking induction of cytoprotective proteins such as ferritin H, MnSOD, and IL-6.

In addition to its role in NF-κB-dependent induction of ferritin H, p300/CBP also serves as a component of the basal transcriptional enhancer of ferritin H, FER1. Our previous results have demonstrated that FER1 is critical to the induction of ferritin H by oxidants (67). Thus, p300 serves to bridge two critical but previously discrete regulatory pathways in ferritin H regulation: that of induction by oxidants and by cytokines. These results implicate p300 as a critical convergence point of inflammatory (cytokine-meditated) and oxidant stress.

We found that mutants that abrogate binding of E1A to the pocket proteins pRb, p107, and p130 remained competent to repress TNF-dependent induction of a FER2-luc gene, although their activity was interfering with p300-dependent pathways in ferritin H regulation: that of induction by oxidants and by cytokines. More broadly, our results imply that recruitment of coactivators is an important mechanism for control of NF-κB-dependent cytoprotective activities.

Acknowledgments—We thank Dr. Warner C. Greene for the kind gift of anti-acetylated K310 RelA antibody. We are grateful to Rong Ma for superb technical assistance.

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