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Localization of rap1 and rap2 proteins in the gelatinase-containing granules of human neutrophils

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The subcellular localization of rap proteins in resting human neutrophils was investigated by immunoblot analysis with specific anti-rap2 and anti-rap1 antibodies of the membrane proteins obtained from distinct subcellular fractions. Rap2 protein was mainly located in gelatinase-containing granules, whereas rap1 protein was detected both in gelatinase-containing granules and in fractions enriched in plasma membrane. Neither rap1 nor rap2 proteins were found in the cytosol or in azurophilic granules. Rap2B, not rap2A, appeared to be the major rap2 protein in human neutrophils. The identification and subcellular localization of rap1 and rap2 proteins at the membranes of gelatinase-rich granules suggest that these proteins could play a role in the regulation of the rapid and selective mobilization of gelatinase-containing granules in human neutrophils.

Rap1 protein; Rap2 protein; Gelatinase-rich granule; Human neutrophil

1. INTRODUCTION

Four distinct granule populations have been reported in human neutrophils, namely: azurophilic granules, specific granules, gelatinase-rich tertiary granules and alkaline phosphatase-rich granules or phosphasomes [1–7]. Recent evidence suggests that gelatinase-containing granules could be a subpopulation of the previously described specific granules [5,8,9]. However, the existence of separate gelatinase-rich tertiary granules is confirmed by a different distribution pattern in subcellular fractionation studies as well as by a differential and prone release of gelatinase marker as compared to other granule markers, including those of specific granules [3,4,5,9–12]. Fusion of the different granule populations with the plasma membrane may occur independently. Thus, mobilization of gelatinase-containing granules can be induced readily under conditions which mobilize slightly the specific granules and hardly mobilize the azurophilic granules [10–12]. As the gelatinase-rich granules contain proteins involved in cell adhesion processes [5,13,14] as well as in diapedesis [3–5,9], it has been postulated that the rapid mobilization of these granules can modulate early neutrophil responses upon cell activation [5]. Degranulation of neutrophils must implicate specific recognition of the plasma membrane fusion site by the granules, bringing membranes into close contact, and subsequent fusion of membranes. The differential degranulation may likely involve the presence of different proteins in the surface of the distinct granule populations, which can regulate the selective mobilization of neutrophil granules. GTP analogues can induce secretion in human neutrophils [15,16], suggesting that GTP-binding proteins are involved in exocytosis in human neutrophils. A distinct pattern of low molecular mass GTP-binding proteins has been described in the different granule populations [17,18]. More recently, rap1 and rap2 proteins have been reported to be located both at the surface of the specific granules and in the plasma membrane [19]. Furthermore, rap1A has also been found colocalized with cytochrome *b* in plasma membrane and specific granules in human neutrophils [20], and evidence indicates a functional association of these two molecules in intact neutrophils, suggesting that rap1A is required for assembly of a functional superoxide-generating system [21,22].

ras-related genes constitute a group of genes encoding a series of proteins that share between 30 to 85% homology with ras proteins [23]. Among the ras superfamily of small GTP-binding proteins, the rap proteins comprise four members: rap1A, rap1B, rap2A and rap2B [23–25]. While rap1 proteins are 70% identical to rap2 proteins, rap1A is 95% identical with rap1B [24], and rap 2A is 90% identical with rap2B [25].

Using specific antibodies to rap1 and rap2 proteins, we herein report that rap1 protein is located both in the

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Abbreviations: GTP, guanine triphosphate; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

plasma membrane and in the membranes of gelatinase-containing granules, and that rap2 protein is mainly located in gelatinase-containing granule membranes with a minor location in fractions enriched in plasma membrane. This rap2 protein seems to correspond predominantly to the rap2B protein as we report here that rap2A is undetectable in human neutrophils.

2. MATERIALS AND METHODS

2.1. *Cell isolation and subcellular fractionation*

Neutrophils were obtained from fresh human peripheral blood by dextran sedimentation and centrifugation on Ficoll–Hypaque, followed by hypotonic lysis of residual erythrocytes as previously described [26]. The final cell preparation contained more than 98% neutrophils, as assessed by Giemsa–Wright stain.

Neutrophils (about 700×10^6) were disrupted by hypotonic shock and homogenization in a Potter–Elvehjem tissue grinder in the presence of 2 mM PMSF as described [9,27]. The postnuclear supernatant (6 ml) was layered onto a 27 ml, 15–40% (w/w) continuous sucrose gradient, with a 1 ml cushion of 60% (w/w) sucrose, and centrifuged in a SW-27 rotor at 25,000 rpm ($113,000 \times g_{max}$) for 15 min at 4°C as previously described [9,27]. At the end of the run, fractions (the first one of 6 ml, containing the cytosol, and the remaining seven of 4 ml each) were collected by pumping 60% (w/w) sucrose into the bottom. Fractions were stored at −20°C in the presence of 2 mM PMSF. Membranes from each fraction were obtained by dilution of the fractions with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and centrifugation in a 30-type rotor at 29,000 rpm ($99,000 \times g_{max}$) for 90 min at 4°C as previously described [9]. Membranes were resuspended in 50 mM Tris-HCl, pH 7.6 containing 2 mM PMSF.

The marker enzymes lactate dehydrogenase, 5′-nucleotidase, gelatinase, lysozyme and peroxidase were assayed as described [4,11]. Alkaline phosphatase was assayed as described [11], in the presence of 1% Triton X-100 to avoid underestimation of latent activity. Lactoferrin was assayed as described [4]. Protein determination was carried out by the Bradford method [28], using BSA as standard.

2.2. *Generation of recombinant rap proteins*

The four known human *rap* genes (1A, 1B, 2A and 2B) were cloned by PCR techniques from total RNA from either human platelets, Jurkat or K562 cells. Independent PCR reactions were performed for each gene in a PHC-2 Techne thermocycler (Cambridge, UK) according to the manufacturer's indications, and using the following primers:

*rap*1A
(forward) 5′GACGAATTCATGCGTGATTACAAG 3′
(backward) 5′GACGAATTCGGCCTAGAGCAGCAG 3′

*rap*1B
(forward) 5′CAGCGAATTCATGCGTGAGTATAA 3′
(backward) 5′CAGCGAATTCGTATTAAAGCAGCT 3′

*rap*2A
(forward) 5′CAGGGAATTCATGCGCGAGTACAA 3′
(backward) 5′CAGGAATTCGTATTGTATGTTACATG 3′

*rap*2B
(forward) 5′GAGGAATTCACGGAGCCATGAGA 3′
(backward) 5′GAGGAATTCCCTCAGTAGGATCAC 3′

The above primers inserted an *Eco*RI restriction site at both ends of the complete coding sequence of each *rap* gene. This strategy allowed to subclone the genes into the bacterial expression vector pRC23 as previously described [29]. High level expression colonies were then selected and the respective proteins were purified following a procedure previously reported [29].

2.3. *Generation of antisera against rap proteins*

Sera were generated in rabbits by injection of either the full length, purified recombinant rap2B protein, or the synthetic peptides corresponding to residues 127–144 or rap1 (NH_2-GKEQGQN-LARQWCNCAFL-COOH) and to residues 164–183 of rap2A (NH_2-MNYAAQPDKDDPCCSACNIQ-COOH) conjugated to BSA with glutaraldehyde as previously described [30].

2.4. *Immunoblot analysis*

Proteins were separated through 12% or 15% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, and subjected to immunological detection basically as described [31]. Low range pre-stained protein molecular mass standards (Bio-Rad, Richmond, CA, USA) were also run in the same gel. After electroblotting and blocking for 1 h in 2% powdered defatted milk in TBS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), the nitrocellulose filters were incubated overnight with the respective specific anti-rap antibodies at a dilution of 1:500 in TBS buffer containing 0.05% Tween 20. Signal was developed either using a goat anti-rabbit immunoglobulin coupled to alkaline phosphatase according to standard techniques [31] or by incubation with [^{125}I]protein A (0.5 μCi/ml) and autoradiography at −80°C. In order to achieve an enhanced visualization of the corresponding band, signal was also developed using a biotin-streptavidin system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions, and using 1.7 mM 3,3′-diaminobenzidine with 0.03% (v/v) H_2O_2 in 50 mM Tris-HCl, pH 7.4, as a substrate solution.

3. RESULTS AND DISCUSSION

3.1. *Specificity of anti-rap1, anti-rap2 and anti-rap2A antibodies*

We raised a polyclonal antiserum directed against a peptidic sequence unique to rap1, corresponding to residues 127–144 of rap1A. This sequence is 94% identical to that of rap1B. The polyclonal antiserum against rap2 was raised from a purified preparation of the recombinant rap2B protein expressed in *Escherichia coli*. The anti-rap2A antiserum was directed against a peptidic sequence unique to rap2A protein, corresponding to residues 164–183 of rap2A, which shows a 20% identity to that of rap1A and a 65% identity to that of rap2B. The specificity of these antisera was assessed with the distinct purified recombinant rap1A, rap1B, rap2A and rap2B proteins (Fig. 1). Antibodies against rap1 only recognized rap1A and rap1B, but not rap2A and rap2B proteins (Fig. 1A). Likewise, antibodies against rap2 only recognized rap2A and rap2B, but not rap1A and rap1B proteins (Fig. 1A). The antiserum against rap2A was highly specific and dit not cross-react with rap2B, rap1A or rap1B proteins (Fig. 1A). In fact, cross-reaction of the anti-rap2A antibodies with rap2B resulted almost negligible when the antiserum was used at a dilution of 1:500 (Fig. 1B). Dilutions of the anti-rap2A antiserum at 1:200 gave identical results (data not shown). None of the antibodies used cross-reacted with ras or rho proteins under similar conditions (data not shown), indicating their specificity to rap proteins.

3.2. *Subcellular distribution of rap1, rap2 and rap2A proteins in resting neutrophils*

Postnuclear fractions prepared from resting human

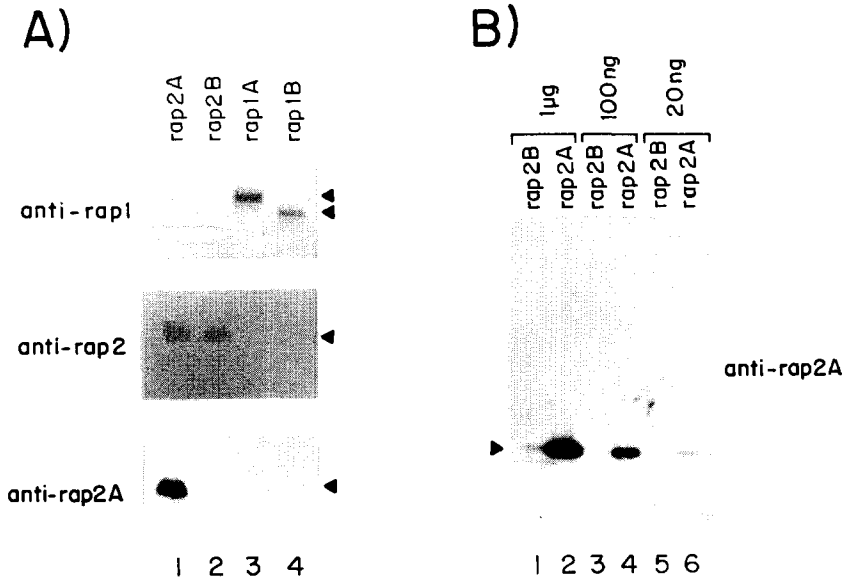

Fig. 1. Characterization of the specificity of the antibodies directed against rap1, rap2 and rap2A proteins by immunoblotting. (A) The purified recombinant rap2A (lane 1), rap2B (lane 2), rap1A (lane 3) and rap1B (lane 4) proteins were run on 12% SDS-polyacrylamide gels (30 ng/lane) and transferred to nitrocellulose filters which were incubated with anti-rap1, anti-rap2 or anti-rap2A antibodies as described in section 2. (B) Different amounts of purified recombinant rap2B and rap2A proteins were run on a 12% SDS-polyacrylamide gel, processed as above and incubated with anti-rap2A antibody. Bands were visualized using [^{125}I]protein A. Arrowheads indicate the positions of the respective rap proteins.

neutrophils were centrifuged in the presence of 2 mM PMSF to obtain soluble, containing cytosol, and membrane fractions. We found that rap1 and rap2 proteins were located in the membrane fraction, as determined by immunoblotting with specific antisera (Fig. 2). Neither rap1 nor rap2 proteins were detected in neutrophil cytosol (Fig. 2). In order to further examine the subcellular localization of rap1 and rap2 proteins, postnuclear fractions obtained from resting neutrophils were separated by rate zonal centrifugation on a continuous sucrose gradient under conditions that resolved cytosol (lactate dehydrogenase), plasma membrane (5′-nucleotidase), gelatinase-rich tertiary granules (gelatinase), specific granules (lysozyme and lactoferrin), and azurophilic granules (peroxidase) as shown in Fig. 3A. Alkaline phosphatase has been localized in a novel light density organelle named phosphasome [6,7]. Nevertheless, we found that alkaline phosphatase, measured in the presence of Triton X-100 to take into account the latent pool of this enzyme activity [7], cofractionated with the plasma membrane marker 5′-nucleotidase. Thus, under the fractionation conditions used, we were unable to separate plasma membrane from the alkaline phosphatase-rich organelle. Gelatinase-rich tertiary granules (fractions 4 and 5) were resolved from specific granules (fractions 5 and 6), as well as from azurophilic granules (fraction 8) and the plasma membrane (fractions 2 and 3). Furthermore, we found by immunoblot analysis, using a specific antigelatinase antibody (kindly provided by M. Nakajima and A. Fabra), that the 92 kDa gelatinase was located mainly in fractions 4 and 5 (data not shown).

To analyze the subcellular localization of rap proteins, we prepared membranes from the distinct subcellular fractions obtained in Fig. 3A and performed immunoblots with the respective antibodies. As shown in

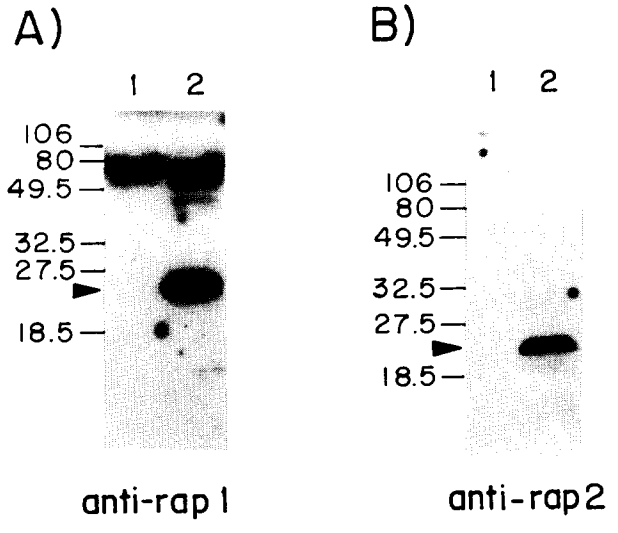

Fig. 2. Immunoblot analysis of rap1 and rap2 proteins in cytosolic and membrane fractions from resting human neutrophils. Postnuclear fractions from resting human neutrophils, prepared as described in section 2, were centrifuged in the presence of 2 mM PMSF at $99{,}000 \times g_{max}$ for 90 min at 4°C, and the cytosolic and membrane fractions collected. Equal amounts of cytosolic (lane 1) and membrane (lane 2) proteins (40 μg/lane) were run on 15% SDS-polyacrylamide gels and analyzed by immunoblotting using specific anti-rap1 (A) and anti-rap2 (B) antibodies, and [^{125}I]protein A for signal development. Arrowheads indicate the positions of the respective rap proteins. The molecular masses (kDa) of protein markers are shown on the left.

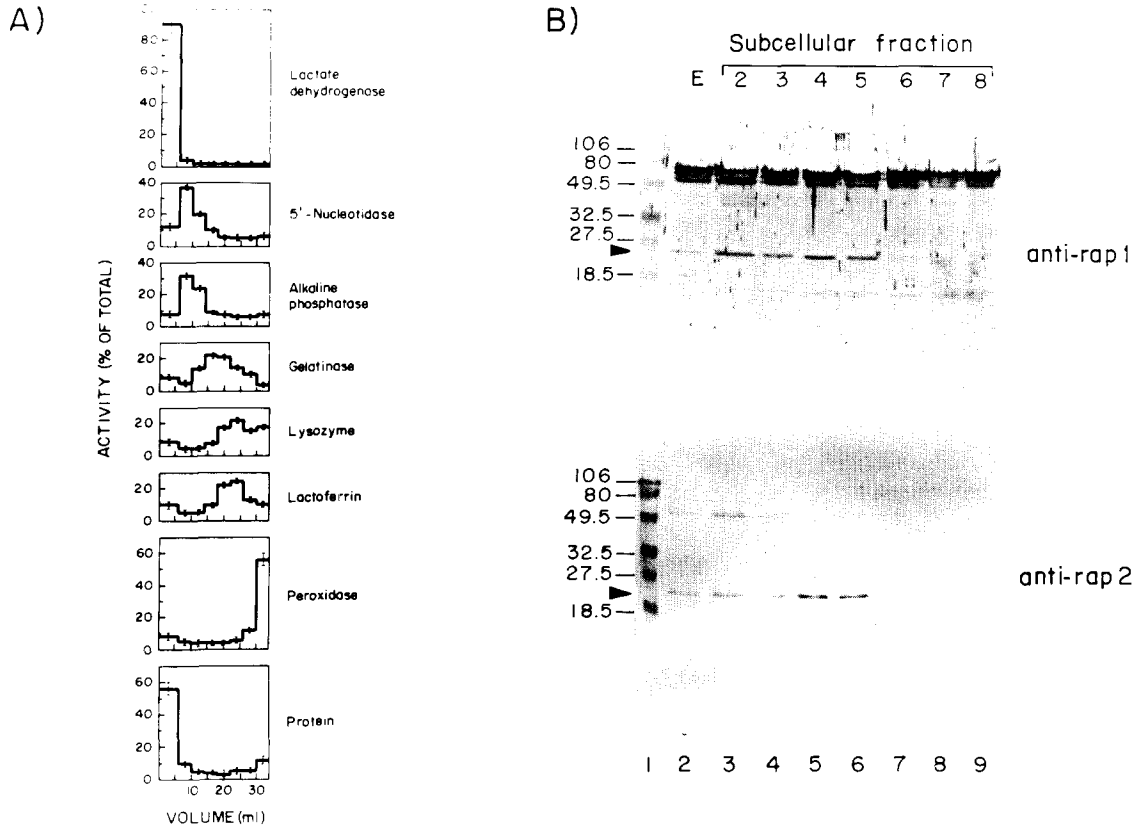

Fig. 3. Subcellular localization of rap1 and rap2 proteins in resting human neutrophils. (A) Subcellular distributions of marker enzymes after gradient centrifugation of postnuclear fractions obtained from resting human neutrophils as described in section 2. Plots of percent of total activity in each fraction (versus volume) are shown. The total amounts of protein, lactoferrin as well as the total enzyme activities for each marker enzyme in all the fractions are taken as 100%. The actual activity values for the distinct enzyme markers in the postnuclear fraction of resting human neutrophils have been previously reported [27]. Values are shown as mean ± S.E. of three independent determinations. The percentages of recovered activities were higher than 80% for all the assayed markers. (B) Equal amounts of membrane proteins (30 μg/lane), prepared from the postnuclear fraction as well as from the subcellular fractions of resting human neutrophils shown in (A) were run on 15% SDS-polyacrylamide gels and analyzed by immunoblotting using specific anti-rap1 and anti-rap2 antibodies as described in section 2. Bands were visualized using an anti-rabbit immunoglobulin coupled to alkaline phosphatase. Lane 1, molecular mass protein markers; lane 2, membranes from postnuclear extract (E); lanes 3–9, membranes isolated from the subcellular fractions 2–8 shown in A. The positions of rap1 and rap2 proteins are indicated with arrowheads. The molecular masses (kDa) of protein markers are indicated on the left.

Fig. 3B, the rap1 protein was mainly localized in fractions enriched in plasma membrane and gelatinase-rich tertiary granules. The rap2 protein was predominantly located in gelatinase-rich tertiary granules, and a lower amount of rap2 protein was detected in fractions enriched in plasma membrane (Fig. 3B). However, no rap1 or rap2 proteins were found in azurophilic granules (Fig. 3B). Although the specific and gelatinase-rich tertiary granules partially overlapped under the fractionation conditions used [4,9], the intracellular localization of both rap1 and rap2 proteins correlated better with the distribution pattern of gelatinase-rich granules than with the distribution pattern of specific granules (Fig. 3). Thus, the present data indicate that both rap1 and rap2 proteins are localized in fractions enriched in plasma membrane and in the membranes of gelatinase-rich granules in resting human neutrophils. Rap2 protein is mainly located at the gelatinase-rich granules, whereas rap1 protein is more evenly distributed between these organelles and the plasma membrane.

As the anti-rap2 antibody was not able to discriminate between rap2A and rap2B proteins, we analyzed the presence of rap2A protein in the subcellular fractions using a highly specific anti-rap2A antibody (Fig. 1). We were unable to detect a specific signal for rap2A in the membranes of the distinct neutrophil subcellular fractions using three different methods to visualize the specific rap2A band, namely incubation with [^{125}I]protein A, with goat anti-rabbit immunoglobulin coupled to alkaline phosphatase or with a biotin-streptavidin system (Fig. 4). These results indicate that the major form of rap2 protein present in human neutrophils is rap2B, and that the anti-rap2 antibody used in the present study recognizes mainly rap2B protein in human neutrophils. Furthermore, the data herin reported indicate that rap2B protein is predominantly located in the

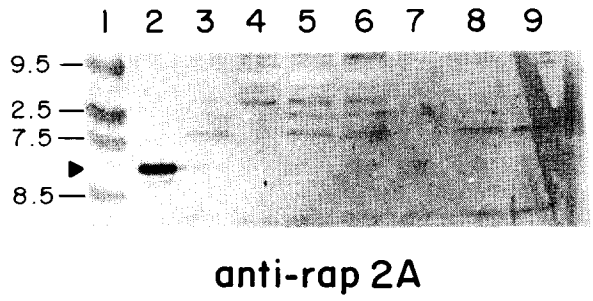

Fig. 4. Subcellular localization of rap2A protein in resting human neutrophils. Equal amounts of membrane proteins (30 μg/lane), prepared from the postnuclear fraction as well as from the subcellular fractions of resting neutrophils shown in Fig. 3A, were run on a 15% SDS-polyacrylamide gel and analyzed by immunoblotting using a specific anti-rap2A antibody and a biotin-streptavidin system for signal development. Lane 1, molecular mass protein markers; lane 2, 50 ng of purified recombinant rap2A; lane 3, membranes from postnuclear fraction; lane 4, cytosol (fraction 1, Fig. 3A); lanes 5–9, membranes isolated from the subcellular fractions 2–6 shown in Fig. 3A. The position of rap2A protein is indicated with an arrowhead. The molecular masses (kDa) of protein markers are indicated on the left.

membranes of gelatinase-rich granules. The polyclonal anti-rap1 antibody used in the present study could not discriminate between rap1A and rap1B proteins. However, it has been recently shown that the human neutrophil rap1 protein is fundamentally, if not exclusively, rap1A [32]. Taken together these data, we suggest that the readily mobilized gelatinase-rich granules contain at their membranes both the rap2B and rap1A proteins. By quantitative Western blot analysis using specific antibodies and purified recombinant rap1A and rap2B proteins as standards, we estimate that rap1A and rap2B proteins are present in the membranes of the gelatinase-rich granules at a level of 1.8 ± 0.3 and 5.6 ± 0.5 μg/mg of membrane protein ($n = 3$), respectively (Fig. 5). Fractions enriched in plasma membrane contained a slightly higher amount of rap1A protein (2.3 ± 0.4 μg/mg of membrane protein) and a lower level of rap2B protein (0.9 ± 0.2 μg/mg of membrane protein). Interestingly, we found that the rap2 protein was often detected as a doublet in immunoblots (Fig. 5B). This doublet has been previously detected in resting platelets [33] and in phorbol ester-stimulated neutrophils [19].

Neither rap1 nor rap2 proteins were detected in the cytosol or in the azurophilic granules, which are sluggishly mobilized in human neutrophils. As we found a partial overlapping between gelatinase-rich granules and specific granules under the subcellular fractionation conditions employed, we cannot rule out that some of the rap1 and rap2 proteins are also located in the specific granule population. Nevertheless, the distribution pattern of rap2 and rap1 proteins correlated better with the marker for gelatinase-containing organelles than with the markers for specific granules. A recent report has shown the localization of rap1 and rap2 proteins in both plasma membrane and specific granules [19]. However, gelatinase activity was not measured in that study, and specific and gelatinase-rich tertiary granules have almost identical densities, being difficult to resolve from each other except under certain fractionation conditions where sedimentation velocity is used [3,4,9]. We could not resolve plasma membrane from the alkaline phosphatase-rich organelle. Thus, it might also be suggested that a portion of the rap1 and rap2 proteins found in the plasma membrane fraction corresponds to the alkaline phosphatase-rich organelle. In this context, the alkaline phosphatase-rich and the gelatinase-rich organelles have been reported to be prone to fuse with the plasma membrane upon gentle stimulation and to precede fusion of specific and azurophilic granules [3,5,7,9–12]. Thus, we could envisage that the presence of rap1A and rap2B in neutrophil intracellular granules is correlated with their readiness to be mobilized.

The presence of rap1A and rap2B in gelatinase-rich granules suggests that these proteins may be involved in the regulation of the rapid and selective mobilization of these organelles upon neutrophil activation, which seems to be of pivotal importance in neutrophil adhesion, diapedesis and migration into the inflammatory sites.

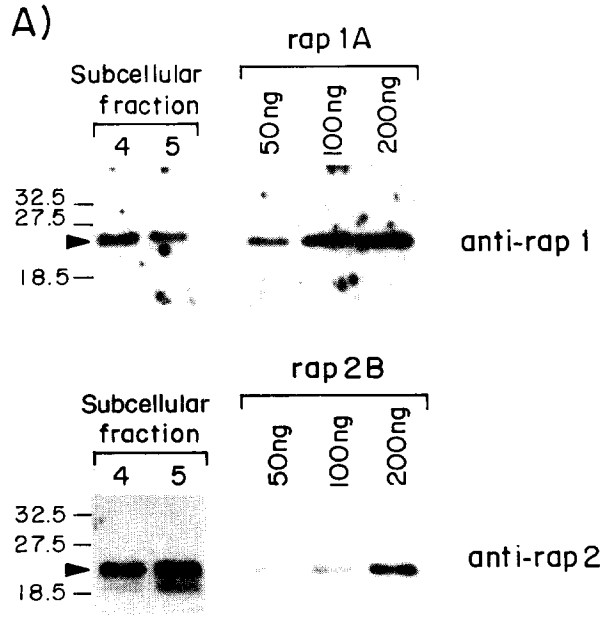

Fig. 5. Quantitative Western blot analysis of rap1A and rap2B proteins. Membrane proteins (40 μg/lane) from the gelatinase-rich granules (fractions 4 and 5, Fig. 3A) were run on 15% SDS-polyacrylamide gels and analyzed by immunoblotting using specific anti-rap1 and anti-rap2 antibodies, and [^{125}I]protein A for signal development. In the same gel, distinct amounts of purified recombinant rap1A and rap2B proteins were also run and processed as above. The relative amounts of rap proteins in each subcellular fraction were determined by cutting out the immunoreactive regions of the nitrocellulose filters and counting in a gamma counter. Arrowheads indicate the positions of rap1 and rap2 proteins. The molecular masses (kDa) of protein markers are shown on the left.

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