Adenovirus E1A regulates lung epithelial ICAM-1 expression by interacting with transcriptional regulators at its promoter

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Morimoto K, Gosselink J, Kartono A, Hogg JC, Hayashi S, Ogawa E. Adenovirus E1A regulates lung epithelial ICAM-1 expression by interacting with transcriptional regulators at its promoter. Am J Physiol Lung Cell Mol Physiol 296: L361–L371, 2009. First published December 26, 2008; doi:10.1152/ajplung.90331.2008.—We focused on the regulation of inflammatory mediator expression by adenovirus E1A in lung epithelial cells and the role of this viral protein in the pathogenesis of chronic obstructive pulmonary disease (COPD). We previously reported that E1A, a well-known regulator of host genes, increased ICAM-1 expression in human bronchial epithelial (HBE) and A549 cells in response to LPS stimulation. In this report, we clarified the mechanism of this regulation. We found NF-κB translocation to the nucleus after LPS stimulation in both E1A-positive and -negative HBE cells. ICAM-1 promoter reporter constructs revealed that a mutation in the proximal NF-κB binding site completely inhibited increased transcription, whereas the mutation in a distal site did not. We analyzed the participation of E1A in transcriptional complex formation at this promoter using chromatin immunoprecipitation. In E1A-positive HBE and A549 cells, LPS stimulation increased ICAM-1 promoter immunoprecipitation with NF-κB p65 and p300 but not activator protein-1 antibodies with a concomitant increase by the E1A antibody. No increase was found in E1A-negative cells except in HBE cells with p65 antibody. The association of E1A with the increased promoter immunoprecipitation with p300 was also observed after TNF-α stimulation of A549 cells. These results suggest that adenovirus E1A regulates the ICAM-1 promoter through its proximal NF-κB binding site, most likely by interacting with the transcriptional complex that forms at this site. E1A regulation of the LPS response may play a role in acute exacerbations as a consequence of bacterial infections in COPD.

gene regulation; inflammation; nuclear factor-κB

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is characterized by chronic cough and sputum production, peripheral airway obstruction, and emphysematous lung destruction. All these symptoms are related to chronic inflammation in airways, parenchyma, and blood vessels where the presence of various inflammatory cells is increased as well as the release of a variety of inflammatory mediators including TNF-α (7), one of the inducers of ICAM-1 (CD54).

ICAM-1 is an inducible cell surface glycoprotein and a well-known counter-receptor for lymphocyte adhesion molecule LFA-1 (CD11a) and the adhesion molecule Mac-1 (CD11b) expressed on the surface of leukocytes such as monocytes and polymorphonuclear leukocyte (9, 44, 45). It is induced on many cell types including airway and alveolar epithelial cells by several cytokines such as interferon-γ, TNF-α, and interleukin-1 (39, 48, 54). ICAM-1 expression by epithelial cells is increased in airway inflammatory processes associated with cigarette smoke-induced chronic bronchitis (8), which is a component of COPD. Furthermore, antibodies against ICAM-1 reduced the adhesion of leukocytes to lung epithelial cells (47).

The cigarette smoking habit is the major cause of this inflammation, however, only a susceptible minority of smokers develops COPD. In addition to tobacco smoke exposure, other risk factors include genetic predisposition, airway hyperresponsiveness, reduced lung growth during development, and exposures to occupational dusts and fumes as well as outdoor and indoor air pollutants (12a). Several studies also suggest that childhood infections are an independent risk factor for the development of COPD (24, 42) and that adenovirus infections are a common cause of bronchiolitis in children (11).

Adenovirus is a double-stranded DNA virus, and group C adenoviruses are significant infective agents of the lower respiratory tract, particularly in young children, with the possible persistence of the viral DNA (15). Integration of the viral DNA into host chromosomes has been documented where at least 12–14% of the adenovirus genome including the E1A gene is found (10, 14). Adenoviral E1A is a well-known transactivator that functions by interacting with numerous cellular transcription factors (26), transcriptional coactivators (3, 27), and cell cycle regulatory proteins (55). The modulation of the activity of NF-κB, a key transcription factor regulating the expression of inflammatory mediator genes (31), by E1A (33, 38, 43) as well as indirect effects of E1A on NF-κB that are mediated by coactivators cAMP response element binding protein (CREB) binding protein (CBP) and the related p300 protein (12, 38) have been demonstrated. Thus the E1A protein is capable of interacting with different components of this multiprotein complex to modulate the expression of genes.

Our working hypothesis is that latent adenoviral infection is one of the factors that amplifies lung inflammation in COPD, and we had previously established greater levels of adenoviral E1A DNA, in the absence of an active infection, in the lungs of smokers with COPD compared with those with normal lung function (28). Also, we have shown that, in advanced emphysema, there is an amplified inflammatory process that is associated with increased ICAM-1 as well as adenovirus E1A expression (40). In a guinea pig model of latent adenovirus infection, the expression of viral E1A
continues after viral replication had ceased at 20 days (51). In this model of latent adenovirus infection, exposure to an inflammatory stimulus, in this case cigarette smoke, increased lung inflammation as demonstrated by an increase in inflammatory cells in both the airways and lung parenchyma as well as an increase in the emphysematous destruction of the lung compared with uninfected controls (30). Therefore, we were interested in the possibility that E1A could affect inflammatory mediator expression, especially that of ICAM-1, in lung epithelial cells.

To determine whether the capacity of E1A to regulate gene transcription of the host cell is the basis of the relationship between ICAM-1 and E1A expression and increased inflammation in lung diseases such as COPD, both A549 cells, as a model of alveolar epithelial cells, and primary human bronchiolar epithelial (HBE) cells expressing adenovirus 5 E1A were developed (16, 22). With respect to the pathology of COPD, the A549 cells represent tissue affected by emphysematous destruction (29), whereas the HBE cells represent the epithelial component of the airways disease (18). In both cells, ICAM-1 expression in response to LPS stimulation was increased by E1A (16, 22), and this was accompanied by increased binding activity of NF-κB (16, 23). These results were supported by an increase in ICAM-1 promoter-driven reporter gene expression in both A549 and HBE cells when E1A was present (16, 17).

The current study further investigates the mechanism by which E1A regulates ICAM-1 expression in lung epithelial cells. Site-directed mutagenesis of the two NF-κB binding sites in the ICAM-1 promoter was applied to determine that the more proximal of the two was required for the E1A-directed increase in promoter activity. Chromatin immunoprecipitation (ChIP) assays showed that E1A interacts with the proteins of the transcription complex, including NF-κB, the coactivator p300, and RNA polymerase II, that assemble at this proximal site to promote transcription of the ICAM-1 gene. Subtle differences in the manner in which E1A regulates the expression of the ICAM-1 gene, such as differences in NF-κB translocation, were noted between A549 and HBE cells. This is the first report of adenovirus E1A upregulating the expression of an inflammatory mediator gene through its interaction with the host transcriptional apparatus at the promoter of the ICAM-1 gene in lung epithelial cells.

MATERIALS AND METHODS

Reagents. LPS from Escherichia coli 0111:B4 was obtained from Sigma (St. Louis, MO). TNF-α was from Calbiochem (La Jolla, CA). Antibodies used were the following: rabbit antibodies to p65, p50, p30, ICAM-1, and the c-Jun subunit of activator protein-1 (AP-1) from Santa Cruz Biotechnology (Santa Cruz, CA) and RNA polymerase II (Upstate, Lake Placid, NY); mouse antibodies to adenovirus 2 E1A (Calbiochem), Toll-like receptor 4 (TLR4; BioCarta, San Diego, CA), GAPDH (Santa Cruz Biotechnology), and control IgG (Upstate).

Cell culture. The HBE cells used in this study have been previously described in detail (16). Briefly, two primary cell lines, HC35 and HC57, from two different patients were transfected with the pS2neo plasmid carrying the adenoviral E1A and E1B genes under the control of their viral promoters. The resulting clones that express E1A but not E1B are referred to as HA35-1 and HA57, respectively. Similar levels of E1A protein expression in these two cell lines have been reported (35). One cell line from the same patient from which HA35-1 was developed was transfected with this plasmid DNA but did not express E1A mRNA or protein. It is referred to as HA35-2 and was used as an E1A-negative control for HA35-1. In addition to this control, HC35 and HC57, primary HBE cells from the two patients who provided these cells, as well as those from two other patients, which are referred to as HC3503 and HC3411, were used as negative controls. The HBE cells were cultured in Bronchial/Tracheal Epithelial Cell Basal Medium (BEGM; Clonetics, San Diego, CA), and the medium was changed every 48–72 h. Subconfluent cells were used at the start of each experiment.

E1A-expressing A549 cells, a human alveolar type II cell line originally from a patient with bronchioloalveolar carcinoma, have been described in detail in our previous report (22). Briefly, the cells had been stably transfected with a plasmid carrying adenovirus 5 E1A gene driven by its own promoter or the empty plasmid as control. We used four different clones, E4 and E11 as E1A-expressing cells and C4 and C8 as E1A-negative cells. The transfectants were maintained in Eagle’s minimum essential medium (Sigma) containing 10% fetal bovine serum (GIBCO, Grand Island, NY) and were under constant selection with 280 μg/ml G418 (GIBCO).

Western blot analysis. After 24-h incubation with or without 10 μg/ml LPS in 10-cm dishes, total proteins were prepared from cells lysed with 1% Nonidet P-40 (NP-40) (GIBCO). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) and 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies to TLR4, GAPDH in 5% skim milk in TBS and 0.1% Tween 20. The membranes were then incubated with the respective horseradish peroxidase-conjugated anti-mouse antibody or anti-rabbit antibody (Dako), and the chemiluminescence kit (Promega, Madison, WI) was used for detection.

Immunocytochemical staining of ICAM-1. E1A-positive and -negative A549 cells grown on sterile coverslips were incubated for 24 h with or without 10 μg/ml LPS before fixing with 100% ethanol. After fixed cells were dried, blocking buffer (Dako protein block) was applied for 15 min, the 50 times diluted ICAM-1 antibody for 1 h, horseradish peroxidase-conjugated anti-rabbit antibody for 1 h, followed by staining using the 3,3’-diaminobenzidine enhanced liquid substrate system tetrahydrochloride (Sigma). Micrographs of the stained cells were taken on a Nikon Eclipse E600 photomicroscope.

Immunofluorescent staining. E1A-negative and -positive HBE cells grown in BEGM on glass coverslips were incubated with or without 10 μg/ml LPS for 1.5 h. Then, the cells were fixed with 10% buffered formalin for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and washed three times with PBS. After 1-h incubation with universal blocking reagent (Sigma), the cells were incubated overnight at 4°C with primary antibodies, either rabbit anti-p65 or p50 antibody, followed by incubation with FITC-conjugated swine anti-rabbit IgG (Dako) for 30 min. Fluorescence microscopy and digital image capture were performed on a Nikon Eclipse TE300 microscope.

Construction of mutated ICAM-1 promoters driving the luciferase reporter. The human ICAM-1 promoter from nucleotides −1336 to −9 relative to the transcription start site at +1 as designated in NT_011295 of the National Center for Biotechnology Information (NCBI) sequence and located at nucleotide 1644581 of this sequence was excised from the plasmid pHBluc1.3 (a kind gift from Dr. C. Stratowa, Bender, Vienna, Austria) and then ligated into the pGL3 basic luciferase plasmid (Promega) between the Kpn I and Xho I sites. The recombinant plasmid is hereafter referred to as pWild-LUC.

Site-directed mutagenesis was used to mutate the distal NF-κB and Ets-1 binding sequences of the wild-type ICAM-1 promoter that was cloned into the pALTER-1 vector (Promega). The oligonucleotide corresponding to nucleotides −495 to −459 relative to the transcription start site used for mutating the distal NF-κB binding sequence located at nucleotides −483 to −474 (Fig. 1) was 5’-GAG GGA GCC CGG GcC GcC TaTa gTG GGC CCC CAC CCA G-3’ where the
lowercase letters represent the mutations. The Ets-1 binding site located at −140 to −129 (Fig. 1) was used as a control since this site responds to growth factor and stress-activated signaling pathways that are involved in cell proliferation, differentiation, and oncogenic transformation (53) and is not expected to affect the LPS response by ICAM-1 promoter. Its mutated sequence corresponding to nucleotides −154 to −121 was 5'-GAA GCC GCC AGC Gaa Gaa Gaa TGA CCC TCT CGG C-3'. After site-directed mutagenesis using the Altered Sites II in vitro Mutagenesis System (Promega) was confirmed as successful by sequence analysis, the mutated ICAM-1 promoters were used to replace the wild-type promoter between EcoRI and Pst I sites of pWild-LUC to create luciferase reporter plasmids driven by each mutant ICAM-1 promoter. The respective plasmids are referred to as pDistal-LUC and pEts-LUC.

To create a mutation in the proximal NF-κB binding sequence located at −171 and −161 of the ICAM-1 promoter (Ref. 46; Fig. 1), PCR with two pairs of primers (Fig. S1, available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site) and pWild-LUC as a template was used. In the first pair, primer PCR1-5' was taken from position −421 to −402 of the ICAM-1 promoter with the following sequence, 5'-AAG CGT GAG AGG GAG GAC TT-3', whereas primer PCR1-3' corresponding to nucleotides −185 to −161 includes the proximal NF-κB binding sequence, which has been mutated to create an Xba I restriction site to give following sequence, 5'-ctA ATc Tag AAG CTA AAG CAA TCG G-3', where the Xba I site is underlined and the altered nucleotides are in lowercase. In the second pair, primer PCR2-5' corresponding to nucleotides −173 to −150 also spans the proximal NF-κB binding sequence but was mutated to create an Xba I restriction site to give following sequence, 5'-GCT Tct AgA TTA gGG AGC TGA AGC GAC TC-3', where the Xba I site underlined and altered nucleotides in lower case. Primer PCR2-3' located between positions 159 and 167 in the pALTER-1 vector sequence was 5’-TGC TAT TGG GTG TT-3’, the two PCR products from the primers pairs were digested with Xba I and ligated to each other. In the next step, PCR with primers PCR1-5' and PCR2-3' amplified the ligated fragment that included the mutated proximal NF-κB binding sequence. This PCR product was digested with Hind III, which has sites at nucleotide −261 relative to the transcription start site and at nucleotide 56 in the pALTER-1 vector (Promega) and used to replace the Hind III fragment of pWild-LUC to create the plasmid pProximal-LUC. This Hind III fragment carrying the mutated proximal NF-κB bonding site was also used to replace the corresponding Hind III fragment in pDistal-LUC to produce a plasmid, pDual-LUC, with mutations in both proximal and distal NF-κB binding sequences. All the constructs were verified by sequencing.

Transient transfection of HBE cells and luciferase assay. Trypsinized HA35-2, HA35-1, and HA57 cells were resuspended at 5.0 × 10⁶ cells per milliliter in Bronchial/Tracheal Epithelial Cell Basal Medium (Clonetics) with 1 mM dextrose and 0.1 mM DTT, whereas primary HBE cells were resuspended at 1.0 × 10⁶ cells per milliliter. The cells were transiently cotransfected with 10 μg of the reporter construct and 4 μg of a β-galactosidase (β-gal) control vector, pCMV-β-gal (a gift from Dr. Luo, University of British Columbia, Vancouver, Canada), by electroporation in 0.4-cm cuvettes at 200 V and 950 μF (HA35-1), 400 V and 950 μF (HA57), 420 V and 950 μF (HA35-2), and 440 V and 1,000 μF (primary HBE cells). These conditions had been determined to give maximal transfection efficiency for the respective cells. The cells were then plated on 12-well culture dishes, allowed to attach for 24 h, and incubated for the next 24 h in fresh BEGM. After 48-h incubation from the time of electroporation, the cells were incubated with or without 10 μg/ml LPS for an additional 24 h before they were lysed with Reporter Lysis Buffer (Promega).

Luciferase activity in the cell lysates was measured using the luciferase assay system (Promega). β-Gal assay (Promega) was performed, and the luciferase activity normalized to the respective β-gal activity was used as a measurement of ICAM-1 promoter activity. Statistical analysis by an ANOVA with post hoc Fisher test was used for comparisons between groups. Values of P < 0.05 were considered statistically significant.

EMSA. After 2-h incubation with or without 10 μg/ml LPS, nuclear proteins were prepared from E1A-negative and -positive HBE cells as described previously (23). Protein concentrations were determined using the Protein Assay System (Bio-Rad), and 10 μg of nuclear extract were used in the EMSA. The sequences of the double-stranded DNA oligonucleotide representing the wild-type proximal NF-κB binding site was 5’-GGA GCG GCC AGC GAa Gat ctA TGA CTA AAG CAA TCG G-3', whereas primer PCR2-3' oligonucleotide with the sequence 5’-CGA TTG CTT TAG CTT CTA GAT TAG GGA GAC TT-3' was used as cold competitors (23).

ChIP assay. After 2- to 2.5-h incubation of E1A-expressing HBE and A549 cells and their respective controls in culture medium containing 10 μg/ml LPS, 100 U/ml TNF-α, or PBS as control in 10-cm dishes, ChIP assays were performed according to the ChIP assay kit (Upstate). Briefly, 1 × 10⁶ cells were fixed with 1% formalin to cross-link transcriptional regulators to the DNA as well as to themselves. The fixed cells were resuspended in 100 μl of SDS lysis buffer supplied with the kit with the addition of 1 μg/ml protease inhibitors aprotinin (Sigma), pepstatin A (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma). The cells were sonicated 3–4 times at 20–30% of maximum power for 10 s using the model VC50T sonicator (Sonics and Materials, Danbury, CT) to shear DNA to 200–1,000 bp and then centrifuged. After preclearing with the Salmon Sperm DNA/Protein A Agarose-50% Slurry provided in the ChIP assay kit, the 10 times diluted supernatant was treated with one of the following antibodies: anti-RNA polymerase II, anti-p65, anti-p300, anti-E1A, anti-c-Jun, or normal mouse IgG as a negative control with following antibodies: anti-RNA polymerase II, anti-p65, anti-p300, anti-E1A, anti-c-Jun, or normal mouse IgG as a negative control with
Reversed by incubation in 192 mM NaCl at 65°C for more than 4 h.

The DNA was then purified by the DNeasy kit (Qiagen, Mississauga, Ontario, Canada) and eluted with 40 μl of AE buffer of the DNeasy kit. Quantitative PCR was performed to quantify the amount of precipitated DNA. To quantify the amount of input DNA, 5% of the 10 times diluted supernatant that initially had been precleared with the Salmon Sperm DNA/Protein A Agarose-50% Slurry was kept without immunoprecipitation by antibodies and then treated to remove the cross-linking before the DNA was purified as above.

Quantitative PCR. PCR primers for amplifying the proximal NF-κB binding site of human ICAM-1 promoter were the following: forward primer, 5’-TTG GAA ATT CCG GAG CTG AA-3’, and reverse primer, 5’-TGC AGT TAT TCT GCG ACT-3’, at nucleotides −171 to −152 and −86 to −106, respectively, relative to the transcription start site. Quantitative PCR on the ABI 7900HT (Applied Biosystems, Foster City, CA) was programmed for 95°C for 15 min for 1 cycle followed by 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s for 40 cycles, and the PCR reactions contained 0.5 μM each primer, SYBR Green master mix (Qiagen), and a 5.5-μl aliquot of the 40 μl of the immunoprecipitated DNA. Similarly, to quantify the amount of input DNA, that is the amount of DNA present before immunoprecipitation with antibodies, PCR primers for amplifying human U6 small nuclear RNA (U6 snRNA) gene promoter (34) with the following sequences were used: forward primer, 5’-CAA GGT CGG GCA GGA AGA G-3’, and reverse primer, 5’-TGC AAA TAT GAA GGA ATC ATG GG-3’. These sequences were taken from the NCBI nucleotide sequence of NT_010194.16 and are located up-stream of this chromosome 15 U6 snRNA gene, which is located between nucleotides 38922640 and 38922596. Both PCR products were electrophoresed on 2% agarose gels to check the product sizes of 86 and 51 bp for the NF-κB and U6 targets, respectively. Also melting curve profiles were obtained to confirm the presence of a single product. The standard curves of the cycle threshold (Ct) plotted against the log value of the initial amount of DNA using genomic DNA from A549 cells had a slope of −3.36 and −3.33 for the promoter of the ICAM-1 and the small nuclear RNA genes, respectively.

The relative log-transformed amount of immunoprecipitated proximal NF-κB site of the ICAM-1 promoter was calculated as follows (32):

\[ Y(x) = \frac{C_{ICAM-1(x)}/\text{slope}_{ICAM-1(x)}}{C_{U6snRNA(x)}/\text{slope}_{U6snRNA}\text{gene}} \]

where \( Y(x) \) represents the log-transformed amount of immunoprecipitated ICAM-1 promoter adjusted for the amount of input DNA (U6 snRNA gene) after treatment \( x \), that is, control or treatment with either LPS or TNF-α; \( C_{ICAM-1(x)} \) represents the Ct value of the ICAM-1 promoter PCR after treatment \( x \); slope\text{ICAM-1(x)} represents the slope of the ICAM-1 promoter standard plot, in this case −3.36; \( C_{U6snRNA(x)} \) represents the Ct value of the U6 snRNA gene PCR after treatment \( x \); and slope\text{U6snRNA}\text{gene} represents the slope of U6 snRNA gene standard plot, in this case −3.33.

For each antibody used in the immunoprecipitations, the \( Y(LPS) \) value after treatment with LPS was compared with that of the untreated control using the paired t-test, and the magnitude of the differences between LPS and control was measured as \( \Delta Y\text{ICAM-1} = Y(LPS) - Y(\text{control}) \). Similarly, \( \Delta Y\text{TNF-α} \) was calculated, and this along with \( \Delta Y\text{LPS} \) was analyzed in comparisons between E1A-negative and -positive A549 cells by the Fisher protected least significant differences test. Values of \( P < 0.05 \) were considered statistically significant. The antilog of the average \( \Delta Y \) was used to calculate fold change between E1A-negative and -positive A549 cells.

**Coimmunoprecipitation of p65 with adenovirus E1A.** Chromatin samples from the ChIP assay (see above) that were treated with the E1A antibody and then precipitated by the Salmon Sperm DNA/Protein A Agarose-50% Slurry had cross-links reversed as above. SDS electrophoresis buffer containing 125 mM Tris·HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.01% bromophenol blue was added, and precipitated proteins were analyzed by Western blotting as described above using the p65 antibody. IgG was used as a control for equal protein loading.

**RESULTS**

**Expression of ICAM-1 and TLR4 in E1A-negative and -positive A549 and HBE cells.** Western analysis showed that LPS stimulation increased the expression of ICAM-1 in both E1A-positive A549 and HBE cells but not in the respective E1A-negative cells (Fig. 2A). Similar results were obtained after ICAM-1 immunocytochemical staining of transfected A549 cells (Fig. S2). Whereas the expression of TLR4, an LPS receptor that signals through the NF-κB pathway, was upregulated in E1A-positive but not E1A-negative A549 cells regardless of LPS stimulation, no significant difference in its expression was found between E1A-negative and -positive HBE cells, and LPS stimulation did not affect this expression (Fig. 2B).

**Translocation of NF-κB from cytoplasm to nucleus after LPS stimulation.** To determine whether p65 and p50 translocate to the nucleus in response to LPS stimulation of our HBE cells, immunofluorescence analysis to track these NF-κB subunits was performed before and 1.5 h after LPS stimulation. In unstimulated cells, p65 and p50 were mainly detected in cytoplasms of both E1A-negative (HA35-2) and E1A-positive (HA35-1 and HA57) HBE cells (Fig. 3, A, C, E, G, I, and K, respectively). As expected, LPS induced the translocation of both p65 and p50 to the nucleus in E1A-positive cells (Fig. 3, F, H, J, and L). However, contrary to expectations, both subunits translocated to the nucleus in E1A-negative HBE cells as well (Fig. 4, B and D). We have previously reported that translocation of NF-κB from cytoplasms to nuclei after LPS stimulation was observed only in E1A-positive A549 cells (23).

Luciferase reporter with mutated NF-κB sites in the ICAM-1 promoter. Figure 1 shows a diagram of human ICAM-1 promoter, which is part of pWild-LUC and shows the location, relative to the transcription initiation site, of the distal and proximal NF-κB binding sites at −483 to −474
bp and −170 to −161 bp, respectively, and the Ets-1 site at −140 to −129 bp.

To determine whether either of the NF-κB binding sites was required for the LPS-induced ICAM-1 gene upregulation in E1A transfected HBE cells (16), four luciferase constructs with either wild-type or mutant distal or proximal NF-κB binding sites or the combination of the two mutant sites in the ICAM-1 promoter were prepared and referred to as pWild-LUC, pDistal-LUC, pProximal-LUC, or pDual-LUC, respectively (Fig. 1). In addition to the above constructs, a fifth plasmid, pEts-LUC, was prepared and referred to as pEts-LUC.

Fig. 3. Translocation of NF-κB from cytoplasm to nuclei after LPS stimulation. The E1A-negative transfected HBE cells, HA35-2 (A–D), and the E1A-positive transfected cells, HA35-1 (E–H) and HA57 (I–L), were incubated with (B, D, F, H, J, and L) or without (A, C, E, G, I, and K) 10 μg/ml LPS for 1.5 h. The localization of p65 (A, B, E, F, I, and J) and p50 (C, D, G, H, K, and L) were determined by immunofluorescent staining with either rabbit anti-p65 antibody or rabbit anti-p50 antibody. Bar = 50 μm.

Fig. 4. Transcriptional activity of the ICAM-1 promoter as determined by a luciferase reporter in E1A-positive and -negative HBE cells. E1A positive HA35-1 (A), positive HA57 (B), negative HA35-2 (C), and negative primary HC35, HC3503, and HC3411 (D) HBE cells were transiently cotransfected with luciferase reporter plasmids driven by the wild-type or indicated mutated ICAM-1 promoters (see Fig. 1 for details) and the pCMV-β-gal control vector, pCMV-β-gal. After 48 h, the cells were incubated with or without 10 μg/ml LPS for 24 h. Luciferase activity in the cell was normalized to the respective β-gal activity to correct the transfection efficiency and then further normalized to that of the corresponding unstimulated pWild-LUC transfected control. Open bars representing unstimulated controls and closed bars, LPS-stimulated, are the means ± SE from 3 independent experiments on each of HA35-1, HA35-2, and HA57 and, for the primary HBE cells, the means ± SE from combined single experiments on each of HC35, HC3503, and HC3411. *Significantly different from the unstimulated control transfected with the same luciferase plasmid (P < 0.05); **significantly different from respective pWild-LUC transfectant (in C, P < 0.025 for Distal vs. Wild and in D, P < 0.005).
was prepared with a mutation in the Ets-1 binding site as a control (Fig. 1).

Proximal NF-κB site of the ICAM-1 promoter required by adenovirus E1A to increase promoter activity in HBE cells. For E1A-positive HBE cells, in agreement with previous results showing increased expression of ICAM-1 mRNA (16) and protein (Fig. 2A) in response to LPS stimulation of these cells, the wild-type ICAM-1 promoter activity was also increased by LPS stimulation, and this increase was not affected by the mutation in the distal NF-κB or Ets-1 binding sites of this promoter (Fig. 4, A and B). In contrast, a mutation in the proximal NF-κB binding site, either alone or together with the mutated distal site, abrogated this increased promoter response (Fig. 4, A and B).

In E1A-negative HBE cells (Fig. 4, C and D), as expected from results where LPS stimulation did not change either basal ICAM-1 mRNA (16) or protein (Fig. 2A) expression, the activity of the wild-type promoter also did not change after LPS stimulation. This lack of response to LPS stimulation was not altered by mutations in the distal or proximal NF-κB binding sites, by the combination of these two, or by the mutated Ets-1 binding site. However, in the transfected but E1A-negative HBE cells, mutations in the distal NF-κB or Ets-1 binding sites increased promoter activity compared with the wild-type promoter regardless of LPS stimulation (Fig. 4C). Results from primary HBE cells support both this increase due to the mutation in the distal NF-κB binding site (Fig. 4D) and the absence of change with the proximal mutation. A similar test of the Ets-1 site in these cells was not possible due to an inadequate number of these primary cells.

Adenovirus E1A increases NF-κB binding to the nucleotide sequence specifying its proximal binding site in the ICAM-1 promoter. Once it was established that, of the two NF-κB binding sites, the proximal one was responsible for increased ICAM-1 promoter activity in E1A-expressing HBE cells, EMSA was applied to determine whether, in response to LPS stimulation of these cells, this transcription factor bound to a double-stranded oligonucleotide representing the sequences from this proximal site and whether other proteins of interest were part of the complex formed by this transcription factor. The results (Fig. 5) showed that LPS stimulation of E1A-negative HC57 cells did not affect the low basal levels of binding of protein to the labeled oligonucleotide. In contrast, binding to form this complex was strong in unstimulated E1A-positive HA57 cells and even stronger when these cells were stimulated with LPS (Fig. 5, NF-κB binding I). Besides this complex, another of higher mobility and thus of smaller size (Fig. 5, NF-κB binding II) was observed only in E1A-positive cells stimulated with LPS. That these two bands represent NF-κB-specific binding was supported by results from the cold competition experiments where excess unlabeled wild-type proximal NF-κB oligonucleotide decreased the binding of proteins to the labeled probe in these two complexes, whereas the nonspecific AP-1 and mutated proximal NF-κB oligonucleotides affected this binding minimally. NF-κB-specific binding was further confirmed by supershift assays where application of antibodies to either the p65 or p50 subunits of NF-κB resulted in the formation of supershifted complexes of lower mobility, whereas a weak supershifted band was present with antibody to E1A and no supershifts when antibodies to CBP, p300, or the respective control IgG antibodies were applied. Similar results were found in comparisons between E1A-positive HA35-1 and control HC35 or E1A-negative HA35-2 (data not shown).

E1A participates in the formation of the transcriptional complex at the ICAM-1 promoter in LPS-stimulated HBE cells. Since our immunofluorescence analyses showed that p65 translocation from the cytoplasm to the nucleus in response to LPS stimulation is independent of the presence of E1A in HBE cells, the dependence of the activity of the ICAM-1 promoter on E1A could result from the capacity of the viral protein to transactivate transcription factors and transcriptional complexes bound to promoters (3, 26, 27). However, only weak participation of E1A in the complex that assembles on the short 38-bp oligonucleotide from the ICAM-1 promoter that includes the proximal NF-κB binding site and none by the other transcriptional coactivators, CBP and p300, could be demonstrated by EMSA (see above). Because the size of probes used for EMSA are usually limited to sequences comprising a single transcription factor binding site, binding of these non-DNA binding proteins to the complexes that form at the ICAM-1 promoter may require an extension of the current probe to include sequences such as the TATA site, which is located 191

[Fig. 5. Nuclear protein binding to the proximal NF-κB binding sequence of the ICAM-1 promoter. Representative autoradiogram from an EMSA using a labeled oligonucleotide probe with the proximal NF-κB binding site of the ICAM-1 promoter to detect NF-κB binding activity in nuclear extracts from E1A-negative HC57 (−) and -positive HA57 (+) cells incubated for 2 h with (+) or without (−) 10 μg/ml LPS is shown. The unlabeled oligonucleotides with the wild-type proximal NF-κB binding site, nonspecific [activator protein-1 (AP-1)] site, or mutated proximal NF-κB binding site were used as a cold competitor. For supershift assays, antibodies against p65, p50, cAMP response element binding protein (CREB) binding protein (CBP), p300, or E1A and, as a negative control, immunoglobulin (IgG, either rabbit or mouse) were used. Supershifted complexes (asterisk) and those specific for NF-κB binding (I and II) are indicated.]
nucleotides upstream of this NF-κB binding sequence. Since both CBP/p300 and E1A bind effectively to the TATA binding protein (TBP) (12), their presence at the ICAM-1 promoter may be stabilized through such interactions but not by the limited complex that forms when only the NF-κB binding sequence is available. Therefore, we turned to a ChIP assay that interrogates fragments of DNA 200–1,000-bp long to investigate whether E1A and the transcriptional coactivator p300, known to interact with E1A (3, 27), are part of the transcriptional complex that forms at the ICAM-1 promoter in E1A-positive HBE cells in response to LPS stimulation. Amplification plot diagrams (Figs. S3 and S6), denaturation curves of the corresponding PCR products (Figs. S4 and S7), tables of details of the samples in the wells of the plates together with their Ct values (Tables S1 and S2), and agarose gels of PCR products from representative ChIP assays (Fig. S5) are presented in the online data supplement.

LPS stimulation of both E1A-positive HA35-1 and E1A-negative HA35-2 HBE cells significantly increased the coimmunoprecipitation of the ICAM-1 promoter with p65 compared with unstimulated cells (Fig. 6, A and B), and these increases were not different between these two cells (Table 1). Also, only in E1A-positive cells, significant increases were observed after immunoprecipitation of p300 and E1A (Fig. 6, A and B). These increases represented changes of 7.7 and 5.7 times, respectively, over the levels of promoter immunoprecipitated in the E1A-negative cell (Table 1). The lack of increase in the E1A binding in the absence of its expression verifies the specificity of the antibody that was used. No change in response to LPS was found in either cell after immunoprecipitation with anti-c-Jun/AP-1 antibody, although basal levels of binding were relatively high, nor with normal mouse IgG (Fig. 6, A and B).

**E1A participates in the formation of the transcriptional complex at the ICAM-1 promoter in LPS-stimulated A549 cells.** Contrary to the findings reported above in HBE cells, NF-κB translocation from cytoplasm to nucleus in response to LPS stimulation in A549 cells is dependent on E1A (23). Although this facilitation of nuclear translocation could explain the increased ICAM-1 mRNA expression in response to LPS stimulation in A549 cells, the participation of E1A in the transcriptional complex that forms as a consequence of NF-κB translocation in these cells may be equally important. To test this, we applied the ChIP assay to these cells as described above for HBE cells, except that in this case LPS stimulation was for 2.5 h since this was the time interval used to detect NF-κB translocation in A549 cells (23). LPS stimulation significantly increased the binding of p65 to the ICAM-1 promoter in E1A-positive E11 cells (Fig. 7A) but not in E1A-negative C4 controls (Fig. 7B). The differences between these E1A-positive and -negative A549 cells were significant and represented fold increases of 11.9 times (Table 1). Similarly, and again only in E1A-positive cells, significant increases were observed after immunoprecipitation of p300 and E1A (Fig. 7, Table 1). As expected, significant increases were also observed, but only in E1A-positive cells, after immunoprecipitation of RNA polymerase II (data not shown). On the other hand, no significant increases were found in these cells after anti-c-Jun/AP-1 antibody or normal mouse IgG immunoprecipitation, although again basal levels of AP-1 binding were high (Fig. 7A). The finding that no significant changes in response to LPS stimulation were observed in E1A-negative C4 A549 cells (Fig. 7B) was expected since p65 nuclear translocation did not occur in these cells (23). When a different pair of E1A-negative and -positive A549 cells (C8 and E4, respectively) was compared, similar results were obtained (Table 1 and data not shown). Further support for the presence of E1A in this NF-κB transcriptional complex came from ChIP of LPS-stimulated A549 cells with the E1A antibody followed by Western blotting of the precipitated proteins using antibody to p65. Results showed that, when E1A was expressed, p65 coimmunoprecipitated with E1A, whereas, as expected, little or no coimmunoprecipitation of p65 was found in E1A-negative cells (Fig. 8A). Furthermore, p65 coimmunoprecipitation in these E1A-expressing cells was increased by LPS stimulation compared with unstimulated cells (Fig. 8B).

**E1A increases the formation of a transcriptional complex on the ICAM-1 promoter at the proximal NF-κB site in A549 cells after TNF-α stimulation.** In A549 cells, TNF-α-stimulated NF-κB nuclear translocation as well as increased ICAM-1 transcription had previously been demonstrated to be independent of adenovirus E1A (22, 23). We applied the ChIP assay to determine whether this E1A independence in response to TNF-α stimulation extends to the binding of the transcriptional regulators to the ICAM-1 promoter. After TNF-α stimulation, no significant difference was found between E1A-positive and
Table 1. ΔY values of ICAM-1 promoter immunoprecipitated from E1A-positive and -negative lung epithelial cells by the chromatin immunoprecipitation assay

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>E1A-Negative or -Positive Cells</th>
<th>ΔY After Immunoprecipitation with the Following Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p65</td>
</tr>
<tr>
<td>LPS</td>
<td>HBE: negative</td>
<td>1.027±0.058</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>1.025±0.040 (1.0)†</td>
</tr>
<tr>
<td></td>
<td>A549: negative C4</td>
<td>−0.167±0.002</td>
</tr>
<tr>
<td></td>
<td>positive E11</td>
<td>0.909±0.02* (11.9)</td>
</tr>
<tr>
<td></td>
<td>A549: negative C8</td>
<td>−0.023±0.003</td>
</tr>
<tr>
<td></td>
<td>positive E4</td>
<td>0.367±0.010* (2.5)</td>
</tr>
<tr>
<td></td>
<td>A549: negative C4</td>
<td>0.479±0.013</td>
</tr>
<tr>
<td></td>
<td>positive E11</td>
<td>0.674±0.014 (1.6)</td>
</tr>
</tbody>
</table>

ΔY = Y_{LPS or TNF-α} − Y_{control} (refer to details in MATERIALS AND METHODS) and represents the means ± SE from 3 independent experiments. *Significantly different from the corresponding adenovirus E1A-negative cell (P < 0.05). †Fold increase in parentheses compared with corresponding E1A-negative cells (refer to details in MATERIALS AND METHODS). HBE, human bronchial epithelial cells.

DISCUSSION

We demonstrated that adenovirus E1A regulates the expression of the ICAM-1 in two types of lung epithelial cells, the A549 cells of alveolar origin and the primary HBE cells of bronchial origin, through similar but not identical mechanisms (see below). Site-directed mutagenesis studies showed that this regulation centers around the promoter of the ICAM-1 gene, more specifically, the more proximal of the two NF-kB binding sites of this promoter. ChIP assays showed that, as a consequence of LPS stimulation, the presence of E1A at this site is increased together with that of the p65 subunit of the transcription factor NF-κB and the coactivator p300. However, the c-Jun subunit of another transcription factor, AP-1, was not. The implied interaction of E1A with NF-κB was supported by the coimmunoprecipitation of these two proteins from LPS-stimulated E1A-expressing HBE cells. Increases in E1A and p300 at this promoter site were also found as a consequence of TNF-α stimulation of A549 cells. The above results are supportive of our previous findings reviewed in the Introduction (15) and suggest a mechanism whereby E1A can regulate ICAM-1 mRNA expression.
Although the ultimate result of the regulation of the ICAM-1 gene by E1A is that of increased expression of ICAM-1 mRNA (16, 22) and protein (Ref. 22 and this study) in response to LPS stimulation in both A549 and HBE cells, subtle differences in achieving this increase have been revealed by the present experiments. In A549 cells, LPS-induced NF-κB translocation requires the presence of E1A (23); in HBE cells, the current study showed that NF-κB translocates to the nucleus in response to LPS even in the absence of E1A. This difference can be partly explained by our current finding that TLR4 expression in A549 cells is increased by E1A, whereas this receptor is expressed in HBE cells regardless of E1A. Why expression is different could be attributed to the fact that TLR4 is regulated differently in these two different cell types, one alveolar in nature, the other bronchiolar. Another possible reason is that A549 cells are a transformed tumor cell line, whereas the HBE cells were originally primary cells of nontumor origin. A further downstream consequence of this difference in TLR4 expression is that the increased presence of p65 at the ICAM-1 promoter is dependent on E1A in A549 cells but not in HBE cells. However, the mere presence of p65 at the promoter is inadequate to activated transcription of the ICAM-1 gene. The coactivator p300 needs to be recruited to this promoter and, in both lung cell types, this happens only when E1A is present. In summary, whereas E1A appears to engage the transcriptional complex to activate ICAM-1 transcription in both alveolar and bronchiolar epithelial cells, the subtle cell type specificity that occurs at this promoter underscores the importance of the molecular differences, which may have consequences in, for example, how cell type-specific therapeutic targets are selected.

Adenovirus E1A is expressed as two main isoforms, 12S and 13S, both of which include CR1 and CR2, two regions conserved between different adenovirus serotypes and which are required for transformation and cell growth regulation (38). A third conserved region, CR3, is present only in the 13S product, has a binding site for the TBP, and is responsible for transcriptional activation of both viral and cellular genes (38). Both A549 and HBE cells used in our studies have the capacity to express both 12S and 13S E1A (16, 22). Examples of transcriptional activation by E1A include activation of the enhancer of k-light chain gene by E1A in fibroblasts (4) where the activation is exerted through the NF-κB binding site of this enhancer (43) and augmentation of the human immunodeficiency virus (HIV) promoter activity by 13S E1A in Jurkat cells (38). These results support our findings of ICAM-1 promoter activation by E1A in response to an inflammatory stimulus. Other studies have demonstrated repression of the interleukin-6 (20) or TNF-α inducible ferritin H (21) promoters when cells were transiently transfected with either 12S or 13S E1A plasmids. However, since the mRNA transcribed from the wild-type 13S plasmid could subsequently be spliced to the 12S mRNA, and since, in both studies, it was not resolved whether 13S transfection yielded the protein product of the expected size, the promoter repression ascribed to 13S protein could be due to the 12S isoform, which is a well-documented repressor of transcription (38). Also, interleukin-6 secretion, in response to LPS or TNF-α stimulation, was suppressed in most NCI-H292 human lung mucoepidermoid carcinoma-derived cells stably transfected to express E1A (50), but, since both E1A isoforms were expressed at equal levels in these cells, the observed repression can again be attributed to the 12S isoform.

The current findings indicate that the most proximal of the two NF-κB binding sites (see Fig. 4) identified in the ICAM-1 promoter (46) is the target of transcriptional activation by E1A in response to LPS stimulation of lung epithelial cells. This finding is consistent with reports by others that the proximal site is required for increased ICAM-1 promoter activity in response to TNF-α stimulation of human umbilical vein endothelial cells (25), Hep G2 human hepatoma cells (19), U-937 cells (49), and A549 cells (6) and, in addition, to PMA stimulation of A549 and Hs913T cells (52). On the other hand, mutating the distal NF-κB binding site resulted in increased ICAM-1 promoter activity, which was unaffected by LPS stimulation or E1A. This identification of a repressor at this site, although consistent with a previous report of a repressor in this region of the ICAM-1 promoter (52), pinpoints the repressor to this specific distal NF-κB binding site. The above results suggest that the targeting of the proximal NF-κB site in the ICAM-1 transcriptional activation by E1A in response to LPS stimulation of lung epithelial cells is, in many respects, similar to the mechanism employed to activate ICAM-1 transcription in response to inflammatory stimulation of other cells; but this may differ in other, more subtle aspects, as discussed below.

Taken together, our results obtained after LPS stimulation suggest a scenario whereby E1A, through its binding site for TBP in its conserved region 3 as well as a p300 binding site in its NH2 terminus (38), increases the activity of the ICAM-1 promoter by recruiting p300 to the proximity of the DNA-bound NF-κB and, as a result, permits transactivation of the basal transcriptional machinery. In our case, whether the E1A recruited p300 achieves transcriptional activation by relaxing the chromatin bound promoter by its inherent or associated histone acetylation capacities (36) or by other means remains to be determined.

We (22) had previously shown that whereas E1A increased the expression of ICAM-1 in response to LPS stimulation of A549 cells, the already increased response to TNF-α was unaffected by E1A. The current finding that p65 binding to the ICAM-1 promoter was not increased after TNF-α stimulation in the presence of E1A is consistent with this previous result. However, increases in the p300 at the promoter, along with E1A, were unexpected. Our results suggest that E1A recruits p300 to the ICAM-1 promoter as a consequence of either LPS or TNF-α stimulation, but, in case of TNF-α stimulation, further recruitment of p300 does not augment ICAM-1 transcription. The TNF-α-stimulated binding of p65 to this promoter in the absence of E1A reflects that found in HBE cells treated with LPS. However, unlike TNF-α, LPS stimulation of lung epithelial cells relies on additional recruitment of p300, in our case by E1A, to increase ICAM-1 promoter activity. This difference most likely reflects differences in the signaling cascades activated by the respective receptors bound by these two inflammatory ligands.

Results from recent studies on an unexpected role of IKKα on promoter activation may shed light on this difference between TNF-α and LPS stimulation. As reviewed by Gloire and coworkers (13), IKKα is a member of the IkB kinase complex that is responsible for the phosphorylation and subsequent degradation of IkBα by the proteasome, and this releases IkBα-bound NF-κB to allow its transcriptional activity to the
nucleus. These investigators (13) found that after TNF-α stimulation of mouse embryonic fibroblast cells, some promoters including that of ICAM-1, but not all NF-κB promoters, specifically that of IkBα, require nuclear IKKα for the removal of chromatin-bound HDAC3, a histone deacetylase that inhibits p65 binding to DNA. This allows the aforementioned translocated NF-κB to bind to the ICAM-1 promoter and thus permits full NF-κB mediated transcription. If a similar scenario applies to A549 cells stimulated with TNF-α, then IKKα activation of p65 binding together with basal coactivation by p300 appears to be sufficient for ICAM-1 transcription, and the additional recruitment of p300 by E1A, in this case, is redundant. Whether activation of IKKα in this manner is relevant to the LPS-stimulated ICAM-1 promoter activity remains to be determined. On the other hand, both TNF-α stimulation of mouse embryo fibroblasts (1, 56) and LPS stimulation of macrophages (37) resulted in the activation of IKKα at the promoters of several NF-κB-dependent genes, including that of IkBα, to phosphorylate histone H3, fulfilling one part of the overall mechanism necessary for maximal gene expression, and thus permit the subsequent acetylation of this histone. In our case, LPS-stimulated p300 recruitment to the ICAM-1 promoter by E1A in lung epithelial cells, but not the already maximally activated TNF-α-stimulated promoter, could enhance histone acetylation at this promoter and consequently activate it.

In conclusion, our results suggest that, in lung epithelial cells in vitro, adenovirus E1A increases the expression of ICAM-1, a mediator relevant to the pathogenesis of COPD, by interacting with transcriptional regulators at the promoter of this gene. We recognize the limitations of single lung epithelial cell systems to model a complex disease process in the human lung. Nonetheless, our results do provide greater insight into one possible molecular mechanism by which the expression of an adhesion molecular that is a key in leukocyte infiltration in chronic lung diseases such as chronic bronchitis and emphysema is increased. Furthermore, the regulation of this response to bacteria products may play a role in the chronic emphysematous destruction of the lung in these patients who are susceptible to bacterial infections (2).

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REFERENCES

REGULATION OF LUNG EPITHELIAL ICAM-1 BY ADENOVIRUS E1A


