in the left frontal cortex (BA 45/47) and showed greater activation as compared with the control task, in the anterior cingulate cortex (BA 32). During recognition, young people showed greater activation than did old people in right BA 7 (compared with matching) and in BA 19 (compared with results from the control task).
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Regulation of Human Leukocyte p21-Activated Kinases Through G Protein–Coupled Receptors

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The Rac guanosine 5′-triphosphate (GTP)–binding proteins regulate oxidant production by phagocytic leukocytes. Two Ste20-related p21-activated kinases (PAKs) were identified as targets of Rac in human neutrophils. Activity of the ~65- and ~68-kilodalton PAKs was rapidly stimulated by chemoattractants acting through pertussis toxin–sensitive heterotrimeric GTP-binding proteins (G proteins). Native and recombinant PAKs phosphorylated the p47^{phox} reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase component in a Rac-GTP–dependent manner. The action of PAKs during phagocyte activation by G protein–coupled pathways may contribute to regulation of NADPH oxidase activity.

The oxidative burst of human phagocytic leukocytes, which is critical to the inflammatory response, is mediated by a multicomponent NADPH oxidase regulated by the small guanosine triphosphatase (GTPase) Rac2 (*1*). Oxidase activation occurs through chemoattractant receptors coupled to pertussis toxin–sensitive G proteins (*2*). Rac guanine nucleotide exchange factors (*3*) and the Bcr GTPase activating protein (*4*) are important components of the activation process. The molecular details of how Rac regulates NADPH oxidase activity remain to be elucidated. Phosphorylation and dephosphorylation events modulate oxidant production, and NADPH oxidase components are substrates in vivo for unidentified kinases (*5*). p65^{pak}, a mammalian protein kinase related to Ste20 kinase of budding yeast (*6*), binds specifically to the GTP-bound forms of Rac and the related GTPase CDC42 (*7*). Rac-GTP stimulates autophosphorylation of p65^{pak} and its catalytic activity toward exogenous substrates.

Human neutrophil cytosolic fractions screened in overlay assays with the active forms of p21 Rho-related GTP-binding proteins revealed two proteins with apparent molecular sizes of 65 and 68 kD that bound Rac-GTP or CDC42-GTP or both, but not RhoA-GTP (Fig. 1A). Guanosine 5′-diphosphate (GDP)–bound forms of Rac (Fig. 1A) and CDC42 did not interact with these targets but did bind to the 28-kD Rho GDP dissociation inhibitor (RhoGDI). Both *Escherichia coli* and baculovirus Sf9 cell-expressed Rac proteins bound p65 and p68. Neither p65 nor p68 was detected in neutrophil membranes when Rac1-GTP was used as a probe (Fig. 1A).

The binding of these cytosolic proteins specifically to Rac-GTP was confirmed with Rac1 and RhoA GST fusion proteins.

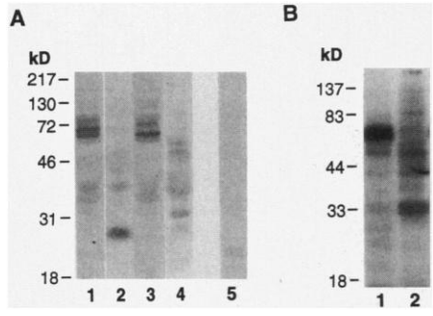

Fig. 1. Detection of kinases binding to activated Rho-family p21s in human neutrophils. (**A**) Nitrocellulose filters containing neutrophil cytosolic and membrane proteins separated by SDS-PAGE were probed with GTP-binding proteins (1 μg/ml) (*18*) isolated as in (*10*, *19*) and labeled with [^{35}S]GTPγS as in (*19*), whereas ^{32}P-labeled GDP forms were prepared by allowing intrinsic hydrolysis of bound [α-^{32}P]GTP to take place. Shown is one of three representative overlays with Rac1-GTPγS (lane 1), Rac1-GDP (lane 2), CDC42-GTPγS (lane 3), and RhoA-GTPγS (lane 4) on cytosolic proteins and Rac1-GTPγS on membrane proteins (lane 5). (**B**) Activated Rac1 (lane 1) and RhoA (lane 2) GST fusion proteins coupled to glutathione-Sepharose beads were incubated with dialyzed neutrophil cytosol for 60 min and washed extensively, and kinase activity was measured (*17*).

Kinase activity assays revealed that both p65 and p68 became phosphorylated when bound to Rac-GTP resin (Fig. 1B). Antibodies to p65^{pak} (PAK1) revealed a 68-kD protein in neutrophil cytosol that comigrated with recombinant human PAK1, whereas antibodies to a homolog that is 79% identical to PAK1 [termed PAK2 (*8*)] detected a 65-kD protein (Fig. 2). The proteins observed by immunoblotting of neutrophil cytosol migrated with the same apparent molecular sizes as the p65 and p68 Rac-binding proteins detected in overlays and were not present in neutrophil membranes. Both p65 and p68 were immunoprecipitated by the respective antibodies to PAK2 and PAK1. Thus, human neutrophils contain kinases closely related or identical to PAK1 and PAK2, and both kinases autophosphorylate when Rac-GTP is present.

Because PAKs are direct targets of Rac, we investigated whether PAKs could be activated under conditions in which Rac activation occurs (9). Proteins from neutrophil lysates stimulated with the chemotactic peptide fMetLeuPhe (fMLP) or phorbol myristate acetate (PMA) were immunoprecipitated with antisera to PAK1 or PAK2 and kinase activity was determined. We observed rapid (30 s to 1 min) and transient

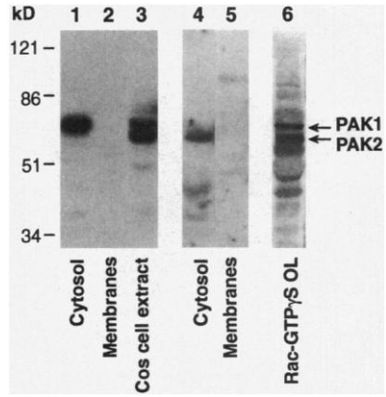

Fig. 2. Detection of p65^{pak}-related kinases in neutrophils by immunoblotting. Cytosol (lanes 1 and 4) and membrane fractions (lanes 2 and 5) of neutrophils transferred onto nitrocellulose were probed with antisera to PAK1 (lanes 1 to 3) and PAK2 (lanes 4 and 5) (*20*). A control lysate of Cos cells overexpressing human PAK1 was used (lane 3); PAK1 partially degraded in this lysate, accounting for the lower band in this lane. The autoradiogram of a Rac1-[^{35}S]GTPγS neutrophil cytosol overlay (OL) (lane 6) is provided for comparison.

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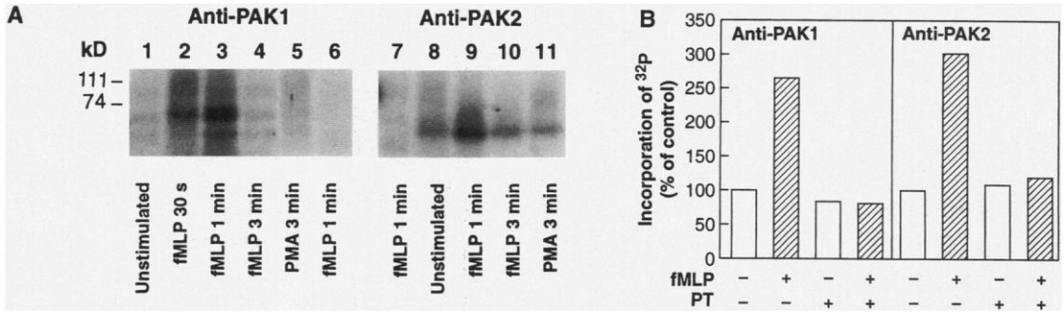

Fig. 3. Activation of PAK kinases in fMLP-stimulated neutrophils. (**A**) Human neutrophils at 1×10^8 cells per milliliter were stimulated with fMLP (1 μM) or PMA (1 μg/ml) at 37°C for the indicated times. Immunoprecipitates from cell lysates with antiserum to PAK1 (anti-PAK1, lanes 1 to 5), antiserum to PAK2 (anti-PAK2, lanes 8 to 11), or preimmune sera (lanes 6 and 7) were analyzed for kinase activation, as detected by PAK autophosphorylation (*21*). The autoradiogram shown is representative of four similar experiments. (**B**) Neutrophils were incubated in the presence or absence of pertussis toxin (PT) (10 μg/ml) for 2 hours before stimulation with fMLP (1 μM) for 1 min. Pertussis toxin treatment inhibited superoxide generation (*15*) by 80 to 90%. Cell lysates were immunoprecipitated as in (A) and assayed for kinase activity with 1 μg myelin basic protein (MBP) as substrate. The incorporation of ^{32}P into MBP was quantitated with the Molecular Dynamics PhosphorImager system. Unstimulated and untreated neutrophils served as the control (100%). The experiment shown is representative of two similar experiments.

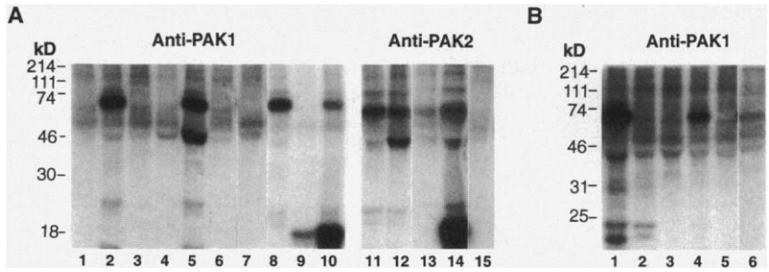

Fig. 4. Activation of PAK kinases in vitro by GTP-binding proteins. Immunoprecipitates with PAK1, PAK2, and preimmune serum were prepared from dialyzed human neutrophil cytosol (*22*) and assayed for kinase activity (*17*) with or without 1 μg of GTP-binding proteins or the substrates MBP (2 μg) and $p47^{phox}$ (1 μg). The autoradiograms shown are representative of two to five independent experiments. (**A**) PAK1 immunoprecipitates were from cytosol alone (lane 1), plus Rac1-GTPγS (lanes 2 and 8), plus 1 mM GTPγS (lane 3), plus $p47^{phox}$ (lane 4), plus $p47^{phox}$ and Rac1-GTPγS (lane 5), plus MBP (lane 9), plus MBP and Rac1-GTPγS (lane 10). Lanes 6 and 7 are control immunoprecipitates with preimmune serum plus Rac1-GTPγS (lane 6) or $p47^{phox}$ plus Rac1-GTPγS (lane 7). PAK2 immunoprecipitates were from cytosol alone (lane 13), plus Rac1-GTPγS (lane 11), plus p47-*phox* and Rac1-GTPγS (lane 12), plus MBP and Rac1-GTPγS (lane 14). Lane 15 shows preimmune serum with Rac1-GTPγS. (**B**) Effect of various Rho-related GTP-binding proteins on PAK autophosphorylation. Shown are PAK1 immunoprecipitates plus Rac1-GTPγS (lane 1), plus Rac1-GDP (lane 2), plus 1 mM GTPγS (lane 3), plus Rac2-GTPγS (lane 4), plus Rac2(D38A)-GTPγS (lane 5), plus RhoA-GTPγS (lane 6).

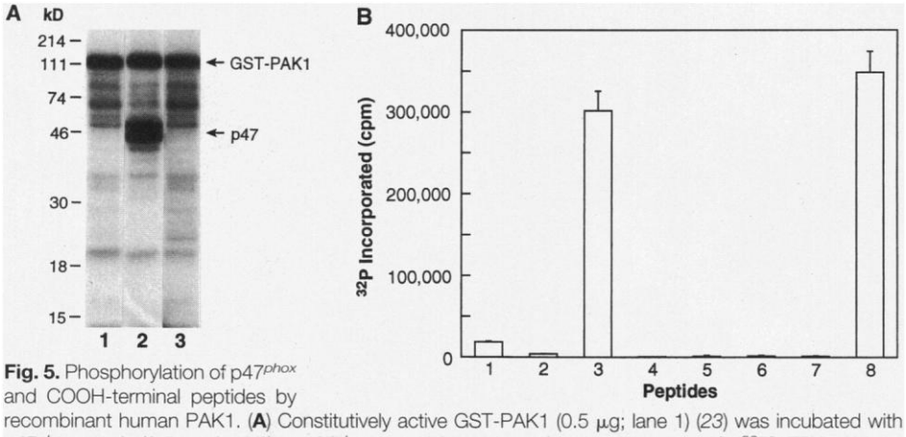

Fig. 5. Phosphorylation of $p47^{phox}$ and COOH-terminal peptides by recombinant human PAK1. (**A**) Constitutively active GST-PAK1 (0.5 μg; lane 1) (*23*) was incubated with $p47^{phox}$ protein (0.5 μg; lane 2) or $p67^{phox}$ protein (1 μg; lane 3) in the presence of [γ-^{32}P]ATP (7 μM; 20 Ci/mmol) in 35 μl of kinase buffer. (**B**) COOH-terminal peptides of $p47^{phox}$ and control peptides (40 μM) were incubated with GST-PAK1 (0.5 μg) as in (A) and assayed for bound radioactivity on phosphocellulose filter units (Pierce). Values, adjusted for autophosphorylation of GST-PAK1, correspond to three independent assays with the standard deviation indicated. Bars 1 to 5 show the $p47^{phox}$ peptides amino acids 300 to 311 (1), 316 to 327 (2), 324 to 331 (3), 336 to 350 (4), and 379 to 390 (5). Control peptides shown are Kemptide (Sigma, bar 6), an epidermal growth factor receptor–derived PKC peptide substrate (bar 7) (*24*), and residues 39 to 50 of histone H4 protein (bar 8).

activation of both PAKs in the fMLP-stimulated neutrophils (Fig. 3A), which is consistent with the rapid translocation of Rac and the rate of superoxide generation observed in response to fMLP (9). Pertussis toxin treatment inhibited fMLP-stimulated PAK activity by 85 to 100% (Fig. 3B). Immunoprecipitates from PMA-stimulated neutrophils at 1-, 3-, 6-, or 10-min time points showed no detectable activation of PAKs (Fig. 3A).

Proteins immunoprecipitated from unstimulated neutrophil cytosol with antisera to PAKs were incubated with activated Rac proteins in the presence of [γ-^{32}P]ATP (adenosine triphosphate), and phosphorylation was examined by autoradiography. Incubation with Rac-GTP resulted in the autophosphorylation of a 68-kD band in anti-PAK1 precipitates and a 65-kD band in anti-PAK2 precipitates (Fig. 4A). Phosphoaminoacid analysis of these bands revealed phosphoserine and phosphothreonine, confirming that the neutrophil PAKs belong to the family of p21-activated serine-threonine kinases. Rac-GDP was inactive, whereas Rac1-GTP and Rac2-GTP both stimulated PAK autophosphorylation (Fig. 4B). A Rac effector domain mutant, Rac2 (D38A), that is not impaired in its GTP-binding abilities but is unable to stimulate superoxide generation (*10*) did not activate PAK1 (Fig. 4B).

Neutrophil PAKs incubated with Rac-GTP had increased activity toward exogenous substrates (Fig. 4A). We tested both recombinant $p47^{phox}$ and $p67^{phox}$ in in vitro kinase assays with immunoprecipitated neutrophil PAK1 and PAK2, and observed $p47^{phox}$ was phosphorylated in the presence of Rac-GTP (Fig. 4A). $p67^{phox}$, actin, RhoGDI, and GDP dissociation stimulator (GDS) were not phosphorylated under these conditions, which indicates that phosphory-

lation was specific. A constitutively active recombinant human PAK1 phosphorylated $p47^{phox}$ and MBP (*11*) in the absence of Rac-GTP, but did not stimulate phosphorylation of $p67^{phox}$ (Fig. 5A). Modification of the NH_2-terminus of PAK may cause a conformational change that activates the kinase catalytic domain.

In vivo phosphorylation of $p47^{phox}$ during neutrophil activation takes place on serine residues (Ser^{303}, Ser^{304}, Ser^{320}, Ser^{328}, Ser^{345}, and Ser^{348}) that are all located in a 14-kD COOH-terminal portion of the protein (*12*). $p47^{phox}$ peptides containing all possible serine phosphorylation sites were tested in kinase assays (Fig. 5B). Substantial incorporation of ^{32}P was only observed with a single peptide containing Ser^{328}. Additional control peptides such as the cyclic adenosine 3′,5′-monophosphate–dependent protein kinase (PKA) substrate Kemptide or a protein kinase C (PKC) peptide substrate were not phosphorylated by PAK. A histone H4 peptide previously shown to be a substrate for an fMLP-stimulated neutrophil kinase (*13*) was phosphorylated to a similar extent as was the $p47^{phox}$ peptide. These peptide data indicate that PAK phosphorylates a physiologically relevant site in $p47^{phox}$; this remains to be confirmed in vivo. The PAKs we have identified have many of the properties of a group of renaturable serine-threonine kinases that participate in NADPH oxidase activation (*14*), including inhibition of PAK activation by phosphatidylinositol 3-kinase inhibitors (*11*). We propose that we have identified the major 63- to 69-kD renaturable neutrophil kinases as PAK1 and PAK2.

We have established that a G protein–coupled receptor can regulate PAKs in mammalian cells. Our findings therefore support a link between the activation of heterotrimeric G proteins and the activity of Rac and related GTPases. Evidence from earlier studies indicates that Rac regulates the NADPH oxidase at the membrane level (*15*), and a direct interaction between Rac and $p67^{phox}$ has been reported (*16*). The present work suggests that PAK-mediated phosphorylation events on $p47^{phox}$ may play an important role in NADPH oxidase regulation.

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20. Noncrossreactive antisera to PAK were raised in rabbits against purified GST (guanosine-S-triphosphate) fusion proteins representing amino acids 175 to 306 of rat $p65^{pak}$ (PAK1) and amino acids 1 to 252 of rat PAK2 (*8*). Amino acid sequence comparison of rat with human PAK1 and PAK2 revealed 98 and 93% identity, which supports the observed cross-reactivity of these antisera with human neutrophil PAKs.
21. Cells were lysed on ice in 50 mM tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1% NP-40, 2.5% glycerol, and 1 mM Na_3VO_4 (pH 7.5), containing the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and pepstatin. Cells were then centrifuged for 10 min at 1000*g*. For immunoprecipitations, incubations with antibody (1:25) were for 2 hours at 4°C, followed by incubation with 60 μl of 1:1 protein A beads for 60 min, then five 1.0 ml lysis buffer washes and two washes with kinase buffer. Kinase assays were as described in (*17*) but with 1.4 μM [γ-^{32}P]ATP (100 Ci/mmol).
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23. The full-length complementary DNA (cDNA) from human PAK1 (*8*) was cloned into pGEX-4T-3 for expression in *E. coli*. The protein was purified by glutathione-Sepharose affinity chromatography, eluted as the intact GST fusion protein, and assessed for kinase activity (*17*). Recombinant PAK1 was 80 to 90% pure as shown by Commassie blue staining.
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Peptide Binding and Presentation by Mouse CD1

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CD1 molecules are distantly related to the major histocompatibility complex (MHC) class I proteins. They are of unknown function. Screening random peptide phage display libraries with soluble empty mouse CD1 (mCD1) identified a peptide binding motif. It consists of three anchor positions occupied by aromatic or bulky hydrophobic amino acids. Equilibrium binding studies demonstrated that mCD1 binds peptides containing the appropriate motif with relatively high affinity. However, in contrast to classical MHC class I molecules, strong binding to mCD1 required relatively long peptides. Peptide-specific, mCD1-restricted T cell responses can be raised, which suggests that the findings are of immunological significance.

CD1 molecules are a heterogeneous family of proteins related to the classical MHC class I proteins both in overall sequence homology and by virtue of their association with β_2-microglobulin (β_2M) (*1*). However, they are as similar to MHC class II as to class I molecules in their α_2 domain, with little apparent similarity to either in the α_1 domain. CD1 molecules are expressed on antigen-presenting cells (*2*) and are recognized by selected T cells (*3*), but an antigen-presenting function for mCD1 (*4*) or its human equivalent hCD1d has not been reported. With less than 40% structural homology in the $\alpha_1\alpha_2$ domain to mCD1 (*5*), hCD1b can present nonpeptidic ligands (mycolic acids) derived from mycobacteria to T cells (*6*), but the structure of

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