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Evidence for a Role for SAM68 in the Responses of Human Neutrophils to Ligation of CD32 and to Monosodium Urate Crystals

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SAM68 (Src-associated in mitosis 68 kDa) is a member of the signal transduction of activator RNA novel gene family coding for proteins postulated to be involved in signal transduction and activation of RNA. It has been implicated through its phosphorylation status in the control of the transition from the G1 to the S phases during mitosis. However, the implication and role of SAM68 in nonproliferative cells are unknown. The present study was initiated to examine the role of SAM68 in the phagocytic responses of the terminally differentiated human neutrophils. The results obtained show that SAM68 is present in human neutrophils and that it is tyrosine phosphorylated in response to stimulation by monosodium urate crystals or by ligation of CD32. Stimulation of neutrophils by these agonists decreases the association of SAM68 with Sepharose-conjugated poly-U beads. Additionally, the amount of immunoprecipitable SAM68 was modulated differentially after stimulation by monosodium urate crystals or by CD32 engagement indicating that the posttranslational modifications and/or protein associations of SAM68 induced by these two agonists differed. The results of this study provide evidence for an involvement of SAM68 in signal transduction by phagocytic agonists in human neutrophils and indicate that SAM68 may play a role in linking the early events of signal transduction to the posttranscriptional modulation of RNA.


The signal transduction of activator RNA (STAR) novel gene family codes for proteins involved in signal transduction and activation of RNA (1, 2). The first STAR member to be characterized was mouse Sam68 (Src-associated in mitosis 68 kDa; Ref. 3). SAM68 may interact with RNA via its central human ribonucleoprotein K homology domain (4–6). Mammalian SAM68 originally was cloned from NIH 3T3 cells transformed by the tyrosine kinase c-Src under the name of GT-Pase-activating protein (GAP)-associated protein p62, because it coimmunoprecipitated with Ras-GAP (6). SAM68 also is thought to participate in the early events of signal transduction by virtue of its five proline-rich regions and its C-terminal tyrosine-rich region. Several lines of evidence now suggest that SAM68 plays a prominent role in signal transduction, in particular via TCR-CD4 stimulation (7–13). SAM68 acts as a downstream target of several tyrosine kinases (Src, Fyn, Lck, Btk, Tec, Zap70, Jak3, and BRK; Refs. 3, 8–10, 12, 14–18, and 76). It has been shown to bind several signaling molecules such as adaptor proteins (Grb2, Grap, and Nck; Refs. 7, 8, 10, and 18–19), phospholipase C (PLC·γ1; Refs. 8, 13, 18, and 20), tyrosine phosphatases (SHP-1; Ref. 8), the phosphatidylinositol-3 kinase regulatory p85 subunit (8, 20), as well as other proteins (Cbl, and p47phox; Refs. 8 and 20). Finally, the association of phosphorylated SAM68 with Ras-GAP implies that it also is involved in regulating the Ras and mitogen-activated protein kinases pathway (9, 12–13, 21–25).

In vitro, the tyrosine phosphorylation of SAM68 decreases its nucleic acid binding properties (26). Recently, it was shown that SAM68 preferentially binds to a UAAA motif (27). The involvement of SAM68 in the regulation of cellular proliferation (3, 14, 17, 19, 28–31) has been suggested, among others, by the observation that the threonine phosphorylation of SAM68 by CDC2 during mitosis permits rapid transition into G1 (31). More recently, SAM68 has been identified as a functional homologue of the HIV-1 Rev protein (32, 33), thereby implicating it in the posttranscriptional regulation of complex retroviruses (34). However, the potential role for SAM68 in nonproliferating cells and during nonmitogenic responses has yet to be investigated in detail.

The ligation of opsonin receptors (and of CD32, in particular) and the addition of monosodium urate (MSU) crystals represent two of the most potent neutrophil agonists. The former are critically involved in the optimization of the phagocytic response of neutrophils (35–37), whereas the latter are the etiological agents of gout, a disease characterized by an acute inflammatory response in which neutrophil recruitment and activation play critical roles (38–40). The signal transduction pathways elicited by these two agonists rely to a major, though only incompletely characterized, extent on the stimulation of tyrosine phosphorylation. It is worthwhile to note that the interaction between MSU crystals and human

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3 Abbreviations in this paper: STAR, signal transduction of activator RNA; SAM68, Src-associated in mitosis 68 kDa; MSU, monosodium urate; GAP, GTPase-activating protein; DFP, di-isopropylfluorophosphate; NP-40, Nonidet P-40; SB, sample buffer; LB, lysis buffer; PVDF, polyvinylidene difluoride; MIP, macrophage inflammatory protein.
neutrophils have recently been shown to be mediated, in part at least, by CD16 and CD11b/CD18 (41).

The aims of the present studies were to investigate the potential involvement of SAM68 in the regulation of the functional responsiveness of human neutrophils. The results obtained establish firstly that SAM68 is expressed in these cells. Stimulation by MSU crystals or by ligation of CD32 leads to its tyrosine phosphorylation. Furthermore, the kinetics of the effects of MSU crystals and of CD32 ligation on the ability to bind to poly-U correlate with the ability of specific Abs to interact with, and immunoprecipitate, SAM68. These results provide direct evidence for an involvement of SAM68 in the responses of human neutrophils to phagocytic agonists and may provide a stimulus-dependent link between the initial events of signal transduction and the regulation of the post-transcriptional modulation of RNA.

Materials and Methods

Antibodies

The anti-SAM68 (sc-333) Ab was purchased from Santa Cruz Biotechnolog- 
y (Santa Cruz, CA); it recognizes an epitope in the C-terminal tyrosine-rich region of SAM68. The anti-phosphotyrosine (UBI 05–321, clone 4G10) and anti-FcγRIII (VIF;RIII, AXL-961 M) Abs were purchased from Upstate Biotechnology (Lake Placid, NY) and Accurate Chemical and Scientific (Westbury, NY), respectively. Anti-FcγRIII Abs (IV.3) were purified from the ascitic fluids of mice inoculated with hybrid-
oma HB 217, which was obtained from the American Type Culture Col-
lection (ATCC, Manassas, VA). F(ab)’2 of Ab IV.3 were prepared essen-
tially as described in the Pierce catalog (Rockford, IL). Briefly, the Abs were digested with pepsin (as pepsin beads) and intact Abs were eliminated by adding protein A and protein G beads. The integrity of the F(ab’)2 was verified by their ability to label intact human neutrophils as determined by flow cytometry.

Reagents

Di-isopropylfluorophosphate (DFP) was purchased from Sigma (St. Louis, MO). Triclinic MSU crystals were kindly provided by Drs. R. de Médicis and A. Lussier (Université de Sherbrooke, Sherbrooke, Quebec, Canada) and prepared as described previously (40). Dextran T-500, Ficoll Paque, Sephadex G-10, protein A Sepharose, and poly-U Sepharose were purch-
ased from Pharmacia Biotech (Dorval, Quebec, Canada).

Cells

Neutrophils were obtained from healthy adult volunteers as described pre-
niously (42) and were resuspended at a concentration of 4 × 10⁵ cells/ml in HBSS containing 1.6 mM calcium and no magnesium, pH 7.4.

Cell stimulation, tyrosine phosphorylation, and immunoblotting

Neutrophil suspensions (4 × 10⁵ cells/ml) were preincubated at room tem-
perature (RT) with 1 mM DFP for 10 min except for Fig. 1, lane 1. Neutro-
phils were stimulated at the same concentration at 37°C with 3 mg/ml of 
MSU crystals for 10 min or for the indicated periods of time. For CD32 stimulation, the cell suspensions were preincubated with 2.5 µg/ml anti-
FγRIII (IV.3) Abs or fragment for 15 min at 4°C and stimulated by cross-
linking the cell-bound Abs with 50 µg/ml or 25 µg/ml F(ab’)2 goat anti-
mouse Fc or Fab Abs (Jackson ImmunoResearch, Mississauga, Ontario, Canada) for 5 min at 37°C or the indicated periods of time. After stimu-
ation, the reactions were stopped by transferring 100 µl of the cell sus-
pensions to an equal volume of boiling 2% Laemmli sample buffer (SB) (1X) is 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% 2-ME, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitrophenylphosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.025% bromophenol blue) and boiled for 7 min. The samples were then subjected to 7.5–20% SDS-PAGE and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Immunoblotting was performed using the 4G10 antiphos-
photyrosine or the anti-SAM68 Abs at a final dilution of 1/4,000 and 
1/1,000, respectively, and revealed by using the renaissance detection sys-
tem (LAS Life Science, Boston, MA) and HRP-conjugated secondary anti-
mouse or anti-rabbit Abs (Jackson ImmunoResearch) at a dilution of 
1/20,000, as described previously (43).

Immunoprecipitation under denaturing conditions

Neutrophils were preincubated and stimulated as described above. Aliquots (500 µl) of the cells were lysed by direct transfer to an equal volume of
boiling 2× lysis buffer (LB) (1X is 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 
1.5% 2-ME, 8.5% glycerol, 2.5 mM orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.025% bromophenol blue) and boiled for 7 min. Immuno-
precipitations were performed as described previously (44). Briefly, the 
lysates were filtered through Sephadex G-10 columns to remove the de-
naturing agents. The filtered lysates were preincubated with protein A-Se-
parose at 4°C for 30 min in the presence of 1% Nonidet P-40 (NP-40), 2 mM orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin. The samples then were immunoprecipitated using 5 µg of anti-SAM68 for 90 min at 4°C on 
the indicated platform. Fifty microliters (30%) slurry of A-Sepharose 
A then was added and the samples were incubated for 1 h at 4°C with constant end-
der-over mixing. The beads were collected and washed four times with LB containing 137 mM NaCl, 1% NP-40 but no SDS, 2-ME, or bromophenol blue. SB (40 µl, 2X) was added to the beads, which were boiled for 7 min. The proteins in the supernatants then were separated by electrophoresis as described above.

The membranes first were blotted with the anti-phosphotyrosine Ab and then incubated for 30 min at 56°C in stripping buffer (1% SDS, 50 mM 2-ME, 31.25 mM Tris-base, pH 6.7). The mem-

Neutrophils were then immunoblotted with the anti-SAM68 Abs.

Nucleic acid binding and immunoprecipitation under nondenaturing conditions

Neutrophils were preincubated and stimulated as described above. After stimulation, the cells were transferred in precooled Eppendorf and rapidly centrifuged, the supernatants were removed, and the cells pellet were re-
purified and incubated for 5 min at 4 × 10⁵ cells/ml at 4°C in a hypo-
tonic LB (final concentrations NP-40 0.1%, Tris-HCl pH 7.5 20 mM, NaCl 10 mM, EDTA 1 mM, orthovanadate 2 mM, aprotinin 10 µg/ml, leupeptin 10 µg/ml, PMSF 2 mM, trypsin inhibitor soybean 50 µg/ml, and DFP 3 

mM). The lysates were centrifuged at 600 × g for 10 min at 4°C. The concentrations of the NaCl and NP-40 were readjusted to 137 mM and 1%, respectively, in the solution fraction. Briefly, for the immunoprecipitations under native conditions, 800 µl of supernatants were used. The samples were immunoprecipitated with 5 µg of anti-SAM68 for 90 min at 4°C or 
60 µl protein A-Sepharose coated with antiphosphotyrosine (4G10) for 3 h at 
4°C on a rotator platform. Fifty microliters (30% slurry) of protein A 
then was added to the anti-SAM68 Abs precipitates and the samples were incubated for 1 h at 4°C with constant end-over-end mixing. The beads were collected and washed four times with an isotonic LB (final concen-
trations NP-40 1%, Tris-HCl pH 7.5 20 mM, NaCl 137 mM, EDTA 1 mM, 
orthovanadate 2 mM, aprotinin 10 µg/ml, leupeptin 10 µg/ml, PMSF 2 mM, 
trypsin inhibitor soybean 50 µg/ml). The nucleic acid binding affinity 
assays were conducted with up to 200 µl of supernatants (8 × 10⁵ cells). The lysates were diluted 1:4 in the isotonic LB described above. The 
lysates were incubated with 50 µl of polyclonal Sepharose-conjugated beads 
for 60 min at 4°C with constant end-over-end mixing. After washing of 
the precipitates with the isotonic LB, SB (40 µl, 2X) was added to the 
beads, which were boiled for 7 min. The samples then were electrophoresed as described above. The specificity of the interaction between SAM68 and 
poly-U beads was determined by preincubating lysates with Sepharose 
beads alone. The membranes were blotted with anti-phosphotyrosine or the 
anti-SAM68 Abs. This lysis procedure was required to exclude nuclear 
SAM68 from the precipitation protocol and also to establish the existence 
of a nonnuclear fraction of SAM68 in human neutrophils. Immunoblotting 
of soluble (nuclear enriched in cytosol, granules, and membranes mark-
ers) or insoluble (nuclear enriched in endosomes, nuclear, cytosquelette 
markers) fractions with nuclear marker Abs such as anti-p62 nucleoporin 
was performed on several lysis protocols and showed that the nuclear 
markers were absent from the soluble fraction (data not shown). Nuclear 
identity also was verified by microscopy.

Statistical analysis

The data were analyzed by using the Wilcoxon signed rank test and 
significance was considered to be attained when p < 0.05.

Results

Presence of SAM68 in human neutrophils

SAM68 is known principally for its implication in the progression through 
the cell cycle, and its role, or even presence, in nondividing 
cells has not been characterized adequately as of yet. The pres-
ence of SAM68 in human neutrophils and its sensitivity to 
proteolytic degradation in cell lysates, a major experimental concern 
in these cells were first examined by immunoblotting samples of 
neutrophil lysates prepared from cells untreated (lane 1) or treated 
(lane 2) with 1 mM DFP (Fig. 1). The lysates were prepared by
Precipitated by addition of Sepharose-protein A beads. As shown, the lysates were incubated with anti-SAM68 Abs (pre-neutralized or not with the immunizing peptide) and the Abs were used either as is or after their preneutralization with the anti-SAM68 Ab. The data shown are representative of at least three independent experiments.

Tyrosine phosphorylation of SAM68 in response to phagocytic agonists

Neutrophils were stimulated by ligation of FcγRIIA (CD32) because of the relevance of this receptor to phagocytosis and because of the known dependence of its signaling mechanisms on the tyrosine phosphorylation pathways (45, 46). After a 10-min DFP pretreatment, neutrophils were stimulated by cross-linking CD32 and the reactions were stopped by transfer of 500 μl of cell suspensions in an equal volume of 2× boiling LB. The denaturing and reducing agents were removed as described in Materials and Methods, the lysates were incubated with anti-SAM68 Abs (pre-neutralized or not with the immunizing peptide) and the Abs were precipitated by addition of Sepharose-protein A beads. As shown in Fig. 2A, ligation of FcγRIIA increased the level of tyrosine phosphorylation of SAM68. Reprobting the membrane with anti-SAM68 Abs demonstrated that equivalent amounts of SAM68 were present in the precipitates of control or stimulated cells (Fig. 2B). The specificity of the detection of SAM68 was established by showing that preneutralization of the Ab with the immunizing peptide inhibited the precipitation of SAM68 and, consequently, its detection with anti-phosphotyrosine or SAM68 Abs. Having observed that ligation of one of the major opsonin receptor (CD32) led to the tyrosine phosphorylation of SAM68, we examined next whether a phagocytic particle the effects of which on the tyrosine phosphorylation patterns in human neutrophils are also well characterized, namely, MSU crystals (40, 42, 45, 47), induced a similar response. The choice of MSU crystals also was motivated by the fact that the pattern of tyrosine phosphorylation they induce includes prominent bands in the 60- to 70-kDa molecular mass region (40, 47). The results of an experiment in which a time-course of stimulation by MSU crystals was monitored are illustrated in Fig. 3. Neutrophils were stimulated by MSU crystals for the indicated times and the denaturated lysates were prepared as described above and in Materials and Methods and immunoprecipitated with anti-SAM68 Abs. Alternatively, precipitation with anti-phosphotyrosine Abs and blotting with the anti-SAM68 Abs was also conducted (data not shown). Both procedures revealed that MSU crystals induced a transient tyrosine phosphorylation of SAM68 that peaked between 5 and 15 min after the addition of the crystals. Recently, it has been shown that the signal transduction pathways initiated by MSU crystals in human neutrophils rely on FcγRIIB (CD16) and CD11b (41). In the next series of experiments, we examined whether the effects of MSU crystals on the tyrosine phosphorylation of SAM68 exhibited a similar dependence. After a DFP pretreatment, the cells were incubated with or without anti-FcγRIIB Abs (VIIcRIIIB) for 1 min at 37°C and then stimulated by the addition of MSU crystals (3 mg/ml, 10 min at 37°C). The cells were lysed under denaturing conditions as described in Materials and Methods and processed for immunoprecipitation with anti-SAM68 Abs and immunoblotting with anti-phosphotyrosine Abs. The results illustrated in Fig. 4 demonstrated that the anti-CD16 Abs (VIIcRIIIB) drastically reduced the tyrosine phosphorylation of SAM68 induced by MSU crystals (compare lanes 2 and 4, Fig. 4A) without affecting the efficiency of the immunoprecipitation (Fig. 4B).
Abs (B). The data shown are representative of two independent conditions and processed for immunoprecipitation (IP) with anti-SAM68 crystals (3 mg/ml, 10 min at 37°C). The cells were lysed under denaturing conditions and processed for immunoprecipitation (IP) with anti-SAM68 Abs and immunoblotting (blot) with an anti-phospho-tyrosine (anti-pY) Ab (A). The same membrane was reprobed after stripping with anti-SAM68 Abs (B). The data shown are representative of two independent experiments.

Detection and preservation of SAM68 in native cell lysates
Neutrophil lysates were prepared next by using a hypotonic LB as described in Materials and Methods. Preliminary experiments established that SAM68 as well as nucleus integrity and the tyrosine phosphorylation profiles were better preserved in the hypotonic LB (data not shown) than in the more traditional isotonic LB (44). Thus, this LB was used in the next series of experiments. The

FIGURE 4. Inhibition of the tyrosine phosphorylation of SAM68 induced by MSU crystals by an anti-FcγRIIIb Ab. After DFP pretreatment, the cells (4 × 10^7/ml) were incubated with or without an anti-FcγRIIIb Ab (VIFcγRIII, 25 μg/ml) for 1 min at 37°C and then stimulated with MSU crystals (3 mg/ml, 10 min at 37°C). The cells were lysed under denaturing conditions and processed for immunoprecipitation (IP) with anti-SAM68 Abs and immunoblotting (blot) with an anti-phospho-tyrosine (anti-pY) Ab (A). The same membrane was reprobed after stripping with anti-SAM68 Abs (B). The data shown are representative of two independent experiments.

A, IP: Anti-SAM68
Blot: Anti-pY

[FIGURE 4]

MSU [3 mg/mL] Anti-FCγRIII [25 μg/mL]

[FIGURE 4] (Ref. 6; data not shown), and

Correlation with the tyrosine phosphorylation of SAM68 under native conditions
The results of the experiments described above indicate that the stimulation of human neutrophils by MSU crystals or by ligation of CD32 leads, albeit with different kinetics, to changes in the presence and integrity of SAM68 in the soluble and in the insoluble fractions of the cells derived from this lysis protocol were analyzed by immunoblotting (Fig. 5A, lanes 3–6). It first should be noted that, as shown in Fig. 1, optimal preservation of SAM68 in the whole-cell lysates (Fig. 5A, lanes 1 and 2) required a preincubation with the protease inhibitor DFP despite the fact that these samples were prepared by direct transfer of cell aliquots into boiling SB. Practically no SAM68 was detectable in the soluble fractions of cell lysates in the absence of DFP in the LB even if the cells were pretreated with the latter (lane 3). Although SAM68 was present in the insoluble fraction recovered under these conditions, evidence of significant proteolysis was observed in this fraction (lane 5). Addition of DFP to the LB allowed the detection of SAM68 in the soluble fractions (lane 4) and significantly reduced the amount of degradation in the insoluble fractions (lane 6). Similar results were obtained by using another protease inhibitor, Pe-fabloc (Boehringer Mannheim, Laval, Québec, Canada), instead of DFP (data not shown).

The distribution of SAM68 in the soluble and insoluble fractions in resting cells and in cells stimulated by MSU crystals was examined next. As illustrated in Fig. 5B, SAM68 was detected under both conditions in the two fractions, and no significant changes in the distribution of SAM68 could be detected in the fractions derived from cells stimulated by MSU crystals. However, slight increases in the amount of anti-SAM68-reactive material were sometimes observed in the soluble fraction of stimulated cells, particularly when cells were lysed by using the isotonic LB (data not shown). It should be pointed out that the addition of Mg^{2+} to the LB affected the distribution of SAM68 and increased the amount detected in the soluble fraction (data not shown). This observation correlated with an increase in tyrosine phosphorylation of SAM68 induced by Mg^{2+} (Ref. 6; data not shown), and with the previously reported decreased affinity of SAM68 for RNA subsequent to its tyrosine phosphorylation (26).

Nucleic acid binding ability of SAM68
To examine whether a link exists between the participation of SAM68 in the early events of signal transduction and its ability to interact with RNAs in response to physiological agonists, we evaluated the affinity of nonnuclear SAM68 for poly-U beads in resting and stimulated cells. Neutrophils were incubated with MSU crystals for various times and lysed in the hypotonic buffer described in Fig. 5 and in Materials and Methods. The soluble fraction (non-nuclear) from the equivalent of 8 × 10^6 cells (200 μl) was incubated with Sepharose-conjugated poly-U beads. The precipitates obtained were analyzed by immunoblotting with the anti-SAM68 Ab. A densitometric analysis of the amounts of SAM68 recovered in the poly-U precipitates is illustrated in Fig. 6. These data show that the stimulation of the cells with MSU crystals led to a monotonic time-dependent decrease of the affinity of SAM68 for poly-U beads. A similar analysis of the affinity of SAM68 for poly-U beads was conducted following the ligation of CD32. The results of these experiments showed that although a decrease in the amounts of SAM68 bound to poly-U beads also was observed on ligation of CD32, the kinetics of this effect were much more transient than those following the addition of MSU crystals, with a significant but short-lived decrease observed at 1 min (data not shown).

Correlation with the tyrosine phosphorylation of SAM68 under native conditions
The results of the experiments described above indicate that the stimulation of human neutrophils by MSU crystals or by ligation of CD32 leads, albeit with different kinetics, to changes in the
ability of nonnuclear SAM68 to bind to poly-U beads. However, this type of experiment does not provide any direct data on any stimulated changes in the physical conformation or the state of association of SAM68 with potential protein partners that may have taken place inside the intact cells. To address this point, soluble fraction from resting and stimulated cells were prepared under native conditions as described in Materials and Methods. These fractions then were used in immunoprecipitation protocols using anti-SAM68 or anti-phosphotyrosine Abs. Additionally, the affinity of SAM68 in these lysates for poly-U beads also was evaluated.

In the first phase of these studies, phosphotyrosine-containing proteins first were precipitated from the lysates prepared under native conditions using an anti-phosphotyrosine Ab. The precipitates then were blotted using the anti-SAM68 Abs. The results of these experiments are illustrated in Fig. 7A. Although MSU crystals increase the level of tyrosine phosphorylation of SAM68 (as monitored under denaturing conditions; Figs. 3 and 4), decreased amounts of SAM68 were recovered in the anti-phosphotyrosine precipitates derived from native lysates (Fig. 7A, left column). It should be pointed out that the immunoblot shown on the left side of Fig. 7A was intentionally overexposed (as compared with that on the right side) to maximize the sensitivity of the detection in the samples treated with MSU crystals. This accounts for the visual differences in the amounts of SAM68 detected in the control lanes on the sides of this panel. It should be pointed out that the immunoprecipitation with anti-phosphotyrosine Abs of SAM68 in the unstimulated cells is not direct proof of the tyrosine phosphorylation of SAM68, as it may coprecipitate in association with other, unidentified, tyrosine phosphorylated protein. In contrast, ligation of CD32 led to an increase in the amounts of SAM68 precipitated with anti-phosphotyrosine Abs (Fig. 7A, right column).

Similar results were obtained when SAM68 was directly precipitated from the native lysates (Fig. 7B). Stimulation by MSU crystals resulted in a decrease in precipitation of tyrosine phosphorylated SAM68, whereas cross-linking of CD32 led to a marked increase of precipitable tyrosine phosphorylated SAM68. A reblot of the SAM68 precipitates indicates that equivalent amounts of SAM68 were loaded onto each lane in the case of stimulation by CD32. However, slightly decreased amounts of SAM68 were precipitated from MSU crystal-stimulated cells.

Despite the marked differences in immunoreactivity of SAM68 derived from MSU crystals or CD32 stimulated cells, a similar decreased affinity of SAM68 for poly-U beads on stimulation by MSU crystals or by ligation of CD32 was observed in these experiments (Fig. 7C). The reduction in the level of SAM68 in the anti-phosphotyrosine or anti-SAM68 immunoprecipitates (Fig. 7, A and B) did not correspond to a loss of the protein because immunoblotting of the supernatants using the anti-SAM68 Abs showed equal quantities of SAM68 (Fig. 5). SAM68 was also preserved in the soluble fraction after a 3-h incubation at 4°C (data not shown).

Discussion
The results of the present study demonstrate firstly that SAM68 is expressed and present in both the nonnuclear and nuclear fractions of human neutrophils and, secondly, that it is a novel tyrosine...
kinase substrate that is phosphorylated in response to the activation of opsonin receptors such as CD16 and CD11b/CD18 (stimulation by MSU crystals; Ref. 41) and CD32. Furthermore, these investigations provide strong evidence that the posttranslational modifications of SAM68 in response to stimulation by MSU crystals and to cross-linking of the CD32 result in significant differences in the immunoreactivity of SAM68. Finally, the ability of SAM68 to bind to poly-U beads (and by extrapolation, its affinity for RNA) also was modulated by these two agonists. Thus, the results of this study provide a link, in the physiological setting of an intact native cell, between the two previously postulated sites of action of SAM68, namely regulation of RNA function and role in the early events of signal transduction.

The successful completion of these studies required the development of several experimental modifications of standard protocols. The extreme lability of SAM68 in neutrophil lysates first should be mentioned, and extreme precautions (including the addition of DFP in preincubation steps as well as in the LB and the direct transfer of the cell aliquots to boiling SB) to limit proteolysis needed to be exercised to adequately and reproducibly preserve this protein. Secondly, the results obtained clearly demonstrate that the immunoreactivity of SAM68 in lysates derived under native conditions differs depending on the agonists used to stimulate the cells.

At present, the subcellular localization of SAM68 is a subject of considerable debate. It originally was described as a nuclear marker in HL-60, and BaF3 cell lines (19). However, poliovirus infection of HeLa cells has been shown to lead to the relocalization of wild-type SAM68 from the nucleus to the cytoplasm (48). In addition, in 293T transfected cells with the SAM68 (P439R) mutant, the resultant SAM68 heterodimers were retained in the cytoplasm (33). Furthermore, SAM68 has been shown to interact with several cytosolic proteins in T cell lines (7–13). Finally, the association of SAM68 with the activated tyrosine kinase Src in mitotic cells has been ascribed to nuclear envelope breakdown (31). The solubilization data shown in Fig. 5 demonstrate that once proteolysis is prevented, SAM68 is present in both the soluble (nonnuclear) and insoluble (nuclear) fractions of resting human neutrophils.

Our experimental conditions allowed us to detect SAM68, not only and as expected in the presumably nuclear fraction, but also in the soluble (presumably nonnuclear) fraction of human neutrophils where it would need to be located to participate in the early events of signal transduction. Additionally, SAM68 also was detected in enucleated neutrophil cytoplasts (data not shown). The relative amounts of soluble and nuclear SAM68 did not change after stimulation of the cells by either MSU (Fig. 5B) crystals or CD32 ligation (data not shown).

In contrast, stimulation by both MSU crystals and CD32 cross-linking led to the tyrosine phosphorylation of SAM68. It is worth noting that the stimulated tyrosine phosphorylation of SAM68 was transient and therefore is likely to involve both tyrosine kinases and phosphatases. Although the specific enzymes involved in this response remain to be identified, several lines of evidence provide initial clues for further investigations. SAM68 originally was described as a Src substrate (3). Interactions of SAM68 with several kinases, including Btk (17), Itk (16), Fyn (8, 18, 49), Lck, and Zap70 (10) have been described. Preliminary experiments also indicate that SAM68 is a substrate for Syk, a kinase intimately involved in the FcγR-mediated signaling pathways (50–53) and that the Syk inhibitor piceatannol (54, 55) interferes with the stimulated changes of the affinity of SAM68 with poly-U beads after stimulation with MSU crystals (data not shown). Additionally, an association between SAM68 and the tyrosine phosphatase SHP-1 (8) also has been described and may play a role in the dephosphorylation of SAM68.

No data are currently available directly linking SAM68 to the regulation of gene expression in neutrophils despite the well-described ability of this cell to synthesize a large variety of proteins on stimulation by several agonists (56–64). As a first approach to this question, we have examined whether SAM68 in these nondividing cells maintained its RNA binding activity, and we used agonists that influence the synthesis and secretion of cytokines and chemokines, namely CD32 ligation and addition of MSU crystals. We adapted a semiquantitative assay of binding of SAM68 to RNA (26) based on its ability to interact with Sepharose-coated poly-U beads. This assay allowed us to determine that SAM68 present in the nonnuclear or soluble fraction of human neutrophils interacts with RNA and that this interaction was modulated on stimulation of the cells with MSU crystals or CD32 engagement. It should be pointed out that the tyrosine phosphorylation data presented in Figs. 2–4 are derived from whole-cell lysates, whereas the poly-U binding and the native immunoprecipitate data (Figs. 5–7) represent the behavior of the fraction of SAM68 present in the soluble fraction of the cells. Therefore, it is difficult at this stage to compare the kinetics of the effects of the agonists studied on these two parameters related to the behavior of SAM68 within the context of an intact, unengineered cell such as peripheral blood neutrophils.

The decreased ability of SAM68 to bind poly-U after stimulation of the cells by MSU crystals also corresponded to its lack of immunoreactivity under native conditions with the anti-SAM68 and anti-phosphotyrosine Abs. It is important to point out that decrease in immunoreactivity was transient and thus cannot be explained on the basis of a degradation of the protein. These results are more likely to be attributable to stimulated association of SAM68 with other proteins. Because the anti-SAM68 Abs recognize epitopes located in the tyrosine-rich C-terminal region of SAM68, it is possible that this region is involved in the formation of multimolecular complexes masking epitopes recognized by the anti-SAM68 and anti-phosphotyrosine Abs. This interpretation is consistent with in vitro data demonstrating a role for this region of SAM68 in its association with proteins involved in leukocyte activation (18, 26). Alternatively, the changes in the abilities of these Abs to recognize SAM68 subsequent to the stimulation of the cells by MSU

**FIGURE 8.** Hypothetical scheme of the involvement of SAM68 in the early events of signal transduction associated with the stimulation of human neutrophils by ligation of CD32 and by MSU crystals.
crystals may be attributable to the serine phosphorylation of the former. Preliminary experiments have provided suggestive evidence for such a posttranslational modification of SAM68 (C.G. and P.H.N., data not shown). Finally, it should be pointed out that no evidence for a translocation of SAM68 from the soluble to the insoluble fraction of our lysates was evident after stimulation by MSU crystals (Fig. 5).

Another unsuspected finding that came out of the present series of experiments was the difference in the effects of stimulation by MSU crystals and by CD32. Both agonists induced a transient increase in the levels of tyrosine phosphorylation of SAM68 and a decrease of its ability to interact with poly-U beads. In contrast, only stimulation by MSU crystals, not by CD32 cross-linking, decreased the ability of the anti-SAM68 and the anti-phosphotyrosine Abs to recognize SAM68 in lysates prepared under native conditions. These results suggest that stimulation by MSU crystals and CD32 cross-linking do not result in the masking of the same epitopes on SAM68 despite apparently equivalent levels of tyrosine phosphorylation as detected by using a denaturing protocol. This may be attributable to the formation of different SAM68-containing multimeric (protein and/or RNA) complexes or steric hindrance by serine/threonine phosphorylation.

It is relevant to point out that recent results indicate that MSU crystals activate human neutrophils after their interaction with CD16 but not CD32 (41). Our present data also indicate that the stimulation of the tyrosine phosphorylation of SAM68 by MSU crystals is similarly mediated by CD16 (Fig. 4). Both CD32 and CD16 are low-affinity Fcγ receptors constitutively expressed on human neutrophils. However, in contrast to CD32, which is a transmembrane protein the cytosolic portion of which contains an immunoreceptor tyrosine-based activation motif, CD16 is a GPI-linked protein (36, 51). The signaling mechanisms associated with CD16, as are those of most GPI-linked protein, are poorly understood but are distinct, at least in part, from those involved in the activation of CD32 (36, 51, 65–70).

The role of SAM68 in the metabolism of RNA presently is poorly understood. It is not known whether SAM68 acts at the level of RNA stability, splicing, or transport. Consistent with a potential role in RNA transport, SAM68 possesses a nuclear localization signal in its C terminus (6, 71) that suggests it may enter the nucleus. In contrast to what has been proposed in T cells (10), the expression of SAM68 does not undergo a nuclear translocation signal in its C terminus that suggests it may enter the nucleus. In contrast to what has been proposed in T cells (10), the expression of SAM68 does not undergo a nuclear translocation. This may be attributable to the formation of different SAM68-containing multimeric (protein and/or RNA) complexes or steric hindrance by serine/threonine phosphorylation.

During an inflammatory response, regulation of the stability of the mRNA of various cytokines and cytokine receptors occurs. For example, in response to LPS, the mRNA of CCR2 (receptor for monocyte chemoattractant protein-1) in THP-1 cells is rapidly degraded by a degradative protein, while at the same time, the mRNA of macrophage inflammatory protein (MIP)-1α is stabilized by the AU-rich element motif (72). Therefore, the response to an agonist may lead to different posttranscriptional regulation of individual mRNA species. Furthermore, in response to various oncoprogenized microorganisms that presumably act via CD16 and CD11b (41), preferential synthesis of IL-8 and IL-1 is observed (58–59, 61). As we have observed that SAM68 responds differentially to stimulation by MSU crystals and CD32, it becomes conceivable that SAM68 contributes, at least in part, to the distinct profiles of chemokine and cytokine synthesis induced by these stimuli which have been previously described (75).

A model summarizing the major findings of the present investigation is shown in the attached scheme (Fig. 8). This figure indicates that SAM68 is present in both the nonnuclear and the nucleus of human neutrophils. After stimulation by MSU crystals (i.e., activation of CD16-CD11b/CD18) or cross-linking of CD32, SAM68 is tyrosine phosphorylated, but not necessarily on the same sites. This leads to hypothetical altered conformational changes and differential association with signaling proteins and ultimately to dissociation (and activation) of different species of RNA. Presently, it is unknown whether tyrosine phosphorylated SAM68 plays additional signaling roles.

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