NFκB is a critical transcription factor involved in modulating cellular responses to environmental injuries. Tyrosine 42 phosphorylation of IκBα has been shown to mediate NFκB activation following hypoxia/reoxygenation (H/R) or perivandate treatment. This pathway differs from the canonical proinflammatory pathways, which mediate NFκB activation through serine phosphorylation of IκBα by the IKK complex. In the present study, we investigated the involvement of c-Src in the redox activation of NFκB following H/R or perivandate treatment. Our results demonstrate that perivandate or H/R treatment leads to tyrosine phosphorylation of IκBα and NFκB 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 IκBα tyrosine phosphorylation and NFκB activation following perivandate or H/R treatment. Overexpression of glutathione peroxidase-1 or catalase, but not Mn-SOD or Cu,Zn-SOD, significantly reduced both NFκB activation and tyrosine phosphorylation of IκBα. In vitro kinase assays further demonstrated that immunoprecipitated c-Src has the capacity to directly phosphorylate GST-IκBα and that this IκBα kinase activity is significantly reduced by Gpx-1 overexpression. These results suggest that c-Src-dependent tyrosine phosphorylation of IκBα and subsequent activation of NFκB is controlled by intracellular H₂O₂ and defines an important redox-regulated pathway for NFκB activation following H/R injury that is independent of the IKK complex.

Reactive oxygen species (ROS)¹ are normal metabolic byproducts 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 NFκB (3).

NFκB family members include p50, p52, p65, and c-RelB, which form homodimeric and heterodimeric transcriptional complexes (4). The activation of NFκB is controlled by a family of IκB repressor proteins (IκBα, IκBβ, and IκBε) that sequester NFκB in the cytoplasm (4). Phosphorylation-dependent inactivation of IκBα proteins leads to the mobilization of NFκB to the nucleus where it can act as a transcription factor. These phosphorylation pathways have been most extensively studied for IκBα and include two distinct mechanisms involving either serine or tyrosine phosphorylation of IκBα. The most comprehensively studied pathway regulating IκBα includes phosphorylation on two serine (32 and 36) residues by the IκB kinase complex (IKK) (5). This phosphorylation leads to ubiquitination of IκBα at nearby lysine residues and degradation by the proteasome. An alternative, less characterized pathway of NFκB activation acts through tyrosine phosphorylation of IκBα at residue 42 (6). In contrast to IKK-mediated serine phosphorylation of IκBα, tyrosine phosphorylation of IκBα is capable of activating NFκB in the absence of ubiquitin-dependent degradation of IκBα. However, it is presently unclear if IKK and/or the IκBα protein-tyrosine kinase (PTK) interactions with IκBα are functionally modulated by prior tyrosine or serine phosphorylation of IκBα, respectively. Experimental evidence appears to suggest that prior tyrosine phosphorylation of IκBα 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 IκBα and the net effect on NFκB transcriptional activation remains an open question. Although the exact identity of the IκB 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 IκBα in T-cells following perivandate treatment (8) and bone marrow macrophages (BMMs) following TNFα stimulus (9). In addition to perivandate, H/R has also been shown to induce tyrosine phosphorylation of IκBα in T-cells in vitro (6) and following I/R injury to the liver in vivo (10).

The tyrosine kinase p56lk is required for IκBα tyrosine phosphorylation and NFκB activation in T-lymphocytes following perivandate treatment (6). Loss of tyrosine kinases p56lk and ZAP-70 in two Jurkat mutants abolished NFκB activation and
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partially suppressed and delayed phosphorylation of Tyr-42 on IxBo in response to pervanadate treatment (11). However, this study in T-cells also demonstrated that tyrosine phosphorylation of IxBo was not sufficient to activate NFkB and suggests that both tyrosine and serine kinases act at multiple levels to dissociate the IxBo/NFkB complex. Furthermore, tyrosine phosphorylation of IxBo is observed in BMMs following TNFa treatment, and this phosphorylation requires c-Src activity (9).

Given the historical dependence of TNFa-mediated activation of NFkB on the IKK complex and serine phosphorylation of IxBo, the functional involvement of IxBo tyrosine phosphorylation in response to TNFa appears to be quite unique to BMMs. Furthermore, the vast majority of studies evaluating the importance of IxBo tyrosine phosphorylation to date have been performed in hematopoietically derived T-cells or BMMs. Thus, the functional relevance of these systems to epithelial models of reperfusion remains an open question. Since c-Src can be directly activated by H2O2 (12), pervanadate (13), hypoxia (14), or hypoxia/reoxygenation (15), its central involvement in ROS-mediated IKK and PTK activation of NFkB appears reasonable. It is also recognized that H2O2 is capable of activating both IKK- and PTK-dependent pathways of IxBo phosphorylation and NFkB activation in T-cells (11).

In the present study, we sought to investigate the involvement of c-Src in the redox-mediated activation of NFkB activation following H/R or pervanadate treatments in an epithelial cell line (HeLa cells). Since both IKK-dependent and independent pathways of NFkB activation have been associated with c-Src activation, we used a number of adenoviral vectors expressing dominant negative mutants of IKKα, IKKβ, and IxBo to selectively test for serine or tyrosine IxBo phosphorylation-depend-ent transcriptional activation of NFkB. In contrast to previous studies, we have utilized an NFkB-responsive luciferase reporter gene to directly assess changes in the transcriptional activation of NFkB. Since the association of tyrosine-phosphorylated IxBo with PI 3-kinase has been suggested in proposed models to alter the transcriptional properties of NFkB 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 IxBo 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 IxBo and NFkB activation is mediated through redox activation of c-Src.

EXPERIMENTAL PROCEDURES

Adenoviral Vectors—Several E1-deleted recombinant adenoviral vec- tors were used to modulate the NFkB pathway and assay for transcriptional induction of NFkB. Previously described vectors included the dominant negative mutants Ad.IKKα(KM) (16), Ad.IKKβ(KA) (16), and Ad.IxBo(S32A/S36A) (17), and the NFkB-responsive luciferase reporter vector Ad.NFkBLuc (16). Ad.BgII was used as an empty vector control (16). Ad.IxBo(Y42F), which expresses the Y42F mutant form of IxBo, was generated by cloning the previously described IxBoY42F cDNA (6) into pAd.CMVlink (18). All adenoviral vectors, except for Ad.NFkBLuc, used the CMV enhancer/promoter to express the transgene. Recom- binant adenosine was purified by two rounds of CsCl centrifugation and desalted prior to use (described). 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 μg/ml penicillin and streptomycin. For the tyrosine phosphorylation assays, HeLa cells were transduced with Ad.IKKα(KM), Ad.IKKβ(KA), Ad.IxBo(S32A/S36A), or Ad.IxBo(Y42F), or Ad.BgII at a multiplicity of infection (MOI) equal to 1000 particles/cell.

For the NFkB luciferase reporter assay, HeLa cells were co-infected with Ad.NFkBLuc at an MOI = 500 particles/cell and Ad.IKKα(KM), Ad.IKKβ(KA), Ad.IxBo(S32A/S36A), or Ad.IxBo(Y42F) at an MOI = 1000 particles/cell. Luciferase reporter assays in SYF and SYF + c-Src cells were performed following infection with Ad.NFkBLuc 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% PBS/DMEM. Typically, experiments were initiated at 24 h post-transduction. Experimental methods used to induce NFkB were performed according to the following protocols.

Pervanadate Treatment—Sodium orthovanadate was prepared fresh in water at a concentration of 500 mM. 40 μl of sodium orthovanadate solution (60% (w/w) H2O2, 30% (w/w) H2O2, 10% (w/w) H2O2 solution) was then added to a phosphate-buffered saline. This mixture was incubated for 5 min at room temperature prior to the addition of catalase (20 μg/ml) to remove the excess H2O2. 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-pervana- date treatments for NFkB 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% N2, 5% CO2, or 95% O2, 5% CO2 was used as hypoxia and reoxygenation medium, respectively. Cells were covered with minimal hypoxia medium and incubated at 37 °C for 5 h in an airight chamber equilibrated with 5% CO2 and 95% N2. The medium was then replaced with a minimal amount of reoxygenation medium and incubated further at 37 °C in a chamber flushed with 5% CO2 and 95% O2. Cells were harvested 6 h after reoxygenation for NFkB activation luciferase assays. Control cells were fed with fresh medium at identical times as the hypoxia/reoxygenation samples, but were exposed to 5% CO2 in atmospheric oxygen.

TNFa Treatment—Mouse recombinant TNFa (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 TNFa until they were harvested at 6 h post-stimulation for luciferase and Western blot analyses. Control cells were fed at the time of treatment with fresh DMEM medium without TNFa.

Western Blotting and IxBa 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 μg of cell lysate was resolved on a 10% SDS-PAGE and then transferred to nitrocellulose membranes using previously described protocols (18). IxBa protein levels were determined by Western blot analysis using an anti-IxBa monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To evaluate IxBa tyrosine phosphorylation, 200 μg of cell lysate was immunoprecipitated using 2 μg of IxBa antibody (Santa Cruz Biotechnology) followed by Western blot analysis using a phosphotyrosine 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-SrcPY739, and anti-c-Src antibodies (Santa Cruz Biotechnology).

NFkB Activation Assays—NFkB transcriptional activity was evaluated using an Ad.NFkBLuc reporter vector as previously described (16). Briefly, cells were infected with Ad.NFkBLuc at an MOI of 500 parti-cles/cell 24 h prior to TNF-a, pervanadate, or H/R treatment. 5 μg 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 lumen and used as an internal control for the transduction efficiency of NFkB. To assess potential global changes in transcription induced by each type of environmental stimuli, that were not dependent on NFkB, several experiments were performed normalizing changes in Ad.NFkBLuc expression to that seen with a control Ad.CMV.LacZ vector (20). In these studies both Ad.NFkBLuc and Ad.CMV.LacZ were co-infected into cells for each condition (MOI = 500 part-icles/cell for each vector) 24 h prior to TNF-a, pervanadate, or H/R treatment. Luciferase activity was then assessed using 5 μg of lysate as described above, and β-galactosidase activity was quantified with 5 μg of lysate using a previously described protocol (21). Luciferase activity was then normalized for β-galactosidase expression in reference to the Ad.Bgl infected (no infection control). Electrophoretic mobility shift as-says for NFkB DNA binding were performed as previously described using a 32P-labeled NFkB oligonucleotide probe (18).

In Vitro Kinase Assays—Two types of in vitro kinase assays (radio-active and non-radioactive) were used to evaluate the activity of immu-
noprecipitated c-Src or IKKβ to phosphorylate GST-IκBα 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 μg of protein was immunoprecipitated with anti-IKKα/β antibodies (Santa Cruz Biotechnology) and protein A-agarose beads. 1 μg of GST-IκBα protein (Santa Cruz Biotechnology) was then added to washed protein A pellets in the presence of 10 μl of kinase buffer (40 mM Hepes, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM Na3VO4, 10 mM MgCl2, 2 mM dithiothreitol, 0.3 mM cold ATP, and 10 μCi of [γ-32P]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 32P) and exposed to x-ray film. Non-radioactive in vitro kinase assays were performed to directly evaluate the extent of tyrosine phosphorylation of GST-IκBα by immunoprecipitated c-Src or IKKβ. These in vitro kinase assays were performed identical to the protocol described above except for the omission of [γ-32P]ATP. In vitro labeled GST-IκBα samples were then evaluated by Western blotting for the extent of tyrosine phosphorylation using antiphosphotyrosine antibody (Santa Cruz Biotechnology).

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

To establish that NFκB activation following H/R occurs through a selective pathway involving tyrosine phosphorylation of IκBα that is independent of the IKK complex, we utilized several dominant negative mutants to modulate IKK activation and IκBα phosphorylation. NFκB transcriptional activity was evaluated using a recombinant adenoviral reporter vector (Ad.NFκBLuc) expressing the NFκB-inducible luciferase gene. As expected and previously reported in epithelial cell lines, the transcriptional induction of NFκB following TNFα treatment was significantly inhibited (p < 0.001) by expression of Ad.IKKβ(β/KA), Ad.IKKα/β(KM), or Ad.IκBα(S32A/S36A) in comparison to Ad.BgII (empty vector control)-transduced cells (Fig. 1A). No inhibition in TNFα-induced NFκB activation was seen following expression of Ad.IκBαY42F. These results confirm the functionality of our vectors to inhibit IKK-mediated TNFα activation of NFκB and demonstrate a lack of functional involvement of IκBα Y42 phosphorylation under these conditions. In contrast to findings with TNFα, IκBα(Y42F) expression significantly inhibited NFκB 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 NFκB was seen following infection with Ad.IKKβ(β/KA), Ad.IKKα/β(KM), or Ad.IκBα(S32A/S36A) mutant vectors. Furthermore, when the induction of NFκB-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 NFκB 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 NFκB transcriptional activation is accompanied by increased DNA binding in nuclear extracts. Cumulatively, our results evaluating IKK and IκBα mutants suggest that IKK-mediated serine 32/36 phosphorylation of IκBα does not play a significant role in regulating NFκB following pervanadate or H/R stimuli in our HeLa cell line model. To directly evaluate whether TNFα imparts selective activation of the IKK complex not observed following H/R or pervanadate treatments, we performed in vitro kinase assays with immunoprecipitated IKKβ to directly evaluate IKK activation and ability to phosphorylate GST-IκBα following each of these stimuli. Results from this analysis are shown in Fig. 1E and demonstrate that TNFα 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 NFκB transcriptional activation by IKK mutants seen following H/R and pervanadate treatments.

Src Inhibitor pp2 Blocks NFκB Activation and IκBα Tyrosine Phosphorylation Following Pervanadate or H/R Treatment—Our results in the HeLa cell model have established that NFκB activation following H/R or pervanadate treatment is independent of IKK and serine phosphorylation of IκBα. We next sought to evaluate candidate upstream factors capable of mediating tyrosine phosphorylation of IκBα and subsequent NFκB 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 TNFα-induced tyrosine phosphorylation of IκBα in BMMs (9). Given the lack of a functional requirement for IκBα tyrosine phosphorylation in the transcriptional induction of NFκB following TNFα in our epithelial cell line model, we investigated whether c-Src might also play a role in NFκB 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 IκBα and subsequent activation of NFκB, 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 NFκB activation (p <
Furthermore, the level of tyrosine phosphorylation of IκBα/IκBαβ following pervanadate or H/R treatment was also significantly reduced in the presence of pp2. These results are consistent with the hypothesis that c-Src is functionally required for both IκBα/IκBαβ tyrosine phosphorylation and subsequent activation of NFκB.

Tyrosine Phosphorylation of IκBα and NFκB Activation Is Significantly Reduced in a c-Src, Fyn, and Yes Triple Knockout Cell Line Following H/R or Pervanadate Treatment—The importance of c-Src in mediating tyrosine phosphorylation of IκBα and NFκB activation was further investigated using a knockout cell line deficient for Src family kinases Src, Fyn, and Yes. These kinases have similar redundant functions and thus, the knockout of a single gene will not completely abolish their activity. In SYF cells, Src, Yes, and Fyn have been all knocked out. The im-

FIG. 1. NFκB activation following pervanadate or H/R treatment is inhibited by IκBα/IκBαβ(Y42F) but not IκBα/IκBαβ(S32A/S36A) or IKK-dominant mutants. HeLa cells were co-infected with Ad.IκBα/IκBαβ(Y42F), Ad.IκBα (S32A/S36A), Ad.IKKα(RA), or Ad.BglII (MOI of 1000 particles/cell) together with Ad.NFκBLuc (MOI of 500 particles/cell) 24 h before experimental analysis. Cells were treated with TNF-α (10 ng/ml) (A), pervanadate (100 μM) (B), or H/R (5 h of hypoxia, 6 h of reoxygenation) (C). Whole cell extracts were harvested 6 h following treatments, normalized for total protein content, and evaluated for NFκB transcriptional activity using a luciferase assay. NFκB transcriptional activity was determined by the mean relative luciferase activity (RLA) (± S.E., n = 6). D, electrophoretic mobility shift assays for NFκB DNA binding in nuclear extracts harvested from HeLa cells following TNF-α (10 ng/ml, 1 h), pervanadate (50 μM, 1 h), or H/R (5 h of hypoxia, 3 h of reoxygenation) treatments. Exposure times are not equivalent for each treatment condition. E, HeLa cells were treated with TNF-α (10 ng/ml) for 30 min, pervanadate (100 μM) for 30 min, or H/R (5 h of hypoxia, 30 min of reoxygenation). IKKβ was immunoprecipitated with anti-IKKβ antibody from 500 μg of protein lysate. The ability of immunoprecipitated IKKβ to directly phosphorylate GST-IκBα fusion protein was evaluated in the presence of [γ32P]-ATP in vitro.
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 NFκB transcriptional activation in comparison to SYF+src cells (p < 0.001) (Fig. 4A). In contrast, there was no significant difference in TNFα-mediated induction of NFκB in either of these two cell lines. These results suggest that c-Src activity is required for NFκB pathways involving tyrosine, but not serine-mediated phosphorylation of IκBα. To conclusively address the requirement for c-Src activity to mediate tyrosine phosphorylation of IκBα, we next evaluated the extent of IκBα tyrosine phosphorylation in both SYF and SYF+src cells following pervanadate or H/R treatment. These studies demonstrated that IκBα tyrosine phosphorylation was significantly reduced in SYF following pervanadate and completely blocked following H/R as compared with SYF+src cells (Fig. 4, B and C). Given the previous demonstration of p56

Finally, 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 NFκB 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 IκBα tyrosine phosphorylation and NFκB activation can be completely blocked in c-Src, Fyn, and Yes knockout cells and fully restored

Fig. 3. Inhibition of c-Src activation blocks NFκB transcriptional activation and IκBα tyrosine phosphorylation following pervanadate or H/R treatment. A. HeLa cells were transduced with Ad.NFκB.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 μM) for 30 min, followed by treatment with pervanadate (100 μM) for 6 h or H/R (hypoxic media; 5% N2, 5% CO2) for 5 h followed by reoxygenation media (95% O2, 5% CO2) 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 NFκB activation using a luciferase assay. Results depict the mean (± S.E., n = 6) relative luciferase activity (RLA). HeLa cells were treated with pervanadate (100 μM) (B) for 30 min or hypoxic media (95% N2, 5% CO2) (C) for 5 h followed by reoxygenation media (95% O2, 5% CO2) for 30 min. One group was pretreated with pp2 (10 μM) for 30 min prior to pervanadate or H/R. Inhibitor (pp2) was continually present during the treatments. Cell lysates were harvested and 200 μg of total protein was immunoprecipitated with anti-IκBα antibody followed by Western blotting with an antiphosphotyrosine antibody or anti-IκBα antibody.

Fig. 4. Tyrosine phosphorylation of IκBα and transcriptional activation of NFκB are significantly reduced in c-Src knockout cell lines. A. SYF cells or SYF+src cells were transduced with Ad.NFκB.Luc (MOI of 500 particles/cell) 24 h prior to initiated experiments. Cells were treated with pervanadate (50 μM) 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. NFκB transcriptional activation was evaluated as the relative luciferase activity. Results depict the mean (± S.E., n = 6) relative luciferase activity (RLA). SYF or SYF+src cells were treated with pervanadate (50 μM) (B) for 15, 30, and 45 min or hypoxic media (95% N2, 5% CO2) (C) for 5 h followed by reoxygenation media (95% O2, 5% CO2) for 15, 30, and 45 min. Both untreated and treated cell lysates were harvested at the indicated time points, 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.
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Fig. 5. c-Src phosphorylates IkBa in vitro. A, SYF cells or SYF-c-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 [γ-32P]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 IkBa 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 IkBa. 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 phosphate IκBα (Fig. 5B). Furthermore, when similar assays were performed using immunoprecipitated IKKβ, no increase in tyrosine phosphorylation of IkBa 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 IkBa. 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 IkBa.

Overexpression of Gpx-1 or Catalase, but Not Mn-SOD or Cu,Zn-SOD, Inhibits Tyrosine Phosphorylation of IkBa 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 IKKα (16) and IKKβ (24) subunits of the IKK complex following environmental stimuli. Moreover, H2O2 has been shown to activate tyrosine phosphorylation of IkBa in T-cells in a manner similar to pervanadate (25). The observed dependence of IkBa 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 IkBa 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 H2O2 and Ad.Mn-SOD or Ad.Cu,Zn-SOD vectors that dismutate superoxide anion (O2·−) into H2O2. Using our in vitro model, we analyzed the role of these antioxidant enzymes in regulating IkBa 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 IkBa 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 IkBα tyrosine phosphorylation or NFκB activation. These findings suggest that H2O2 is an important redox component in NFκB activation mediated through IkBα 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 IkBa, 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.BgII-infected control (Fig. 7). Furthermore, in this assay, transient treatment with 1 mM H2O2 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 IkBa tyrosine phosphorylation. Overexpression of H2O2 scavengers is able to efficiently reduce c-Src kinase activity, IkBa tyrosine phosphorylation, and NFκB activation following H/R or pervanadate injury.

DISCUSSION

The physiologic significance of IkBa tyrosine phosphorylation in mediating NFκB transcriptional activation has remained one of the poorly understood aspects of this well-stud-
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Fig. 6. Overexpression of Gpx-1 or catalase, but not Mn-SOD or Cu,Zn-SOD, inhibits NFκB activation and tyrosine phosphorylation of IκB following pervanadate or H/R treatment. HeLa cells were co-infected with Ad.BglII, Ad.Gpx-1, Ad.Catalase, Ad.Cu,Zn-SOD, or Ad.MnSOD (MOI of 500 particles/cell) for 24 h prior to initiating experimental treatments described below. Cells were treated with pervanadate (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) (± S.E., n = 6). For evaluation of IκB 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-IκB antibody followed by Western blot analysis with an antiphosphotyrosine antibody or anti-IκB antibody.

Fig. 7. Overexpression of Gpx-1 reduces c-Src kinase activity following pervanadate treatment. HeLa cells were infected with Ad.BglII 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-IκB fusion protein was then evaluated in the presence of [γ-32P]ATP in vitro.
following peroxanadate or H/R treatments, while confirming the selectivity of NFkB by TNFα as mediated through serine phosphorylation of IκBα. These functional studies evaluating the transcriptional activation of NFkB 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, peroxanadate 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 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 treatment (12, 25), and stimuli such as angiotensin II and c-Src have been shown to associate with one another (30). 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 treatment (12, 25), and stimuli such as angiotensin II and c-Src have been shown to associate with one another (30). 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 treatment (12, 25), and stimuli such as angiotensin II and c-Src have been shown to associate with one another (30).

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