Redox Regulation of Adenovirus-Induced AP-1 Activation by Overexpression of Manganese-Containing Superoxide Dismutase

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Adenovirus gene therapy is a promising tool in the clinical treatment of many genetic and acquired diseases. However, it has also caused pathogenic effects in organs such as the liver. The redox-sensitive transcription factors AP-1 and NF-κB have been implicated in these effects. To study the mechanisms of adenovirus-mediated AP-1 and NF-κB activation and the possible involvement of oxidative stress in adenovirus transduction, rats were injected with either replication-defective recombinant adenovirus with DNA containing the cytomegalovirus promoter region only (AdCMV), adenovirus containing human manganese-containing superoxide dismutase (MnSOD) cDNA (AdMnSOD), or vehicle. Compared to vehicle and AdCMV transduction, MnSOD gene transfer yielded a fivefold increase in liver MnSOD activity 7 days postinjection. Gel shift assay showed that AdCMV transduction induced DNA binding activity for AP-1 but not NF-κB. MnSOD overexpression abolished this activation. Western blotting analysis of c-Fos and c-Jun suggested that up-regulation of c-fos and cjun gene expression does not directly contribute to the induction of AP-1 activation. Glutathione/glutathione disulfide ratios were decreased by adenovirus transduction and restored by MnSOD overexpression. The AP-1 binding activity that was induced by AdCMV was decreased by immunoprecipitation of Ref-1 protein. Ref-1 involvement was confirmed by restoration of AP-1 binding activity after the immunoprecipitated Ref-1 protein had been added back. AP-1 DNA binding activity was also elevated in control and AdMnSOD-injected rats after addition of the immunoprecipitated Ref-1 protein. These data indicate that cellular transduction by recombinant adenovirus stimulates AP-1 DNA binding activity. Furthermore, our results suggest that MnSOD overexpression decreases AP-1 DNA binding activity by regulating intracellular redox status, with the possible involvement of Ref-1 in this redox-sensitive pathway.

Gene therapy is a promising tool for the clinical treatment of many genetic and acquired diseases. The success of gene therapy relies largely on the delivery systems that transfer target genes into cells and lead to gene expression. Recombinant adenoviruses have been developed as one of these delivery systems. These recombinant adenoviruses are in general replication defective, because a large portion of the genes (such as E1 and E3 genes) in these viruses have been replaced by foreign genes. This system provides many advantages over other conventional delivery systems, including (i) the ability to produce extremely efficient gene transduction with high levels of recombinant gene expression in a variety of cellular targets, including both quiescent and dividing cells (22), (ii) the possibility of large-scale production, and (iii) the ability of the virus to be engineered to accommodate a broad range of transgene sizes.

However, in recent years, problems associated with recombinant adenovirus gene therapy have arisen (21, 30, 34). One of the major problems is cytotoxicity following injection with adenovirus in vivo. For example, systemic application of the first generation of adenovirus resulted in liver damage and necrosis (20). The exact mechanisms by which infection with the replication defective virus can cause cytotoxicity are not clear. However, systemic symptoms that have been observed after in vivo transduction of recombinant adenovirus, such as shock, fever, and inflammation, are similar to the in vivo stress response noted in many other pathological conditions. Therefore, it is reasonable to speculate that recombinant adenovirus infection can result in a stress response at both the systemic and cellular levels. Importantly, these stress responses may play a role in the cytotoxicity observed with adenoviral administration.

Eukaryotic organisms respond to stress by increasing stress response gene expression. Several signal transduction cascades are usually involved in the activation of stress response proteins. NF-κB and AP-1 are widely recognized as two of the early-response transcriptional factors that participate in these signal transduction cascades (19, 31). NF-κB and AP-1 are sensitive to changes in cell environment and activate their target genes by binding to specific motifs on the regulatory regions of stress response genes. Therefore, it is tenable to postulate that DNA binding activity of NF-κB and AP-1 can be induced by adenovirus transduction. In fact, a recent study demonstrated that NF-κB and AP-1 were up-regulated by recombinant adenovirus transduction (24). However, the mechanisms responsible for induction of NF-κB and AP-1 adenovirus have not been delineated.

The NF-κB DNA binding complex is composed of homo-
dimers or heterodimers of the NF-κB family members (i.e., p50 and p65). The activation of NF-κB is controlled by its inhibitory protein, IκB. In most cells, NF-κB is sequestered in an inactive, cytoplasmic complex by binding to IκB. Many stress factors can stimulate IκB kinase (IKK)-mediated phosphorylation of IκB, leading to its ubiquitination and degradation (27). The removal of IκB allows the NF-κB complex to be translocated to the nucleus and act as a transcriptional activator. In a different pathway, AP-1 activation takes place at both the transcriptional and posttranscriptional levels. First, the transcription of AP-1 family members, Jun and Fos, can be up-regulated after stress (9). A second mechanism for AP-1 activation includes redox regulation of Fos and Jun DNA binding activity (1). Redox factor 1 (Ref-1) is thought to function in this pathway (10). Ref-1, which was first found to be a DNA repair enzyme, apurinic/apyrimidinic endonuclease (12), is also now known to be a redox-sensing signal transduction protein and to participate in the modulation of several transcription factors, such as NF-κB (12) and AP-1 (36).

NF-κB and AP-1 are two transcriptional factors known to respond directly to oxidative stress. Oxidative stress has been linked to pathological cell death from many insults, such as ischemia-reperfusion, trauma, and hyperthermia, concomitant with induction of NF-κB and AP-1 activation in these conditions (3). Therefore, oxidative stress may also participate in the adenovirus-induced stress response. To study this possibility, we manipulated the in vivo levels of manganese-containing superoxide dismutase (MnSOD) and modified the activation of AP-1 and NF-κB. MnSOD, which is found in mitochondria, is one of the primary antioxidant enzymes that catalyze the dismutation of superoxide to hydrogen peroxide. Hydrogen peroxide is further reduced to water by catalase or one of the peroxidases. Thus, overexpression of MnSOD creates a mechanism for reactive oxygen species (ROS) removal and, in turn, modulates intracellular redox status.

The overall purpose of the present study was to test the hypothesis that AP-1 and NF-κB DNA binding activity can be induced by in vivo recombinant adenovirus transduction. We also speculated that if oxidative stress is involved in this induction, AP-1 and NF-κB DNA binding activity could be modulated by MnSOD overexpression through a redox regulatory mechanism. Finally, we postulated that Ref-1 may be part of this regulation pathway. Our results indicated that in vivo administration of adenovirus stimulated AP-1 but not NF-κB DNA binding activity in the liver of rats. This activation does not appear to be due to the up-regulation of c-fos and c-jun gene expression. Instead, the induction of oxidative stress by adenovirus most likely contributed to the activation of AP-1 DNA binding. Furthermore, our findings suggest that overexpression of MnSOD suppresses adenovirus-induced AP-1 DNA binding activity by regulating intracellular redox status, with the possible involvement of Ref-1 in this redox-sensitive pathway.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (350 to 450 g; 24 months old; National Institute on Aging) were used in these experiments. Rats were housed in The University of Iowa Animal Care Facility, and all experimental procedures conformed to institutional animal care guidelines. Animals were maintained at 22 to 24°C on a 12- to 12-hour light-dark cycle and provided food (standard rat chow) and water ad libitum.

Adenovirus gene transfer. Replication-defective recombinant adenovirus type 5 with the E1 region replaced with DNA containing the cytomegalovirus (CMV) promoter region only (AdCMV) or with a specific cDNA (e.g., MnSOD) after the CMV promoter were provided by the Gene Transfer Vector Core at The University of Iowa. For all experiments, the adenovirus concentration was 10^{12} particles per ml in phosphate-buffered saline (PBS) buffer with 3% sucrose. The number of infectious units of the adenovirus was typically 10^{10} PFU/ml.

To examine the efficiency of recombinant adenovirus transduction and gene expression in the liver, recombinant adenovirus containing β-galactosidase (β-Gal) cDNA was used as a reporter gene. Fischer 344 rats anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) were given either recombinant adenovirus containing LacZ cDNA (n = 12) or vehicle (3% sucrose in PBS; n = 3) via carotid catheter injection and then housed for 5, 7, 10, or 14 days. On the designated day, tissues were harvested from both the small and large lobes of the liver and processed for X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining.

In subsequent experiments, rats were given either AdCMV (empty vector; n = 3), an adenovirus containing human MnSOD cDNA (AdMnSOD; n = 3), or vehicle alone (3% sucrose in PBS; n = 3) via carotid catheter injection. Each rat received 1 ml of adenovirus (10^{10} infectious units) and was returned to its individual cage. On the designated experimental day, animals were given an overdose of sodium pentobarbital (80 mg/kg intraperitoneally). Livers were collected, rinsed in PBS, and processed immediately for an experiment or frozen in liquid nitrogen.

X-Gal staining for β-Gal activity. X-Gal staining for β-Gal activity was performed as previously described (6). Tissues (0.125 cm^3) were harvested from both the small and large lobes of the liver, fixed in 4% paraformaldehyde fixative solution, incubated at 4°C in PBS containing 2 mM MgCl₂ and 30% sucrose, and embedded in tissue-freezing medium. Cryostat sections (10 μm) were cut and mounted on glass slides. The slides were then stained with X-Gal solution and counterstained with fast red to permit calculation of the percentage of cells expressing the β-Gal protein as a result of LacZ cDNA uptake and expression. The numbers of cells expressing β-Gal (blue) and not expressing β-Gal (red) were counted by microscopic viewing. Several fields from each slide were counted in order to obtain an accurate estimation of cell number. Final results were expressed as the percent β-Gal-positive cells from an average of three animals in each group.

Immunohistochemistry. Formalin-fixed tissues were processed for immunohistochemistry as previously described (14, 15). Sections (4 μm) cut from paraffin blocks were evaluated for immunoreactive MnSOD proteins using immunoperoxidase staining techniques and antibodies specific for MnSOD. Two slides were prepared from both a large and small lobe of each liver sample for the staining. The specificity of the antibodies has been described elsewhere (37). Normal rabbit serum was substituted for the MnSOD antibody as a negative control.

Tissue preparation. Liver samples were prepared for Western blotting and enzyme activity measurements as previously described (14). Frozen liver samples were homogenized in liquid nitrogen, homogenized in 50 mM phosphate buffer with a homogenizer, and sonicated with a Vibra cell sonicator (Sonicus & Materials). Total protein concentrations were determined by the Bradford assay (Bio-Rad).

Western blot analysis. Equal amounts of total protein or nuclear extracts were separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis according to the method of Laemmli (23). The separated proteins were then transferred onto nitrocellulose membranes and blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS). The nitrocellulose membranes were incubated overnight at 4°C with an antibody (1:1,000 for c-Fos and c-Jun, 1:25 for Ref-1) specific for the proteins of interest. After three washings with TTBS, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (1:10,000) in TTBS for 1 h at room temperature. Blots were stained with the chemiluminescent ECL method (Amersham Life Sciences), and immunoreactive signals were visualized by exposure to an X-ray film (Kodak).

MnSOD activity assay. SOD activity was measured by the modified nitroblue tetrazolium (NBT) method described by Spitz and Oberley (35). This is an indirect assay based on a competition reaction between SOD and the superoxide indicator molecule NBT. The rate of increase in the absorption at 560 nm over a 5-min period indicates the reduction of NBT by superoxide. The competitive inhibition of this reaction is an indicator of total SOD activity. In the assay, the xanthine-xanthine oxidase system was used to generate superoxide. Various amounts of total protein were added to the reaction until maximal inhibition was obtained, as determined by spectrophotometry. Total SOD activity was determined by the amount of protein necessary for half-maximal inhibition of the
NBT assay. MnSOD activity was quantified in the presence of 5 mM NaCN, which inhibits only copper- and zinc-containing SOD (CuZnSOD) activity. One unit of activity was defined as the concentration of SOD that reduced the NBT reaction to one-half of the maximum.

**SOD activity gel assay.** The native activity gel assay described by Beauchamp and Fridovich (5) was used. Fifty micrograms of total protein from each sample was separated in a native 12% polyacrylamide gel. The gel was then stained by incubation with 2,4-dinitrophenylhydrazine (2 mM) for 20 min in the dark. After the gel had been exposed to a fluorescent light, the aichromatic bands corresponding to SOD activity appeared on a dark blue background.

**Preparation of nuclear extracts.** Nuclear extracts from rat liver were prepared using a modification of the method of Hattori et al. (16). All procedures were carried out at 4°C. Freshly harvested liver tissues were minced on ice, weighed, and placed in homogenizing buffer (0.3 M sucrose, 10 mM HEPES [pH 7.6], 10 mM KCl, 0.74 M spermidine, 0.15 M spermine, 0.1 M EDTA, 0.1 M EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, and one complete protease inhibitor cocktail tablet [Boehringer Mannheim] per 50 ml of buffer). The buffer-tissue mixture (2 ml of buffer per g of tissue) was homogenized in a Beckman ultracentrifuge at 105,000 g for 150 min. The supernatant, containing the cytoplasmic fraction, was decanted, and buffer was removed. Each nuclear pellet was resuspended in 38.5 ml of polyallomer tubes. Homogenates were centrifuged in a Beckman ultracentrifuge at 35,730 g for 40 min at 4°C. Nuclear membranes were then pelleted by spinning the nuclear lysate in an ultracentrifuge at 35,730 × g for 11 min. The resultant supernatants were collected as nuclear fractions. Their total protein contents were determined using the Bradford assay.

**EMSA.** Nuclear extract (5 μg; 5 μl) was combined with electrophoretic mobility shift assay (EMSA) buffer (250 mM KCl, 100 mM HEPES [pH 7.9], 25 mM MgCl2, 1.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT), briefly homogenized in a Dounce homogenizer, and gently rotated for 40 min at 4°C. Nuclear membranes were then pelleted by spinning the nuclear lysate in an ultracentrifuge at 35,730 × g for 11 min. The resultant supernatants were collected as nuclear fractions. Their total protein contents were determined by using the Bradford assay.

**GSH and GSSG measurement.** The method for glutathione (GSH) measurement was adapted from previously described techniques (2, 13). Total GSH of samples was measured based on the colorimetric reaction of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) with GSH to form TNB (5-thio-2-nitrobenzoic acid). The rate of TNB formation, which was proportional to GSH concentration, was monitored spectrophotometrically at 412 nm. Cellular glutathione disulfide (GSSG) was reduced to GSH by the specific GSSG reductase (GR). To prepare liver samples, ground tissues were homogenized in ice-cold 5% 5-sulfosalicylic acid. The measurement of total GSH was started by mixing 700 μl of 6 mM DTNB in phosphate buffer (pH 7.4). The samples were reacted with water to make a total volume of 150 μl and added to the reaction solution. The assay was initiated by addition of 50 μl of GR solution. The rate of TNB formation was monitored at 412 nm. The total GSH of a sample was calculated from a standard curve of GSH concentration versus rate (change in absorbance/time). The GSSG level was determined by the same DTNB assay when GSH was masked by 2-vinylpyridine. In brief, 2 μl of 2-vinylpyridine was added to 100 μl of sample prepared as noted above for total GSH measurement. After a 90-min incubation on ice, GSSG was measured as described for the total GSH assay.

**RNAase protection assay.** Total RNA was isolated from liver tissues harvested as indicated above by phenol-guanidium extraction methods. RNAse protection assays were performed using a RibonQuant multiple probe RNAse protection assay kit (PharMingen, San Diego, Calif.) according to the manufacturer’s instructions. Steady-state mRNA levels of a panel of 11 liver cytokines were determined. These cytokines included interleukin 1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), TNF-β, and gamma interferon.

**Statistical analysis.** Data are presented as means ± standard errors of the means. Analysis of variance-Tukey’s multiple comparison was used to determine the statistical significance of the data at a P level of ≤0.05.

**RESULTS**

**Adenovirus gene transfer efficiency.** The efficiency of recombinant adenovirus transduction and gene expression in the liver was evaluated by injecting rats with adenovirus containing β-Gal cDNA as a reporter gene. Microscopic analysis of liver tissue sections revealed that at day 5 posttransduction, 96% of hepatocytes were expressing β-Gal protein (Fig. 1). This level of expression lasted through day 10 posttransduction. By day 14, approximately 50% of the cells were still expressing the protein. These results are consistent for both the large and small lobes of the liver.

**Verification of MnSOD overexpression.** Seven days after recombinant adenovirus transduction, liver samples were harvested from rats (three rats per group) in the following three groups: (i) vehicle injection (control), (ii) transduction with AdCMV, and (iii) transduction with AdMnSOD. MnSOD levels were examined by Western blot analysis and MnSOD activity assays. All three animals in the AdMnSOD group had a large increase in MnSOD protein expression compared to the control and AdCMV groups (Fig. 2A), with small variations in MnSOD protein levels among individual rats. However, on average, no significant differences were noted between the control and the AdCMV Biolabs. Densitometry analysis demonstrated an approximately fourfold increase in MnSOD expression in the AdMnSOD group compared with the control and AdCMV groups (data not shown).

The overexpression of MnSOD was further measured using both activity assay and activity gel techniques. Figure 2B summarizes the results of the MnSOD activity gel assays. All three animals transduced with AdMnSOD had large increases in MnSOD activity levels. The MnSOD bands of control and AdCMV groups were not detectable, due to the fact that rat MnSOD does not produce bands under the conditions used for the activity gel assays. Only the exogenous human MnSOD expressed from adenovirus transduction was detectable. There were no differences among samples from the three treatment groups for the CuZnSOD activity as demonstrated by activity gel assays (Fig. 2B). The levels of catalase and GSH peroxidase were also measured by activity gel assays. No changes in the activities of these antioxidant enzymes were found among the samples (data not shown).

MnSOD activity was also determined by the NBT competition assay (Fig. 2C) to further examine whether AdMnSOD transduction would affect endogenous MnSOD activity. Animals transduced with AdMnSOD had a fourfold increase in MnSOD activity compared to the control group (P < 0.05). This level of increase in MnSOD activity was consistent with

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the increase in MnSOD protein levels as measured by Western blotting. The AdCMV group had a MnSOD activity level similar to that of the control group.

Immunohistochemical staining for MnSOD protein by specific MnSOD antibody was also performed in all rats to examine the zonal distribution of MnSOD in hepatocytes of the liver. Figure 3 shows a representative slide of MnSOD immunostaining from each group. The immunostaining of MnSOD was predominantly intensified in hepatocytes of the AdMnSOD group (Fig. 3C). Sections from control and AdCMV animals had only light staining of MnSOD, representing endogenous levels of rat MnSOD. These immunohistochemistry findings were consistent with the results obtained in Western blotting experiments.

Response of AP-1 and NF-κB DNA binding activities to adenovirus transduction and MnSOD overexpression. DNA binding activities of AP-1 and NF-κB were determined in nuclear extracts from adenovirus-transduced livers and control animals using EMSA. EMSA resulted in different DNA binding patterns for AP-1 and NF-κB (Fig. 4). The DNA binding activity of AP-1 was greatly induced in the AdCMV group compared with the basal level in the control group. However, this elevated AP-1 DNA binding activity was strongly suppressed in livers from rats overexpressing MnSOD (Fig. 4A). In contrast to the AP-1 response, there were large variations among all animals in NF-κB DNA binding activity (Fig. 4B), which may reflect the endogenous difference of individual animals. Adenovirus transduction did not change the DNA bind-
ing activity of NF-κB, nor did MnSOD overexpression have any effect on NF-κB DNA binding activity (Fig. 4B).

A supershift experiment was then performed to verify that the bands shown in the EMSA were the AP-1 and NF-κB complexes. Two nuclear extracts from control animals (rats 1 and 3) were incubated with either an antibody specific to c-Jun or antibodies specific to NF-κB p50 and p65 before these extracts were subjected to EMSA. As shown in Fig. 5A, a clear decrease in intensity in the band for normal AP-1 complex was observed. Simultaneously, a new band migrated higher than the normal AP-1 binding complex, representing the AP-1 complex supershifted by anti-c-Jun antibody. Similar results were observed in the case of antibody to p50 for the NF-κB supershift experiment. However, p65 antibody failed to supershift the NF-κB complex, indicating that the observed NF-κB complex band consisted only of p50-p50 homodimer.

Response of c-Jun and c-Fos protein expression after adenovirus transduction and MnSOD expression. The signaling pathways that may regulate the response of AP-1 to adenovirus transduction were evaluated by measuring c-Fos and c-Jun protein levels by Western blot analysis, since the Fos and Jun families are two major components of AP-1 protein. Results indicated that the overall expression of both c-Fos and c-Jun proteins varied greatly among individual animals in all three groups (Fig. 6). Therefore, it is unlikely that the up-regulation of c-jun and c-fos gene expression was responsible for the induction of AP-1 DNA binding activity by AdCMV transduction and restoration of its activity by overexpression of MnSOD.

FIG. 2. AdMnSOD, but not AdCMV, yielded high-level expression of human MnSOD. Rats were infected with adenovirus (10^10 infectious units), and livers were harvested at day 7 after the infection. (A) Western blotting analysis demonstrated an increase in MnSOD protein in all three AdMnSOD-transduced rats. (B) Activity gel assay showed intense exogenous MnSOD activity bands in AdMnSOD-transduced rats but not control or AdCMV-transduced rats. Each lane was loaded with samples from individual rats. (C) NBT activity assay demonstrated a significant increase of MnSOD activity in AdMnSOD-transduced rats but no differences in control and AdCMV-transduced rats. n = 3 for each group. * P < 0.05

FIG. 3. Photomicrographs of immunohistochemical preparations demonstrating increased staining for MnSOD in the livers of AdMnSOD-transduced rats. Livers were harvested at day 7 after transduction. (A) A representative liver section from control rats stained with an antibody specific for MnSOD shows a very light brown color, indicating a low level of constitutive MnSOD protein. (B) A representative liver section from an AdCMV-transduced rat shows low-level MnSOD staining that is close to the intensity of controls. (C) A representative liver section from an AdMnSOD-transduced rat shows a dramatic increase in MnSOD staining, which is predominantly located in hepatocytes. Liver sections from AdMnSOD-transduced rats were also stained with normal rabbit serum and showed no brown staining (not shown). Three animals from each group were examined, and the micrographs shown are representative of the whole group.
Redox regulation of AP-1. The ratio of GSH to GSSG is considered an important parameter for evaluation of intracellular redox status (32). Therefore, GSH/GSSG ratios were measured for animals in the three groups (Fig. 7). AdCMV transduction caused a fivefold decrease in GSH/GSSG ratio, indicative of a significantly increased intracellular oxidizing environment, whereas MnSOD overexpression significantly reversed the GSH/GSSG ratio from the level of adenovirus transduction to control levels. These changes in the GSH/GSSG ratio were related to the changes in AP-1 DNA binding activity, suggesting that a cellular redox component may have participated in activation of AP-1 DNA binding in the liver of animals injected with adenovirus but not overexpressing MnSOD.

Ref-1 is involved in the regulation of AP-1 DNA binding activity. It has been reported that Ref-1 is associated with the redox regulation of AP-1 activation (17, 38). We examined whether Ref-1 is involved in the pathway of redox regulation of AP-1 DNA binding activity by adenovirus transduction and MnSOD overexpression. Ref-1 protein was immunodepleted by an antibody specific to Ref-1 from nuclear extracts of AdCMV-injected animals, and then these nuclear extracts were subjected to EMSA. AP-1 DNA binding ability was decreased after Ref-1 depletion (Fig. 8A, rat 5, lane 6) compared to the assay without Ref-1 depletion (lane 4). When Ref-1 was added back to the EMSA, the AP-1 DNA binding ability was restored (Fig. 8, lane 7). When the immunoprecipitated Ref-1 was added to nuclear extracts from MnSOD-overexpressing animals (rats 8 and 7), increases in AP-1 DNA binding activities (Fig. 8, lanes 9 and 11) from low binding levels (lanes 8 and 10) were observed. Addition of immunoprecipitated Ref-1

FIG. 4. DNA binding activity of AP-1, but not NF-κB, was activated by adenovirus transduction and suppressed by MnSOD. Rats were transduced with either AdCMV or AdMnSOD cDNA and compared to vehicle-treated controls. Livers were harvested at day 7 after transduction. Each nuclear extract (5 μg) was analyzed by gel mobility-shift assay using AP-1-specific (A) or NF-κB-specific (B) 32P-labeled oligonucleotides. NS, nonspecific binding; FP, free probes. Three animals were examined for each group, and each lane was loaded with samples from individual rats. The experiments were repeated three times, and representative pictures are shown.

FIG. 5. AP-1 and NF-κB DNA binding activities were supershifted by specific c-Jun or p50 antibodies reacting with nuclear extracts from two control animals. Each nuclear extract (5 μg) from the experiment represented in Fig. 4 was incubated with 5 μL of antibody (ab) specific for AP-1 (c-Jun) (A) or NF-κB (p50 and p65) (B) 2 h before EMSA analysis. NS, nonspecific DNA binding; FP, free probe. The experiments were repeated three times, and representative pictures are shown.
to a nuclear extract from a control animal (rat 3) also induced AP-1 activation (Fig. 8, lane 3) from its basal level (lane 2). Immunoprecipitated Ref-1 alone did not bind to the oligonucleotides with the consensus AP-1 binding sequence (Fig. 8, lane 5), and its binding was similar to that of the free probe (lane 1). These results were repeated using another nuclear extract in the AdCMV group (rat 6) (data not shown). In order to eliminate the possibility that c-Jun and c-Fos may be coprecipitated with Ref-1 protein, the presence of c-Jun and c-Fos in immunoprecipitated pellets was checked by Western blotting using c-Jun- and c-Fos-specific antibodies. No detectable c-Jun or c-Fos protein was noted (data not shown). To confirm that Ref-1 was indeed immunoprecipitated, the presence of Ref-1 protein in nuclear extract, pellet, and supernatant (rat 5) was examined by Western blotting. As shown in Fig. 8B, an intense Ref-1 protein band was present in the nuclear extract but disappeared in the supernatant after immunoprecipitation. This Ref-1 band was also in the pellet of the immunoprecipitate. These data suggest that Ref-1 is involved in the pathway of redox regulation of AP-1 binding activation by adenovirus transduction and MnSOD overexpression.

An inflammatory response was not involved in the up-regulation of AP-1 activation. To examine whether inflammation plays a role in the up-regulation of AP-1 activation, RNase protection assays were used to determine steady-state mRNA levels of a panel of 11 liver cytokines. These cytokines included IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, TNF-β, and gamma interferon. All the cytokines were below detection limits except IL-1α and -β, which had high steady-state levels of mRNA. However, no differences were observed among AdCMV-transfected, AdMnSOD-transfected, and control animals (data not shown).
DISCUSSION

Virus-induced stress responses are an important issue in many types of viral infections. For example, there is evidence that cellular heat shock proteins are induced during adenovirus transductions (18, 29). In the present study, we demonstrated that replication-defective recombinant adenovirus induced a significant increase in AP-1 DNA binding activity. This result clearly indicates that a stress response can be induced in vivo, even though the adenovirus used is replication defective.

The mechanisms of virus-induced activation of transcription factor responses are still under active investigation. Our results indicate that AP-1 DNA binding activity was up-regulated, but not NF-κB DNA binding activity, suggesting that the regulation of a specific signal transduction pathway is stimulated after recombinant adenovirus transduction, rather than just a generalized stress response.

It is known that AP-1 activation is controlled at several different levels (11). Our data indicate that adenovirus transfection did not induce the expression of c-fos and c-jun genes. Therefore, redox regulation of AP-1 activation was hypothesized. The fact that MnSOD, a primary antioxidant enzyme, suppressed induction of AP-1 activation by adenovirus clearly demonstrated ROS involvement with both recombinant adenovirus transduction and the nature of AP-1 redox regulation.

Importantly, GSH/GSSG ratio measurements showed that adenovirus transfection increased the oxidation of GSH and decreased the GSH/GSSG ratios in the livers of rats, while MnSOD overexpression restored the GSH/GSSG ratio to normal levels. GSH is believed to be the most abundant redox balance buffer in vivo (32). Therefore, the shift from reduced GSH to GSSG indicates the presence of oxidative stress and an oxidative environment in vivo. The change of GSH/GSSG ratios by adenovirus transduction and MnSOD overexpression strongly suggests the presence of a redox regulation mechanism in the activation of AP-1 DNA binding by recombinant adenovirus transfection.

It is known that redox regulation of AP-1 activation involves conserved cysteine residues (Fos Cys-154 and Jun Cys-272) in its DNA binding domain (1), and investigators have suggested the involvement of Ref-1 protein in the stimulation of AP-1 DNA binding through a redox regulation pathway (17, 38).

Ref-1 is a bifunctional protein, serving as a DNA repair enzyme and as a regulator protein for several transcription factors, including AP-1, in a redox-dependent manner. Genetic analysis has identified a cysteine residue at position 65 in the redox regulatory domain of Ref-1 protein that is critical for the redox activation of AP-1 DNA binding (38). This residue is required for the direct interaction between Ref-1 and c-Jun through Cys-272 on c-Jun protein in vitro (36). We observed a decrease in AP-1 DNA binding activity after immunodepletion of Ref-1. Also, this reduction was restored by adding back the immunoprecipitated Ref-1 protein. However, immunoprecipitated Ref-1 alone did react with the oligonucleotides containing the consensus AP-1 binding sequence. These results demonstrate that Ref-1 must be physically present with AP-1 protein in the stimulation of AP-1 activation after adenovirus transfection. In addition, AP-1 activation was also elevated after the nuclear extracts from the control and MnSOD-overexpressing animals had been supplemented with the immuno-precipitated Ref-1 protein from adenovirus-transfected animals. These data indicate that adenovirus transfection altered Ref-1 protein so that it could react with AP-1 protein and elevate AP-1 DNA binding ability in the samples that had low AP-1 binding. Overall, our results strongly demonstrate that redox regulation is one important mechanism for the adenovirus induction of AP-1 activation in vivo, with the possible involvement of Ref-1 protein.

In contrast to AP-1 activation, we did not observed induction of NF-κB by adenovirus transfection at 7 days postinjection. This observation conflicts with reports of NF-κB induction by recombinant adenovirus from other groups (8, 24, 26). One major reason for the observed difference in transcription factor induction may be that in all cases where NF-κB was up-regulated by adenovirus transduction, it was also accompanied by induction of cytokines such as TNF-α and IL-1. These cytokines induce NF-κB activation by phosphorylation of IKKs (27). When IKKs are phosphorylated, they can lead to the degradation of the NF-κB inhibitor, IκB. This in turn causes the components of NF-κB, p50 and p65, to be dissociated from IκB and translocate into the nucleus. The translocations of p50 and p65 are key steps during NF-κB activation. Therefore, the fact that we did not observe an induction of cytokines at the 7-day point after adenovirus transduction could well be why NF-κB was not activated at the same time point. Secondly, we speculate that the duration of NF-κB activation may be transient. A biphasic induction of NF-κB activation is usually observed after adenovirus infection (25). The first early-phase induction is often only minutes after the infection, probably related to interaction of the viral particles and cell receptors. A second phase is usually seen approximately 3 days postinfection and is thought to be due to foreign gene expression. In the present study, we measured NF-κB activity only at 7 days after infection. If the NF-κB gene is a rapid early-responding gene, it is very possible that its activation had returned to control levels in our experiments.

NF-κB is also believed to be directly regulated by oxidative stress. However, the precise steps by which ROS mediates activation of NF-κB are not well defined. It is thought that the control of IκB phosphorylation is the key step in NF-κB redox activation, which indicates that the direct reaction of ROS with either IKK or a protein upstream of IKK is essential for redox regulation. The specific ROS that can induce NF-κB activation need to be elucidated as well. While research has shown that H₂O₂ can activate the NF-κB pathway (28), one study indicated that this activation is cell dependent (3). Shi et al. (33) recently demonstrated that hydroxyl radical, rather than H₂O₂, is the primary signal for the activation of NF-κB in macrophages and Jurkat cells. These observations provide additional support for our results, which suggest that there are differences in the mechanisms between NF-κB and AP-1 activation in vivo.

Unlike some other reports suggesting that an inflammatory response is the direct cause of the stress response after recombinant adenovirus infection (4, 7), our results from the RNase protection assay, which has a high level of sensitivity, did not show increased cytokine expression at 7 days after administration of adenovirus. It is very possible that cytokines were elevated at early time points and had returned to normal 7 days postinjection. Therefore, a kinetic analysis of cytokine regula-
tivation is warranted in future studies. Besides cytokine regulation of transcription factors, Lieber et al. (24) have demonstrated that the induction of NF-κB activation immediately after recombinant adeno virus transduction is independent of Kupffer cell presence in the liver. Our study has also identified a signaling mechanism other than inflammatory cytokine stimulation, which is often associated with the recombinant adenovirus transduction, that can lead to liver toxicity and total body stress responses. Our results suggest that oxidative stress may be partly responsible for the toxicity of adenovirus transduction. Therefore, maintenance of redox balance during and after adenovirus transduction may be beneficial for adeno virus-mediated gene therapy. The present study contributes to an understanding of the signaling regulation mechanisms related to recombinant adenovirus transduction.

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