Involvement of TRAF4 in Oxidative Activation of c-Jun N-terminal Kinase*

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We previously found that the angiogenic factors TNFα and HIV-1 Tat activate an NAD(P)H oxidase in endothelial cells, which operates upstream of c-Jun N-terminal kinase (JNK), a MAPK involved in the determination of cell fate. To further understand oxidant-related signaling pathways, we screened lung and endothelial cell libraries for interaction partners of p47phox and recovered the orphan adapter TNF receptor-associated factor 4 (TRAF4). Domain analysis suggested a tail-to-tail interaction between the C terminus of p47phox and the conserved TRAF domain of TRAF4. In addition, TRAF4, like p47phox, was recovered largely in the cytoskeleton/membrane fraction. Coexpression of p47phox and TRAF4 increased oxidant production and JNK activation, whereas each alone had minimal effect. In addition, a fusion between p47phox and the TRAF4 C terminus constitutively activated JNK, and this activation was decreased by the antioxidant N-acetyl cysteine. In contrast, overexpression of the p47phox binding domain of TRAF4 blocked endothelial cell JNK activation by TNFα and HIV-1 Tat, suggesting an uncoupling of p47phox from upstream signaling events. A secondary screen of endothelial cell proteins for TRAF4-interacting partners yielded a number of proteins known to control cell fate. We conclude that endothelial cell agonists such as TNFα and HIV-1 Tat initiate signals that enter basic signaling cassettes at the level of TRAF4 and an NAD(P)H oxidase. We speculate that endothelial cells may target endogenous oxidant production to specific sites critical to cytokine signaling as a mechanism for increasing signal specificity and decreasing toxicity of these reactive species.

The vascular endothelium is generally well supplied with oxygen and produces significant quantities of oxidants when stimulated in vivo or in vitro (1, 2). As in other cell types, such tightly regulated oxidant bursts appear to transduce a variety of signals. Mechanical forces, growth factors, and cytokines stimulate oxidant production by endothelial cells, leading to migration, proliferation, apoptosis, or adhesion protein expression (3–5). However, the relatively broad biochemical reactivity of these oxidants poses a potential problem for signal specificity. As an example, a number of studies now support the participation of oxidants in both proliferative (6–8) and apoptotic (9, 10) pathways, depending on stimulus and context. The basis for the divergent responses to oxidants is not clear.

Endothelial cells possess an NAD(P)H oxidase (11) thought to participate in a number of these signal pathways. Inhibitors of this oxidase suppress growth factor, TNFα,1 HIV-1 Tat, and shear cessation-induced signaling (2, 4, 12, 13), and dominant negative Rac1 disrupts TNFα signaling (5) in endothelial cells. Recently, both cytochrome subunits of the oxidase, p22phox and gp91phox, were cloned from rat and human endothelial cells (14, 15). We subsequently cloned the oxidase adapter subunit p47phox from HUVEC, demonstrating its participation in TNFα signaling (12). Unexpectedly, endogenous p47phox was found to be constitutively associated with the cytoskeleton of unstimulated ECV-304 cells, contrasting the free cytosolic location of p47phox in unstimulated neutrophils. Because most signaling proteins are associated with the cytoskeleton at some point in their activation cycle, the strong association of p47phox with the endothelial cytoskeleton suggested specific localization of the oxidase with cytoskeletonally anchored signaling complexes. Indeed, cytoskeletal disruption derailed both oxidase activation and downstream signaling (12). Spatial targeting of the oxidase may therefore potentially confer signal specificity to these evanescent radicals.

To identify potential vicinal signaling elements associated with the endothelial NAD(P)H oxidase, we screened lung and HUVEC libraries for p47phox-interacting proteins and recovered the orphan adapter TRAF4. This interaction appears to participate in downstream activation of JNK by the oxidase-activating endothelial agonists TNFα and HIV-1 Tat.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All PCR amplifications for subcloning or mutagenesis were performed with Pfu Turbo (Stratagene). The bait vector pGBKKT7-p47 was created by a single base mutation of p47phox (T to C at +2), creating a new NcoI site. The NcoI-EcoRI fragment containing the coding region and 3′-UTR of p47phox was then subcloned into pGBK7 (CLONTECH) in-frame with the GAL4-BD. Full-length TRAF4 was PCR-amplified from a HUVEC library (Stratagene) between the EcoRI and SalI sites. It was directly ligated into the expression vector pCI (Promega) to create pCI-T4 and into the yeast shuttle vector pGBKT7 to create pGBK7-T4. The C-terminal TRAF domain of TRAF4 was excised from the library prey plasmid pACT2-T4 using EcoRI and PstI and ligated into pCIneo-FLAG (16) to create pCINF-T4(CT). pGBK7T-p47(1–205) was constructed by removing the C-terminal BamHI-BamHI fragment from pGBK7-T-p47, and pGBK7-T-p47(205–390) was

1 The abbreviations used are: TNF, tumor necrosis factor; HIV, human immunodeficiency virus; JNK, c-Jun N-terminal kinase; UTR, untranslated region; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; TRAF4, TNF receptor-associated factor 4; HUVEC, human umbilical vein endothelial cell.
obtained by isolation of the N-terminal Sall-BamHI fragment of p47\textsuperscript{phox}\ and ligation into pGBK7. pGBK7-p47 (1-346) was derived by excision of a Small-Small segment from pGBK7-p47. pGBK7-p47 (1-298) was derived by complete restriction of pGBK7-p47 with EcoRI, partial restriction with NsiI, T4 polymerase end fill-in, gel purification, and blunt-end ligation to release the plasmid. pGBK7-p47 (1-346) was obtained by PCR deletion of p47 (1-346) from pGBK7-p47 and frame correction by NcoI restriction, end fill-in, and blunt-end resealing. GAL4-DB fusions for p47 (1-28052/H11003) were stably transformed with pGBK7-peptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. Cells were sonicated for 5 s, and centrifuged at 6000 g for 5 min. GAL4-AD fusions with TRAF4-(153-345) and TRAF4-(308-470) were produced by PCR amplification of segments between EcoRI and SalI sites, with insertion of appropriate stop codons, followed by ligation into pGBK7. GAL4-AD fusions with TRAF4-(299-307) and TRAF4-(308-470) were produced by PCR amplification of segments between EcoRI and XhoI sites and ligation into pGADT7 (CLONTECH). The coding region of p47\textsuperscript{phox} was PCR-amplified between EcoRI and SalI sites and ligated into pCIneo-FLAG to yield pCINF-p47, and between two EcoRI sites with ligation into pGEX-2TK to yield pGEX-p47. HA-JNK2 was derived by reversal of HA-JNK2/2/AFP mutant (gift from Dr. Lynn Heasley) back to wild type with PCR mutagenesis, and HA-JNK1 was a gift from Dr. Stephen Dreskin (17). pGAD424-TRAF1 and pGAD424-TRAF2 were gifts from Dr. Preet Chaudry. The frame of the former construct was corrected prior to use. The fusion construct pCINF-p47-T4 was produced by PCR amplification of p47\textsuperscript{phox}-(1-389) between EcoRI sites and ligation into pCINF-T4 (CT) between the FLAG tag and TRAF4 C terminus. p47-GFP was constructed as previously described (12). All constructs were confirmed by direct sequencing.

**Microscopy—HUVEC** were transfected with p47-GFP and plated onto fibronectin-coated slides. After 24 h, cells were fixed and permeabilized (12), counterstained with rhodamine-phalloidin (5 units/ml), and examined using a Zeiss Axiovert S100TV LSM 410 laser-scanning system. **TRAF4 and Oxidant Signaling**—Approximately 2 μg of GST or GST-p47 was used per 200 μl of binding reaction. Full-length TRAF4 was transcribed and translated in vitro from pC1-T4 (TNT Quick Coupled, Promega) using [35]S methionine, and 5 μl (3 μCi) of translation mix was added to each binding reaction. In some reactions, p47 (299-390) was in vitro transcribed and translated from pGBK7-p47 (299-390) without isotope and added directly to the binding reaction simultaneously with labeled TRAF4.

**Adenoviral Construction—** A silent G360C mutation was introduced in p47\textsuperscript{phox} to eliminate a potential PI-Cell site. The entire p47\textsuperscript{phox} cDNA was excised with XbaI and KpnI and ligated into pSHuttle, and the expression cassette was then subcloned into the backbone pAdeno-X (CLONTECH). The linearized adenoviral DNA was then transfected into HEK-293 cells and replication-incompetent viruses harvested and titred.

**JNK Activity**—JNK activity of Fx cells was assessed using a traditional immunoprecipitation kinase technique using anti-JNK1 (Santa Cruz Biotechnology, C-17) and GST-Jun (Santa Cruz Biotechnology) (2). Equivalent capture of JNK was assessed with immunoblot using a pan-specific anti-JNK (JNK-FL, Santa Cruz Biotechnology). To increase HUVEC transfection efficiency, passage 2 (100 ng/ml, Peprotech) or HIV-1 Tat, prepared as a GST fusion as previously described (2). The JNK activity of anti-HA immunoprecipitates was then assessed.

**RESULTS**

**TRAF4 Interacts with p47\textsuperscript{phox}—** We found first that similar to the situation with ECV-304 cells (12), p47\textsuperscript{phox} appears to constitutively associate with the actin cytoskeleton of HUVEC. p47-GFP colocalized with submembranous lateral actin bundles and with actin microspikes. Magnification ×100.

**Fig. 1.** HUVEC were transfected with p47-GFP and counterstained with rhodamine-phalloidin to visualize F-actin. p47-GFP colocalized strongly with lateral actin bundles and with actin microspikes. Magnification ×100.

![Image](https://via.placeholder.com/150)

**GST Pull-down—** Direct interactions were confirmed in vitro (18). BL21-RP E. coli (Stratagene) were transformed with either pGEX-2TK or pGEX-p47, induced for 3 h at 37°C, and the GST proteins were captured on glutathione-Sepharose (Amersham Biosciences, Inc.). Approximately 2 μg of GST or GST-p47 was used per 200 μl of binding reaction. Full-length TRAF4 was transcribed and translated in vitro from pC1-T4 (TNT Quick Coupled, Promega) using [35]S methionine, and 5 μl (3 μCi) of translation mix was added to each binding reaction. In some reactions, p47 (299-390) was in vitro transcribed and translated from pGBK7-p47 (299-390) without isotope and added directly to the binding reaction simultaneously with labeled TRAF4.

**Coprecipitation—** Phoenix-293 (Fx) cells electroporated with indicated vectors were incubated in lysate 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 Na:\textsubscript{2}VO\textsubscript{4}, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C, sonicated for 5 s, and centrifuged at 6000 × g for 20 min at 4°C. Immunoprecipitation was then performed using antibodies against FLAG (Sigma), pelleting protein G-agarose (Amersham Biosciences, Inc.) conjugates at 1500 × g. Immunoblots were performed with antiserum recognizing the C terminus of TRAF4 (Santa Cruz Biotechnology, C-20) or FLAG. Cytoskeletal fractions were collected as previously described (12), and cytosolic fractions were recovered by acetone precipitation of cell extracts.

**Yeast Mating—** AH109 yeast stably transformed with GAL4-AD-TRAF4 constructs were tested first for autonomous transactivation; negative colonies were mated with Y187 yeast stably transformed with various GAL4-BD-p47 deletion constructs. Diploids were replated on medium selecting for both plasmids and tested for lacZ expression with a filter lift assay. Negative controls were Tyr-187 transformed with empty pGBK7 and pGBK7-lamin C; the positive control was AH109 transformed with holo-GAL4 (pCL1, CLONTECH). Positive interactions were identified by development of a blue color within 1 h, negative interactions remained white for >24 h.

**Coproclpitation—** Phoenix-293 (Fx) cells electroporated with indicated vectors were incubated in lysate 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 Na:\textsubscript{2}VO\textsubscript{4}, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C, sonicated for 5 s, and centrifuged at 6000 × g for 20 min at 4°C. Immunoprecipitation was then performed using antibodies against FLAG (Sigma), pelleting protein G-agarose (Amersham Biosciences, Inc.) conjugates at 1500 × g. Immunoblots were performed with antiserum recognizing the C terminus of TRAF4 (Santa Cruz Biotechnology, C-20) or FLAG. Cytoskeletal fractions were collected as previously described (12), and cytosolic fractions were recovered by acetone precipitation of cell extracts.
this clone was bidirectionally sequenced and found to encode the C-terminal 210 amino acid residues of TRAF4. To accomplish a more endothelial-specific screen, 1.9 × 10⁶ clones were screened from the HUVEC library, and only 10 colonies were found to survive auxotrophic selection. Of these, four were lacZ-positive and two were subsequently found to be true positives. These clones were identical by restriction digest analysis. Sequence of two were subsequently found to be true positives. These clones were identical by restriction digest analysis. Sequence of one revealed the C-terminal 287 residues of TRAF4. Yeast mating demonstrated lack of binding of p47phox to full-length TRAF1 and 2 (not shown).

When overexpressed in Fx cells, TRAF4 preferentially remained with the detergent-insoluble fraction, with a smaller portion of the protein found in the detergent-soluble fraction (Fig. 2a), mimicking the cytoskeletal distribution of p47phox (12). When coexpressed, TRAF4 was found to specifically coprecipitate with FLAG-p47 (Fig. 2b). TRAF4 was also found to coprecipitate with p47-GFP, using anti-GFP, though at reduced efficiency (not shown). In addition, full-length TRAF4 coprecipitated with the C-terminal TRAF domain of TRAF4 (Fig. 2c), suggesting self-association through this domain.

The C terminus of p47phox Interacts with the TRAF Domain of TRAF4—Two-hybrid interactions were used to determine interacting domains of p47phox and TRAF4. The C terminus of p47phox (299–390), containing a variant proline-rich (PR) sequence, an arginine-rich basic motif, and a C-terminal PR site, was both necessary and sufficient for interaction with TRAF4 (Fig. 3a). Interestingly, the extreme C terminus (347–390) of p47phox was necessary but by itself insufficient, and the adjacent segment (299–345) was also necessary but insufficient either by itself or attached to the rest of the protein N-terminal to it. The GST pull-down assay confirmed a direct interaction of p47phox with full-length TRAF4 in vitro (Fig. 3b). In addition, in vitro-translated p47-(347–390) competed with full-length p47phox for TRAF4 binding, consistent with a specific interaction of the C terminus of p47phox with TRAF4.

The smaller of the TRAF4 library inserts obtained encoded residues 261–470, corresponding to both the 6th exon and the TRAF domain of TRAF4, indicating an interaction of p47phox with this domain. The TRAF domains of TRAFs 1–6 contain a C-terminal β-sheet-predominant subdomain (TRAF-C) preceded by a shorter coiled-coil subdomain (TRAF-N). By homology and secondary structure prediction, these regions span residues 308–470 and 267–307, respectively, of TRAF4 (20). Yeast mating studies suggested that the isolated TRAF-N and TRAF-C domains were each insufficient to bind p47phox, whereas the entire TRAF domain bound p47phox (Fig. 4).

TRAF4 Participates in JNK Activation—Overexpression of either p47phox or full-length TRAF4 alone in Fx cells did not appreciably affect JNK activity. In contrast, overexpression of both p47phox and TRAF4 increased JNK activity greater than 4-fold over control, suggesting a functional as well as physical interaction between the two proteins (Fig. 5a). Similarly, coexpression of p47phox and TRAF4 increased DCF oxidation, consistent with an increase in oxidant production. To demonstrate that interaction of p47phox with the TRAF4 TRAF domain was sufficient for JNK activation, we fused this TRAF domain to the C terminus of p47phox. Overexpression of the fusion protein increased JNK activity in HUVEC (Fig. 5b), whereas overexpression of either p47phox or the TRAF4 TRAF domain alone did not. This activation was decreased by the antioxidant N-acetyl cysteine (NAC).

To further implicate TRAF4-p47phox interactions in endogenous endothelial cell signaling pathways, we investigated the effect of TRAF4 p47phox binding domain overexpression on signaling by TNFα and HIV-1 Tat, two agonists that activate endothelial cell JNK through p47phox-dependent oxidant production (2, 12). Overexpression of this TRAF4 truncation consistently decreased activation of both HA-JNK1 and HA-JNK2 by TNFα and HIV-1 Tat in HUVEC (Fig. 6), whereas full-length TRAF4 did not affect JNK activation (not shown).

Association of TRAF4 with Other Signaling Proteins—A secondary screen of the HUVEC library using full-length TRAF4 was performed on 5 × 10⁶ AH109 transformants. Of 92 His⁺/Ade⁺/lacZ⁺ clones, 73 were thought to be unique by PCR and digest pattern. These clones were isolated, passaged through E. coli, and 71 clones were confirmed by mating back to Y187/pGBKKT7-T4 and lacZ expression testing. These clones were all...
sequenced and found to represent 23 unique genes with coding regions in-frame with GAL4-AD, including 4 extracellular proteins, 4 nuclear proteins, and 3 unpublished cDNAs. Of the remaining 12 genes, 8 encoded proteins involved in the determination of cell death and/or proliferation (Table I).

FIG. 3. TRAF4 binds to p47 (299–390). a, AH109 yeast stably transformed with full-length GAL4-BD-p47 were mated with Y187 harboring truncations of GAL4-AD-TRAF4 and interactions detected by lacZ expression. The entire TRAF domain (261–470) bound p47, whereas the TRAF-N and TRAF-C regions were each necessary but insufficient for p47 interaction.

b, GST pull-down assay demonstrated direct binding of [35S]methionine-labeled TRAF4 with GST-p47. Partial competition for capture of TRAF4 was seen with low levels of in vitro translated p47-(299–390).

FIG. 4. AH109 yeast stably transformed with full-length GAL4-BD-p47 were mated with Y187 harboring truncations of GAL4-AD-TRAF4 and interactions detected by lacZ expression. The entire TRAF domain (261–470) bound p47, whereas the TRAF-N and TRAF-C regions were each necessary but insufficient for p47 interaction.

FIG. 5. TRAF4 and p47phox activate JNK. a, Fx cells were infected with either Ad-p47 or Ad-lacZ and then transfected with either pCI-T4 or vector alone (pCI) 24 h later. After an additional 24 h, JNK activity was assessed by IP kinase, and oxidant production by DCF fluorescence. Overexpression of both p47phox and TRAF4 increased JNK activity and DCF fluorescence, whereas either alone had no significant effect. b, HUVEC were cotransfected with HA-JNK2 and either pCI, pCINF-p47, pCINF-T4(CT), or pCINF-p47-T4(CT). Upper panel shows immunoblot of p47-T4(CT) with anti-FLAG, showing fusion protein at ~74 kDa. JNK activity was assessed by IP kinase 24 h after transfection. Transfection of HUVEC with the fusion construct pCINF-p47-T4(CT) constitutively activated JNK, whereas addition of NAC (5 mM x 16 h) decreased this activation. Histograms represent means of three experiments.
Tat and TNF-α both activated HA-JNK1 (a) and HA-JNK2 (b). JNK activation by Tat and TNF-α was reduced by overexpression of the TRAF4 C terminus. Histograms represent means of three experiments.

**DISCUSSION**

TRAF4 is the least well understood of the traditional TRAF family members. Originally identified in a differential screen of metastatic breast cancer lymph nodes (21), it was subsequently shown to be widely expressed in normal human tissues, especially actively dividing epithelium (22). Its closest relative in both domain organization and amino acid sequence is the Dro sophila adapter DTRAF1, which interacts with Misshapen (Msn), a MAP4K acting upstream of JNK (23). The biological function of TRAF4, however, is poorly understood, beyond its ability to prevent dimerization of the neurotrophin receptor p75NTR (24) and to contribute to normal tracheal development in mice (25).

Full-length TRAF4 demonstrated a preference for the detergent-insoluble cell fraction, a distribution similar to that of p47phox in endothelial cells (12). The constitutive association of p47phox with the cytoskeleton demonstrated in ECV-304 cells in this prior study and in HUVEC in the present study stands in marked contrast to its behavior in neutrophils. In the latter cell type, p47phox migrates from a cytosolic location to the cytoskeleton upon stimulation (26, 27). The constitutive association of p47phox with the endothelial cytoskeleton suggests that the oxidase may exist in a preformed but inactive complex in endothelial cells. Abrupt cessation of flow, for instance, causes oxidant production by endothelial cell NADPH oxidase within 15 s (28). Although the basis for such constitutive cytoskeletal association is not known, there are clear differences in cytoskeletal structure between adherent endothelial cells and suspended neutrophils. Adhesion-dependent reorganization of the actin cytoskeleton may create or expose p47 binding sites or initiate partial phosphorylation of p47phox, resulting in cytoskeletal localization. Notably, adhesion primes neutrophils for a robust respiratory burst upon TNF-α stimulation in a mechanism dependent upon actin polymerization (29). A similar mechanism may operate in adherent endothelial cells.

The isolated TRAF domain of TRAF4 also demonstrated a preference for the detergent-insoluble fraction. Because the TRAF4 TRAF domain also bound p47phox, these data do not allow us to determine whether TRAF4 is primarily associated with the cytoskeleton or whether this localization arises secondarily from its association with p47phox. The retrieval of α-actinin as a potential TRAF4-interacting partner (Table I) is consistent with the former possibility. This situation may be somewhat different from the localization of TRAF5, because the zinc finger motifs of this latter protein rather that its TRAF domain appear to confer detergent insolubility (30). It is equally plausible that TRAF4 and p47phox each have independent cytoskeletal association domains.

The C-terminal tail of p47phox from residues 299–390 comprised the minimum TRAF4 binding domain we identified. This region encompasses a variant proline-rich site (299–302), a basic region (314–347), and a type II polyproline motif (362–368). In addition, this tail harbors three basic peptides corresponding to residues 305–319, 325–339, and 373–387, which independently block activation of the NADPH oxidase in vitro in a reconstituted phagocyte membrane system (31). GAL4-AD fusions containing either the variant proline-rich and basic regions or the C-terminal polyproline motif were each necessary but insufficient for binding TRAF4 in the two-hybrid assay, suggesting either a cooperative interaction or a tertiary structure requiring both halves of the C-terminal tail.

Our data also indicate an interaction of p47phox specifically with the C-terminal TRAF domain of TRAF4, and suggest that both TRAF-N and TRAF-C domains are necessary for this interaction. The TRAF domain also appeared to be sufficient for TRAF4 self-association, consistent with prior studies suggesting homomultimerization of TRAF proteins through the TRAF domain (32, 33). Besides self-association, the TRAF domains of TRAFs 2, 3, and 6 are also capable of binding downstream signaling elements such as cIAP-1/2, TRADD, receptor-interacting protein (RIP), NF-κB-inducing kinase (NIK), MIP-3, and TTRAP (34–39). Following this paradigm, the NAD/PH oxidase may lie downstream of TRAF4. Consistent with this interpretation, we found that ectopic expression of both TRAF4 and p47phox in Fx cells act cooperatively to stimulate activation of JNK. Further, forced association of p47phox and the TRAF4 TRAF domain through genetic fusion caused activation of JNK in HUVEC. In the latter experiment, the C-terminal TRAF domain of the fusion protein may have associated with endogenous endothelial cell TRAF4, thus recruiting p47phox to full-length TRAF4. Alternatively, the TRAF domain of TRAF4 may be sufficient to initiate distal signaling in the presence of p47phox, a possibility we have not excluded.

In endothelial cells, TNFα and HIV-1 Tat are strong JNK activators that recognize distinct receptors yet appear to both signal through the NADPH oxidase (2, 12). Interestingly, we found that overexpression of the p47phox binding TRAF4 TRAF domain interrupted signaling initiated by these two endothelial cell agonists in HUVEC. The isolated TRAF domains of TRAF2, 3, 5, and 6 have been demonstrated to block upstream signals originating from the TNFα, LTβ, and IL-1 receptors (37, 40, 41), but we are not aware of any reports in which the TRAF4 TRAF domain acts as a dominant negative for extra-cellular ligand-induced signaling. In addition, TRAF4 does not appear to mimic other TRAF members, as it does not, for instance, heterotrimerize with TRAF1, 2, or 3, and does not...
bind to TNFR1 or TNFR2 (22, 32). These data raise the possibility that the ring and/or zinc fingers of TRAF4 may link p47phox with upstream elements necessary for TNFα- and Tat-dependent signals, whereas the C-terminal TRAF domain may link p47phox with distal events leading to JNK activation.

Both TNFα and HIV-1 Tat affect angiogenesis and vascular remodeling and therefore influence cell fate. Tat, for instance, triggers either proliferative or apoptotic pathways in endothelial cells (42, 43), and TNFα initiates opposing death and survival pathways in endothelium (5). Notably, therefore, our screen of endothelial proteins interacting with TRAF4 recovered a number of proteins involved with proliferation and apoptosis. UBC9, for instance, binds to the death domains of TRAF4 and can mediate either apoptosis or proliferation (54, 56, 57).

Of interest, TRAF4 is robustly expressed in mature granulocytes and can mediate either apoptosis or proliferation (54, 56, 57).

Activation by the extracellular ligands TNFα and VEGF, the latter sharing a receptor with Ang-1 and Ang-2 (50), has been associated with hyperproliferation and angiogenesis (49). Similarly, VRP mediated serine exposure during apoptosis (46) and NRAGE mediates apoptosis. UBC9, for instance, binds to the death domains of TRAF4 and can mediate either apoptosis or proliferation (54, 56, 57).

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TABLE I

| Protein | Abbreviation | No. of clones | GenBankTm |
|---------|--------------|--------------|-----------|
| Melanoma-associated antigen | MAGE | 1 | AF200348 |
| NADP-dependent malic enzyme | BCKDK | 1 | X79440 |
| Non-smooth muscle α-actinin | UBC9 | 5 | X06427 |
| α-Ketocetyl dehydrogenase kinase | Hic-5 | 2 | NM 021105 |
| Ubiqutin conjugase 9 | TARBPP2 | 1 | NM 004178 |
| Phospholipid scramblase | ArgBP-2 | 1 | NM 021089 |
| Hydrogen peroxide-inducible clone-5 | NRAGE | 2 | NM 005868 |
| Tar-binding protein 2 | HCR | 1 | NM 019052 |
| Arg/Abi binding protein 2 | VRP | 2 | NM 007063 |

Vascular Rab-GAP/TBC domain-containing protein
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