HIV-1 Tat Activates Dual Nox Pathways Leading to Independent Activation of ERK and JNK MAP Kinases*

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Human immunodeficiency virus, type 1 Tat is known to exert pleiotropic effects on the vascular endothelium through mitogen-activated protein (MAP) kinases, although the signaling pathways leading to MAP kinase activation are incompletely understood. We focused on proximal pathways potentially governing downstream MAP kinase activity by Tat. Within 2 min, Tat activated both Ras and Rho GTPases in endothelial cells, leading to ERK phosphorylation by 10 min. Notably, Rac1 was necessary for downstream activation of RhoA and both Rac1 and RhoA acted upstream of the Ras/ERK cassette. Antioxidants and the oxidase inhibitor diphenylene iodonium blocked ERK phosphorylation, but specific interference with the canonical Nox2 oxidase had no effect on ERK. Instead, knock down of the novel oxidase Nox4 completely suppressed Tat-dependent Ras and ERK activation downstream of Rac1 and RhoA. Conversely, interference with Rac1, PAK1, and Nox2 blocked JNK phosphorylation, whereas RhoA(N19) and Nox4 knock down did not. Further, knock down of Nox2, but not Nox4, blocked Tat-induced cytoskeletal rearrangement, whereas knock down of Nox4, but not Nox2, blocked Tat-dependent proliferation. Rac1, therefore, bifurcates Tat signaling, leading to concurrent but separate Nox4-dependent Ras/ERK activation, and Nox2-dependent JNK activation. Tat signaling, therefore, provides an example of Nox-specific differential control of MAP kinase pathways.

Human immunodeficiency virus, type 1 (HIV-1)2-infected individuals exhibit a variety of vasculopathic conditions manifested in both pre- and post-anti-retroviral eras (1, 2). Both large and small vessels display marked endothelial hyperproliferation, death, and monolayer disorganization, leading to aneurysms, occlusions, and telangiectasias (3–7). Strikingly, HIV-1-infected patients also develop an unusually aggressive form of the vascular neoplasm Kaposi sarcoma, suggesting a prominent contribution of HIV-1 in determining the behavior of Kaposi sarcoma cells.

Because HIV-1 is not a vasculotropic virus, the endothelium is thought instead to be the target of either host- or virus-derived soluble mediators. An important example of the latter is the viral transactivator Tat that is released into the circulation where it acts as an endothelial agonist. Of the five structural domains, the most important in this capacity are the cysteine-rich, basic, and C-terminal domains, the last of which contains an integrin binding RGD motif (8, 9). Tat exerts both proliferative and apoptotic effects on endothelial cells (10, 11), increases invasion in vitro and angiogenesis in vivo (10, 12), and facilitates Kaposi sarcoma development through direct effects on Kaposi sarcoma cells (13). In large part, such protein effects are mediated through activation of the mitogen-activated protein (MAP) kinases that are known to control basic cell fate decisions. In particular, Tat activates both ERK and JNK MAP kinases, although the upstream activators have been less well studied (14, 15). Notably, Tat activates an NADPH oxidase upstream of JNK in endothelial cells, suggesting activation of a pathway common to mitogens and cytokines (15, 16). This oxidant-related pathway is consistent with evidence of oxidant production in vivo by the vasculature in both murine and human AIDS (17).

Like other angiogenic factors, Tat also stimulates cytoskeletal rearrangements leading to junctional separation and increased motility (12, 18). Previously, we found that Tat caused dramatic actin rearrangements through activation of the Ste20 kinase p21/cdc42/Rac1-activated kinase1 (PAK1) and downstream phosphorylation of p47(phox), the adapter for the Nox2 oxidase (9). The principal activators of PAK1, Rac1 and Cdc42, are both members of the Rho GTPase family that coordinate cell shape changes with proliferative or cytostatic effects in response to a variety of stimuli. Recently, immobilized Tat was found to activate Rac1 and RhoA, confirming the ability of Tat to activate these proximal molecular switches (12, 19). We, therefore, sought to map in greater detail the activation by soluble Tat of the Rho and Ras family GTPases upstream of the MAP kinases and to further define the participation of the NADPH oxidase in this process. We found that Tat initiates a novel cascade of GTPase activation bifurcating at Rac1, leading to PAK1- and Nox2-dependent JNK activation down one pathway and RhoA-, Nox4-, and K-Ras-dependent activation of ERK down a separate path.

EXPERIMENTAL PROCEDURES

Tat Purification and Stimulation—pGEX-Tat-86 from HIV-1 was obtained from the AIDS Research and Reference

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The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; MAP, mitogen-activated protein; PAK1, p21/cdc42/Rac1-activated kinase1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cells; siRNA, small interfering RNA.
Reagent Program. Glutathione S-transferase (GST)-Tat was produced in BL21-RP *Escherichia coli* (Stratagene) and purified on GST-Sepharose (GE Healthcare) as described previously (15). The GST fusion has been found to have bioactivity equivalent to that of the purified Tat peptide (9). GST fusions of Tat(C22A, C25A, and C27A) and Tat(R49A, R52A, R53A, R55A, R56A, and R57A) were produced as described previously (9). pGEX-Tat(D80E) was created by PCR mutagenesis and GST-Tat(D80E) was produced as above. Parallel preparations of purified GST were used in isomolar equivalents as the control agonist. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and grown to passage 4–5 in endothelial cell growth medium and then serum-starved in 0.1% serum for 16 h prior to stimulation with GST-Tat (1 μg/ml, equivalent to 260 ng/ml Tat). For Tat binding studies, adenovirally transduced HUVEC were treated with GST-Tat at either 37 or 4°C for 10 min and then washed extensively with cold phosphate-buffered saline, lysed, and subjected to immunoblot with anti-Tat (AIDS Research and Reference Reagent Program).

**GTPase Activity**—Rho and Ras activation were assessed using a pulldown technique. The Rho-binding domain and Cdc42/Rac-interactive binding domains of rhotekin and PAK1 were subcloned into pGEX-2TK as described (20). The Ras-binding domain of Raf was a gift from Rhonda Souza. Fusion proteins were expressed in BL21-RP *E. coli* (Stratagene) and purified on GST-Sepharose (GE Healthcare). Following stimulation with GST-Tat, HUVEC were lysed and the 10,000 × g supernatants were pulled down with the respective fusions and immunoblotted for Cdc42 (Santa Cruz Biotechnology), Rac1 (BD Biosciences), RhoA (Santa Cruz), or pan-Ras (BD Biosciences). Aliquots of lysates were also precipitated in ice-cold acetone (1:1) prior to pull down for assessment of total GTPase by immunoblot. Blots were quantified with densitometry, and the ratios of active to total GTPase were assessed and analyzed using analysis of variance with the Tukey multiple comparison test.

**Immunoblot**—HUVEC were washed twice in phosphate-buffered saline and extracted in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice, sonicated for 5 s, and centrifuged at 10,000 × g for 20 min at 4°C. Immunoblots were performed for phospho-ERK or phospho-JNK (Cell Signaling), and then blots were stripped and reprobed for total ERK (Cell Signaling) or total JNK (Santa Cruz Biotechnology). To assess knockdown, whole cell lysates were immunoblotted for K-Ras, Nox4 (Santa Cruz Biotechnology), or Nox2 (BD Biosciences), and then stripped and reprobed for actin (Chemicon). Blots were quantified with densitometry, and the ratios of phosphorylated to total kinase were assessed and analyzed using analysis of variance with the Tukey multiple comparison test.

**Transduction and Knockdown**—HUVEC were grown to 50–70% confluence prior to adenoviral transduction. After infection for 2 h, HUVEC were washed and allowed to recover for 24 h prior to stimulation with GST-Tat. Multiplicity of infection for each virus was titrated to protein expression level.

**Functional End Points**—Proliferation was assessed by cell count and by bromodeoxyuridine incorporation using a sandwich enzyme-linked immunosorbent assay (Roche Applied Science). HUVEC were serum-starved overnight, stimulated for 30 min with GST-Tat or GST alone, washed, and incubated in 0.1% serum-containing medium for 24 h prior to lysis and bromodeoxyuridine measurement. Cell death was assessed by measurement of DNA fragmentation using an enzyme-linked immunosorbent assay kit (Roche Applied Science). HUVEC in 24-well plates were serum-starved overnight prior to a 30-min incubation with either GST-Tat or GST. 24 h later, wells were harvested for either cell counts or cell death enzyme-linked.

**FIGURE 1.** GST-Tat activates Rho and Ras GTPases. A, HUVEC were stimulated with either GST-Tat (1 μg/ml) or GST (1 μg/ml) for the indicated times. Immunoblots for active and total GTPases are shown. Bottom panels, whole cell lysates were immunoblotted for active (phosphorylated) or total ERK. *Bar graph* shows the mean ± S.E. of three replicates. *, *p < 0.05 compared with time zero.

Construction of Ad-lacZ, Ad-PAK1(K298A), Ad-p47(W193R), and Ad-p67(V204A) was described previously (9, 21). Ad-Rac1(N17) and Ad-RhoA(N19) were gifts from Kaikobad Irani and Christopher Chen. Ad-Ras(N17) was obtained from Vector Biolabs. Small interfering RNA (siRNA) corresponding to nucleotides 1291–1309 of human Nox2 and 409–427 (coding region numbering) of human Nox4 with UU tails were synthesized by Dharamcon and transfected using TransIT-TKO (Mirus). Experiments were performed and knockdown assessed 48 h after siRNA transfection.
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FIGURE 2. GST-Tat activates Rac1 and RhoA upstream of Ras. A, HUVEC were transduced with either lacZ or Ras(N17) through adenoviral infection. 24 h later, cells were stimulated with either GST-Tat or GST alone for 10 min and then immunoblotted for phospho- and total ERK. A–H, bar graphs show the mean ± S.E. of three to five replicates. *, p < 0.05 compared with GST-treated control; †, p < 0.05 compared with GST-Tat-treated control. Side panels show levels of transgene expression; blot was reprobed for actin in bottom panel. B, HUVEC were transfected with siRNA against K-Ras or luciferase (control); 48 h later, cells were stimulated with either GST-Tat or GST for 10 min and then immunoblotted for phosphorylated or total ERK. Side panel shows immunoblot for K-Ras.

C, HUVEC were transduced with lacZ, RhoA(N19), or Rac1(N17). 24 h later, cells were stimulated with either GST-Tat or GST alone for 10 min and then immunoblotted for phosphorylated and total ERK. Bottom panels, immunoblots for RhoA and Rac1, showing level of transgene expression.

D, HUVEC were transduced as in panel C and then stimulated with either GST or GST-Tat for 2 min. Ras activation was completely suppressed by Rac1(N17) or RhoA(N19).

E, HUVEC were transduced with lacZ or Rac1(N17). 24 h later, cells were stimulated with GST or GST-Tat for 2 min and then harvested for active RhoA pull down.

F, HUVEC were transduced with lacZ or RhoA(N19) for 24 h and then stimulated with GST or GST-Tat for 2 min before assessing Rac1 activity. G, HUVEC were transduced with Rac1(N17) or RhoA(N19) as indicated. 24 h later, cells were treated with GST-Tat for 10 min and then extensively washed and immunoblotted with anti-Tat. Cells in the last lane were chilled to 4 °C for 15 min prior to addition of GST-Tat. Blot is representative of two independent experiments.

H, HUVEC were treated with GST or GST-Tat (wild type), GST-Tat(C22A, C25A, and C27A), GST-Tat(R49A, R52A, 53A, 55A, 56A, and 57A), or GST-Tat(D80E) for 10 min, and ERK phosphorylation was assessed. Bottom panel, cells were stimulated for 2 min, and then Rac1-GTP was assessed as above.
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Tat Activates a Rac1-RhoA-Ras Pathway Upstream of ERK Phosphorylation—When stimulated with extracellular Tat, HUVEC respond within minutes with marked cytoskeletal rearrangements (9). Accordingly, within 2 min of exposure to GST-Tat, we found that Cdc42, Rac1, and RhoA were all activated when compared with GST alone (Fig. 1A). Activation was transient, reaching basal levels by 10 min. In parallel, activation of Ras was also seen within 2 min of GST-Tat. ERK phosphorylation occurred by 10 min and was sustained for at least 30 min, returning to basal levels by 60 min (Fig. 1B).

We next examined the role of Ras and Rho in Tat-dependent ERK activation. Ras (N17) completely blocked ERK phosphorylation by GST-Tat, as did knock down of K-Ras (Fig. 2, A and B), consistent with the known Ras/Raf/MEK/ERK cassette. In addition, RhoA (N19) and Rac1 (N17) each abolished both ERK phosphorylation and Ras activation (Fig. 2, C and D), placing RhoA and Rac1 upstream of Ras. Further, Rac1 (N17) blocked RhoA activation by GST-Tat, whereas RhoA (N19) had no effect on Rac1 (Fig. 2, E and F). Thus, Rac1 activation is necessary for RhoA activity, which in turn acts upstream of Ras-dependent ERK phosphorylation. However, because both Rac1 and RhoA control actin dynamics, their respective dominant negatives may conceivably interfere with receptor engagement and endocytosis rather than downstream signaling. Thus, we also assessed GST-Tat association with cells in the presence of these mutants and found that neither Rac1 (N17) nor RhoA (N19) interfered with GST-Tat association with HUVEC (Fig. 2G). In contrast, incubation of cells with GST-Tat at 4 °C, which would be expected to block internalization of Tat but not nonspecific binding, significantly reduced GST-Tat association with HUVEC.

Because HIV-1 Tat is a multidomain agonist, we next sought to determine whether a specific domain is sufficient for ERK activation. Alanine substitutions within the cysteine-rich region (Cys-22, -25, and -27), thought to be important in Tat dimerization, completely blocked ERK phosphorylation (Fig. 2H). However, alanine substitutions within the basic domain (Arg-53, -55, -56, and -57) and disruption of the C-terminal RGD motif (D80E) also completely blocked ERK activation. Consistent with the role of Rac1 as a proximal mediator of Tat-induced ERK activation, all three mutants failed to activate Rac1 (Fig. 2H). Thus, these three Tat domains, each of which has been implicated in endothelial cell activation, all appear necessary for ERK activation.

RESULTS

Tat Activates a Rac1-RhoA-Ras Pathway Upstream of ERK Phosphorylation—When stimulated with extracellular Tat, HUVEC were pretreated with Mn-TMPyP (100 μM, 60 min) or PD098059 (20 μM, 30 min) before stimulation with GST or GST-Tat for 10 min prior to immunoblot for phosphorylated or total ERK. A–D, bar graphs show the mean ± S.E. of three to five replicates, *, p < 0.05 compared with GST-treated control; †, p < 0.05 compared with GST-Tat-treated control. B, cells were pretreated with diphenylene iodonium ([DPI], 10 μM, 30 min), stimulated with GST or GST-Tat for 10 min, and then immunoblotted for phosphorylated or total ERK. C, HUVEC were adeno virally transduced with lacZ, p67(VA), PAK1(KA), or p47(WR) for 24 h, stimulated with GST or GST-Tat for 10 min, and then immunoblotted for phosphorylated or total ERK. Side panels, immunoblots for p67(phox), PAK1, and p47(phox), showing transgene expression. D, HUVEC were transfected with siRNA against Nox2 or luciferase (control). 48 h later, cells were stimulated with either GST or GST-Tat for 10 min and immunoblotted for phosphorylated or total ERK. Immunoblot for Nox2 is shown in side panel.
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Tat Activates ERK through Nox4—Tat activates the JNK MAP kinase through an NADPH oxidase (9, 15, 16), so we questioned whether the same pathway leads to Ras and ERK activation. Both the antioxidant Mn-TMPyP and the oxidase inhibitor diphenylene iodonium blocked GST-Tat-induced ERK phosphorylation (Fig. 3, A and B), consistent with a role for an NADPH oxidase upstream of ERK activation. As expected, the MEK1 inhibitor PD098059 suppressed both basal and Tat-dependent ERK phosphorylation. However, antagonists of Nox2 failed to significantly affect ERK phosphorylation (Fig. 3C). These antagonists included p67(V204A), containing a mutation in the activation motif of p67phox, the kinase dead mutant PAK1(K298A), and p47(W193R), a mutant Nox2 adapter defective in p22phox binding. Knock down of endogenous Nox2 itself also had no effect on Tat-dependent ERK phosphorylation, confirming the lack of participation of the Nox2 complex in ERK signaling (Fig. 3D).

The principal NADPH oxidases of endothelial cells are Nox2 and Nox4, with HUVEC expressing no detectable Nox1 (22). We therefore examined Nox4, whose activation appears to be upstream of JNK activation (9). Indeed, knock down of Nox2 blocked JNK phosphorylation following GST-Tat stimulation (Fig. 5B), while having no effect on ERK (Fig. 3D). In addition, Rac1(N17) and PAK1(K298A) suppressed JNK phosphorylation, consistent with a key role for Nox2 (Fig. 5, C and D). Conversely, Nox4 knock down had no effect on JNK phosphorylation despite its effect on ERK (Figs. 5E and 4A). Thus, Nox2 and Nox4 independently mediate activation of JNK and ERK, respectively. Further, RhoA(N19) did not affect GST-Tat-dependent JNK phosphorylation (Fig. 5F), suggesting that RhoA commits Tat signaling down a Nox4/Ras pathway to ERK. Finally, each of the three Tat domain mutants failed to cause JNK phosphorylation (Fig. 5G), consistent with JNK activation downstream of Rac1.

Nox4 Mediates Tat-dependent Proliferation, whereas Nox2 Mediates Cytoskeletal Rearrangements—Stimulation of HUVEC with GST-Tat increased cell number after 24 h by 32% (16,700 ± 420 versus 22,000 ± 1800 cells/well, p = 0.02). We further found that cell increases were due to both an increase in proliferation, as assessed by bromodeoxyuridine incorporation, and an even more prominent decrease in cell death, as assessed by DNA fragmentation (Fig. 6A). Although knock down of Nox2 had no effect on either proliferation or cell death, Nox4 knock down completely suppressed GST-Tat-induced proliferation and cell death effects without changing basal values. Pathway specificity was further suggested by suppression of the cell death protection by PD098059, but not by the dominant negative JNK1-APF mutant (Fig. 6, B and C).

Actin rearrangements consistent with endothelial cell activation are also stimulated by Tat and include membrane ruffling and stress fiber dissolution (9). We previously found that Tat-dependent ruffling was suppressed by Nox2 antagonists; indeed, knock down of Nox2 caused dramatic quantitative and qualitative reductions in membrane ruffling (Fig. 6D). In contrast, Nox4 knock down had no effect on GST-Tat-dependent ruffling, again consistent with independent control of distinct pathways by discrete Nox proteins. Interestingly, JNK1-APF, when cotransfected with actin-green fluorescent protein, had no effect on GST-Tat-induced ruffling, suggesting that the cytoskeletal rearrangements occur downstream of Rac1 and Nox2, but independent of JNK (Fig. 7).

DISCUSSION

In this study, we mapped activation pathways of the Ras and Rho GTPases by HIV-1 Tat and found evidence for independent activation of dual Nox paralogs leading to differential ERK and JNK MAP kinase activation. Several novel features of these pathways were observed. First, Ras is typically activated early in
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A. HUVEC were stimulated with GST-Tat for the indicated times. B, HUVEC were transfected with siRNA against Nox2 or luciferase. 48 h later, cells were stimulated with GST or GST-Tat for 10 min and immunoblotted for phosphorylated or total JNK. Bar graphs show the mean ± S.E. of three to five replicates. *, p < 0.05 compared with GST-Tat-treated control; †, p < 0.05 compared with GST-Tat-treated control. C and D, cells were transduced with lacZ, Rac1(N17), or PAK1(K298A) for 24 h, stimulated with GST or GST-Tat for 10 min, and then immunoblotted for phospho- or total JNK. E, HUVEC were transfected with siRNA against Nox4 or luciferase. 48 h later, cells were stimulated with GST or GST-Tat for 10 min and immunoblotted for phosphorylated or total JNK. F, HUVEC were transduced with lacZ or RhoA(N19) for 24 h, stimulated with GST or GST-Tat, and immunoblotted for phospho- or total JNK. G, HUVEC were treated with GST or GST-Tat (wild type), GST-Tat(C22A, C25A, and C27A), GST-Tat(R49A, R52A, R53A, R55A, R56A, and R57A), or GST-Tat(D80E) for 10 min, and JNK phosphorylation was assessed.

FIGURE 5. GST-Tat activates JNK through Nox2. A, HUVEC were stimulated with GST-Tat for the indicated times. B, HUVEC were transfected with siRNA against Nox2 or luciferase. 48 h later, cells were stimulated with GST or GST-Tat for 10 min and immunoblotted for phosphorylated or total JNK. Bar graphs show the mean ± S.E. of three to five replicates. *, p < 0.05 compared with GST-Tat-treated control; †, p < 0.05 compared with GST-Tat-treated control. C and D, cells were transduced with lacZ, Rac1(N17), or PAK1(K298A) for 24 h, stimulated with GST or GST-Tat for 10 min, and then immunoblotted for phospho- or total JNK. E, HUVEC were transfected with siRNA against Nox4 or luciferase. 48 h later, cells were stimulated with GST or GST-Tat for 10 min and immunoblotted for phosphorylated or total JNK. F, HUVEC were transduced with lacZ or RhoA(N19) for 24 h, stimulated with GST or GST-Tat, and immunoblotted for phospho- or total JNK. G, HUVEC were treated with GST or GST-Tat (wild type), GST-Tat(C22A, C25A, and C27A), GST-Tat(R49A, R52A, R53A, R55A, R56A, and R57A), or GST-Tat(D80E) for 10 min, and JNK phosphorylation was assessed.

cascades initiated by mitogens or integrins, leading to downstream Rac1 and RhoA activation (23, 24). In contrast, we found that activation of Rac by Tat appears to invert this sequence by requiring involvement of both Rac1 and RhoA upstream of Ras. The mechanism of Ras activation is unclear, although conditionally active mutants of the RhoA effector ROCK-II have recently been shown to activate Ras (25).

Second, Ras activation by Tat requires the novel NADPH oxidase Nox4. Although little is known about Nox4-dependent signaling, this oxidase appears to be necessary for differentiation of certain cells such as cardiac myocytes and myofibroblasts (26, 27). Interestingly, overexpression of Nox4 causes senescence in 3T3 cells (28). Ras is well known to induce senescence in nontransformed cells; thus, our data suggest a possible mechanism for Nox4-dependent senescence. A further link with this pathway is the functional association of Nox4 with insulin receptor signaling, broadly associated with cell and organismal senescence and Ras activation throughout metazoa. Nox4 is activated by insulin, leading to inactivation of the protein tyrosine phosphatase 1B and thus enhancement of insulin receptor pathways (29). Similar to Ras, however, Nox4 signaling may be highly context-dependent, as Nox4 expression is common in tumor cell lines (30).

Third, our data suggest regulation of Nox4 activity at some level by the Rho GTPases Rac1 and RhoA. Nox4 has been shown to possess constitutive activity independent of Rac1 and the known adapters and organizers p47phox, p67phox, NOXO1, and NOXA1 (31). However, agonists such as arachidonic acid, angiotensin II, and insulin cause an abrupt increase in oxidant production within 5 min, which is suppressed by either Nox4 knock down or truncated mutants of Nox4, indicating rapid post-translational regulation of Nox4 activity (29, 32). In the case of arachidonic acid and angiotensin II, Rac1(N17) blocks Nox4-dependent oxidant production, consistent with control of Nox4 activity by Rac1 (32). It seems unlikely that this represents a direct activation, as Rac1 binds p67phox, unnecessary for Nox4 activity; however, activation mechanisms of Nox4 at this point remain unclear.

Lastly, despite the common production of superoxide and hydrogen peroxide by both Nox2 and Nox4, our data suggest that the two oxidases are capable of independently controlling activation of JNK and ERK by Tat. Nox2 has clearly been linked with JNK activation by Tat (9, 15, 16), tumor necrosis factor (16, 33), and atrial natriuretic peptide (22); conversely, involvement of Nox2 in ERK activation is much less well documented. Assignment of different Nox proteins to different signaling pathways may in part explain the presence of multiple Nox genes in virtually all plants, animals, and primitive multicellular species that have been studied (34). The present study suggests that Nox specificity cannot be simply explained on the basis of
FIGURE 6. **Nox4 and Nox2 mediate independent cellular events.** A, HUVEC were transfected with siRNA against luciferase, Nox2, or Nox4; 48 h later, serum-starved cells were stimulated with either GST or GST-Tat for 30 min. 24 h later, cells were assessed for bromodeoxyuridine incorporation (upper panel) or DNA fragmentation (lower panel). A–F, bar graphs show the mean ± S.E. of three to five replicates. *, p < 0.05 compared with GST-treated control; †, p < 0.05 compared with GST-Tat-treated control. B, HUVEC were treated with either vehicle (Me2SO) or PD098059 (20 μM) 30 min prior to and during a 30-min treatment with GST or GST-Tat. DNA fragmentation was assessed 24 h later. C, HUVEC were transfected with either empty vector or JNK1-APF and 24 h later stimulated with GST or GST-Tat for 30 min. DNA fragmentation was assessed 24 h later. D, HUVEC were transfected with siRNA against luciferase, Nox2, or Nox4 and subsequently transfected with actin-green fluorescent protein. 24 h later, cells were stimulated with GST or GST-Tat and membrane ruffling scored. Ruffles appear as dense linear actin structures on multiple peripheral edges. Knock down of Nox2, but not Nox4, diminished ruffling. E, HUVEC were cotransfected with actin-green fluorescent protein and JNK1-APF; 24 h later, cells were stimulated with GST or GST-Tat and membrane ruffles scored. Dominant negative JNK had no effect on GST-Tat-induced ruffling.
differential expression in various cells or tissues or of selective response to different agonists. The preservation of MAP kinase specificity despite simultaneous activation of dual Nox proteins suggests signaling specificity beyond that allowed by the actual oxidants produced. The basis for such Nox-dependent specificity is presently unknown.

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**FIGURE 7. Differential activation of MAP kinases by distinct Nox pathways.** Schematic depicting proposed pathways downstream of Tat. Nox2 acts upstream of JNK, but not ERK, whereas Nox4 acts upstream of ERK, but not JNK.