

## Tyrosine Phosphorylation of I $\kappa$ B $\alpha$ Activates NF $\kappa$ B through a Redox-regulated and c-Src-dependent Mechanism Following Hypoxia/Reoxygenation\*

Received for publication, July 7, 2002, and in revised form, November 8, 2002  
Published, JBC Papers in Press, November 11, 2002, DOI 10.1074/jbc.M206718200

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NF $\kappa$ B is a critical transcription factor involved in modulating cellular responses to environmental injuries. Tyrosine 42 phosphorylation of I $\kappa$ B $\alpha$  has been shown to mediate NF $\kappa$ B activation following hypoxia/reoxygenation (H/R) or pervanadate treatment. This pathway differs from the canonical proinflammatory pathways, which mediate NF $\kappa$ B activation through serine phosphorylation of I $\kappa$ B $\alpha$  by the IKK complex. In the present study, we investigated the involvement of c-Src in the redox activation of NF $\kappa$ B following H/R or pervanadate treatment. Our results demonstrate that pervanadate or H/R treatment leads to tyrosine phosphorylation of I $\kappa$ B $\alpha$  and NF $\kappa$ B transcriptional activation independent of the IKK pathway. In contrast, inhibition of c-Src by pp2 treatment or in c-Src (–/–) knockout cell lines, demonstrated a significant reduction in I $\kappa$ B $\alpha$  tyrosine phosphorylation and NF $\kappa$ B activation following pervanadate or H/R treatment. Overexpression of glutathione peroxidase-1 or catalase, but not Mn-SOD or Cu,Zn-SOD, significantly reduced both NF $\kappa$ B activation and tyrosine phosphorylation of I $\kappa$ B $\alpha$ . *In vitro* kinase assays further demonstrated that immunoprecipitated c-Src has the capacity to directly phosphorylate GST-I $\kappa$ B $\alpha$  and that this I $\kappa$ B $\alpha$  kinase activity is significantly reduced by Gpx-1 overexpression. These results suggest that c-Src-dependent tyrosine phosphorylation of I $\kappa$ B $\alpha$  and subsequent activation of NF $\kappa$ B is controlled by intracellular H<sub>2</sub>O<sub>2</sub> and defines an important redox-regulated pathway for NF $\kappa$ B activation following H/R injury that is independent of the IKK complex.

Reactive oxygen species (ROS)<sup>1</sup> are normal metabolic by-products and intermediates found in many physiological processes. Three major sources of intracellular ROS include the xanthine/xanthine oxidase system, receptor-coupled NADPH

oxidase at the cellular membrane, and the mitochondrial electron transport system (1, 2). ROS have been increasingly recognized as critical components in disease and stress-induced cellular injuries such as ischemia/reperfusion (I/R), UV irradiation, and inflammation. These ROS can lead to direct cellular damage and can also act as intracellular second messengers to modulate signal transduction pathways. One such redox-regulated transcription factor is NF $\kappa$ B (3).

NF $\kappa$ B family members include p50, p52, p65, and c-RelB, which form homodimeric and heterodimeric transcriptional complexes (4). The activation of NF $\kappa$ B is controlled by a family of I $\kappa$ B repressor proteins (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ ) that sequester NF $\kappa$ B in the cytoplasm (4). Phosphorylation-dependent inactivation of I $\kappa$ B proteins leads to the mobilization of NF $\kappa$ B to the nucleus where it can act as a transcription factor. These phosphorylation pathways have been most extensively studied for I $\kappa$ B $\alpha$  and include two distinct mechanisms involving either serine or tyrosine phosphorylation of I $\kappa$ B $\alpha$ . The most comprehensively studied pathway regulating I $\kappa$ B $\alpha$  includes phosphorylation on two serine (32 and 36) residues by the I $\kappa$ B kinase complex (IKK) (5). This phosphorylation leads to ubiquitination of I $\kappa$ B $\alpha$  at nearby lysine residues and degradation by the proteasome. An alternative, less characterized pathway of NF $\kappa$ B activation acts through tyrosine phosphorylation of I $\kappa$ B $\alpha$  at residue 42 (6). In contrast to IKK-mediated serine phosphorylation of I $\kappa$ B $\alpha$ , tyrosine phosphorylation of I $\kappa$ B $\alpha$  is capable of activating NF $\kappa$ B in the absence of ubiquitin-dependent degradation of I $\kappa$ B $\alpha$ . However, it is presently unclear if IKK and/or the I $\kappa$ B $\alpha$  protein-tyrosine kinase (PTK) interactions with I $\kappa$ B $\alpha$  are functionally modulated by prior tyrosine or serine phosphorylation of I $\kappa$ B $\alpha$ , respectively. Experimental evidence appears to suggest that prior tyrosine phosphorylation of I $\kappa$ B $\alpha$  on Tyr-42 may prevent interactions with the IKK complex and inhibit serine phosphorylation on Ser-32/Ser-36 (7). Hence, the existence of reciprocal interactions between IKK- and PTK-mediated phosphorylation of I $\kappa$ B $\alpha$  and the net effect on NF $\kappa$ B transcriptional activation remains an open question. Although the exact identity of the I $\kappa$ B tyrosine kinase has not yet been demonstrated using *in vitro* reconstitution assays, both PI 3-kinase and c-Src have been demonstrated to associate with tyrosine phosphorylated I $\kappa$ B $\alpha$  in T-cells following pervanadate treatment (8) and bone marrow macrophages (BMMs) following TNF $\alpha$  stimulus (9). In addition to pervanadate, H/R has also been shown to induce tyrosine phosphorylation of I $\kappa$ B $\alpha$  in T-cells *in vitro* (6) and following I/R injury to the liver *in vivo* (10).

The tyrosine kinase p56<sup>lck</sup> is required for I $\kappa$ B $\alpha$  tyrosine phosphorylation and NF $\kappa$ B activation in T-lymphocytes following pervanadate treatment (6). Loss of tyrosine kinases p56<sup>lck</sup> and ZAP-70 in two Jurkat mutants abolished NF $\kappa$ B activation and

\* This work was supported by National Institutes of Health Grants P50 HL60316 (to G. H.) and DK51315 (to J. F. E.) and the Center for Gene Therapy funded by NIDDK (P30 DK54759). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: ROS, reactive oxygen species; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RIPA, radioimmune precipitation assay buffer; MOI, multiplicity of infection; PI, phosphatidylinositol; GST, glutathione S-transferase; PTK, protein tyrosine kinase; CMV, cytomegalovirus; H/R, hypoxia/reoxygenation; SOD, superoxide dismutase; BMM, bone marrow macrophages; I/R, ischemia/reperfusion.

partially suppressed and delayed phosphorylation of Tyr-42 on I $\kappa$ B $\alpha$  in response to pervanadate treatment (11). However, this study in T-cells also demonstrated that tyrosine phosphorylation of I $\kappa$ B $\alpha$  was not sufficient to activate NF $\kappa$ B and suggests that both tyrosine and serine kinases act at multiple levels to dissociate the I $\kappa$ B $\alpha$ /NF $\kappa$ B complex. Furthermore, tyrosine phosphorylation of I $\kappa$ B $\alpha$  is observed in BMMs following TNF $\alpha$  treatment, and this phosphorylation requires c-Src activity (9). Given the historical dependence of TNF $\alpha$ -mediated activation of NF $\kappa$ B on the IKK complex and serine phosphorylation of I $\kappa$ B $\alpha$ , the functional involvement of I $\kappa$ B $\alpha$  tyrosine phosphorylation in response to TNF $\alpha$  appears to be quite unique to BMMs. Furthermore, the vast majority of studies evaluating the importance of I $\kappa$ B $\alpha$  tyrosine phosphorylation to date have been performed in hematopoetically derived T-cells or BMMs. Thus, the functional relevance of these systems to epithelial models of ischemia/reperfusion remains an open question. Since c-Src can be directly activated by H<sub>2</sub>O<sub>2</sub> (12), pervanadate (13), hypoxia (14), or hypoxia/reoxygenation (15), its central involvement in ROS-mediated IKK and PTK activation of NF $\kappa$ B appears reasonable. It is also recognized that H<sub>2</sub>O<sub>2</sub> is capable of activating both IKK- and PTK-dependent pathways of I $\kappa$ B $\alpha$  phosphorylation and NF $\kappa$ B activation in T-cells (11).

In the present study, we sought to investigate the involvement of c-Src in the redox-mediated activation of NF $\kappa$ B activation following H/R or pervanadate treatments in an epithelial cell line (HeLa cells). Since both IKK-dependent and independent pathways of NF $\kappa$ B activation have been associated with c-Src activation, we used a number of adenoviral vectors expressing dominant mutants of IKK $\alpha$ , IKK $\beta$ , and I $\kappa$ B $\alpha$  to selectively test for serine or tyrosine I $\kappa$ B $\alpha$  phosphorylation-dependent transcriptional activation of NF $\kappa$ B. In contrast to previous studies, we have utilized an NF $\kappa$ B-responsive luciferase reporter gene to directly assess changes in the transcriptional activation of NF $\kappa$ B. Since the association of tyrosine-phosphorylated I $\kappa$ B $\alpha$  with PI 3-kinase has been suggested in proposed models to alter the transcriptional properties of NF $\kappa$ B dimers (8, 11), direct functional assessment of activation may be more informative than assessing DNA binding. Triple knockout cell lines (c-Src $^{-/-}$ , Fyn $^{-/-}$ , Yes $^{-/-}$ ) with and without c-Src were also used to confirm the dependence of I $\kappa$ B $\alpha$  tyrosine phosphorylation on c-Src. Furthermore, recombinant adenoviral vectors expressing various ROS scavengers were used to test whether activation of these pathways contained redox-sensitive components. Results from these studies indicate that tyrosine phosphorylation of I $\kappa$ B $\alpha$  and NF $\kappa$ B activation is mediated through redox activation of c-Src.

#### EXPERIMENTAL PROCEDURES

**Adenoviral Vectors**—Several E1-deleted recombinant adenoviral vectors were used to modulate the NF $\kappa$ B pathway and assay for transcriptional induction of NF $\kappa$ B. Previously described vectors included the dominant negative mutants Ad.IKK $\alpha$ (KM) (16), Ad.IKK $\beta$ (KA) (16), Ad.I $\kappa$ B $\alpha$ (S32A/S36A) (17), and the NF $\kappa$ B-responsive luciferase reporter vector Ad.NF $\kappa$ BLuc (16). Ad.BglII was used as an empty vector control (16). Ad.I $\kappa$ B $\alpha$ (Y42F), which expresses the Y42F mutant form of I $\kappa$ B $\alpha$ , was generated by cloning the previously described I $\kappa$ B $\alpha$ Y42F cDNA (6) into pAd.CMVlink (18). All adenoviral vectors, except for Ad.NF $\kappa$ BLuc, used the CMV enhancer/promoter to express the transgene. Recombinant adenovirus was purified by two rounds of CsCl centrifugation and desalted prior to use as described (19).

**Cell Culture, Adenoviral Transduction, and Treatments**—HeLa, SYF, and SYF+c-Src cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100  $\mu$ g/ml penicillin and streptomycin. For the tyrosine phosphorylation assays, HeLa cells were transduced with Ad.IKK $\alpha$ (KM), Ad.IKK $\beta$ (KA), Ad.I $\kappa$ B $\alpha$ (S32A/S36A), Ad.I $\kappa$ B $\alpha$ (Y42F), or Ad.BglII at a multiplicity of infection (MOI) equal to 1000 particles/cell. For the NF $\kappa$ B luciferase reporter assay, HeLa cells were co-infected

with Ad.NF $\kappa$ BLuc at an MOI = 500 particles/cell and Ad.IKK $\alpha$ (KM), Ad.IKK $\beta$ (KA), Ad.I $\kappa$ B $\alpha$ (S32A/S36A), or Ad.I $\kappa$ B $\alpha$ (Y42F) at an MOI = 1000 particles/cell. Luciferase reporter assays in SYF and SYF+c-Src cells were performed following infection with Ad.NF $\kappa$ BLuc alone at an MOI = 500 particles/cell. Adenoviral infections were performed for 2 h in DMEM without FBS followed by the addition of an equal volume of 20% FBS, DMEM and continued incubation for 22 h. Virus-containing media was replaced at 24 h post-infection with 10% FBS/DMEM. Typically, experiments were initiated at 24 h post-transduction. Experimental methods used to induce NF $\kappa$ B were performed according to the following protocols.

**Pervanadate Treatment**—Sodium orthovanadate was prepared fresh in water at a concentration of 500 mM. 40  $\mu$ l of sodium orthovanadate and 5  $\mu$ l of 30% (w/w) H<sub>2</sub>O<sub>2</sub> was then added to 455  $\mu$ l phosphate-buffered saline. This mixture was incubated for 5 min at room temperature prior to the addition of catalase (200  $\mu$ g/ml) to remove the excess H<sub>2</sub>O<sub>2</sub>. The pervanadate solution (final concentration 40 mM) was further incubated for 5 min at room temperature, immediately diluted in DMEM and applied to cells. Cells were harvested at 6 h post-pervanadate treatments for NF $\kappa$ B activation using luciferase assays or as indicated. Control cells were fed with identical fresh medium that was devoid of pervanadate.

**Hypoxia/Reoxygenation**—DMEM (devoid of glucose or FBS) (Invitrogen) equilibrated in 95% N<sub>2</sub>, 5% CO<sub>2</sub> or 95% O<sub>2</sub>, 5% CO<sub>2</sub> was used as hypoxia and reoxygenation medium, respectively. Cells were covered with minimal hypoxia medium and incubated at 37  $^{\circ}$ C for 5 h in an airtight chamber equilibrated with 5% CO<sub>2</sub> and 95% N<sub>2</sub>. The medium was then replaced with a minimal amount of reoxygenation medium and incubated further at 37  $^{\circ}$ C in a chamber flushed with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. Cells were harvested 6 h after reoxygenation for NF $\kappa$ B activation luciferase assays. Control cells were fed with fresh medium at identical times as the hypoxia/reoxygenation samples, but were exposed to 5% CO<sub>2</sub> in atmospheric oxygen.

**TNF $\alpha$  Treatment**—Mouse recombinant TNF $\alpha$  (R&D systems, Minneapolis, MN) was diluted in fresh DMEM medium (10 ng/ml final concentration) and applied to cells at the time of treatment. Cells remained exposed to TNF $\alpha$  until they were harvested at 6 h post-stimulation for NF $\kappa$ B activation luciferase assays. Control cells were fed at the time of treatment with fresh DMEM medium without TNF $\alpha$ .

**Western Blotting and I $\kappa$ B $\alpha$  Phosphorylation Assays**—Cells were lysed in RIPA buffer (0.15 M NaCl, 50 mM Tris pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS), and the protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). 5  $\mu$ g of cell lysate was resolved on a 10% SDS-PAGE and then transferred to nitrocellulose membrane using previously described protocols (18). I $\kappa$ B $\alpha$  protein levels were determined by Western blot analysis using an anti-I $\kappa$ B $\alpha$  monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To evaluate I $\kappa$ B $\alpha$  tyrosine phosphorylation, 200  $\mu$ g of cell lysate was immunoprecipitated using 2  $\mu$ g of I $\kappa$ B $\alpha$  antibody (Santa Cruz Biotechnology) followed by Western blot analysis using antiphosphotyrosine antibody (Santa Cruz Biotechnology) and standard protocols (10). Phosphorylated forms of c-Src or total c-Src were detected using anti-c-SrcPY416, anti-c-SrcPY139, and anti-c-Src antibodies (Santa Cruz Biotechnology).

**NF $\kappa$ B Activation Assays**—NF $\kappa$ B transcriptional activity was evaluated using an Ad.NF $\kappa$ BLuc reporter vector as previously described (16). Briefly, cells were infected with Ad.NF $\kappa$ BLuc at an MOI of 500 particles/cell 24 h prior to TNF $\alpha$ , pervanadate, or H/R treatment. 5  $\mu$ g of total protein from each sample was assayed for luciferase activity using manufacturer's protocols (Promega, Madison, WI) in a luminometer as previously reported (16). Luciferase activity was assessed as relative light units and used as an indicator for the transcription induction of NF $\kappa$ B. To assess potential global changes in transcription induced by each type of environmental stimuli, that were not dependent on NF $\kappa$ B, several experiments were performed normalizing changes in Ad.NF $\kappa$ BLuc expression to that seen with a control Ad.CMVLacZ vector (20). In these studies both Ad.NF $\kappa$ BLuc and Ad.CMVLacZ were co-infected into cells for each of the conditions examined (MOI = 500 particles/cell for each vector) 24 h prior to TNF $\alpha$ , pervanadate, or H/R treatment. Luciferase activity was then assessed using 5  $\mu$ g of lysate as described above, and  $\beta$ -galactosidase activity was quantified with 5  $\mu$ g of lysate using a previously described protocol (21). Luciferase activity was then normalized for  $\beta$ -galactosidase expression in reference to the Ad.BglII infected (no injury) control. Electrophoretic mobility shift assays for NF $\kappa$ B DNA binding were performed as previously described using a <sup>32</sup>P-labeled NF $\kappa$ B oligonucleotide probe (18).

**In Vitro Kinase Assays**—Two types of *in vitro* kinase assays (radioactive and non-radioactive) were used to evaluate the ability of immu-

nonprecipitated c-Src or IKK $\beta$  to phosphorylate GST-I $\kappa$ B $\alpha$  *in vitro* following different environmental stimuli. For radioactive *in vitro* kinase assays, HeLa, SYF, or SYF+c-Src cells were washed in ice-cold PBS and lysed in 1 ml of ice-cold RIPA buffer (0.15 M NaCl, 50 mM Tris, pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The protein concentration was then determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). 500  $\mu$ g of protein was immunoprecipitated with anti-c-Src or anti-IKK $\beta$  antibodies (Santa Cruz Biotechnology) and protein A-agarose beads. 1  $\mu$ g of GST-I $\kappa$ B $\alpha$  protein (Santa Cruz Biotechnology) was then added to washed protein A pellets in the presence of 10  $\mu$ l of kinase buffer (40 mM Hepes, 1 mM  $\beta$ -glycerophosphate, 1 mM nitrophenolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.3 mM cold ATP, and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP) and incubated at 30 °C for 30 min. The reaction was terminated by the addition of protein-loading buffer (with SDS) and boiled at 98 °C for 5 min. Samples were then centrifuged to remove the agarose beads, and the supernatant was loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membrane (which reduces the background of free [ $\gamma$ -<sup>32</sup>P]ATP) and exposed to x-ray film. Non-radioactive *in vitro* kinase assays were performed to directly evaluate the extent of tyrosine phosphorylation of GST-I $\kappa$ B $\alpha$  by immunoprecipitated c-Src or IKK $\beta$ . These *in vitro* kinase assays were performed identical to the protocol described above except for the omission of [ $\gamma$ -<sup>32</sup>P]ATP. *In vitro* labeled GST-I $\kappa$ B $\alpha$  samples were then evaluated by Western blotting for the extent of tyrosine phosphorylation using antiphosphotyrosine antibody (Santa Cruz Biotechnology).

## RESULTS

*Transcriptional Activation of NF $\kappa$ B Following Pervanadate or H/R Treatment Requires I $\kappa$ B $\alpha$  Tyr-42 Phosphorylation and Is Independent of the IKK Complex and I $\kappa$ B $\alpha$  Serine Phosphorylation*—NF $\kappa$ B activation can occur through at least two mechanisms that control I $\kappa$ B $\alpha$  phosphorylation on either tyrosine 42 or serine 32/36. Proinflammatory stimuli such as TNF $\alpha$  are well suited to activate NF $\kappa$ B through the I $\kappa$ B kinase complex (IKK) that mediates serine phosphorylation of I $\kappa$ B $\alpha$  and ubiquitin-dependent degradation of I $\kappa$ B $\alpha$ . In contrast, NF $\kappa$ B activation in the liver following ischemia/reperfusion (I/R) injury (10), and in T-cells following H/R (6), occurs in the absence of I $\kappa$ B $\alpha$  degradation and is associated with an increase in tyrosine phosphorylation of I $\kappa$ B $\alpha$ . To better define the mechanisms involved in NF $\kappa$ B activation following I/R injury, we developed an *in vitro* epithelial cell line model system capable of modulating NF $\kappa$ B activity through tyrosine or serine phosphorylation of I $\kappa$ B $\alpha$  following H/R, pervanadate, or TNF $\alpha$  treatments.

To establish that NF $\kappa$ B activation following H/R occurs through a selective pathway involving tyrosine phosphorylation of I $\kappa$ B $\alpha$  that is independent of the IKK complex, we utilized several dominant negative mutants to modulate IKK activation and I $\kappa$ B $\alpha$  phosphorylation. NF $\kappa$ B transcriptional activity was evaluated using a recombinant adenoviral reporter vector (Ad.NF $\kappa$ BLuc) expressing the NF $\kappa$ B-inducible luciferase gene. As expected and previously reported in epithelial cell lines, the transcriptional induction of NF $\kappa$ B following TNF $\alpha$  treatment was significantly inhibited ( $p < 0.001$ ) by expression of Ad.IKK $\beta$ (KA), Ad.IKK $\alpha$ (KM), or Ad.I $\kappa$ B $\alpha$ (S32A/S36A) in comparison to Ad.Bg/II (empty vector control)-transduced cells (Fig. 1A). No inhibition in TNF $\alpha$ -induced NF $\kappa$ B activation was seen following expression of Ad.I $\kappa$ B $\alpha$ Y42F. These results confirm the functionality of our vectors to inhibit IKK-mediated TNF $\alpha$  activation of NF $\kappa$ B and demonstrate a lack of functional involvement of I $\kappa$ B $\alpha$  Y42 phosphorylation under these conditions. In contrast to findings with TNF $\alpha$ , I $\kappa$ B $\alpha$ (Y42F) expression significantly inhibited NF $\kappa$ B transcriptional activation following pervanadate ( $p < 0.001$ ) or H/R ( $p < 0.001$ ) treatments (Fig. 1, A and B). No significant alterations in pervanadate or H/R-mediated activation of NF $\kappa$ B was seen following infection with Ad.IKK $\beta$ (KA), Ad.IKK $\alpha$ (KM), or Ad.I $\kappa$ B $\alpha$ (S32A/S36A) mutant vectors. Furthermore, when the

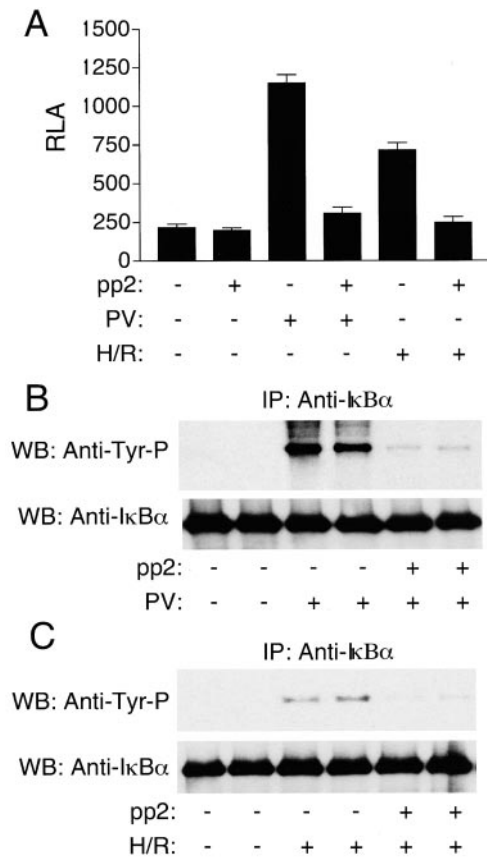
induction of NF $\kappa$ B-mediated luciferase expression was normalized to changes in expression of an irrelevant internal control LacZ transgene under the control of the CMV promoter, the patterns and changes for each of the environmental stimuli and dominant mutants tested were not significantly altered (Fig. 1, A–C). These data demonstrate that global changes in the overall transcriptional state of cells cannot account for the specific alterations induced by the various dominant mutants for a given stimulus.

To confirm that changes in transcriptional activation of NF $\kappa$ B mirrored those seen in DNA binding, electrophoretic mobility shift assays were performed for each of the various stimuli. These results shown in Fig. 1D confirm that NF $\kappa$ B transcriptional activation is accompanied by increased DNA binding in nuclear extracts. Cumulatively, our results evaluating IKK and I $\kappa$ B $\alpha$  mutants suggest that IKK-mediated serine 32/36 phosphorylation of I $\kappa$ B $\alpha$  does not play a significant role in regulating NF $\kappa$ B following pervanadate or H/R stimuli in our HeLa cell line model. To directly evaluate whether TNF $\alpha$  imparts selective activation of the IKK complex not observed following H/R or pervanadate treatments, we performed *in vitro* kinase assays with immunoprecipitated IKK $\beta$  to directly evaluate IKK activation and ability to phosphorylate GST-I $\kappa$ B $\alpha$  following each of these stimuli. Results from this analysis are shown in Fig. 1E and demonstrate that TNF $\alpha$  treatment stimulates higher levels of IKK activity as compared with H/R and pervanadate treatments. However, activation of IKK was also observed at lower levels following both H/R and pervanadate treatments, suggesting that some overlap in signaling may exist. This apparent overlap may be due to pervanadate and H/R activation of cytokines, which restimulate cells through the IKK pathway. These findings substantiate the small non-significant, but observed, partial inhibition of NF $\kappa$ B transcriptional activation by IKK mutants seen following H/R and pervanadate treatments.

*Src Inhibitor pp2 Blocks NF $\kappa$ B Activation and I $\kappa$ B $\alpha$  Tyrosine Phosphorylation Following Pervanadate or H/R Treatment*—Our results in the HeLa cell model have established that NF $\kappa$ B activation following H/R or pervanadate treatment is independent of IKK and serine phosphorylation of I $\kappa$ B $\alpha$ . We next sought to evaluate candidate upstream factors capable of mediating tyrosine phosphorylation of I $\kappa$ B $\alpha$  and subsequent NF $\kappa$ B activation. Src family kinases are widely recognized for their importance in regulating stress response genes in response to redox-regulated stimuli such as H/R (15, 22). Furthermore, it has been reported that c-Src activity was necessary for TNF $\alpha$ -induced tyrosine phosphorylation of I $\kappa$ B $\alpha$  in BMMs (9). Given the lack of a functional requirement for I $\kappa$ B $\alpha$  tyrosine phosphorylation in the transcriptional induction of NF $\kappa$ B following TNF $\alpha$  in our epithelial cell line model, we investigated whether c-Src might also play a role in NF $\kappa$ B activation following H/R or pervanadate treatment.

Consistent with the activation of c-Src following H/R or pervanadate treatment, we observed an increase in both Tyr-416- and Tyr-139-phosphorylated forms of activated c-Src (Fig. 2). H/R treatment demonstrated a greater increase in both phosphorylated forms while pervanadate treatment more selectively increased the Tyr-416-phosphorylated form of c-Src. These findings suggest that indeed c-Src is activated by both pervanadate or H/R treatment and is consistent with the previously reported redox-mediated involvement in the activation of c-Src (15). To assign functional importance to c-Src in the tyrosine phosphorylation of I $\kappa$ B $\alpha$  and subsequent activation of NF $\kappa$ B, we next evaluated the effect of the pp2 c-Src inhibitor. Pretreatment of HeLa cells with pp2 significantly inhibited both pervanadate- and H/R-induced NF $\kappa$ B activation ( $p <$

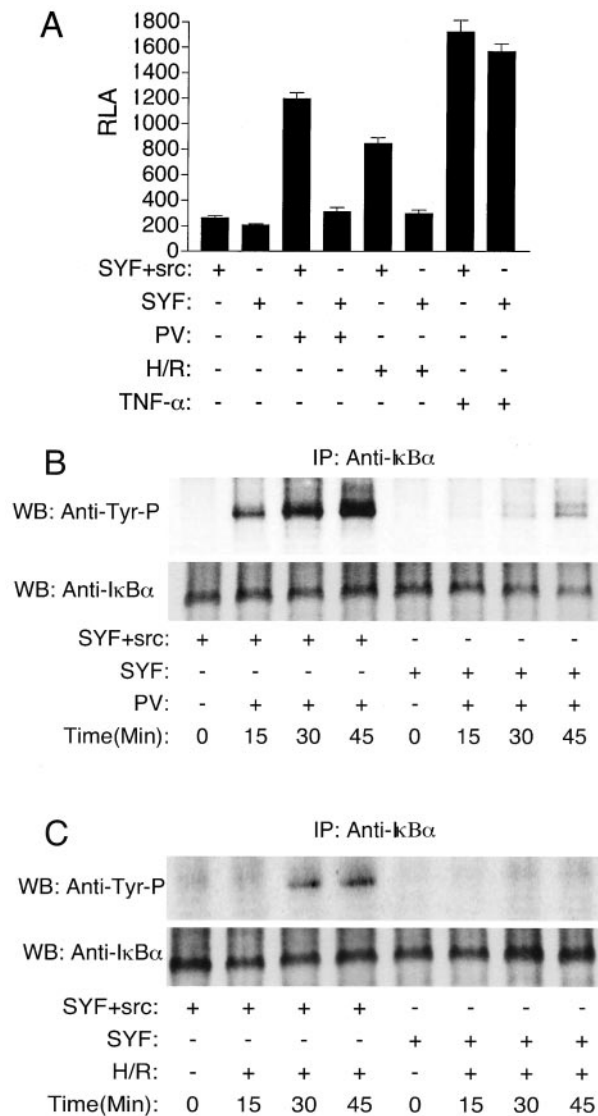




**FIG. 3. Inhibition of *c-Src* activation blocks  $\text{NF}\kappa\text{B}$  transcriptional activation and  $\text{I}\kappa\text{B}\alpha$  tyrosine phosphorylation following pervanadate or H/R treatment.** A, HeLa cells were transduced with Ad.NF $\kappa\text{B}$ Luc (MOI of 500 particles/cell) 24 h prior to the initiation of experimental treatment. Cells were then exposed to fresh media with and without pp2 (10  $\mu\text{M}$ ) for 30 min, followed by treatment with pervanadate (100  $\mu\text{M}$ ) for 6 h or H/R (hypoxic media; 5%  $\text{N}_2$ , 5%  $\text{CO}_2$ ) for 5 h followed by reoxygenation media (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) for 6 h. pp2 inhibitor was continually present during pervanadate and H/R treatments. Whole cell extracts were harvested into lysis buffer, normalized for total protein content, and evaluated for  $\text{NF}\kappa\text{B}$  activation using a luciferase assay. Results depict the mean ( $\pm$  S.E.,  $n = 6$ ) relative luciferase activity (RLA). HeLa cells were treated with pervanadate (100  $\mu\text{M}$ ) (B) for 30 min or hypoxic media (95%  $\text{N}_2$ , 5%  $\text{CO}_2$ ) (C) for 5 h followed by reoxygenation media (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) for 30 min. One group was pretreated with pp2 (10  $\mu\text{M}$ ) for 30 min prior to pervanadate or H/R. Inhibitor (pp2) was continually present during the treatments. Cell lysates were harvested and 200  $\mu\text{g}$  of total protein was immunoprecipitated with anti- $\text{I}\kappa\text{B}\alpha$  antibody followed by Western blotting with an antiphosphotyrosine antibody or anti- $\text{I}\kappa\text{B}\alpha$  antibody.

out to establish null *Src* mutant activity (23). In SYF+src cell lines, the *c-Src* activity was reintroduced into the SYF background. Thus, by comparing these two cell lines, one can elucidate *c-Src* function.

Results evaluating the SYF cell line demonstrated a complete loss of pervanadate- and H/R-induced  $\text{NF}\kappa\text{B}$  transcriptional activation in comparison to SYF+src cells ( $p < 0.001$ ) (Fig. 4A). In contrast, there was no significant difference in TNF $\alpha$ -mediated induction of  $\text{NF}\kappa\text{B}$  in either of these two cell lines. These results suggest that *c-Src* activity is required for  $\text{NF}\kappa\text{B}$  pathways involving tyrosine, but not serine-mediated phosphorylation of  $\text{I}\kappa\text{B}\alpha$ . To conclusively address the requirement for *c-Src* activity to mediate tyrosine phosphorylation of  $\text{I}\kappa\text{B}\alpha$ , we next evaluated the extent of  $\text{I}\kappa\text{B}\alpha$  tyrosine phosphorylation in both SYF and SYF+src cells following pervanadate or H/R treatment. These studies demonstrated that  $\text{I}\kappa\text{B}\alpha$  tyrosine phosphorylation was significantly reduced in SYF following pervanadate and completely blocked following H/R as com-



**FIG. 4. Tyrosine phosphorylation of  $\text{I}\kappa\text{B}\alpha$  and transcriptional activation of  $\text{NF}\kappa\text{B}$  are significantly reduced in *c-Src* knockout cell lines.** A, SYF cells or SYF+src cells were transduced with Ad.NF $\kappa\text{B}$ Luc (MOI of 500 particles/cell) 24 h prior to initiated experiments. Cells were treated with pervanadate (50  $\mu\text{M}$ ) for 6 h or H/R (5 h of hypoxia, 6 h of reoxygenation), harvested into lysis buffer, normalized for total protein content, and subjected to luciferase assays.  $\text{NF}\kappa\text{B}$  transcriptional activation was evaluated as the relative luciferase activity. Results depict the mean ( $\pm$  S.E.,  $n = 6$ ) relative luciferase activity (RLA). SYF or SYF+src cells were treated with pervanadate (50  $\mu\text{M}$ ) (B) for 15, 30, and 45 min or hypoxic media (95%  $\text{N}_2$ , 5%  $\text{CO}_2$ ) (C) for 5 h followed by reoxygenation media (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) for 15, 30, and 45 min. Both untreated and treated cell lysates were harvested at the indicated time points, and 200  $\mu\text{g}$  of total protein was immunoprecipitated with anti- $\text{I}\kappa\text{B}\alpha$  antibody, followed by Western blot analysis with an antiphosphotyrosine antibody or anti- $\text{I}\kappa\text{B}\alpha$  antibody.

pared with SYF+src cells (Fig. 4, B and C). Given the previous demonstration of p56<sup>Lck</sup> function in the activation of  $\text{I}\kappa\text{B}\alpha$  tyrosine phosphorylation following pervanadate treatment (11), the residual phosphorylation seen in our *c-Src*, *Fyn*, and *Yes* knockout cell lines may be due to redundant *Lck* function. However, our studies evaluating  $\text{NF}\kappa\text{B}$  activation following pervanadate treatment suggest that this residual phosphorylation may not be functionally active. In contrast, our studies evaluating H/R demonstrate for the first time that  $\text{I}\kappa\text{B}\alpha$  tyrosine phosphorylation and  $\text{NF}\kappa\text{B}$  activation can be completely blocked in *c-Src*, *Fyn*, and *Yes* knockout cells and fully restored

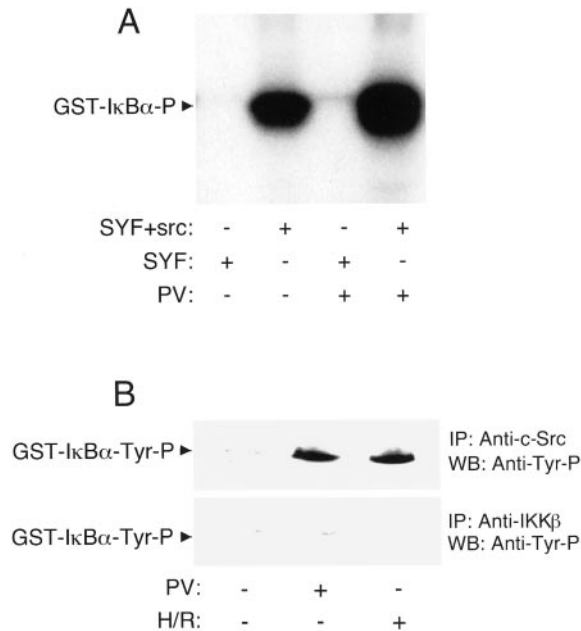


FIG. 5. **c-Src phosphorylates IκBα in vitro.** A, SYF cells or SYF+src cells were treated with pervanadate (50 μM) for 30 min and then harvested in RIPA buffer. c-Src was immunoprecipitated with anti-c-Src antibody from 500 μg of protein lysate. The ability of immunoprecipitated c-Src to directly phosphorylate GST-IκBα fusion protein was then evaluated in the presence of [ $\gamma$ - $^{32}$ P]ATP *in vitro*. Labeled GST-IκBα fusion protein was detected by SDS-PAGE and autoradiography. B, HeLa cells were treated with pervanadate (100 μM, 30 min), or H/R (5 h of hypoxia, 30 min of reoxygenation) and evaluated for c-Src activity using a cold *in vitro* kinase assay. c-Src or IKKβ was immunoprecipitated with anti-c-Src or anti-IKKβ antibody from 500 μg of protein lysate. The ability of immunoprecipitated c-Src or IKKβ to directly tyrosine-phosphorylate GST-IκBα fusion protein was evaluated by Western blotting with antiphosphotyrosine antibody. Immunoreactivity was detected by ECL and autoradiography.

by c-Src activity alone. These findings suggest that other Src family kinases (*i.e.* Lck, Lyn, etc.) play a minor role in mediating the activation of this pathway in epithelial cells following H/R.

**c-Src Phosphorylates IκBα in Vitro**—Having demonstrated that c-Src activity is required for tyrosine phosphorylation and NFκB activation, we tested whether c-Src could be the tyrosine kinase that is directly responsible for tyrosine phosphorylation of IκBα. We used an *in vitro* kinase assay to evaluate c-Src tyrosine kinase activity in SYF cells or SYF+c-src cells following 30 min of pervanadate treatment. Our results presented in Fig. 5A demonstrate that immunoprecipitated c-Src from untreated SYF+c-src cells has the ability to phosphorylate a GST-IκBα fusion protein. Furthermore, as anticipated, the extent of GST-IκBα phosphorylation is significantly increased following PV treatment. Similar assays using SYF cell lysates demonstrated no significant GST-IκBα phosphorylation at baseline, or following pervanadate treatment, and serve as negative controls for the specificity of c-Src immunoprecipitation and kinase function. To conclusively demonstrate that c-Src tyrosine phosphorylates GST-IκBα, we performed cold *in vitro* kinase assays and evaluated the phosphorylated GST-IκBα substrate by Western blotting with antiphosphotyrosine antibody. These results demonstrated that both H/R and pervanadate treatments of HeLa cells activates the ability of immunoprecipitated c-Src to tyrosine phosphorylate IκBα (Fig. 5B). Furthermore, when similar assays were performed using immunoprecipitated IKKβ, no increase in tyrosine phosphorylation of IκBα was observed over baseline untreated controls. Cumulatively, these results suggest that c-Src activation fol-

lowing H/R and pervanadate treatment is required for tyrosine phosphorylation of IκBα. They also suggest that c-Src is likely the direct tyrosine kinase responsible for this phosphorylation event. However, we cannot rule out the possibility that other tyrosine kinases associated with c-Src are not also involved in tyrosine phosphorylation of IκBα.

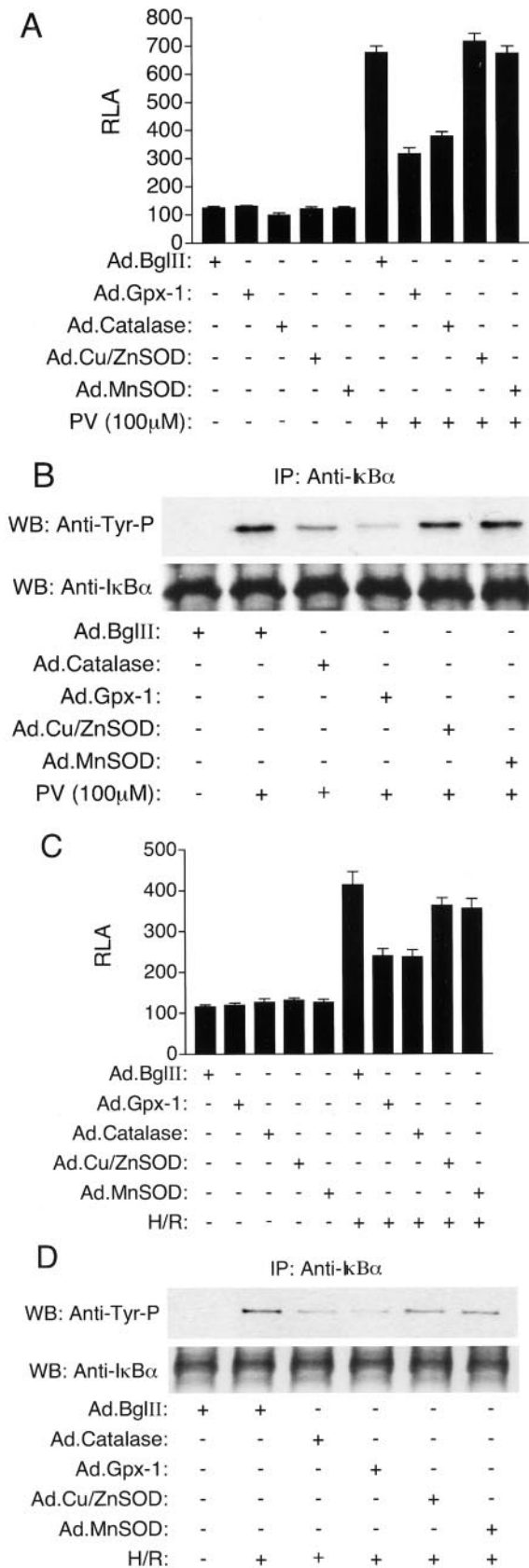
**Overexpression of Gpx-1 or Catalase, but Not Mn-SOD or Cu,Zn-SOD, Inhibits Tyrosine Phosphorylation of IκBα and NFκB Activation Following Pervanadate or H/R**—Activation of NFκB is widely recognized to be dependent on the redox environment within the cell. In the context of IKK-mediated activation of NFκB, ROS have been demonstrated to be a critical component in the activation of both IKKβ (16) and IKKα (24) subunits of the IKK complex following environmental stimuli. Moreover, H<sub>2</sub>O<sub>2</sub> has been shown to activate tyrosine phosphorylation of IκBα in T-cells in a manner similar to pervanadate (11). Given the fact that H<sub>2</sub>O<sub>2</sub> has been shown to activate c-Src (25) and the observed dependence of IκBα tyrosine phosphorylation and NFκB transcriptional activation on c-Src activity in our H/R models, we next sought to investigate whether ROS were a signal component of IκBα tyrosine phosphorylation following H/R.

To investigate the redox-dependence of NFκB transcriptional activation following pervanadate or H/R treatment, we manipulated the intracellular redox environment using a set of recombinant adenoviruses that encoded various ROS-scavenging enzymes. These included Ad.Catalase or Ad.GPx-1 vectors that degrade H<sub>2</sub>O<sub>2</sub>, and Ad.Mn-SOD or Ad.Cu,Zn-SOD vectors that dismutate superoxide anion (O<sub>2</sub><sup>-</sup>) into H<sub>2</sub>O<sub>2</sub>. Using our *in vitro* model, we analyzed the role of these antioxidant enzymes in regulating IκBα tyrosine phosphorylation and NFκB transcriptional activation. Results from these studies demonstrated a significant inhibition ( $p < 0.001$ ) in both pervanadate (Fig. 6A) and H/R (Fig. 6C) induction of NFκB transcriptional activity following expression of GPx-1 or catalase. Consistent with this NFκB activation data, tyrosine phosphorylation of IκBα following pervanadate or H/R treatments was also significantly inhibited by GPx-1 or catalase overexpression (Fig. 6, B and D). In contrast, overexpression of either Cu,Zn-SOD or Mn-SOD failed to alter IκB tyrosine phosphorylation or NFκB activation. These findings suggest that H<sub>2</sub>O<sub>2</sub> is an important redox component in NFκB activation mediated through IκB-α tyrosine phosphorylation.

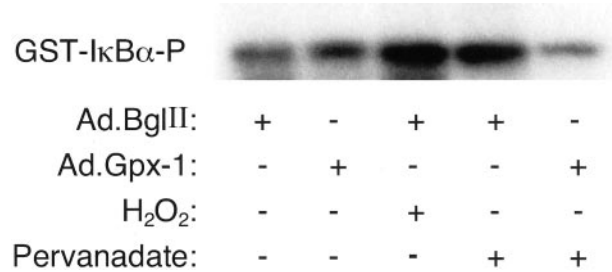
**GPx-1 Overexpression Reduces c-Src Kinase Activity**—Having demonstrated that Gpx-1 overexpression is able to reduce NFκB activity as well as tyrosine phosphorylation of IκBα, we next investigated whether GPx-1 expression acts to directly inhibit activation of c-Src using an *in vitro* kinase assay. Results from these experiments in HeLa cells demonstrated a significant inhibition in the ability of c-Src to phosphorylate GST-IκBα following pervanadate treatment in the presence of Ad.GPx-1 infection as compared with Ad.Bg/II-infected control (Fig. 7). Furthermore, in this assay, transient treatment with 1 mM H<sub>2</sub>O<sub>2</sub> for 30 min also significantly activated c-Src kinase function as previously demonstrated. In summary, our data demonstrate that intracellular hydrogen peroxide (or hydroxyl radical products) mediates NFκB activation through regulation of c-Src-dependent IκBα tyrosine phosphorylation. Overexpression of H<sub>2</sub>O<sub>2</sub> scavengers is able to efficiently reduce c-Src kinase activity, IκBα tyrosine phosphorylation, and NFκB activation following H/R or pervanadate injury.

## DISCUSSION

The physiologic significance of IκBα tyrosine phosphorylation in mediating NFκB transcriptional activation has remained one of the poorly understood aspects of this well-stud-



**FIG. 6. Overexpression of Gpx-1 or catalase, but not Mn-SOD or Cu,Zn-SOD, inhibits NFκB activation and tyrosine phosphorylation of IκBα following pervanadate or H/R treatment.** HeLa cells were co-infected with Ad.BglIII, Ad.Gpx-1, Ad.Catalase, Ad.Cu,Zn-SOD, or Ad.Mn-SOD (MOI of 1000 particles/cell) together with Ad.NFκBLuc (MOI of 500 particles/cell) for 24 h prior to initiating experimental treatments described below. Cells were treated with per-



**FIG. 7. Overexpression of GPx-1 reduces c-Src kinase activity following pervanadate treatment.** HeLa cells were infected with Ad.BglIII or Ad.Gpx-1 (MOI of 1000 particles/cell) for 24 h prior to initiating experimental treatments. Cells were then treated with pervanadate (100 µM) or hydrogen peroxide (1 mM) for 30 min and then lysed in RIPA buffer. c-Src was immunoprecipitated with anti-c-Src antibody from 500 µg of protein lysate. The ability of immunoprecipitated c-Src to directly phosphorylate GST-IκBα fusion protein was then evaluated in the presence of [ $\gamma$ -<sup>32</sup>P]ATP *in vitro*.

ied transcription factor. To date, all studies evaluating the functional regulation of IκBα tyrosine phosphorylation and NFκB activation have been performed in T-cells or BMMs. Although the tyrosine phosphatase inhibitor pervanadate has been shown to be a significant activator of this pathway, natural physiologic stimuli that induce IκBα tyrosine phosphorylation have remained elusive. However, TNFα treatment of BMMs has recently been shown to activate NFκB recruitment to the nucleus in an IκBα tyrosine phosphorylation-dependent manner (9). This TNFα-induced pathway of NFκB activation in BMMs appears to differ significantly from the classical IKK-dependent pathway involving serine phosphorylation of IκBα that is active in other epithelial-derived cell types. Other non-hematopoietic systems have increasingly demonstrated components of IκBα tyrosine phosphorylation in models of *in vitro* and *in vivo* injury (10, 26, 27). The unique fingerprint of this pathway appears to be the ability of an IκBα PTK to activate NFκB in the absence of IκBα proteolytic degradation. In the present study, we have sought to clarify several issues regarding the involvement of this IκBα PTK pathway in mediating NFκB transcriptional activation in epithelial-derived cells following H/R.

Using comparative cell line models to evaluate both IκBα serine and tyrosine phosphorylation-dependent pathways of NFκB transcriptional activation, our studies have further characterized an IKK-independent pathway that regulates NFκB through c-Src activation. Studies using IκBα phosphorylation mutants and SYF knockout cells demonstrated that c-Src-mediated transcriptional activation of NFκB functionally requires tyrosine, but not serine, phosphorylation of IκBα. Using this HeLa cell model system, we found no functional requirement for IκBα tyrosine phosphorylation in the transcriptional activation of NFκB following TNFα stimulus and no evidence for tyrosine phosphorylation of IκBα following TNFα treatment. Furthermore, our studies evaluating IKK-dominant mutants demonstrate, for the first time, the lack of IKK involvement in PTK-mediated pathways of NFκB transcriptional activation

vanadate (100 µM) (A) for 6 h or H/R (5 h of hypoxia/6 h of reoxygenation) (C). Whole cell extracts were normalized for total protein content and subjected to luciferase assays. NFκB transcriptional activity was assessed as the mean relative luciferase activity (RLA) ( $\pm$  S.E.,  $n = 6$ ). For evaluation of IκBα phosphorylation, cells were treated with pervanadate (100 µM) (B) for 30 min or H/R (6 h of hypoxia/30 min of reoxygenation) (D). Both untreated and treated samples were harvested into lysis buffer, and 200 µg of total protein was immunoprecipitated with anti-IκBα antibody followed by Western blot analysis with an antiphosphotyrosine antibody or anti-IκBα antibody.

following pervanadate or H/R treatments, while confirming the selective activation of NFκB by TNFα as mediated through serine phosphorylation of IκBα. These functional studies evaluating the transcriptional activation of NFκB following three independent stimuli suggest that little overlap, if any, exists in IKK and PTK pathways controlling the IκBα-NFκB complex.

Several similarities and differences between our present studies and those in BMMs are worth noting. First, pervanadate appears to be a universal activator of NFκB-requiring IκBα tyrosine phosphorylation, with consistent results observed in HeLa cells, T-cells, and BMMs. Second, unlike HeLa cells, treatment of BMMs with TNFα results in significant activation of NFκB in a manner dependent on IκBα phosphorylation of tyrosine 42 (9). This difference underscores the importance of cell type-specific dependences in the activation of NFκB and IκBα protein tyrosine kinases. Third, our current studies are the first to directly evaluate the transcriptional activation of NFκB using an NFκB-responsive reporter gene. To date, all assays for NFκB activation following stimulation of IκBα tyrosine phosphorylation had been performed using NFκB DNA binding.

Intracellular production of ROS has been implicated in the regulation of numerous signal transduction cascades and in the activation of NFκB following ischemia/reperfusion injury (1, 28). Furthermore, c-Src can be directly activated by hydrogen peroxide treatment (12, 25), and stimuli such as angiotensin II can induce c-Src activity, which is inhibited by antioxidants (29). The association of c-Src activation following H/R (15) has also suggested that c-Src may act as a redox sensor in the activation of NFκB. However, other pathways of NFκB activation involving pro-inflammatory stimuli such as TNFα and LPS also have redox-sensitive activation components, which are associated with the IKK complex (16, 24). These studies have suggested that superoxide formation may be the primary initiating ROS involved in activation of the IKK complex. Hence, the pathways for redox activation of NFκB are quite diverse and likely regulated by the spatial relationship of both specific ROS and the signaling components involved. Our findings in the present study have shed additional light on the redox diversity of NFκB activation involving tyrosine phosphorylation of IκBα. The demonstration that both Gpx-1 and catalase, but not Mn-SOD or Cu,Zn-SOD, are capable of inhibiting H/R or pervanadate-induced IκBα tyrosine phosphorylation and NFκB activation, suggests a preference for H<sub>2</sub>O<sub>2</sub> and/or hydroxyl radicals (as a product of H<sub>2</sub>O<sub>2</sub>) in the activation of the IκBα tyrosine kinase. These findings are consistent with previous reports demonstrating the direct activation of IκBα tyrosine phosphorylation by H<sub>2</sub>O<sub>2</sub> in T-cells (11).

Recent evidence has suggested that the p85 subunit of PI3-kinase associates with tyrosine 42 phosphorylated IκBα but not with unphosphorylated IκBα (8). The catalytic p110 subunit also appears to be critical in the activation of NFκB. The function of p110 may involve phosphorylation of NFκB and/or dissociation of the IκBα: NFκB complex. Since both PI 3-kinase and c-Src have been shown to associate with one another (30) and both maintain redox-sensitive components in their activation (31–33), it is plausible that c-Src may act on this PI 3-kinase complex to mediate NFκB activation. It has been previously demonstrated that c-Src is at least partially required for IκBα tyrosine phosphorylation in BMMs following TNFα treatment (9). However, these studies performed in c-Src (–/–) BMMs demonstrated a delay only in the induction of p50/p65 heterodimers in the nucleus following the TNFα treatment. In comparison to our present studies using SYF cells with a c-Src, Fyn, and Yes, knockout background, we find a

more complete block in both IκBα tyrosine phosphorylation and NFκB transcriptional activation following both H/R and pervanadate treatment than previously described. Furthermore this block was completely reversed by reconstitution of only c-Src activity. These findings highlight the functional redundancy of Src family kinases and conclusively demonstrate that c-Src is fully capable of mediating NFκB activation through IκBα tyrosine phosphorylation. Our studies, for the first time, have also successfully reconstituted IκBα tyrosine phosphorylation with immunoprecipitated c-Src in an *in vitro* kinase assay. Furthermore, the activity of c-Src IκBα tyrosine kinase activity was modulated in response to H/R and pervanadate treatments in a redox-dependent fashion. These findings provide the most conclusive evidence to date that c-Src is able to directly tyrosine phosphorylate IκBα and that this phosphorylation event is required for NFκB activation following H/R or pervanadate treatments. The physiologic relevance of redundant Src family kinases in the activation of NFκB still remains unclear. However, the redox-regulated mechanisms that control activation of NFκB by Src family kinases may be a particularly relevant therapeutic target for organ damage following I/R injury.

**Acknowledgments**—We thank NIDDK, National Institutes of Health funding of the DERC for tissue culture supplies. We thank Dr. J. F. Peyron (Pasteur, France) for providing the IκBα(Y42F) cDNA. We gratefully acknowledge the editorial assistance of Dr. Reitu S. Agrawal and Kevin Wyne.

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