Rac Activation Induces NADPH Oxidase Activity in Transgenic COS*phox* Cells and Level of Superoxide Production is Exchange Factor-Dependent

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Summary

Transient expression of constitutively active Rac1 derivatives, (G12V) or (Q61L), was sufficient to induce phagocyte NAPDH oxidase activity in a COS-7 cell model in which human cDNAs for essential oxidase components, gp91*phox*, p22*phox*, p47*phox* and p67*phox*, were expressed as stable transgenes. Expression of constitutively active Rac1 in "COS*phox*" cells induced translocation of p47*phox* and p67*phox* to the membrane. Furthermore, translocation of p47*phox* was induced in the absence of p67*phox* expression, even though Rac does not directly bind p47*phox*. Rac effector domain point substitutions (A27K, G30S, D38A, Y40C), which can selectively eliminate interaction with different effector proteins, impaired Rac1V12-induced superoxide production. Activation of endogenous Rac1 by expression of constitutively active Rac-GEF derivatives was sufficient to induce high-level NADPH oxidase activity in COS*phox* cells. The constitutively active form of the hematopoietic-specific GEF, Vav1, was the most effective at activating superoxide production, despite detection of higher levels of Rac1-GTP upon expression of constitutively active Vav2 or Tiam1 derivatives. These data suggest that Rac can play a dual role in NADPH oxidase activation, both by directly participating in the oxidase complex and by activating signaling events leading to oxidase assembly, and that Vav1 may be the physiologically relevant GEF responsible for activating this Rac-regulated complex.

Introduction

The phagocyte NADPH oxidase plays an essential role in innate immunity by catalyzing the production of reactive superoxide in conjunction with phagocytosis to facilitate pathogen killing and digestion (1,2). Genetic defects in essential NADPH oxidase components result in chronic granulomatous disease (CGD), a condition characterized by recurrent, often life-threatening infections (3). Binding of inflammatory mediators to specific phagocytic cell surface receptors triggers assembly of the multicomponent NADPH oxidase. Tight regulation of NADPH oxidase activation is required because excessive or inappropriate superoxide release can damage adjacent host tissue, as has been implicated in pathologic inflammatory conditions (4,5).

The active oxidase is comprised of two integral membrane subunits, gp91^{phox} and p22^{phox}, which form flavocytochrome b_{558}, and three cytosolic components, p47^{phox}, p67^{phox}, and Rac, which must translocate to the membrane to activate electron flow through the flavocytochrome (1). In whole cells, p47^{phox} and p67^{phox} associate via reciprocal SH-3 domain interactions (6,7). Membrane translocation of both p47^{phox} and p67^{phox} is dependent upon phosphorylation of p47^{phox} (1-3), which undergoes a conformational change (8) to expose an SH-3 domain that binds to a proline-rich region in the p22^{phox} flavocytochrome subunit (9-14). p47^{phox} translocates to the membrane in the absence of p67^{phox} expression but p67^{phox} remains cytosolic in the absence of p47^{phox} in phagocytes derived from p67$^{phox\ -/-}$ or p47$^{phox\ -/-}$ CGD patients, respectively (9,15). Analysis of NADPH oxidase activation in cell-free

systems has shown that the addition of p47*phox* plus an anionic amphiphile, which is thought to substitute for phosphorylation events, is required for the high affinity binding of p67*phox* and Rac to flavocytochrome b_{558} (16,17), although this requirement can be overcome by the addition of high concentrations of Rac and p67*phox* (16,18-20). Thus, p47*phox* functions as an essential adaptor protein linking and orienting p67*phox* with the flavocytochrome subunits (20).

A second important event required for oxidase activation is Rac activation and membrane translocation (1,2). The mechanisms by which Rac regulates the NADPH oxidase are incompletely understood. In cell-free oxidase reconstitution assays, either the GTP-bound form of Rac1 or Rac2 is essential for high-level superoxide production (21,22). Rac-GTP binds the N-terminal TPR (tetratrico repeat) region of p67*phox* and may also interact directly with the flavocytochrome in the assembled oxidase complex (23-29). In human neutrophils, hematopoietic-specific Rac2 is the major isoform, and ubiquitously-expressed Rac1 is a minor isoform (25). Recent *in vivo* evidence for Rac regulation of phagocyte oxidase function has come from analysis of $Rac2^{-/-}$ mice (30,31) and from a patient with recurrent bacterial infections who was found to express a dominant-negative form of Rac2 (32,33).

Guanine nucleotide exchange factors (GEFs) activate Rac by catalyzing the exchange of bound GDP for GTP (34,35). Rac-GTP can then interact with numerous downstream effector proteins, activating biochemical pathways that include those regulating actin remodeling, cell cycle progression, and gene expression, in addition to NADPH oxidase activity in phagocytes (36,37). Rac has a low rate of intrinsic GTP hydrolysis, which is accelerated by GTPase activating proteins (GAPs), causing it to revert to an inactive GDP-bound conformation (36).

Two Rac point mutants, (Q61L) or (G12V), are deficient in GTPase activity and therefore constitutively active (36,38,39).

Specific point substitutions in the Rac effector domain (amino acids 26 – 45), which changes conformation upon GTP binding, lead to selective defects in binding downstream effector proteins. Point mutations (A27K) or (G30S) abrogate Rac interaction with p67*phox* and NADPH oxidase activity in cell-free assays (40,41). Effector domain point substitutions (D38A) or (Y40C) abrogate Rac/Cdc42 interaction with target effector proteins containing a conserved Rac/Cdc42-binding sequence known as a CRIB motif, including the p21 activated kinases (PAKs) (36,42,43). p67*phox* interacts with Rac via a sequence interspersed in its N-terminal TPR motifs rather than via a CRIB motif (40) and has normal affinity for a C40 Rac derivative (43). Mutation or deletion of the Rac "insert domain" (amino acids 124 – 135), a surface-exposed α-helical region, impairs NADPH oxidase activity in arachidonic acid activated cell-free assays (29,44). The Rac insert domain is not involved in binding to p67*phox* but rather is postulated to directly interact with the flavocytochrome (27,29).

To develop genetic approaches for characterizing signaling pathways regulating phagocyte NADPH oxidase assembly and activation, we recently developed a readily transfectable cell model in which human cDNAs for all four essential oxidase components, gp91*phox*, p22*phox*, p47*phox* and p67*phox*, were expressed as stable transgenes in COS-7 cells (45). COS-7 cells express the Rac1 but not the Rac2 isoform. The transgenic "COS*phox*" cells exhibit high level NADPH oxidase activity induced using either phorbol myristate acetate (PMA) or arachidonic acid, two soluble stimuli commonly used to activate the neutrophil

NADPH oxidase. Agonist-elicited superoxide production by the COS*phox* cells was significantly inhibited by transient expression of dominant negative Rac mutants or by overexpression of the inhibitory Rac-binding protein, RhoGDI. These results supported an important role for Rac in regulating the NADPH oxidase in COS*phox* cells, consistent with the genetic data in murine and human neutrophils.

In the current study we show that Rac activation in COS*phox* cells, either by expression of constitutively active Rac exchange factors or by expression of constitutively active Rac derivatives, is sufficient to induce membrane translocation of p47*phox* and p67*phox* and activate the NAPDH oxidase, in the absence of any other stimulus. Constitutively active Vav1 was more effective at activating superoxide production than Vav2 or Tiam1. Selective Rac effector domain point substitutions, which disrupt interaction with different downstream effector proteins, inhibited Rac1V12-induced NADPH oxidase activity, supporting a role for multiple effectors in mediating Rac-GTP oxidase activation. Our data suggest that Rac can regulate phagocyte NADPH oxidase activity both as a direct participant in the oxidase complex and also as an activator of signaling events leading to oxidase assembly.

Experimental Procedures

Materials. Chemicals were purchased from Sigma unless otherwise stated. Phosphate buffered saline (PBS), penicillin/streptomycin, trypsin/EDTA, glycerol, Lipofectamine Plus and Dulbecco's modified Eagle's low glucose medium (DMEM) were purchased from Gibco BRL. Mouse monoclonal antibodies against Rac1 (clone 23A8) and Myc-epitope tag 9E10 were

purchased from Upstate, Inc. (Lake Placid, NY). Polyclonal rabbit serum raised against p47*phox* and p67*phox* was generously provided by David Lambeth (Emory University). Paul Heyworth (Scripps Research Institute) kindly provided mouse monoclonal antibody against p47*phox*.

Expression Vectors. Full-length cDNAs for human Rac1, Rac2, and Cdc42 were subcloned downstream of a Myc-epitope tag in pRK5 (Pharmingen). PCR mutagenesis was used to introduce (G12V) or (Q61L) point mutations in Rac or Cdc42 cDNA templates. A polyglutamate-tag was subcloned downstream of the Myc-epitope tag and upstream of the mutant Rac (G12V) cDNAs. Rac1 (Δ124-135/A136) cDNA was generated by PCR as described (44). Rac1 (V12/Δ124-135/A136) was generated by restriction digest and ligation of a Bsu36 I/Pst I fragment, encompassing the C-terminal region of Rac1 (Δ124-135/A136), into pRK5 expression vector containing the N-terminal region of Rac1 (G12V). Rac1 double mutants, (V12/ K27), (V12/S30), (V12/A38), (V12/C40), (V12/N130), (V12/E132), and (V12/R134), were generated using the QuikChange mutagenesis kit (Stratagene) to introduce each of the second mutations into a Rac1V12 template. All mutant Rac cDNAs were sequenced in entirety. Human p47*phox* cDNA was subcloned into pRK5 and human p67*phox* cDNA was subcloned upstream of a Myc-epitope tag in pcDNA3 (Invitrogen). Amino-truncated cDNAs for mouse Vav1ΔN-186 and human Vav2ΔN-191, each subcloned in pAX142 (46,47), were graciously provided by Channing Der (University of North Carolina). Amino-truncated cDNA for human Tiam1ΔN-401 (encoding a 135kD derivative) subcloned in pCANmyc (48) was generously provided by Gideon Bollag (Onyx Pharmaceuticals).

Cell Culture and Transfection. COS-7 cell lines were previously established which stably expressed human cDNAs encoding gp91*phox*, p22*phox*, p47*phox*, and p67*phox*, or

combinations of these, as stable transgenes (45). Transgenic COS-7 cell lines were grown in DMEM plus 10% heat inactivated fetal calf serum (Hyclone), 50 units/ml penicillin, 50µg/ml streptomycin, 0.2 mg/ml hygromycin (Calbiochem), 1.8 mg/ml neomycin and 1 µg/ml puromycin. COS-7 cell lines were transiently transfected using Lipofectamine Plus per manufacturer's instructions. Transfection efficiency averaged 25 – 35%, as determined by immunohistochemistry to detect expression of a Myc-epitope tag. Cells were harvested 21 hours post-transfection by incubating with trypsin/EDTA for 5 minutes at 37C, as previously described (45).

Measurement of NADPH Oxidase Activity. Superoxide production by whole COS cells, harvested by brief trypsinization as described above, was measured in a quantitative kinetic assay based on the superoxide dismutase-inhibitable reduction of cytochrome *c*. Assays were performed at 37C using a Thermomax microplate reader (Molecular Devices) as described previously (45,49) except no stimulus was added. Superoxide production was quantified using an extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ for cytochrome *c*. The maximum rate of superoxide generation over a five-minute interval was calculated using SOFTMAX version 2.0. Alternatively, qualitative assessment of NADPH oxidase activity in single cells was performed using the nitroblue tetrazolium (NBT) test; cells were incubated for 30 min at 37C in PBSG containing NBT. Cells were then fixed with methanol, stained with 0.2% safrinin, and counted at 400x magnification.

Indirect Immunofluorescence and Confocal Microscopy. Cells were seeded on glass coverslips and transfected with constitutively active Rac derivatives the following day. After overnight serum starvation, cells were fixed with 4% paraformaldehyde, permeabilized with

0.05% Triton X-100, and blocked with 2% bovine serum albumin (BSA) + 1% normal goat serum (NGS) in PBS. Cells were stained with polyclonal p47*phox* or p67*phox* antisera plus monoclonal Myc Ab (to detect expression of transgenic Rac derivatives) diluted in 2% BSA/1% NGS/PBS for 30 minutes at 37C. Cells were washed thoroughly with PBS, then incubated with FITC-conjugated goat-anti-rabbit and Cy3-conjugated goat-anti-mouse secondary antisera (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in 2% BSA/1% NGS/PBS. Alternatively, cells were stained with p47*phox* monoclonal antibody followed by FITC-conjugated goat-anti-mouse Ab. Coverslips were mounted on glass slides with1% DABCO/ 50% glycerol. Focal planes were imaged at 0.4μm intervals using the Zeiss 510 laser scanning confocal microscope (100X PlanApo 1.4 NA objective) equipped with LSM510 digital imaging software. Three adjacent focal planes were averaged using Metamorph software. Color images were converted to greyscale with Adobe Photoshop 6.0. As a control, Rac1L61-activated COS*phox* cells were incubated with fluorophore-conjugated secondary antibodies only and these cells exhibited negligible background staining (not shown). As controls for combined primary and secondary antibody background staining, Rac1L61-activated COS cell lines that did not express p47*phox* were stained with p47*phox* antibody and processed in parallel with the test samples described above. Likewise Rac1L61-activated COS cell lines that did not express p67*phox* were stained with p67*phox* antibody and processed as described. The level of non-specific background staining in the control cells was well below the level of specific staining in the test cells (not shown).

Analysis of Protein Expression and Membrane Translocation Assay. Cells were harvested as described above. Whole cell lysates were prepared and analyzed by

immunoblotting as previously described (45). To examine membrane localization, cells were disrupted in a Dounce homogenizer and fractionated by high-speed centrifugation over a discontinuous 20%/38% sucrose gradient, as previously described (45). Densitometric analysis of films was performed using the Eagle Eye II Still Video System and associated software (Stratagene). Protein dilutions and multiple exposures were analyzed to ensure that this analysis was performed in the linear range.

Rac Activation Assay. PAK1 p21-binding domain (PBD-GST) was expressed in *Escherichia coli* strain BL21 and purified as described (50). 21 hours post-transfection, COS cells were detached with trypsin, washed, and resuspended in PBS at 5 x 10^6 cells/ 0.5 ml. 20µg PAK1 PBD-GST recombinant protein was added and cells were lysed by the addition of 2x Mg^{2+} lysis/wash buffer (MLB from Upstate) supplemented with 20µg/ml chymostatin, 2mM PMSF, 10µM leupeptin, 5 mM Na_3VO_4 and 25 mM NaF. The lysate was cleared, 30µl glutathione-Sepharose 4B beads (Amersham Pharmacia) were added and the binding reaction was incubated 1 hour at 4C. Beads were pelleted and washed 3x with MLB then finally resuspended in 30µl Laemmli sample buffer. Aliquots of supernatant and pulldown samples were electrophoresed and the proteins analyzed as described above. Rac detected in the supernatant was assumed to be Rac-GDP and that in the pulldown Rac-GTP, with the sum representing total Rac.

Statistical Analysis. Results were analyzed using Instat software. The effects of transfecting different cDNAs were compared using the Student t-test or ANOVA and Student-Newman-Keuls multiple comparisons test, as indicated. P-values ≤ 0.05 were considered significant. Data are expressed as mean ± SE.

Results

Expression of constitutively active Rac1 induces superoxide production in COS^{phox} cells. We previously developed a heterologous cell model in which human cDNAs for all four essential oxidase components, gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}, were expressed as stable transgenes in COS-7 cells (45). COS-7 cells express the Rac1 but not the Rac2 isoform. The transgenic "COS^{phox}" cells produced superoxide at a rate of 11 ± 0.87 nmol/10^7 cells/min (Vmax, n=43) in response to 0.4 μg/ml phorbol myristate acetate (PMA) and at a rate of 35 ± 3.5 nmol/10^7 cells/min (n=11) in response to 100μM arachidonic acid. This compared favorably to human neutrophils, which, when tested under the same conditions, exhibited PMA-elicited Vmax of 47 ± 3.4 nmol/10^7 cells/min and arachidonic acid-elicited Vmax of 41 ± 4.3, both n=5 (45).

In the current study, we found that high-level superoxide production could be induced in the COS^{phox} cells by transient expression of constitutively active Rac1 (Q61L) or (G12V) derivatives, in the absence of any other stimulus (Fig 1A). COS^{phox} cells transfected with Rac1L61 produced superoxide at 13 ± 0.5 nmol/10^7 cells/min (n=4), whereas cells transfected with Rac1V12 produced superoxide at 5.8 ± 0.8 nmol/10^7 cells/min (n=10), consistent with the L61 derivative having higher affinity for both GTP and p67^{phox} (38,39). Using nitroblue tetrazolium (NBT) to detect superoxide production by individual cells, we determined that 29% ± 2.5 (n=3) of Rac1L61-transfected cells were NBT-positive. The percentage of NBT-positive cells was comparable to the transfection efficiency of 25 – 35%, determined by

immunohistochemistry to detect expression of a Myc-epitope tag. Had the Rac1L61 been expressed in all of the cells instead of ~30% of the cells, the measured rate of Rac1L61-elicited superoxide production in the cytochrome *c* reduction assay would have been comparable to that elicited by arachidonic acid and significantly higher than that elicited by PMA. Expression of wild type Rac in COS*phox* cells did not induce phagocyte NADPH oxidase activity over background observed with transfection of empty vector (Fig. 1A). Superoxide production was not elicited by closely related (70% homologous) Cdc42 containing an activating (Q61L) mutation (Fig 1A). Transgenic Rac expression exceeded that of endogenous Rac1 by about 20-fold on a per mg total protein basis (Fig 1B). The overexpressed, epitope-tagged Rac migrated as two bands (Fig 1B) representing post-translationally isoprenylated and unprocessed forms of Rac (unpublished data). Cdc42L61 was expressed at similar levels to the Rac derivatives (data not shown).

p47phox expression is required for optimal Rac1L61-induced superoxide production in COSphox cells. Although p47*phox* is essential for NADPH oxidase assembly in activated neutrophils or in standard cell-free assays, superoxide production under certain cell-free conditions can be obtained in the absence of p47*phox* when sufficiently high concentrations of purified recombinant Rac and p67*phox* are added either to vesicles prepared from detergent solubilized phagocyte membrane or to purified, relipidated flavocytochrome (16,18-20,29). To determine whether activated-Rac induced superoxide production in the COS*phox* cells had a requirement for p47*phox* expression, we transiently transfected a COS cell line, which stably expressed gp91*phox*, p22*phox*, and p67*phox*, with vectors for expression of either Rac1L61, or

Rac1L61 and p47*phox*, or Rac1L61 and p67*phox*. p67*phox* was stably expressed in the COS-91,22,67 cells at a level comparable to that in human neutrophils on a per mg total protein basis. Transient transfection of p67*phox* further increased its level of expression by about 2.5-fold on a per mg total protein basis, or by ~7.5-fold in the cells in which it was expressed, based on the transient transfection efficiency of ~30%. Expression of Rac1L61 induced some superoxide production when p67*phox* was overexpressed under these conditions, consistent with results obtained under some cell-free conditions using purified NADPH oxidase components. However, substantially higher levels of superoxide were produced when COS-91,22,67 cells were transfected with p47*phox* and Rac1L61 (Fig 2), demonstrating that p47*phox* was an important participant in activated-Rac induced NADPH oxidase activity in the COS cell model, as also observed in neutrophils and when using standard cell-free assay conditions.

Specific mutations in the Rac effector and insert domains impair Rac1V12-induced NADPH oxidase activation. To investigate the mechanisms by which Rac-GTP induces NADPH oxidase activation, we examined whether specific effector or insert domain mutations, which selectively eliminate interactions with different target effector proteins, would affect the ability of Rac1V12 to activate the phagocyte NADPH oxidase in COS*phox* cells. Rac1V12 was chosen for these experiments rather than Rac1L61 because the L61 mutation has been shown to override inhibitory effector domain mutations in some instances (38).

Effector domain point mutations at either amino acid 27 or 30, which abrogate binding to p67*phox* (40), had the greatest impact on Rac1V12-activated superoxide production (Fig 3A). In fact, cells expressing Rac1V12/K27 or Rac1V12/S30 generated no superoxide over low-level

background amounts observed with empty vector-transfected cells, supporting the importance of the interaction between Rac and p67*phox* for NADPH oxidase activity. However, effector domain point substitution Y40C, which abrogates Rac interaction with CRIB-domain–dependent effector proteins without affecting affinity for p67*phox*, also reduced Rac1V12-induced NADPH oxidase activity by over 2-fold (Fig 3A). A similar reduction was observed with the (D38A) derivative (Fig 3A), which has reduced affinity both for p67*phox* and likely for CRIB-domain dependent effectors (24,42). Insert domain deletion (Δ124-135) or point substitutions K132E or L134R had a smaller inhibitory effect on Rac1V12-dependent activation of superoxide production (Fig 3A). For these experiments, approximately 7-fold less of the expression vector was used, compared to the experiments shown in Fig 1, to reduce the relative level of recombinant Rac1 protein expression. Transfection efficiency was ~20% under these conditions. Equivalent expression of the Rac derivatives was confirmed by immunoblotting (Fig 3B). Levels of the transgenic Rac derivatives exceeded endogenous Rac1 expression by about 3-fold on a per mg total protein basis, as determined by densitometric analysis of immunoblots (Fig 3B). Normalizing for transfection efficiency, the level of transgenic Rac exceeded endogenous by ~15-fold in cells which expressed the transgenic Rac derivatives.

The effect of further over-expressing selected Rac1 derivatives was examined by transfecting with a ~7-fold higher amount of each expression vector. Under these conditions, the Rac1V12/S30 derivative, which does not bind p67*phox*, still did not induce superoxide production over background observed with empty vector (Fig 3C), despite substantially increased transgenic protein expression (Fig 3D). The Rac1V12/C40 derivative, which binds p67*phox* but not CRIB-domain dependent effectors, was 2-fold less effective than Rac1V12 at

activating superoxide production, even when further overexpressed (Fig 3C, D), supporting the concept that effectors other than p67*phox* participate in Rac-GTP induced oxidase activation. At the higher protein expression level, the insert region deletion derivative elicited superoxide production that was comparable to Rac1V12 (Fig 3C). Thus, the moderately inhibitory effect of insert region deletion on activated-Rac induced superoxide production was overcome by higher expression of Rac1V12/Δinsert in the COS*phox* cells.

Expression of activated Rac is sufficient to induce p47phox and p67phox membrane translocation. NADPH oxidase activity in COS*phox* cells expressing constitutively active Rac derivatives was substantially dependent on p47*phox* expression (Fig 2), similar to phagocytes where p47*phox* expression is important for p67*phox* membrane translocation and absence of p47*phox* results in CGD (1). To verify that the high level superoxide production induced by constitutively active Rac in COS*phox* cells was associated with membrane translocation of p47*phox* and p67*phox*, we examined protein localization in the COS*phox* cells by subcellular fractionation and by indirect immunofluorescence and confocal microscopy.

Two independent experiments showed a 3- to 4-fold increase in the levels of p47*phox* and p67*phox* associated with the membrane fraction in COS*phox* cells transfected with Rac1V12 compared to cells transfected with empty vector (Figs 4A & B). This assay was performed on a bulk population of cells in which transient transfection efficiency was ~30%, as determined by immunohistochemistry to detect expression of the Myc-epitope tag on transgenic Rac. Densitometric analysis of cytosol and membrane fractions (Fig 4A and additional data not

shown) indicated that ~3% of cellular p47*phox* and p67*phox* were membrane-associated in the bulk COS*phox* cell population transfected with Rac1V12. Thus, adjusting for transfection efficiency, the levels of p47*phox* and p67*phox* membrane association were ~10% in the COS*phox* cells which expressed Rac1V12. These levels were comparable to the relative amount of p47*phox* and p67*phox* membrane association detected in activated neutrophils (9,51,52) or COS*phox* cells stimulated with the phorbol-ester PMA (45).

As a second, independent method for determining p47*phox* and p67*phox* localization, we used indirect immunofluorescence and confocal microscopy. In non-transfected or empty vector transfected COS*phox* cells, p47*phox* and p67*phox* were detected primarily in the cytosol**;** the cellular edges appeared soft and poorly defined because little p47*phox* or p67*phox* was associated with the plasma membrane (Fig 5A & B, respectively). Transiently expressed constitutively active Rac1 derivatives (L61 or V12) concentrated along the plasma membrane and induced significant membrane ruffling (Fig 5D & F), as expected (53). Increased plasma membrane association of p47*phox* and p67*phox* was apparent in COS*phox* cells expressing constitutively active Rac derivatives, as evidenced by a bright, clearly defined cellular border (Figs 5C & E), in marked contrast to the empty vector transfected cells (Fig 5A & B) or non-transfected cells on the same slide (e.g. Fig 5E). Cytosolic depletion of p47*phox* and p67*phox* immediately adjacent to the plasma membrane was typically also apparent in cells expressing constitutively active Rac (Fig 5C & E).

In intact neutrophils, agonist-elicited translocation of p47*phox* is dependent on

phosphorylation but does not require co-expression of p67*phox* (9). To determine whether expression of constitutively active Rac could induce membrane translocation of p47*phox* in the absence of p67*phox*, a COS cell line which stably expressed only gp91*phox*, p22*phox* and p47*phox* was transiently transfected with constitutively active Rac derivatives and examined by confocal microscopy. In contrast to empty vector-transfected cells, which resembled Fig 5A, membrane-associated p47*phox* was apparent in COS-91,22,47 cells expressing Rac1V12 (Fig 5G). This result indicates that expression of activated Rac was sufficient to induce membrane translocation of p47*phox* independent of p67*phox* expression, despite the fact that Rac does not directly bind p47*phox* (23,54). Rac1L61 and Rac1V12/S30 also induced p47*phox* membrane translocation in COS-91,22,47 cells (data not shown). p47*phox* membrane localization was less apparent but still detectable in COS-91,22,47 cells expressing Rac1V12/C40 (data not shown), consistent with the significantly decreased, but not absent, oxidase activity in Rac1V12/C40 transfected COS*phox* cells (Figs 3A and C).

p67*phox* does not translocate to the membrane in neutrophils derived from CGD patients who lack p47*phox* expression (9). To determine whether overexpression of the constitutively active form of Rac could induce membrane translocation of p67*phox* in the absence of p47*phox*, the COS cell line which stably expressed only gp91*phox*, p22*phox* and p67*phox* was transiently transfected with activated Rac derivatives and examined by confocal microscopy. p67*phox* membrane translocation was apparent in COS-91,22,67 cells expressing Rac1L61 (Fig 5H), indicating that high-level Rac-GTP expression could induce p67*phox* membrane translocation

in the absence of transgenic p47^{phox} expression, possibly through direct p67^{phox} interaction with overexpressed Rac-GTP. Consistent with this, some low-level p67^{phox} co-localization with transiently expressed Rac1L61 was also observed in COS cells which stably expressed only p67^{phox} but not the flavocytochrome subunits (data not shown). Rac1V12 and Rac1V12/C40 derivatives also induced membrane translocation of p67^{phox}, whereas little to no membrane localization of p67^{phox} was observed in COS-91,22,67 cells expressing Rac1V12/S30, as expected (data not shown).

Activation of endogenous Rac1 by transgenic expression of Rac-GEFs is sufficient to activate the NADPH oxidase in COS^{phox} cells. To determine whether activation of endogenous Rac1 is sufficient to induce superoxide production in COS^{phox} cells, we transiently expressed N-terminal truncated, constitutively activated forms of Rac guanine nucleotide exchange factors (GEFs), Vav1, Vav2, or Tiam1. The expression levels of these three GEFs could not be directly compared to each other as they did not all have epitope tags. Each stimulated significant superoxide production by the COS^{phox} cells (Fig 6A). Vav1 elicited superoxide production at a rate of 11 ± 1.5 nmol/10^7 cells/min (n=7), which was comparable to the Rac1L61-induced Vmax of 13 ± 0.5, n=4, and significantly higher than the Rac1V12-elicited Vmax of 5.8 ± 0.8, n=10 (Fig 1A). Vav1-induced superoxide production could be completely inhibited by co-expression of a dominant negative Rac1(T17N) derivative (data not shown), suggesting that the activated GEFs stimulated superoxide production through activation of Rac. Levels of p47^{phox}, p67^{phox}, and Rac1 membrane association were increased in cells transfected with Vav1ΔN-186

compared to empty vector transfected control cells (Fig 4A and B), consistent with the assembly of an active NADPH oxidase complex at the membrane of COS*phox* cells transfected with constitutively active Rac-GEFs.

Activated Rac pull down assays confirmed that N-terminal truncated exchange factors each activated endogenous Rac1 in the COS*phox* cells, with an observed rank order of effectiveness of Tiam1>Vav2>Vav1 (Fig 6B). Interestingly, although higher levels of activated Rac1 were detected upon transfection of either Tiam1 or Vav2, the constitutively active form of the hematopoietic-specific GEF, Vav1, was the most effective at activating superoxide production (Fig 6A). Activated Rac represented ~1% of total endogenous Rac1 in cells transfected with activated Tiam1, as determined by densitometric analysis of immunoblots (not shown). Since transient transfection efficiency was ~30%, activated Rac probably represented closer to 3% of endogenous Rac1 in the transfected cells which expressed activated Tiam1. In parallel determinations, activated Rac represented ~1% of endogenous Rac1 in cells which expressed activated Vav2, and ~0.2% of endogenous Rac1 in cells which expressed activated Vav1. Less than 0.01% of endogenous Rac was activated in cells transfected with empty vector. The level of Rac-GTP detected in GEF-transfected cells was at least two orders of magnitude less than that detected in Rac1L61 or Rac1V12 transfected cells (not shown), suggesting that a modest increase in the overall level of activated Rac was sufficient to activate the NADPH oxidase in COS*phox* cells.

Discussion

At least two separate events are required for assembly of the phagocyte NADPH oxidase

in intact cells: 1) phosphorylation of p47*phox* followed by membrane translocation of p47*phox* and p67*phox*, and 2) activation and membrane translocation of Rac. How Rac activation contributes to regulation of the NADPH oxidase has not been fully elucidated. It is well established that Rac-GTP is a component of the active oxidase complex, binding to both p67*phox* and also likely the flavocytochrome (1,23-29,55). In this study, we show that in COS*phox* cells, activation of Rac, either by expression of V12 or L61 Rac derivatives or by expression of constitutively active Rac exchange factors, can itself drive membrane translocation of p47*phox* and p67*phox* and assemble a functional NADPH oxidase in the absence of any other stimulus. This contrasts with assembly of the NADPH oxidase in standard cell-free assay conditions, where activated Rac (added as Rac-GTPγS) is insufficient for initiating superoxide production unless an active conformation of p47*phox* is induced by the addition of an anionic amphiphile (1,13,14,16). Optimal Rac-GTP–induced superoxide production required p47*phox* expression, as is the case for agonist-elicited oxidase activity in phagocytes and in another heterologous intact cell model based on the K562 chronic myelogenous leukemia cell line (56). Tyrosine kinase inhibitors have been reported to partially inhibit agonist-elicited membrane translocation of Rac in intact human neutrophils, while having a minimal effect on p47*phox* and p67*phox* membrane translocation (55). However, our results are not inconsistent with these observations, as residual Rac translocation may have been sufficient to activate p47*phox* and p67*phox* translocation in this study. In addition, non-Rac dependent pathways for inducing NADPH oxidase assembly may also exist. We therefore propose that Rac can play a dual role in NADPH oxidase activation in intact cells, both as a direct participant in the assembled oxidase and also as

an activator of signaling events leading to p47*phox* and p67*phox* membrane translocation.

It is important to note that the COS*phox* cells are a model system that may lack proteins which could influence NAPDH oxidase assembly in response to activated Rac in phagocytes. For example, p40*phox* is not expressed in COS cells. In resting neutrophils, p40*phox* is associated with p67*phox* and one third to one half of cellular p47*phox* in a high molecular weight cytosolic complex of undetermined stoichiometry, and translocates to the membrane upon cellular activation (57). p40*phox* can also bind cytoskeletal proteins and phosphotidylinositol-3'phosphate (58-61). p40*phox* is not required for superoxide production in cell-free NADPH oxidase assays nor is it required for activation of the NADPH oxidase in heterologous K562 cells stimulated by either PMA or through a transgenic formyl peptide receptor (57,62) In assays using either cell-free conditions or the K562 model, the addition of p40*phox* has been variously reported to exert either a positive or negative influence or have no effect at all on oxidase activity (58,63-68). Hence, the role of p40*phox* in regulating NADPH oxidase activity and whether or not it might influence the response to Rac activation is unclear, but will be investigated in future studies.

In transgenic COS cells, expression of constitutively active Rac or constitutively active Rac-GEFs was sufficient to direct membrane translocation of p47*phox* and p67*phox*. Expression of constitutively active Rac1 derivatives also stimulated p47*phox* translocation to the plasma membrane, even in the absence of p67*phox* expression, although Rac-GTP does not directly bind to p47*phox* (23,54). In neutrophils, current evidence indicates that p47*phox*

membrane translocation requires phosphorylation of multiple serine residues, including serines 303, 304 and 328, in order to expose an SH-3 domain necessary for p47*phox* association with a proline-rich region on the p22*phox* flavocytochrome subunit (8-11,14). The kinases which phosphorylate p47*phox* *in vivo* are unknown, although multiple protein kinases, including protein kinase C (PKC) and members of the PAK and MAPkinase families can phosphorylate p47*phox* *in vitro* (69). Rac is known to activate numerous downstream effector proteins, including phospholipase C-β2 (PLC-β2), as well as serine/threonine kinases, which regulate diverse signaling pathways (36,37), and we postulate that these can contribute directly or indirectly to p47*phox* membrane translocation. Whether activated Rac and its downstream effector proteins induce p47*phox* phosphorylation in COS*phox* cells is currently under investigation. In preliminary studies we have found that the protein kinase C (PKC) selective inhibitor GF109203x reduced Rac1V12-, Rac1L61-, and Vav1ΔN-186-induced superoxide production by ~25% (data not shown). Expression of constitutively active Rac1V12 or Rac1L61 derivatives was also sufficient to induce p67*phox* membrane translocation in the absence of p47*phox* expression, probably through direct p67*phox* interaction with overexpressed Rac-GTP.

Rac mutations that can selectively disrupt interaction with different effector proteins impaired Rac1V12-induced COS*phox* NAPDH oxidase activity, suggesting that multiple effectors mediate Rac-GTP induced oxidase activation. Point substitutions (A27K) or (G30S), which prevent Rac interaction with p67*phox* (40,41), abrogated Rac1V12-elicited superoxide production in COS*phox* cells, confirming the importance of the interaction between Rac and

p67*phox* for NADPH oxidase activity. The effector domain point substitution (Y40C), which abrogates Rac interaction with CRIB-domain dependent effector proteins (36,42) without affecting affinity for p67*phox* (43), reduced Rac1V12-induced NADPH oxidase activity by over 2-fold, supporting the concept that Rac-GTP targets in addition to p67*phox* can mediate oxidase activation in intact cells**.** Deletion of the insert domain (Δ124-135) reduced Rac1V12-induced superoxide production by 40%, although with sufficient overexpression there was no inhibitory effect, suggesting that the insert domain primarily increases the affinity of Rac-GTP for the NADPH oxidase complex in intact cells, confirming observations from cell-free assays (44,70).

Transient expression of N-terminally truncated, constitutively active Rac-GEF derivatives, Vav1, Vav2 or Tiam1, was sufficient to induce NADPH oxidase activity in COS*phox* cells. Rac activation assays showed that expression of these GEFs activated at most 3% of endogenous Rac1, significantly less than the level of Rac1-GTP detected with transfection of V12 or L61 Rac1 derivatives. This suggests that Rac activation in a physiologically relevant range is sufficient to activate the NADPH oxidase. As a comparison, the chemoattractant fMet-Leu-Phe (fMLP) activates between 2 and 10% of the total Rac in human neutrophils (50,71).

Interestingly, the constitutively active form of the hematopoietic-specific GEF, Vav1, was the most effective at activating superoxide production, although higher levels of Rac1-GTP were detected upon transfection of either Vav2 or Tiam1 derivatives. These results support the emerging concept that GEFs both regulate the activity of small GTPases and link their activation to specific functional responses (72). That different GEFs are not functionally redundant but may help direct small GTPases toward specific downstream signaling pathways may account for the large number of identified GEFs – at least eight activate Rac *in vitro* (72). For example,

Bokoch and colleagues reported that Tiam1 potently activates Pak1 but not Jun N-terminal kinase (JNK) in COS-7 cells, even though both kinases are strongly activated by Rac-GTP (73). Our results are also consistent with evidence suggesting differential regulation of downstream functions by the hematopoietic-specific Vav1 and widely expressed Vav2 isoforms, which are large multi-domain proteins and only 55% homologous (74). In Jurkat T cells, antigen-induced activation of NFAT- or NFκB-dependent transcriptional pathways is strongly potentiated by overexpression of Vav1 but not Vav2 (75,76). Furthermore, studies of Vav-deficient mice indicate that Vav1 and Vav2 differentially regulate various lymphocyte functions (74,77,78). The Vav GEFs are activated by tyrosine phosphorylation and by interaction between their pleckstrin homology (PH) domain and phosphatidyl inositol 3' kinase (PI3K) lipid products (74). Based on observations that fMLP-elicited Rac activation in human neutrophils is sensitive to tyrosine kinase and PI3K inhibition (50,55,71), Vav1 has been proposed to regulate phagocyte NADPH oxidase activation downstream of the Gi-coupled fMLP receptor. Our data support a physiological role for Vav1 versus a subset of other Rac GEFs in promoting NADPH oxidase activation.

In conclusion, our data suggest that Rac can play a dual role in NADPH oxidase activation, both as a direct participant in the oxidase complex and also as an activator of signaling events leading to oxidase assembly. This model is supported by evidence that expression of constitutively active Rac derivatives was sufficient to induce NADPH oxidase activity in COS^{phox} cells, in the absence of any other stimulus. Expression of Rac1V12 drove membrane translocation of p47^{phox} in the absence of p67^{phox} expression, even though Rac-GTP does not directly bind p47^{phox}. Furthermore, a Rac1V12/C40 derivative, which binds

p67*phox* but not CRIB-domain dependent effector proteins, was two-fold less effective than Rac1V12 at activating the NADPH oxidase in COS*phox* cells. Taken together, this data supports a model in which Rac can promote NADPH oxidase assembly through activation of signaling pathways mediated by multiple effectors. In the COS*phox* cells, activation of endogenous Rac1 by transient expression of constitutively active Rac-GEF derivatives was also sufficient to induce robust NADPH oxidase activity. Although higher levels of activated Rac1 were detected upon expression of Vav2 or Tiam1, Vav1 elicited the highest rate of superoxide production, suggesting that hematopoietic-specific Vav1 may more efficiently activate pathways leading to superoxide production. These observations were made in a genetically tractable heterologous cell model, and further studies are underway to determine if similar mechanisms operate in neutrophils.

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Figure Legends

Figure 1 – Superoxide production in COS*phox* cells induced by expression of constitutively

active Rac derivatives. (A) COS^{phox} cells were transiently transfected with either empty pRK5 vector, wt Rac1, Rac1V12, Rac1L61, or Cdc42L61. 0.2µg of each expression construct plus 1.8 µg empty pRK5 vector was transfected per 60mm plate. Twenty-one hours post-transfection, cells were harvested for analysis and tested in the ferricytochrome *c* reduction assay without addition of any stimulus. Data represent mean ± SE, n = 3-10, *Rate of superoxide production was significantly greater than background observed with empty vector. Also, the rate of superoxide production differed significantly between cells expressing Rac1L61 and Rac1V12; ANOVA followed by Student-Newman-Keuls multiple comparisons test, $p<.001$. (B) Whole cell lysates (10µg) were separated by 12% SDS-PAGE, transferred to nitrocellulose and probed with monoclonal Ab to Rac1. Transgenic Rac1L61 migrated more slowly than endogenous Rac1 due to the Myc-epitope tag. Rac1V12 migrated even more slowly due to both Myc- and polyglutamate-epitope tags. Results are representative of at least three independent experiments.

Figure 2 – Effect of p47^{phox} expression on Rac1L61-induced superoxide production in transgenic COS cell lines. A COS cell line which stably expressed gp91^{phox}, p22^{phox} and p67^{phox} was grown in 60mm dishes and transiently transfected with either (0.7 µg Rac1L61 + 1.4 µg empty pRK5), (0.7 µg each Rac1L61, p47^{phox} and pRK5), or (0.7 µg each Rac1L61, p67^{phox} and pRK5). Twenty-one hours post-transfection cells were harvested and tested in the ferricytochrome *c* reduction assay without any stimulus. Data represent mean ± SE, n=3. *Rate of superoxide production differs significantly, two-tailed Student t-test, $p< 0.05$.

Figure 3 – Superoxide production in COS*phox* cells expressing constitutively active Rac effector or insert domain mutants. (A) COS*phox* cells grown in 60mm plates were transiently transfected with 2µg empty pRK5 expression vector or with 1.97 µg empty pRK5 vector + 0.03µg of the indicated Rac derivatives. Twenty-one hours post-transfection cells were harvested and tested in the ferricytochrome *c* reduction assay without addition of any stimulus. Data represent mean ± SE, n=5, *Rate of superoxide production differed significantly from that of Rac1V12-transfected cells, ANOVA followed by Student-Newman-Keuls multiple comparisons test, $p < 0.05$. (B) Whole cell lysates (10µg) from cells transfected as in (A) were separated by 12% SDS-PAGE, transferred to nitrocellulose and probed with monoclonal Rac1 Ab. Transgenic Rac migrated more slowly than endogenous Rac due to the epitope tag. Results are representative of five independent experiments. (C) COS*phox* cells in 60mm plates were transiently transfected with 2µg empty pRK5 expression vector or with the indicated Rac derivatives diluted 1:10 with empty pRK5 vector. Twenty-one hours post-transfection the cells were harvested and tested in the ferricytochrome *c* reduction assay without any stimulus. Data represent mean ± SE, n=4. * Rate of superoxide production differed from that of Rac1V12 transfected cells, ANOVA followed by Student-Newman-Keuls multiple comparisons test, $p < 0.05$. (D) Whole cell lysates from cells transfected as in (C) were separated and transferred to nitrocellulose, as described above, then probed with monoclonal Rac1 Ab. Transgenic Rac migrated more slowly than endogenous Rac1 due to the epitope tag. Results are representative of four independent experiments.

Figure 4. Subcellular fractionation and immunoblot analysis of p47*phox* and p67*phox*

localization in COS^{phox} cells expressing constitutively active Rac1 or Vav1 derivatives. (A) COS^{phox} cells in 100mm plates were transfected with 4µg empty pRK5 vector, or 0.4µg Rac1V12 cDNA plus 3.6µg empty pRK5 vector, or 4µg Vav1ΔN-186 cDNA. Transient transfection efficiency averaged 30%. Twenty-one hours post-transfection cells were harvested by brief trypsinization, disrupted in a Dounce homogenizer, and fractionated over a discontinuous 20%/38% sucrose gradient. Membrane fractions (5×10^5 cell equivalents) and cytosol fractions (6×10^4 cell equivalents) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against NADPH oxidase subunits, p47^{phox}, p67^{phox}, Rac1 and p22^{phox}. Vav1ΔN-186 expression was also confirmed by immunoblotting (not shown). Blots are representative of two independent experiments. (B) The levels of p47^{phox} and p67^{phox} expression in membrane fractions were quantified by densitometric analysis of immunoblots. Data were normalized against p22^{phox} expression and represent the mean of two independent experiments.

Figure 5 – Immunofluorescence and confocal microscopy analysis of p47^{phox} and p67^{phox} localization in transgenic COS cell lines expressing constitutively active Rac1 derivatives. COS cell lines were grown overnight on glass cover slips in 12-well plates, then transiently transfected with 0.9 µg empty pRK5 vector + 0.1µg cDNA (either Rac1V12 or Rac1L61). Twenty-one hours post-transfection cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and blocked with 2% BSA/1% normal goat serum/PBS. Cells were incubated with both mouse monoclonal Ab against Myc-epitope 9E10 (to detect transgenic Rac)

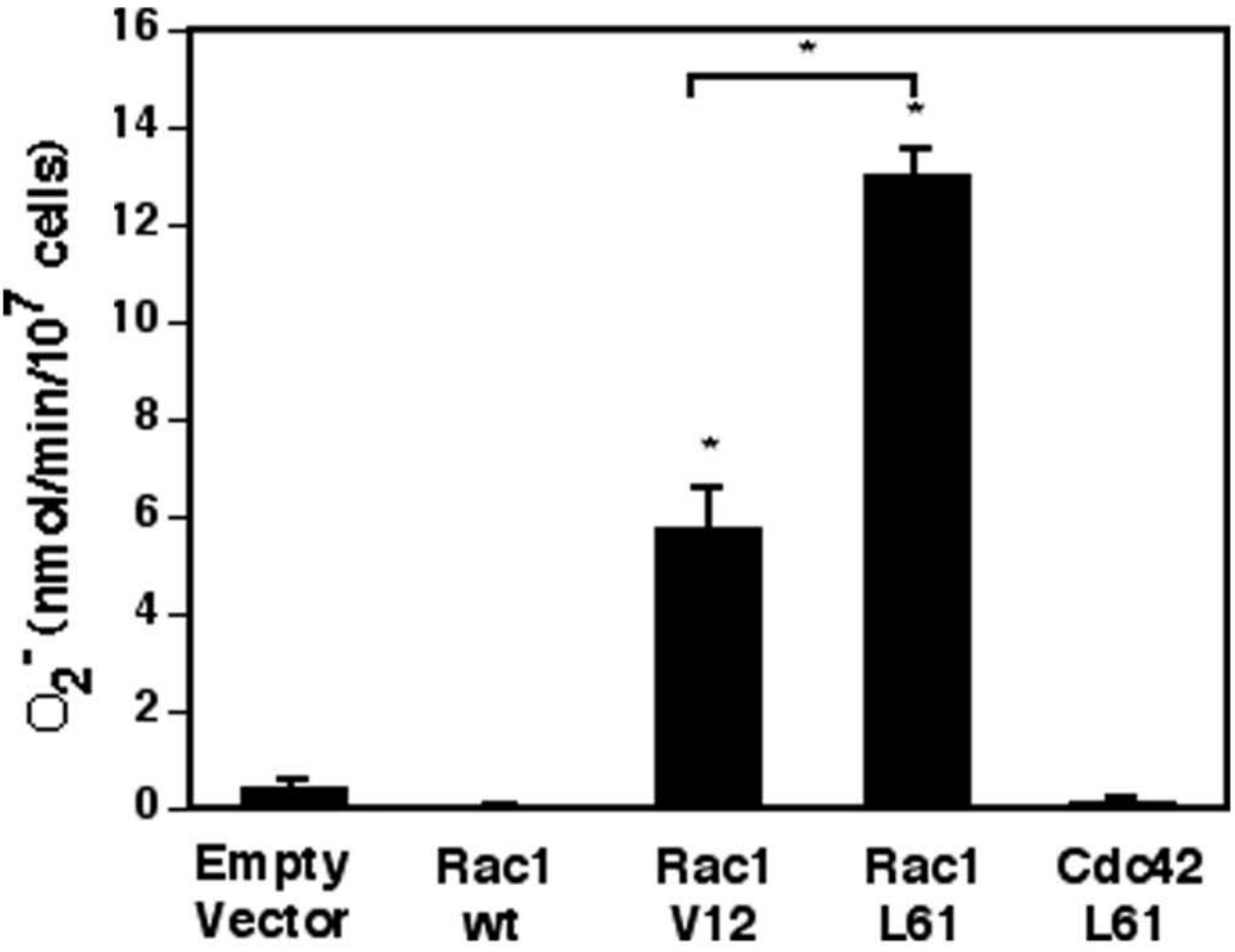
and with rabbit polyclonal Ab against either p47*phox* or p67*phox*. Cells were then washed and incubated with both Cy 3-conjugated goat anti-mouse Ab and FITC-conjugated goat anti-rabbit Ab. Alternatively, cells were incubated with mouse monoclonal Ab against p47*phox* followed by FITC-conjugated goat anti-mouse Ab. Focal planes spaced at 0.4μm intervals were imaged with a Zeiss 510 laser scanning confocal microscope (100X PlanApo 1.4 NA objective) equipped with LSM510 digital imaging software. Three adjacent focal planes were averaged using Metamorph software. Color images were converted to gray-scale using Adobe Photoshop 6.0. The bar in panel H represents 10μm; the field of view is the same size in all panels. (A) p47*phox* localization in empty vector-transfected COS*phox* cells. (B) p67*phox* localization in COS*phox* cells which do not express transgenic Rac. (C & D) p47*phox* and Rac1L61 localization, respectively, both in the same field of view of transfected COS*phox* cells. (E and F) p67*phox* and Rac1L61 localization, respectively, both in the same field of view of transfected COS*phox* cells. Arrows indicate the one cell which expressed Rac1L61 in this field of view. (G) p47*phox* localization in two COS 91,22,47 cells expressing Rac1V12. (H) p67*phox* localization in COS 91,22,67 cells. An asterisk marks the only cell which expressed Rac1L61 in this field of view.

Figure 6 – Superoxide production and Rac activation in COS*phox* cells expressing constitutively active Rac-GEF derivatives. COS*phox* cells were transiently transfected with constructs encoding Vav1ΔN-186, Vav2ΔN-191, Tiam1ΔN-401, or with empty pRK5 expression vector (2μg DNA per 60mm plate). (A) 21 hours post-transfection cells were harvested and tested in the ferricytochrome *c* reduction assay without any stimulus. Data represent mean ± SE, n = 4-7.

*p<.05, **p<.01, rate of superoxide production differs significantly, ANOVA followed by Student-Newman-Keuls multiple comparisons test. (B) Cells were lysed in the presence of PAK1 PBD-GST and incubated with glutathione-Sepharose 4B beads. Beads were pelleted and washed then resuspended in Laemmli buffer. Aliquots of the supernatant containing Rac1-GDP (1×10^5 cell equivalents) and the pulldown containing Rac1-GTP (5×10^6 cell equivalents) were loaded on 12% gels and separated by SDS-PAGE, transferred to nitrocellulose, and probed with Rac1 monoclonal Ab. Results are representative of two independent experiments.

Figure 1

a


16
14
12
10
8
6
4
2
0
O_2^- (nmol/min/10^7 cells)
*
*
*
Empty Vector
Rac1 wt
Rac1 V12
Rac1 L61
Cdc42 L61

b

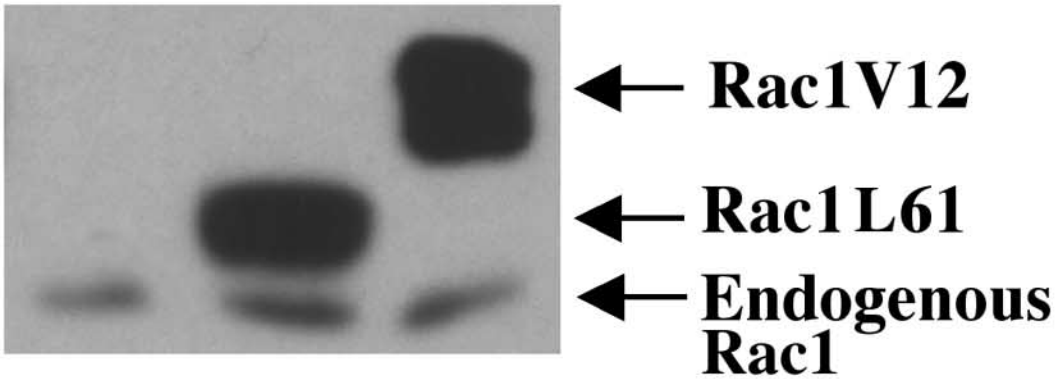

Rac1V12
Rac1 L61
Endogenous Rac1

Figure 2

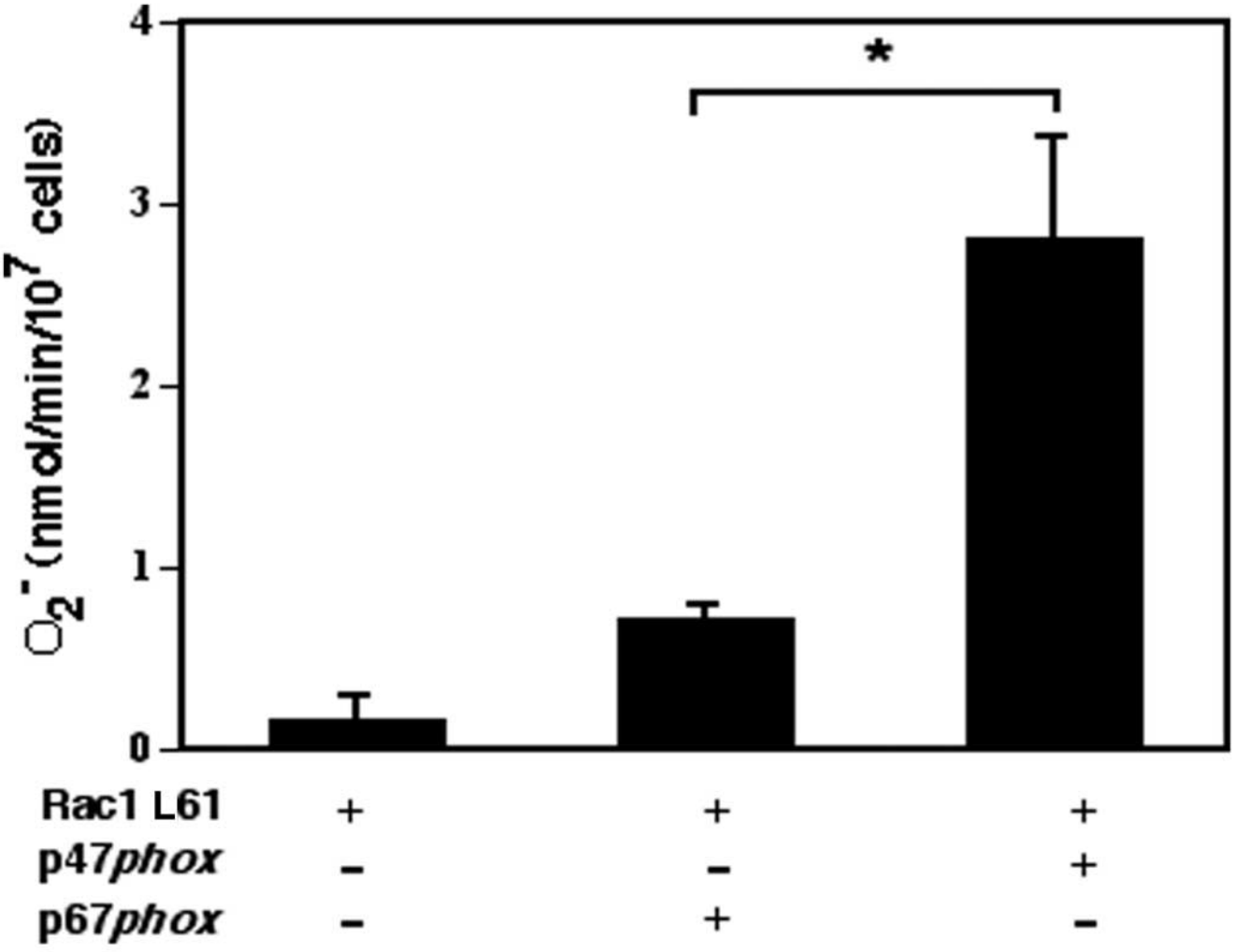


Figure 3

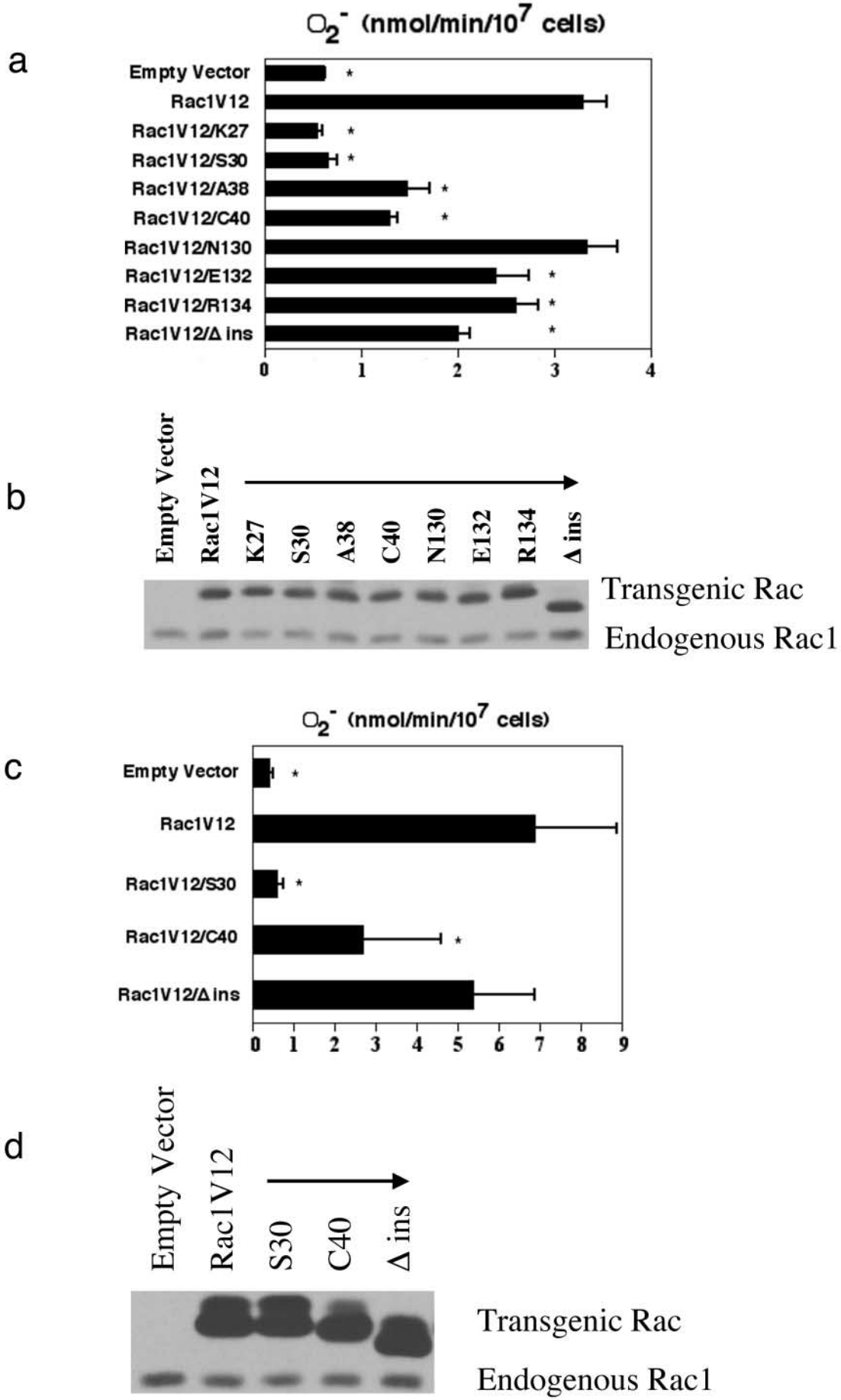
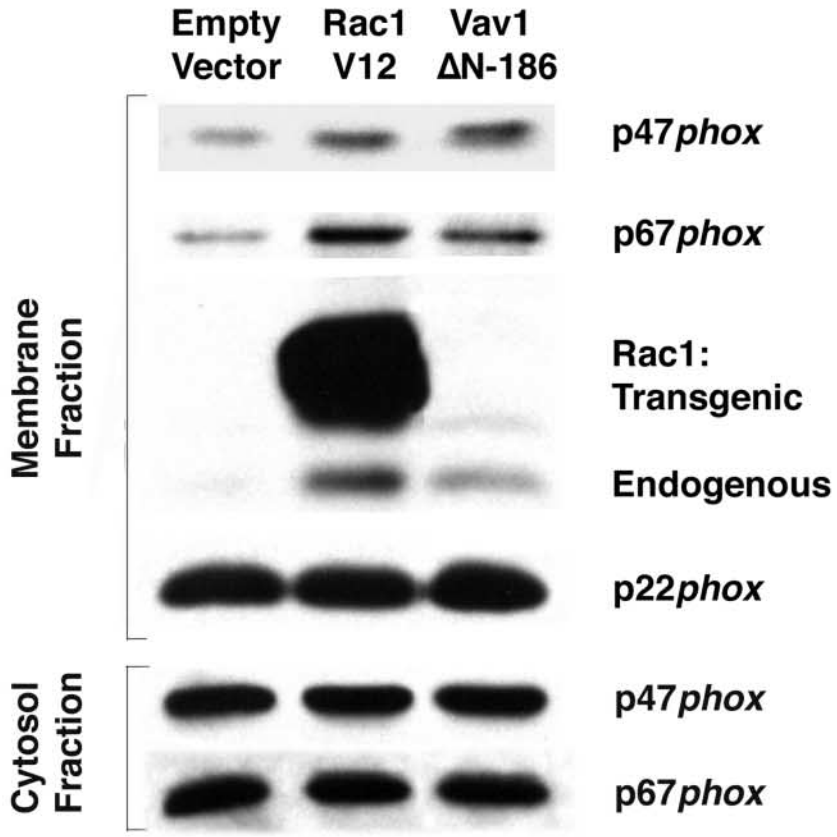

a
O_2^- (nmol/min/10^7 cells)
Empty Vector
Rac1V12
Rac1V12/K27
Rac1V12/S30
Rac1V12/A38
Rac1V12/C40
Rac1V12/N130
Rac1V12/E132
Rac1V12/R134
Rac1V12/Δ ins
0
1
2
3
4
b
Empty Vector
Rac1V12
K27
S30
A38
C40
N130
E132
R134
Δ ins
Transgenic Rac
Endogenous Rac1
c
O_2^- (nmol/min/10^7 cells)
Empty Vector
Rac1V12
Rac1V12/S30
Rac1V12/C40
Rac1V12/Δ ins
0
1
2
3
4
5
6
7
8
9
d
Empty Vector
Rac1V12
S30
C40
Δ ins
Transgenic Rac
Endogenous Rac1

Figure 4

a


Empty Vector
Rac1 V12
Vav1 ΔN-186
Membrane Fraction
Cytosol Fraction
p47phox
p67phox
Rac1: Transgenic
Endogenous
p22phox
p47phox
p67phox

b

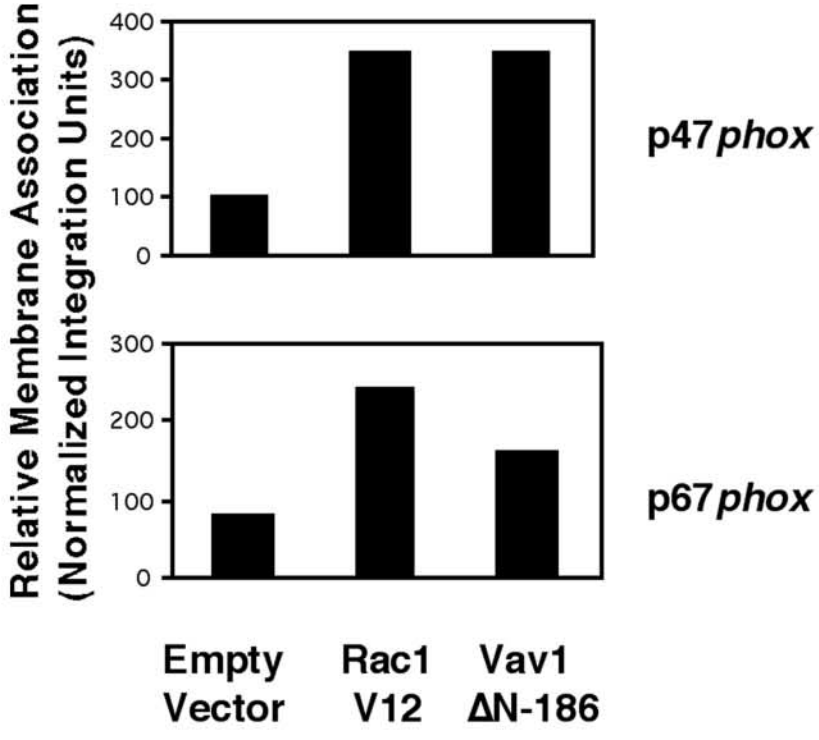

Relative Membrane Association (Normalized Integration Units)
400
300
200
100
0
300
200
100
0
p47phox
p67phox
Empty Vector
Rac1 V12
Vav1 ΔN-186

Fig 5

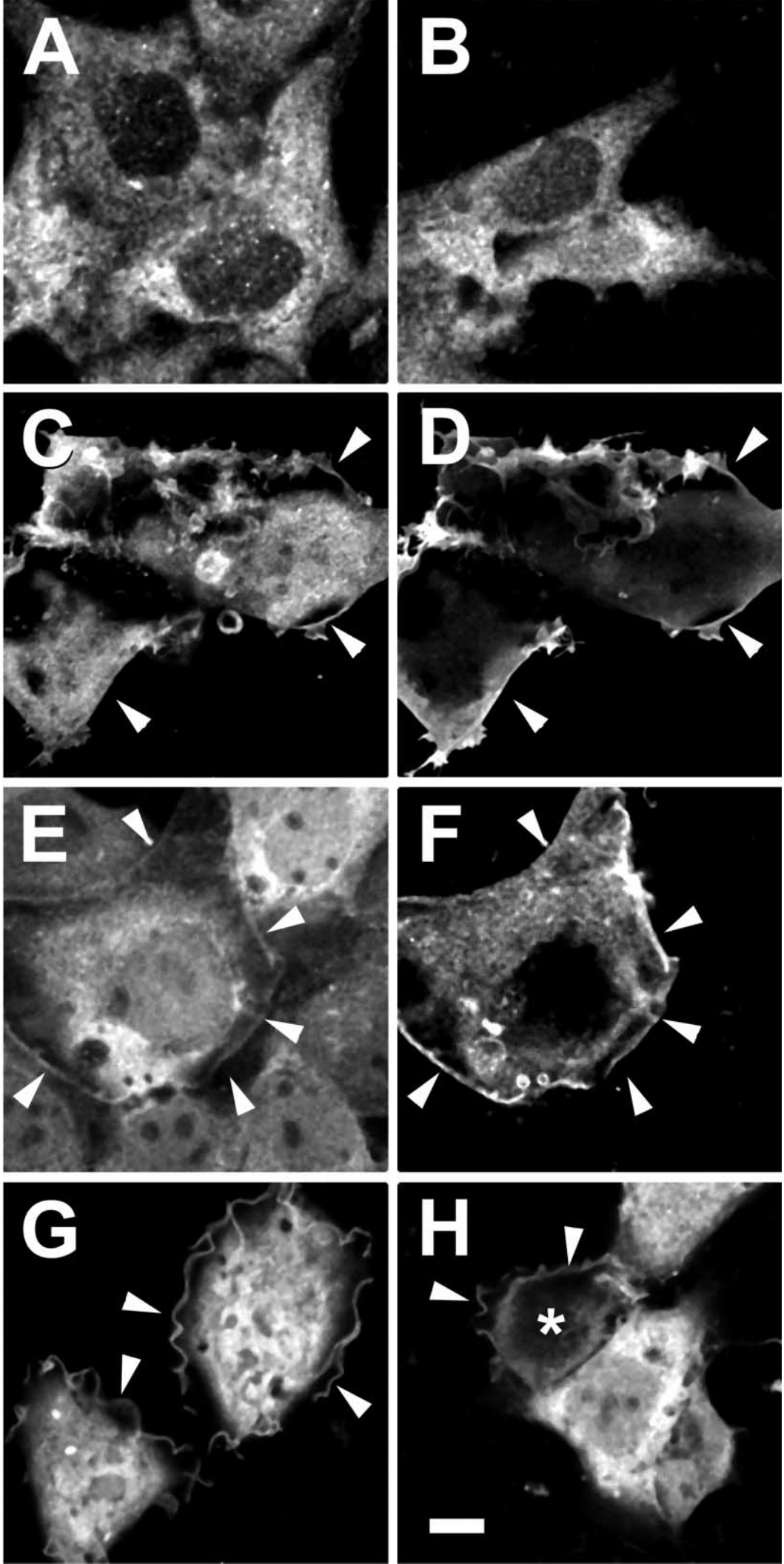
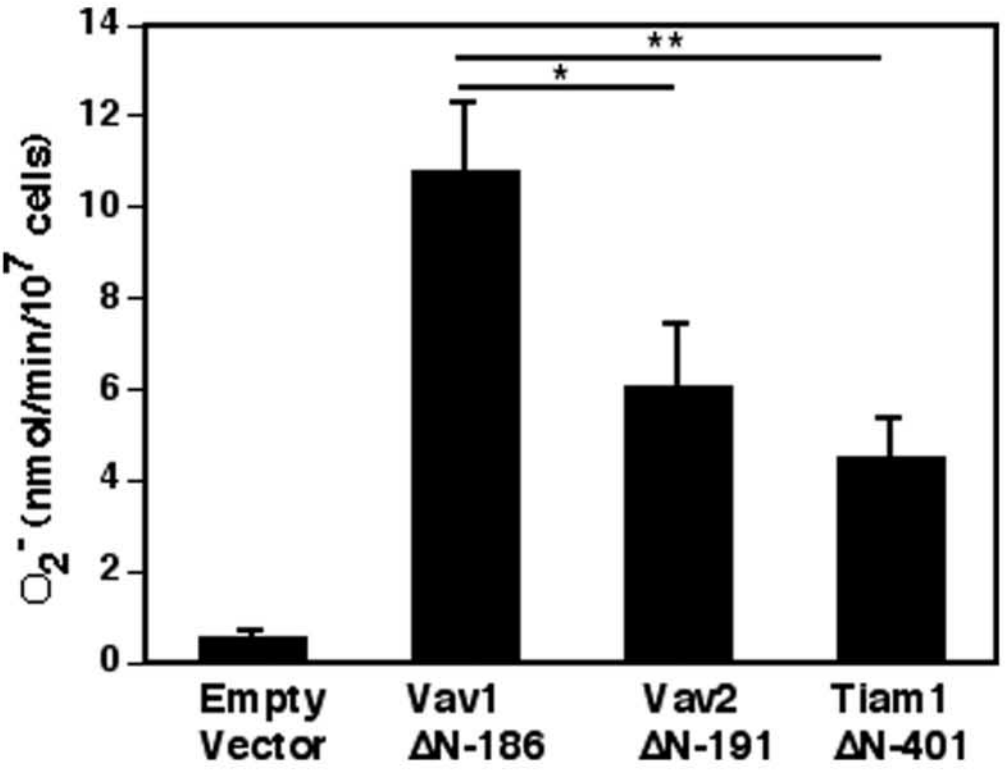
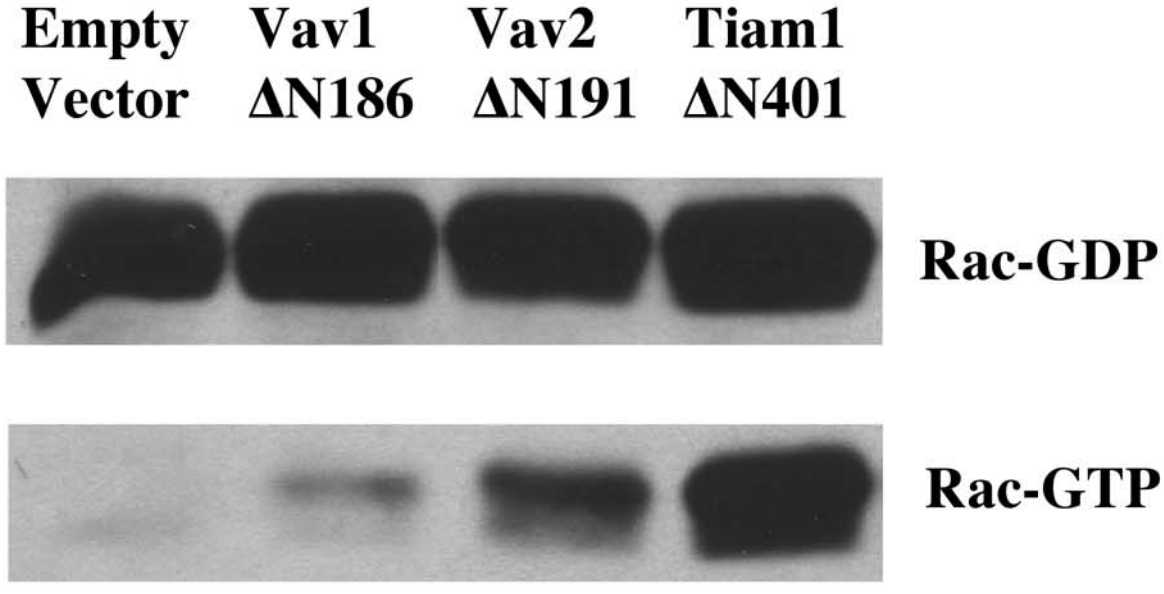


Figure 6

a


14
12
10
8
6
4
2
0
O_2^- (nmol/min/10^7 cells)
**
*
Empty Vector
Vav1 ΔN-186
Vav2 ΔN-191
Tiam1 ΔN-401

b


Empty Vector
Vav1 ΔN186
Vav2 ΔN191
Tiam1 ΔN401
Rac-GDP
Rac-GTP

Antibody: p47^{phox}

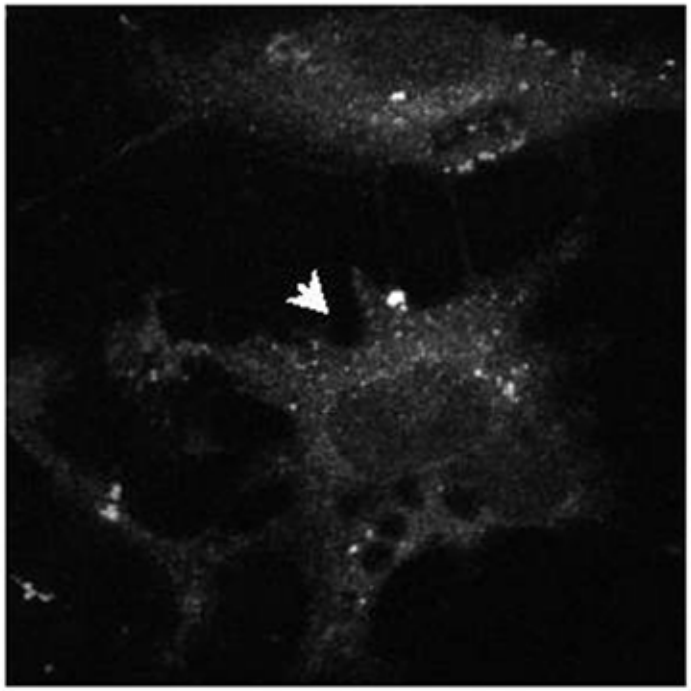


Fig R-5. Background staining from p47^{phox} primary antibody and FITC-conjugated secondary antibody in COS-91,22 cells transfected with Rac1L61. The arrow indicates the Rac-transfected cell. Compare to specific staining of p47^{phox} shown in Figures R-1 and R-2 and to Figures 5C and G in the revised submission.

Antibody: p67^{phox}

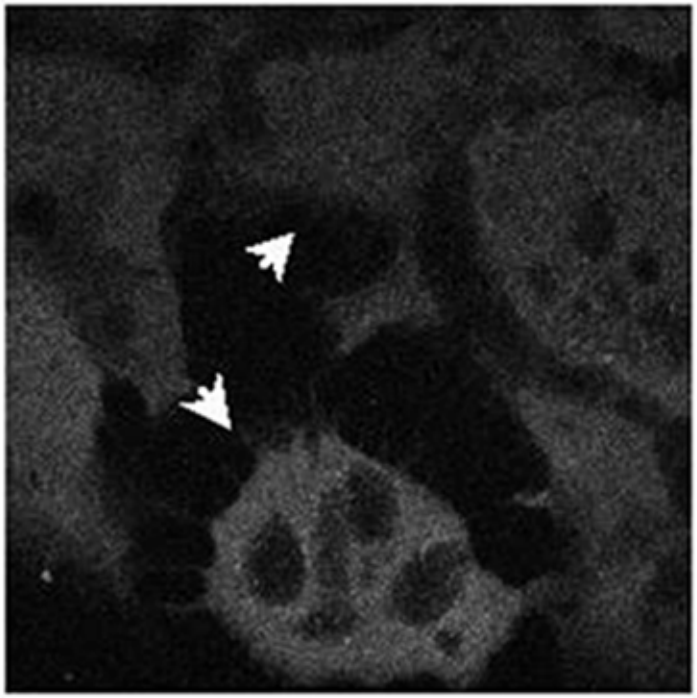


Fig R-6 Background staining from p67^{phox} primary antibody and FITC-conjugated secondary antibody in COS-91,22,47 cells transfected with Rac1V12. Arrows indicate Rac-transfected cells. Compare to specific staining of p67^{phox} shown in Figs 5E and H in the revised submission.