Association of rap1 and rap2 Proteins with the Specific Granules of Human Neutrophils

TRANSLOCATION TO THE PLASMA MEMBRANE DURING CELL ACTIVATION*

Isabelle Maridonneau-Parini§ and Jean de Gunzburg†
From INSERM U-332, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75014 Paris, France and INSERM U-248, Faculté de Médecine Lariboisière-Saint Louis, 75010 Paris, France

Activation of human neutrophils involves the degranulation of specific and azurophil granules. This process is GTP-dependent and the presence of small GTP-binding proteins (SGBPs) has been detected in the two granule populations. At present, none of these SGBPs has been definitely identified. In order to characterize some of these proteins and obtain further insights as to their potential role in degranulation processes, we have used specific antibodies directed against the ras-related rap1 and rap2 proteins. By immunoblot analysis, we observed that rap2p is predominantly located in specific granules, whereas rap1p is detected both in specific granules and a fraction enriched in plasma membranes. Neither rap1p nor rap2p was found in the cytosol or in azurophil granules. Similarly, by indirect immunofluorescence, we observed that cytoplasmic granules were stained with anti-rap1p antibodies and anti-rap2p antibodies, and the plasma membrane was labeled with both antibodies but more distinctly with anti-rap1p than with anti-rap2p antibodies. rap1p and rap2p are tightly bound to the membrane of specific granules since they cannot be extracted by high salt or alkaline buffers. In addition, treatment of intact specific granules with pronase induced the degradation of rap proteins suggesting that they are exposed to the cytoplasmic face of the granules. Degranulation of neutrophils consists of the translocation and subsequent fusion of granules with the plasma membrane. Activation of this process induced the accumulation of rap proteins in the plasma membrane as observed by subcellular fractionation and indirect immunofluorescence experiments; this was not associated with the appearance of a soluble form of these proteins, showing that they remain membrane-bound during this process. The identification and subcellular localization of rap1p and rap2p at the surface of specific granules and the observation that they translocate to the plasma membrane upon cell stimulation without appearance of soluble forms constitute an important step toward the understanding of their physiological functions in human neutrophils.

Stimulation of neutrophils with a variety of agents induces microbicidal responses which include degranulation of at least two granule populations, specific and azurophilic (1). Fusion of specific and azurophilic granules with the plasma membrane and/or the phagosome may occur independently (1–3). Different opsonic factors can influence which granules fuse to the phagosomes (2), and soluble stimuli such as phorbol esters and calcium ionophores induce the fusion of specific granules with the plasma membrane with little or no fusion of the azurophil granules (3). From these observations, one would expect that the differential regulation of degranulation involves the presence of distinct regulatory proteins located at the surface of azurophil and specific granules. Guanine nucleotides have been shown to stimulate the degranulation of neutrophils suggesting that GTP-binding proteins are involved in this process at a step distal to the activation of plasma membrane-associated large GTP-binding proteins (4). A growing number of ras-related small GTP-binding proteins (SGBPs) has been described in mammalian cells; some of them are implicated in the regulation of vesicular traffic between intracellular compartments (5–7). Therefore, SGBPs would be good candidates to control the differential exocytosis of specific and azurophil granules. Indeed, different subsets of SGBPs with distinct M, have been detected in the two granule populations by GTP-binding experiments (8, 9). At present, none of these granule-associated SGBPs has been positively identified.

Few SGBPs have been characterized in human neutrophils (10, 11): rap1 protein (rap1p) was purified from neutrophil membranes (10), and proteins of the rab family were detected in the membrane and the cytosol but not in the granules (11). Additionally, an SGBP, possibly rap1p, was shown to be associated with cytochrome b559, a component of the superoxide-generating enzyme NADPH oxidase, in membranes from stimulated human neutrophils (12). Since in resting neutrophils, cytochrome b559 is mostly located in specific granules (13), one could expect to find rap1p in these granules, and it could be imagined to play a role in degranulation or in intercalation of cytochrome b559 in the plasma membrane, at the end of the NADPH oxidative respiratory chain.

Among the superfamily of SGBPs, rap proteins (rap1p is 95% identical with rap1pBp (14, 15); rap2p is 90% identical with rap2pBp (16)) are the most closely related to the products of ras protooncogenes, each sharing about 50% sequence identity.

* This work was supported in part by a grant from the Association de la Recherche contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence and reprint requests should be addressed. Tel.: 33-1-40-51-64-52. Fax: 33-1-40-51-77-49.

1 The abbreviations used are: SGBP, small GTP-binding protein; BSA, bovine serum albumin; fMLP, f-methionylleucylleucylheptapeptide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMS, phosphate-buffered saline; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; PMA, phorbol 12-myristate-13-acetate; rap1p, rap1 protein; rap2p, rap2 protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylenediamine(tetraacetyl)tetraacetic acid; GTPyS, guanosine 5'-3-O-(thio)tri-phosphate.
homology with p21ras. Overexpression of the Krev-1 gene (that encodes rap1Ap) has been shown to revert the phenotype of K-ras-transformed fibroblasts (17). rap1 proteins are present in most mammalian tissues, but their tissue and subcellular distributions differ from those of p21ras (18–20). This suggests that rap1 proteins exert their own specific functions in addition to potential antagonistic actions to those of p21ras (5, 18), depending on the cell type and intracellular location.

rap2 proteins are also membrane-associated (21). In contrast to rap1p, they are not substrates for protein kinase A (16, 21, 22) and do not interfere with the growth-promoting action of ras proteins (23).

In this study, we have used specific antibodies to investigate the subcellular locations of rap1 and rap2 proteins in resting and activated human neutrophils. We report that rap proteins are found both in the plasma membrane and the specific granules from resting cells, rap2 protein (rap2p) being predominantly associated with the granules, and that, following degranulation, rap proteins accumulate in the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Neutrophil Isolation and Stimulation**—Human neutrophils were purified from the blood of healthy donors by Dextran T-500 sedimentation and centrifugation through Ficoll-Hypaque as previously described (24). They were resuspended at a density of 1 × 10^7 cells/ml in minimal Eagle’s medium supplemented by 20 mM Hepes, pH 7.4, at 37°C and stimulated with PMA or the calcium ionophore A23187 (Sigma) at 30 min. Degranulation was assessed by measuring the intra- and extracellular contents of lysozyme as described (25).

**Preparation of Subcellular Fractions**—Subcellular fractionation of resting or activated human neutrophils was performed as previously described (11, 13). Briefly, neutrophils were resuspended in relaxation buffer (210 mM NaCl) and cultured in a nitrogen bomb at 375 p.s.i. and centrifuged in order to remove debris, nuclei, and intact cells. The supernatant was subjected to centrifugation on a discontinuous isosonic Percoll gradient; four fractions corresponding to the cytosol, the plasma membrane-enriched fraction, and the specific and the azurophil granules were collected and washed free of Percoll by centrifugation at 245,000 g for 60 min. Cytosolic proteins were concentrated by precipitation with 25% trichloroacetic acid. For proteolytic degradation experiments, intact specific granules were rapidly centrifuged at 15,000 g for 10 min.

In some experiments, purified specific granules resuspended in PBS containing protease inhibitors were disrupted by 8 consecutive freeze-thaw cycles and sonicated at 4°C (four 3-a pulses) in the presence of 1 mM NaCl. After centrifugation at 200,000 g for 60 min at 4°C, the supernatant (S1) was removed, and the pellet was further extracted for 15 min at 4°C with 0.1 M Na2CO3, pH 11.5, and centrifuged, yielding supernatant S2 and the final granule membranes depleted of peripheral proteins.

**Assays for Specific Markers in Subcellular Fractions**—The granule and membrane fractions obtained from 5 × 10^6 cells were resuspended in 1 ml of 0.34 M sucrose, 10 mM Pipes, pH 7.2. Except for alkaline phosphatase assay, equal aliquots from each fraction were supplemented with Triton X-100 (0.2%, final concentration) and assayed for protein or enzymatic markers.

Alkaline phosphatase, a marker for plasma membrane when measured in the absence of detergent (27), was assayed using p-nitrophenylphosphate (Sigma) as substrate. The absorbance was measured at 410 nm (28).

Vitamin B12-binding protein, a marker for the specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for the specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma). Vitamin B12-binding protein, a marker for specific granules, was assayed as previously described (29) using [57Co]cyanocobalamin (Sigma).
were disrupted and fractionated by centrifugation on a discontinuous Percoll gradient (13) which has been shown to efficiently separate azurophil from specific granules and specific granules from the membrane fraction (microsomes) (8, 13, 37). This was further confirmed by the assay of markers for azurophil granules (β-glucuronidase), specific granules (vitamin B<sub>12</sub> binding protein), plasma membrane (alkaline phosphatase), endoplasmic reticulum (sulfatase C), and Golgi apparatus (galactosyltransferase) (Table I). rap proteins were detected neither in the cytosol nor in azurophil granules (Fig. 2). Both rap1p and rap2p were found in the specific granules and in the fraction containing the plasma membrane, al-
neutrophils (Fig. 3). Using antibodies directed against Golgi (CTR 433 or anti-rab6 (39, 40)) or endoplasmic reticulum proteins (1D3 (41)), we failed to observe any specific staining in neutrophils whereas these antibodies efficiently stained the Golgi apparatus and the endoplasmic reticulum in other phagocytes such as promyelocytic HL60 cells or human monocytes (data not shown). Although we cannot exclude that a minor fraction of rap proteins could be associated with the Golgi apparatus and the endoplasmic reticulum, from the results described in Figs. 2 and 3, we can conclude that rap1p and rap2p are predominantly localized in the specific granules and in the plasma membrane of human neutrophils.

We further investigated the positioning of rap proteins on the specific granules. Purified specific granules were lysed by repeated freeze-thaw cycles followed by sonication, and their membranes were subjected successively to high salt and alkaline extractions. Under these conditions, both rap1p and rap2p remained membrane-associated, attesting that they are tightly bound to the granule membrane (Fig. 4A). In order to investigate whether rap proteins are located on the external (cytoplasmic) or internal face of the granule membrane, we assessed the capacity of proteolytic enzymes to digest rap1p and rap2p in intact or detergent-permeabilized granules. Purified specific granules were exposed to pronase in the presence or the absence of the nonionic detergent Nonidet P-40. As shown on Fig 3B, both rap1p and rap2p were degraded independently of the absence or the presence of detergent, whereas lactoferrin, an intragranular protein, was accessible to pronase only after granule permeabilization by Nonidet P-40 (Fig. 4B). These data indicate that both rap1p and rap2p are exposed to the cytoplasmic side of the specific granule membrane.

Translocation of rap Proteins to the Plasma Membrane upon Degranulation—The subcellular localization of rap proteins was examined in stimulated neutrophils. Table I shows that stimulation did not modify the partition of protein and enzymatic markers between the subcellular fractions. We used two specific granule secretagogues (3) to activate human neutrophils: the phorbol ester PMA and the calcium ionophore A23187. The extent of degranulation, as the result of granule fusion with the plasma membrane, was assessed by measuring the release of lysozyme in the extracellular medium. In resting cells, the release of lysozyme was 7.7 ± 1.6% of the total lysozyme cell content (mean ± S.D., n = 3). Cell stimulation with 0.5 μg/ml PMA or 5 μM A23187 for 30 min induced degranulation to a similar extent (42 ± 8% versus 45 ± 11%, mean ± S.D., n = 3). In parallel experiments, resting and stimulated neutrophils were fractionated. When compared to resting cells, the amount of rap1p or rap2p in the membrane fraction was increased in both PMA- and A23187-stimulated cells: 43 ± 12% and 51 ± 15% increase in rap1p, respectively; 106 ± 26% and 115 ± 33% increase in rap2p, respectively (mean ± S.D., n = 3) (see Fig. 5, compare lanes 3 with lanes 4). None of these stimulating agents produced the release of rap proteins into soluble compartments since rap proteins were not detected in the cytosol (Fig. 5, lanes 5 and 6) nor in the extracellular medium (Fig. 5, lanes 7). As expected, the levels of rap1p and rap2p in the specific granules which did not fuse with the plasma membrane during activation were similar to those in granules from resting cells (Fig. 5, lanes 1 and 2).

An additional characteristic of PMA stimulation is that rap2p was detected as a doublet in the membrane and granule fractions from stimulated neutrophils (Fig. 5, lanes 2 and 4). In both fractions, the band migrating slightly slower in SDS-PAGE corresponded to roughly half of the total rap2p content. Appearance of this doublet was detectable 5 min after PMA addition (data not shown), suggesting that it might represent some form of post-translational modification of the protein. Although rap2p has been reported not to be a substrate of cyclic AMP-dependent protein kinase A or protein kinase C (16, 21, 22), we investigated whether, in human neutrophils, the upper band could represent a phosphorylated form of rap2p. However, this was not the case since: (i) immunoprecipitation of rap2p after labeling intact neutrophils with [32P]orthophosphate and stimulation with PMA did not result in the incorporation of 32P into the protein, (ii) treatment of membranes from PMA-activated neutrophils with alkaline or acid phosphatase did not reduce the intensity of the upper band of the doublet, and (iii) using an anti-phosphotyrosine antiserum in Western blotting experiments, no immune signal co-migrating with rap2p was detected (data not shown). The
brane in PMA- and A23187-stimulated neutrophils.

The results are representative of three independent experiments.

Arrows show the presence of rap1p and rap2p at the plasma membrane.

Arrows show the presence of rap1p and rap2p at the plasma membrane.

Fig. 5. Translocation of rap proteins to the plasma membrane in PMA- and A23187-stimulated neutrophils. Resting neutrophils or neutrophils stimulated in parallel with 0.5 μg/ml PMA or 5 μM A23187 for 30 min were fractionated. Specific granules from resting and activated cells (lanes 1 and 2), membranes from resting and activated cells (lanes 3 and 4), cytosol from resting and activated cells (lanes 5 and 6), and the extracellular medium from stimulated cells (lanes 7) were analyzed by immunoblotting. Extracellular proteins were concentrated by precipitation with 25% trichloroacetic acid prior to SDS-PAGE. Equal amounts of protein (25 μg) were loaded on the gels. M, standards are shown on the left (×10^3). All of the material used in this experiment originated from the same donor. The results are representative of three independent experiments.

Appearance of a rap2p form with a slower electrophoretic mobility was not directly linked to the process of degranulation since neither A23187 (Fig. 5) nor other secretagogues such as fMLP or serum-opsonized zymosan (data not shown) induced the appearance of a rap2p form with a slower electrophoretic mobility. At present, we have no explanation for this phenomenon, which was only observed when neutrophils were stimulated with PMA.

Translocation of rap proteins to the plasma membrane was confirmed by immunofluorescence experiments using PMA-stimulated neutrophils. The results shown in Figs. 3 and 6 were performed in parallel using cells collected from the same donor. As shown on Fig. 6, the plasma membrane was more strongly stained with anti-rap1p (A) and anti-rap2p (B) than in resting neutrophils (Fig. 3, A and B). In addition, less cytoplasmic granule staining was observed (Fig. 6, A and B) than in resting cells (Fig. 3) as part of the granules has fused with the plasma membrane.

Fig. 6. Subcellular localization of rap1 and rap2 proteins in PMA-stimulated neutrophils by indirect immunofluorescence. Adherent human neutrophils were stimulated with 0.5 μg/ml PMA for 15 min and were subjected to indirect immunofluorescence with affinity-purified anti-rap1 (A) and anti-rap2 (B) antibodies as described in the legend of Fig. 3 (original magnification ×4000). Arrows show the presence of rap1p and rap2p at the plasma membrane.

Several proteins associated with the membranes of specific granules have been previously described such as Mo-1, p150-95, cytochrome b556, plasminogen activator, and receptors for fMLP, vitronectin, fibronectin, and laminin (13, 46-48). Among these proteins that represent an intracellular storage pool of cell surface proteins, some have been more precisely located. All of them are associated with the intragranular side of the membrane (46-48). In contrast, we report here that rap proteins are exposed to the cytoplasmic face of neutrophil specific granules as expected for proteins synthesized as cytosolic precursors that become membrane-bound after a series of post-translational modifications (19, 21, 49, 50). This localization is that of proteins involved in the docking/fusion processes (5-7). The absence of rap proteins in azurophilic granules is in agreement with the observation that different subsets of SGBPs with distinct Mr, are detectable in the two granule populations (8, 9). Association of rap proteins with the specific granules suggests that they could play a role in the selective mobilization of this granule subtype, in their fusion with the plasma membrane, or in the targeting of granular proteins to the plasma membrane. Quinn et al. (12) have reported that cytochrome b556 is associated with a ras-like protein, possibly rap1p (12), in stimulated neutrophils.

Discussions

This is the first report that, based on the use of specific antibodies, positively identifies the presence of two SGBPs, rap1p and rap2p, on the external face of neutrophil specific granules. rap proteins are also present in the plasma membrane, but absent from azurophil granules. Both proteins are translocated to the plasma membrane upon degranulation, and no soluble form is detectable either in resting or stimulated neutrophils.

Rap proteins have been found in several subcellular locations: rap1p has been detected in the cytosol (42) and the membrane of platelets (43, 44), in neutrophil membranes (10), in the cytoplasmic region of most types of neuronal cell bodies (20), in the Golgi apparatus of rat fibroblasts (18), rap2p has been found in a light density structure morphologically related to the endoplasmic reticulum in Chinese hamster ovary cells (19). Here, we report that both proteins are associated with the specific granules and the plasma membrane of human neutrophils. At present, this heterogeneous subcellular distribution of rap proteins is not understood. The polyclonal anti-rap1p antibodies that we used are directed against a peptide common to rap1Ap and rap1Bp. Therefore, it is possible that these localization discrepancies could result from differential expression of rap1Ap and rap1Bp in these different cell types. In addition, if both proteins are expressed in a single cell type, they may partition between distinct subcellular locations.

This hypothesis is supported by the identification of a cytosolic and a plasma membrane-associated GTPase-activating protein specific for rap1p in the promyelocytic HL60 cell (45), a neutrophil progenitor cell line (11). From the results described in the present report, we can conclude that rap proteins are absent in the cytosol of resting and activated neutrophils, and, although we cannot exclude that a minor fraction of rap proteins could be associated with the Golgi apparatus and the endoplasmic reticulum, we demonstrate that rap1p and rap2p are predominantly localized in the specific granules, a subcellular structure which only exists in neutrophils, and in the plasma membrane.
Since cytochrome b556 is mainly located in the specific granules in resting neutrophils (13), it is possible to imagine that, upon degranulation, these two granular proteins associate and translocate to the plasma membrane where rap1p may control the targeting of cytochrome b at the end of the respiratory chain NADPH oxidase.

It has been previously observed, in isolated synaptosomes, that the SGBP rab3Ap is released from the membrane of synaptic vesicles during calcium-dependent exocytosis (51). However, upon stimulation of neutrophils with A23187 or PMA which induce cell activation through distinct pathways (52), rap proteins translocate to the plasma membrane without the appearance of soluble forms. Considering the model which describes the role of SGBPs in intracellular vectorial transport (53), it appears that these proteins have to recycle between membrane-bound and soluble forms: SGBPs are bound to the membrane of vesicles originating from a donor compartment, they control the docking/fusion with the acceptor compartment, they are released from the vesicle membranes, and they recycle via the cytosol to the original donor compartment. This model also involves recycling of the SGBP between DPF-bound (inactive) forms and GTP-bound (active) forms. The non-hydrolyzable GTP analog GTP-S inhibits the SGBP recycling and blocks the intracellular vesicle transport (5-7). Degranulation of human neutrophils in response to an external stimulus exhibits two major differences with this intracellular transport model (53): (i) mature neutrophils are unable to renew their granule population (38); therefore, one would expect that, in neutrophils, the recycling of SGBPs from the acceptor to the donor compartment may be unnecessary; (ii) exocytosis is not blocked, but rather is stimulated by the nonhydrolyzable analog GTP-S (4). In the absence of SGBP recycling, GTP-S would maintain SGBPs in their active form and stimulate rather than inhibit the process of degranulation. For these reasons, our observation that rap proteins do not appear in the cytosol of stimulated human neutrophils should be compatible with their potential involvement in the regulated exocytosis of specific granules. Experiments are currently in progress to investigate the role of rap proteins in degranulation and/or insertion of granular proteins in the neutrophil plasma membrane.

In conclusion, the subcellular localizations of rap1p and rap2p have been identified in resting and activated neutrophil granules. Their presence at the surface of specific granules and their translocation to the plasma membrane upon stimulation should represent an essential step in the definition of their functional activities in neutrophils.

Acknowledgments—We are indebted to A. Tavitian and F. Russo-Marie for their constant interest and support. We gratefully acknowledge J. Breton-Gorius for the gift of anti-lactoferrin antiserum, M. Bornens for the gift of anti-Golgi CTR353 antibodies, B. Goud for anti-rab5 antibodies, S. Fischer for anti-phosphotyrosine antibodies and helpful suggestions, I. Lerosey for providing purified recombinant rap2p, A. Zahraroui for purified rap proteins, S. Fuller for monoclonal antibodies 1D3, and A. Parini, B. Wessler, and B. Goud for their critical reading of the manuscript.

REFERENCES
rap Proteins in Human Neutrophil Specific Granules