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Rab27a regulates exocytosis of tertiary and specific granules in human neutrophils[1]

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FOOTNOTES

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[3] Abbreviations used in this paper: PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

Abstract

The correct mobilization of cytoplasmic granules is essential for the proper functioning of human neutrophils in host defense and inflammation. In this study, we have found that peripheral blood human neutrophils expressed high levels of Rab27a, whereas Rab27b expression was much lower. This indicates that Rab27a is the predominant Rab27 isoform present in human neutrophils. Rab27a was upregulated during neutrophil differentiation of HL-60 cells. Subcellular fractionation and immunoelectron microscopy studies of resting human neutrophils showed that Rab27a was mainly located in the membranes of specific and gelatinase-enriched tertiary granules, with a minor localization in azurophil granules. Rab27a was largely absent from CD35-enriched secretory vesicles. Tertiary and specific granule-located Rab27a population was translocated to the cell surface upon neutrophil activation with phorbol 12-myristate 13-acetate (PMA) that induced exocytosis of both tertiary and specific granules. Specific Abs against Rab27a inhibited Ca^{2+} and GTP-γ-S-, and PMA-induced exocytosis of CD66b-enriched tertiary and specific granules in electropermeabilized neutrophils, whereas secretion of CD63-enriched azurophil granules was scarcely affected. Human neutrophils lacked or expressed low levels of most Slp/Slac2 proteins, putative Rab27 effectors, suggesting that additional proteins should act as Rab27a effectors in human neutrophils. Our data indicate that Rab27a is a major component of the exocytic machinery of human neutrophils, modulating the secretion of tertiary and specific granules that are readily mobilized upon neutrophil activation.

Introduction

Polymorphonuclear neutrophils (PMN)[3] constitute 54% to 70% of circulating white cells in humans and are the cornerstone of the cell-mediated microbicidal activity of innate immunity, phagocytosing and killing damaging pathogens (1). Neutrophils also play a crucial role in inflammation, and overexuberant activation of these cells may lead to extensive degranulation and release of cytoplasmic granule contents, which may be fatal in septic shock, acute lung injury, and other serious inflammatory disorders (2, 3). Central to the physiological role of neutrophils are their three major characteristic cytoplasmic granules, namely primary or azurophil granules, secondary or specific granules and gelatinase-rich tertiary granules (4, 5), which differ in their respective contents and readiness for mobilization. Azurophil granules, mainly involved in phagocytosis, contain a large number of lytic enzymes and are sluggishly mobilized upon neutrophil activation. Specific and tertiary granules contain several proteins involved in the adhesion and extravasation of human neutrophils, and are prone to fuse with the plasma membrane. Due to the particularly high tendency of tertiary granules to be exocytosed upon neutrophil activation and to their distinctive constituents, mobilization of tertiary granules is suggested to constitute a regulatory mechanism for a number of early functional responses in human neutrophils (6), including respiratory burst (7, 8), acidification (9), adhesion (10, 11), extravasation (12, 13), and priming (14). In addition, neutrophils contain the so-called secretory vesicles (15), which are also prone to be exocytosed, and, together with secondary and tertiary granules, constitute a reservoir of plasma membrane proteins that are translocated to the cell surface upon neutrophil activation (4, 5). Thus, there is a hierarchical mobilization of cytoplasmic granules in human neutrophils, which must be tightly regulated to avoid damage to the surrounding tissue if secreted in an uncontrolled manner, and to keep a

proper cell function in the surveillance of host organism. A regulated secretory pathway is then critical in human neutrophils, where synthesized products are first stored in distinct cytoplasmic organelles and then released to the extracellular medium or into a phagocytic vacuole when cells are appropriately stimulated. Each store organelle is mobilized towards the cell surface (exocytosis) or to the phagocytic vacuole (phagocytosis) at a distinct speed and kinetics in accordance with the biological function of the organelle products. Regulatory mechanisms underlying mobilization of neutrophil granules are complex and ill-defined, and some molecules involved in this process have only recently begun to be recognized. A number of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) have been identified as regulators of granule fusion and exocytosis in human neutrophils (16-19). In addition, neutrophils are rich in low-molecular-mass GTP-binding proteins (20, 21), including Rab proteins (22-25), which have been implicated in the regulation of vesicular traffic in the secretory pathways of several cell types (26). Rab proteins are GTPases that form the largest family within the Ras superfamily of small GTPases with more than 60 members in human cells (27, 28), and promote docking and fusion of vesicles between specific pairs of vesicle donor and acceptor membranes (29). Rab27a is believed to play a central role in regulated secretion in a wide range of secretory cells (30-32) and is the first Rab protein that has been shown to be directly associated with a human disease. Mutations of the *rab27a* gene cause type 2 Griscelli syndrome in humans (33-36), a rare autosomal recessive immunodeficiency that results in defects in the transport of at least two types of specialized lysosome-related organelles: melanosomes in melanocytes and lytic granules in cytotoxic T lymphocytes (27, 37, 38). Analysis of *rab27a*-deficient *ashen* mice (a model for human Griscelli syndrome) has revealed involvement of Rab27a in the vesicle-docking step in a number of

secretory cells, including cytotoxic T-lymphocytes (37, 38) and pancreatic β-cells (39). Type 2 Griscelli syndrome is characterized by impaired melanosome transport, which causes partial albinism of hair and skin, and by immunological abnormalities that are responsible for the poor prognosis of the disease. The capacity of lymphocytes and NK cells from these patients to lyse target cells is impaired or absent, as a result of their inability to secrete the content of their lytic granules (35-37). In addition, decreased respiratory burst and chemotaxis responses in neutrophils have been reported in some Griscelli syndrome patients (34). Rab27a protein has been recently found to be expressed in human neutrophils (40, 41), but its subcellular localization and function have not been clearly established. In the present study, we have characterized the expression, subcellular localization and function of Rab27a in human neutrophils by using different experimental approaches, showing the involvement of Rab27a in the exocytosis of tertiary and specific granules in human neutrophils.

Materials and Methods

Antibodies

Anti-Rab27a mouse mAb was purchased from BD Transduction Laboratories (Lexington, KY). Anti-Slp1, anti-Slp2-a, anti-Slp3-a, anti-Slp4-a, anti-Slp5, anti-Slac2-a, anti-Slac2-c, and anti-Rab27b rabbit polyclonal Abs were prepared as described previously (42, 43). The specificity of each Ab was checked by immunoblotting with recombinant T7-tagged Slp1-5 and Slac2-a/c (or FLAG-tagged Rab27a/b) expressed in COS-7 cells (44). Lack of cross-reaction between Abs to Rab27a and Rab27b was assessed previously (43). An additional anti-Rab27a rabbit poyclonal Ab was prepared as described previously (45). Both anti-Rab27a mAb and polyclonal Ab behaved similarly in Western blot and electron microscopy analyses. Anti-CD35 mAb was from

Immunotech (Marseille, France). Rabbit anti-human lactoferrin Ab was purchased from Cappel Laboratories (Cochranville, PA). Rabbit anti-human myeloperoxidase Ab was purchased from DAKO (Glostrup, Denmark). Rabbit anti-gelatinase Ab (46) was generously provided by Dr. N. Borregaard (National University Hospital, Copenhagen, Denmark). Anti-CD20 mAb was a kind gift of Dr. M. Romero (Hospital Rio Hortega, Valladolid, Spain). Specific mAbs against human CD63 (clone CLB-gran/12,435) and CD66b (clone CLB-B13.9) were from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands). Biotinylated anti-mouse and anti-rabbit IgG were from Amersham (Buckinghamshire, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin was from DAKO. P3X63 myeloma culture supernatant, kindly provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), was used as a negative control.

Cell culture

The human acute myeloid HL-60 cell line, the promyelocytic leukemia NB4 cell line, as well as the NK-like cell line YT-Indy, were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere of air/CO_2 (19/1). Neutrophil differentiation of HL-60 cell line was induced by adding 1.3% (v/v) DMSO as previously described (47).

Neutrophil isolation and activation

Neutrophils were obtained from fresh human peripheral blood by dextran sedimentation and Ficoll-Hypaque centrifugation, followed by hypotonic lysis of residual erythrocytes as previously described (48). Freshly isolated human neutrophils were resuspended at

3-5 × 10^6 cells/ml in HEPES/glucose buffer (150 mM NaCl , 10 mM HEPES, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose, pH 7.5), and incubated at 37°C for 10 min with 100 ng/ml phorbol 12-myristate 13-acetate (PMA). Release of gelatinase, lactoferrin, and peroxidase following neutrophil activation was determined as previously described (6, 9, 12).

Subcellular fractionation

Resting neutrophils were resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF, and then disrupted by repeated freeze-thaw. Homogenates were centrifuged at 1200 rpm in a Sorvall T 6000D centrifuge for 10 min, and the supernatant, representing the postnuclear extract, was saved. After centrifugation of the postnuclear extract at 45000 rpm in a TLA rotor for 90 minutes at 4°C using an Optima TL Ultracentrifuge (Beckman Instruments, Palo Alto, CA), supernatant (soluble fraction) and pellet (membrane fraction), resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF, were saved.

To prepare the distinct subcellular fractions, freshly prepared neutrophils (~3-6 x 10^8) were gently disrupted as described previously (7, 9), and the postnuclear fraction (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 at 25000 rpm in a Beckman L8-70B ultracentrifuge using a SW27 rotor (16). Fractions (4-ml each, save fraction 1-cytosol-, 6-ml) were collected by pumping 60% (w/w) sucrose into the bottom, and 2 mM PMSF was added at each fraction. Subcellular fractions were assayed for marker proteins, namely lactate dehydrogenase (cytosol), HLA (plasma membrane), latent alkaline phosphatase (secretory vesicles), gelatinase (tertiary granules), lactoferrin (specific granules) and peroxidase (azurophil granules) as described previously (17). Secretory

vesicles were not resolved from the plasma membrane under the fractionation conditions used (7, 16). Membranes from each fraction were obtained by diluting the fractions with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and centrifugation at 45000 rpm for 90 min at 4ºC using a 70 Ti type rotor (Beckman Instruments). Pellets were then resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF, and stored at -20ºC until use.

Western blotting and immunoprecipitation

Proteins were separated by SDS-7.5%, SDS-10% or SDS-14% polyacrylamide gels and then immunoblotted (17). After blocking for 3 h at room temperature in 4% powdered defatted milk in TBS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween 20, blots were incubated overnight with the respective primary Abs, and then Ab reactivity was monitored with biotinylated anti-mouse IgG or anti-rabbit IgG and streptavidin-horseradish peroxidase conjugate, using an enhanced chemiluminescence detection system (Amersham). Immunoprecipitation assays were conducted from solubilized and biotinylated proteins as described previously (49).

Electropermeabilization and immunofluorescence flow cytometry

Neutrophils were permeabilized immediately before use as reported previously (16, 50, 51). In brief, 5×10^6 cells were washed with PBS, resuspended in 0.5 ml of ice-cold electropermeabilization buffer (120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES, pH 7.0), transferred to a BTX cuvette and subjected to two discharges of 5 kV/cm, 25 μF and 72 Ω using a BTX electroporator (Biotechnologies & Experimental Research, San Diego, CA). The cells were stirred gently between the two pulses, using a plastic pipette. Permeabilized cells were immediately transferred to plastic tubes

containing buffer or the Abs used in the study, and incubated for 5 min at room temperature to allow incorporation of Abs into electroporated neutrophils. Electropermeabilized neutrophils were then incubated for 5 min with 5 μg/ml cytochalasin B at 37°C, followed by stimulation with 1 μM Ca^{2+} (0.1 mM $CaCl_2$, 5.37 mM $MgCl_2$, 5 mM hydroethyl EDTA, 10 mM glucose) and 50 μM GTP-γ-S for 10 min at 37ºC. The free Ca^{2+} concentration was checked by Fura-2 measurement. In some cases, cells were stimulated by incubation with 100 ng/ml PMA for 10 min at 37°C without cytochalasin B pretreatment. Cells were then placed on ice, fixed with 1% paraformaldehyde and processed for immunofluorescence flow cytometry. Control untreated electropermeabilized cells were run in parallel. Antigen cell surface expression was measured in paraformaldehyde-fixed neutrophils as described previously (16) using a BD Biosciences FACScalibur flow cytometer.

RT-PCR

Total RNA (5 μg), primed with oligo-dT, was reverse-transcribed into cDNA at 37ºC for 2 h using a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions in a final volume of 20 μl. A 25-μl PCR mixture contained 250 ng of cDNA template, 20 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 5 units of Taq DNA polymerase (Roche, Basel, Switzerland). PCR reactions were performed in a GeneAmp PCR system model 9600 (Perkin-Elmer, Norwalk, CT). The primers used are listed in Table 1, where the nucleotide numbers indicate the primer location in the corresponding sequences obtained from the GenBank/EMBL database. PCR amplification was as follows: 1 cycle at 95ºC for 5 min as an initial denaturation step, then denaturation at 95ºC for 30 s, annealing for 30 s at distinct temperatures as shown in Table 1, and extension at 72ºC

for 60 s (35 cycles), followed by further incubation for 15 min at 72ºC (1 cycle). PCR products were electrophoresed on 2-2.5 % agarose gels in 1 X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide staining.

PCR products were either extracted from the gel using Concert™ kit (Gibco-BRL) or cloned into the pCR®2.1 vector, using the TA-TOPO cloning kit (Invitrogen, San Diego, CA) following the manufacturer's indications, and sequenced in an automatic sequencer.

For semiquantitative analysis, the number of cycles was reduced (20-28 cycles) to achieve the linear phase of amplification. Amplification of human *β-actin* (GenBank accession number: NM_001101) was used as an internal control. The sense and anti-sense primers for *β-actin* cDNA amplification were 5'-AATATGGCACCACACCTTCTACA-3' and 5'-CGACGTAGCACAGCTTCTCCTTA-3'. This primer pair amplified a 403-bp fragment.

Immunoelectron microscopy

Resting human neutrophils were fixed for 24 h in 2% paraformaldehyde in 0.1M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described (52). Forty-five-nanometer cryosections were cut at -120ºC using diamond knives (Drukker International B.V., Cuijk, The Netherlands) in an ultracryomicrotome (Leica, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids (53). The grids were placed on 35-mm petri dishes containing 2% gelatin. For double immunolabeling, the procedure described by Slot *et al.* (54) was followed with 10- and 15-nm protein-A conjugated colloidal gold probes (Electron

Microscopy Laboratory, Utrecht University, Utrecht, The Netherlands). After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands). Negative controls, prepared by replacing the primary Ab by a nonrelevant Ab, showed no staining.

Statistical analysis

Statistical evaluation of the effect of anti-Rab27a Ab on neutrophil secretion was performed by Students's *t*-test. The criterion for statistical significance was taken as $p < 0.05$.

Results

Rab27a expression in human neutrophils

By using different sets of primers (Table 1) we found that human peripheral blood neutrophils expressed *rab27a* mRNA by RT-PCR (Fig. 1) and subsequent sequencing, whereas the level of *rab27b* transcripts was negligible (data not shown). Two consecutive PCR runs were required to scarcely detect *rab27b* amplification. Subsequent cloning and sequencing of the respective amplicons confirmed that both *rab27* isoforms were expressed, but as indicated above at very different levels. *Rab27a* mRNA was also readily detectable in the NK-like cell line YT-Indy (Fig. 1), the human acute myeloid HL-60 cell line and the human promyelocytic cell line NB4 (data not shown), but *rab27b* mRNA expression was absent in these cell types. In agreement with the RT-PCR data, we found that human neutrophils contained high amounts of Rab27a protein, whereas the level of Rab27b protein was much lower (Fig. 2). Rab27b protein was only detected after protracted autoradiogram exposure (Fig. 2A). Thus, these data

indicate that Rab27a is the predominant isoform expressed in human neutrophils. The use of both a very specific anti-Rab27a mAb (Fig. 2) and a rabbit polyclonal Ab against Rab27a (Fig. 3A) detected this protein as a band of 27-29 kDa. Interestingly, Rab27a expression was particularly high in human neutrophils when compared to other *rab27a*-expressing cells (Fig. 3A). The HL-60 cell line has been largely used a cell culture model for human neutrophils (47). The expression of Rab27a was increased during differentiation of HL-60 cells with DMSO towards neutrophils (Fig. 3B). These results show that human neutrophils express high levels of Rab27a, suggesting a major role of this protein in mature neutrophils.

Subcellular localization of Rab27a in resting and activated human neutrophils

Two different specific anti-Rab27a Abs (a mAb and a rabbit polyclonal Ab), recognized a band of about 27-29 kDa in the postnuclear extract and in the membrane fraction of human neutrophils, but not in the soluble fraction containing the cytosol (Fig. 4A, and data not shown), indicating that Rab27a was membrane-bound. To determine the subcellular localization of Rab27a in resting human neutrophils, we performed subcellular fractionation assays that resolved cytosol, plasma membrane, as well as tertiary, specific and azurophil granules (Fig. 4B). Under these experimental conditions, secretory vesicles, identified by latent alkaline phosphatase, were not resolved from the plasma membrane (7, 16). We found that Rab27a was located mainly in the membranes prepared from subcellular fractions 4-6, enriched in tertiary and specific granules, with a minor location in fraction 8, enriched in azurophil granules (Fig. 4C). When human neutrophils were activated with PMA that released tertiary and specific granules (81% gelatinase and 68% lactoferrin secretion, respectively), but not primary granules (less than 5% secretion) (6, 7, 17), the tertiary/specific granule location of Rab27a was

translocated to the plasma membrane (fraction 2), whereas the azurophil granule-located Rab27a remained in the last fraction (fraction 8) of the subcellular fractionation (Fig. 4C). A comparison of the subcellular fractionation distribution of Rab27a in resting neutrophils (Fig. 4C) suggests that Rab27a is slightly more abundant in tertiary granules (fractions 4-5) than in specific granules (fractions 5-6).

Ultrastructural localization of Rab27a in human neutrophils

To get a better insight on the subcellular localization of Rab27a, resting neutrophils were immunolabeled for Rab27a and analyzed by immunogold electron microscopy. Gold label was detected predominantly on the membrane of some granules (Fig. 5A). A small amount of gold label was found in vesicles and Golgi (Fig. 5A), that could be related to the low macromolecule synthesis capacity displayed by these cells (55, 56). Quantitation of the relative percentage of gold particles in the distinct subcellular structures of neutrophils showed that most of Rab27a was located in granules (66.4%), with a minor proportion in vesicles (31.6%) and a very low amount on plasma membrane (2.0%). To further identify the Rab27a-positive vesicles, we double labeled neutrophils with Abs against anti-Rab27a and anti-CD35, this latter as a marker for secretory vesicles. CD35 was present in both vesicles and plasma membrane (Fig. 5B) as a result of the endocytic origin of these secretory vesicles together with their proneness to be secreted (57). Granules positive for Rab27a (small gold particles, arrows in Fig. 5B) were devoid of CD35 labeling, and no CD35-rich vesicles were positive for Rab27a (Fig. 5B). After counting 200 positive vesicles, we found 54% of them were labeled only for CD35, 41% labeled only for Rab27a and 5% labeled for both CD35 and Rab27a. These data agree with our above subcellular fractionation analysis and indicate that Rab27a is largely absent from CD35- and latent alkaline

phosphatase-rich secretory vesicles. Cryosections of resting human neutrophils were also double labeled with Abs against Rab27a and markers for the different cytoplasmic granules, namely gelatinase (tertiary granules), lactoferrin (specific granules), or myeloperoxidase (azurophil granules), and analyzed by immunogold electron microscopy. Interestingly, Rab27a was mainly located on the membrane of tertiary and specific granules, with a minor presence in myeloperoxidase-positive granules (Fig. 6, A-C). The degree of colocalization of Rab27a with the distinct granule markers is shown in Fig. 6D, after analyzing at least 300 positive granules for Rab27a. Because labeling with anti-Rab27a Ab was weaker than labeling with the corresponding granule markers and only one section was examined for each granule, we analyzed colocalization only in Rab27a-positive granules in order to avoid that the less abundant granule constituent, i.e. Rab27a, could be missed in a particular section of the same granule. These results demonstrate the presence of Rab27a in both tertiary and specific granules, which are readily exocytosed upon cell activation, with a minor location in azurophil granules. Rab27a was more abundant in tertiary granules than in specific granules, about 80% of the Rab27a-positive granules were also enriched in gelatinase (Fig. 6D). Thus, our results suggest that practically all the tertiary granules contain Rab27a in their membranes.

Involvement of Rab27a in neutrophil exocytosis

We next analyzed whether Rab27a has a functional role in neutrophil exocytosis. Human mature neutrophils are non-dividing end cells with poor survival after isolation and show a low macromolecule biosynthesis capacity (47, 55, 58, 59). Consequently, exogenous gene expression or gene down-regulation in these cells is challenging. In addition, there is a lack of transfectable human models of leukocytes to analyze

secretion of tertiary and specific granules as human cell lines, such as HL-60 or NB4, lack these granules when differentiated towards the granulocytic lineage (60, 61). Thus, we used permeabilized neutrophils for the analysis of tertiary and specific granule secretion in human neutrophils. To this aim, we prepared electropermeabilized neutrophils that were able to undergo exocytosis of cytoplasmic granules upon cell activation with Ca^{2+} and GTP-γ-S (16, 50, 51), and allowed rapid access of antibodies into the cytoplasm (16). More than 95% of electropermeabilized neutrophils, following two discharges of 5 kV/cm, were rendered permeable to exogenously added antibodies (16). Degranulation was analyzed by measuring upregulation of the granule membrane markers CD63 and CD66b at the cell surface, as a measure of neutrophil degranulation in electropermeabilized neutrophils (16, 51). This method has been previously shown to monitor efficiently neutrophil degranulation in electropermeabilized neutrophils (16-18, 50, 51). Following paraformaldehyde fixation of electropermeabilized neutrophils, we monitored the expression of both CD63 and CD66b only at the cell surface, allowing us to determine neutrophil degranulation (16). Upregulation of CD63 parallels secretion of azurophil granules, whereas CD66b upregulation parallels secretion of both tertiary and specific granules (17). Incubation of electropermeabilized neutrophils with anti-Rab27a Abs largely inhibited CD66b upregulation (≅70% inhibition, $p < 0.01$) after cell activation with Ca^{2+} and GTP-γ-S, whereas the effect on CD63 upregulation was rather low (≅20% inhibition) (Fig. 7A) and was not statistically significant ($p > 0.05$). PMA induced secretion of only tertiary and specific granules, but not of azurophil granules, in electropermeabilized neutrophils (Mollinedo, F. and Martin-Martin, B., unpublished observations), and we found that preincubation of human neutrophils with anti-Rab27a Abs led to a high inhibition of the CD66b upregulation (≅76% inhibition, $p < 0.01$) induced by PMA (Fig. 7B). Incubation of electropermeabilized neutrophils with an

unrelated and irrelevant Ab, such as anti-CD20 mAb, or with P3X63 myeloma culture supernatant or rabbit preimmune serum, used as a negative controls, had no effect on neutrophil degranulation (Fig. 7). These data indicate that anti-Rab27a Ab inhibits secretion of CD66b-containing tertiary and specific granules.

Expression of Slp/Slac2 proteins in human neutrophils

Rab proteins promote membrane trafficking through interaction of the GTP-bound form of Rab with specific effector molecules (26). To date, a group of Rab27 effectors, collectively named Slp/Slac2 or exophilins, have been identified that share a common N-terminal homologous Rab-binding region (31, 32, 62). Despite Rab27a protein was readily detectable in neutrophil extracts (Figs. 2 and 3), we were unable to detect Slp/Slac2 proteins in human neutrophils (Fig. 8A). Under our immunoblot conditions (Fig. 8, A and B), neither Slp nor Slac2 proteins were detected in human neutrophils, suggesting that additional Rab27 effectors other than Slp/Slac2 proteins may function in human neutrophils. RT-PCR, followed by sequencing, as well as immunoprecipitation assays allowed identification of Slp1 (or JFC1) and Slp3-a in human neutrophils (Fig. 8, C and D). This agrees with the previously reported expression of JFC1 in human neutrophils (63). Overall, our data suggest that neutrophils are rather deficient in most Slp/Slac2 proteins, or express very low amounts of these proteins.

Discussion

The data reported here demonstrate that Rab27a is present in tertiary and specific granules of human neutrophils, and it regulates their secretion upon cell activation. Our ultrastructural and biochemical data indicate that tertiary granules are particularly enriched in Rab27a. A minor location of Rab27a is found in the azurophil granules,

where it hardly affects their mobilization. The isoform Rab27b is also expressed in human neutrophils, but at a much lower extent than Rab27a. Thus, Rab27a protein is the predominant isoform in neutrophils, where it is highly expressed. Our data agree with a recent proteomic analysis of the three major neutrophil granules that showed a higher presence of Rab27a in gelatinase granules, albeit it could be detected in the three major granule populations (40). A very recent report has claimed that Rab27a is distributed mainly in a minor population of myeloperoxidase-containing granules that were assumed to be azurophil granules (41). However, a close examination of the results reported by Munafo *et al.* (41) showed that Rab27a was mainly present in low-density granules, enriched in VAMP-2 and readily exocytosed upon PMA stimulation. These latter features correspond more likely to tertiary and specific granules, which are low-density organelles (4, 5), are enriched in VAMP-2 (17), and are readily secreted following PMA activation (6, 7, 17). In contrast, azurophil granules are high-density granules, do not contain VAMP-2 and are not mobilized after PMA incubation (4-7, 17). At the first identification of tertiary granules as a novel entity (8), it became apparent that they contained low levels of some azurophil granule markers, and this might explain the above claim for the presence of Rab27a in low-density myeloperoxidase-containing granules. Our present findings, together with previous reported evidences, indicate that Rab27a is abundantly expressed in human neutrophils, with a predominant location in specific and, above all, gelatinase-rich tertiary granules, and regulates the exocytosis of these granules. Interestingly, we did not find Rab27a in the CD35-rich secretory vesicles, which have been previously reported to contain Rabs 3a, 4, and 5a (24), thus suggesting a rather selective role of Rab27a in the secretion of particular human neutrophil granules.

Tertiary and specific granules are readily mobilized at the early stages of neutrophil activation, and they might be so-called secretory granules, in contrast to azurophil granules, which are sluggishly mobilized following neutrophil activation and are more involved in phagocytosis. The rapid mobilization of tertiary and specific granules upon cell activation, together with their characteristic protein constituents involved in diapedesis, chemotaxis and superoxide anion generation, suggest a major role of these two granules in the early stages of neutrophil activation and inflammation (6, 12, 14). Thus, the role of Rab27a in the exocytosis of tertiary and specific granules might explain the reported defects in respiratory burst and chemotaxis in some type 2 Griscelli syndrome patients (34).

We found that Rab27a was upregulated during neutrophil differentiation of HL-60 cells. However, HL-60 cells do not form specific and tertiary granules during neutrophil differentiation (60, 64), despite they retain the ability to express a number of proteins typically located in these organelles in mature neutrophils when driven into granulocytic maturation (17, 18, 65). In this regard, the expression of Rab27a during DMSO-induced neutrophil differentiation of HL-60 cells resembles the high induction of CD11b expression during HL-60 differentiation (47, 65), even though most of this protein is located in specific and tertiary granules in mature resting human neutrophils (10, 11). This observation suggests a different location of CD11b in neutrophil-differentiated HL-60 cells, namely, cell surface (47). On these grounds, Rab27a should be present in neutrophil-differentiated HL-60 cells in a distinct location as compared to mature human peripheral blood neutrophils. Munafo *et al.* (41) have recently found that HL-60 cells transfected with Rab27a-specific siRNA showed a dramatic decrease in the level of expression of Rab27a and a significant inhibition in secretion, suggesting a role for this protein in myeloid cell exocytosis.

We found that the GTP-binding Rab27a was membrane-bound in human neutrophils, which is in agreement with its usual membrane localization through geranylgeranylation (66, 67). The involvement of Rab27a in the regulated exocytosis of lysosome-related organelles and secretory granules is dependent on its interaction with a wide array of effectors (31, 32), most of them are classified within the collective name of Slp/Slac2 proteins or exophilins, that share an N-terminal Rab27a-binding region (31, 32, 62). However, we failed to detect expression of most Slp/Slac2 proteins, except Slp1 (JFC1) and Slp3-a, in human neutrophils. Nevertheless, expression of Slp1/JFC1 and Slp3-a was scarce, and they were only detected by RT-PCR or immunoprecipitation. Slp1/JFC1 has been previously identified in neutrophils and assigned to play a role in the respiratory burst of neutrophils (63). In addition, Slp1/JFC1 was found to coimmunoprecipitate with Rab27a in human neutrophil lysates (41). These results might agree with a predominant location of Rab27a in tertiary granules as these organelles are enriched in the cytochrome *b* required for superoxide anion generation (8), and they are readily mobilized to the plasma membrane where Slp1/JFC1 has been located (63). In this regard, fusion of tertiary granules with plasma membrane primes neutrophils for respiratory burst (14). However, the lack of correlation between the relatively high abundance of Rab27a and the low expression of Slp/Slac2 proteins suggests that additional proteins should act as Rab27a effectors in human neutrophils. Rab proteins have been suggested to interact with SNARE proteins to ensure appropriate membrane fusion processes (68-70). This could be an attractive possibility as human neutrophils express a high number of SNARE proteins (16-19, 48, 71, 72) that could act as putative Rab27a effector candidates. Both Rab (73, 74) and SNARE proteins (75) might become major regulators of secretory processes during the immune response. Another putative candidate for a Rab27a effector molecule in neutrophils might be Munc13-4, as

previously demonstrated in other cell systems, including platelets, mast cells, cytotoxic lymphocyts and NK cells (76-78), but this remains to be elucidated.

Rab27a is expressed in a broad range of specialized secretory cells, including exocrine, endocrine, ovarian, and hematopoietic cells, most of which undergo regulated exocytosis (30, 79-81). The regulation of the secretion of tertiary and specific granules by Rab27a reported here suggests a major role for Rab27a in inflammation. Our present data, together with the rather widespread presence of Rab27a in cells of the immune system (38, 74), suggest that Rab27a is a key player in secretory processes occurring in both innate and adaptative immune systems. The present findings on the role of Rab27a in the regulation of the exocytosis of tertiary and specific granules in human neutrophils might explain, at least in part, some of the clinical manifestations of type 2 Griscelli syndrome regarding defects in neutrophil function.

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Disclosures

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Table 1

Gene	Sequence	Annealing temperature
rab27a (accession number: NM_004580)		
set 1		
(forward; nt 426-443):	5’-GCCACTGGCAGAGGCCAG-3’	62 ºC
(reverse; nt 679-698):	5’-GAGTGCTATGGCTTCCTCCT-3’	
set 2		
(forward; nt 426-443):	5’-GCCACTGGCAGAGGCCAG-3’	62 ºC
(reverse; nt 896-911):	5’-CTCGAGTCAACAGCCACATGCC-3’	
rab27b (accession number: U57093)		
set 1		
(forward; nt 273-290):	5’-TCTTCAGGGAAAGCATTT-3’	53 ºC
(reverse; nt 526-545):	5’-CAGTTCCCGAGCTTGCCGTT-3’	
set 2		
(forward; nt 273-290):	5’-TCTTCAGGGAAAGCATTT-3’	53 ºC
(reverse; nt 731-749):	5’-GGCTCGAGCTAGCAGATACATTTCTTC-3’	
slp1 (accession number: NM_032872)		
(forward; nt 300-318):	5’-CTTGCCCTTCATTGACTCC-3’	60ºC
(reverse; nt 549-566):	5’-TGGTCGCTGATCTTGAGC-3’	
slp2 (accession number: NM_032943)		
(forward; nt 2477-2498):	5’-GTAAAAAAACAGCGTTCAGACC-3’	52ºC
(reverse; nt 3205-3223):	5’-CTCCCAGAGAGCAACTTCC-3’	
slp3 (accession number: XM_087804)		
(forward; nt 1781-1802):	5’-AATCCGTATGTGAAGACCTACC-3’	65ºC
(reverse; nt 2383-2400):	5’-AAGCTTGACTGCCTCAGC-3’	
slp4 (accession number: NM_080737)		
(forward; nt 2050-2070):	5’-CCCTGAATCCTCACTACAACC-3’	60ºC
(reverse; nt 2778-2799):	5’-TGTCTTTACTAACCAACCCTGC-3’	
slp5 (accession number: NM_138780)		
(forward; nt 1438-1456):	5’-ACTATCAGCCATACCCAGC-3’	55ºC
(reverse; nt 2023-2041):	5’-AATTCAAACGAACTCCTCC-3’	
slac2-a (accession number: NM_024101)		
(forward; nt 347-365):	5’-GACACTGCCCATCTGAACG-3’	55ºC
(reverse; nt 769-790):	5’-ATCTGAGTCTCCCTCGAAGTCG-3’	
slac2-c (accession number: NM_015460)		
(forward; nt 681-701):	5’-CAGAAGGACATAGTGTGATGG-3’	60ºC
(reverse; nt 1353-1370):	5’-GACACAGCTTGCTCAAGG-3’	

Figure Legends

FIGURE 1. Expression of small GTPase *rab27a* mRNA in human neutrophils. Expression of *rab27a* gene in human peripheral blood neutrophils (PMN) and the NK-like cell line YT-Indy by RT-PCR. PCR amplification of *β-actin* was used as an internal control.

FIGURE 2. Expression of Rab27a/b protein in human neutrophils. (A) Similar amounts of recombinant FLAG-tagged Rab27a/b in COS-7 cells (lane 1) and human neutrophil lysates (80 μg protein; lane 2) were loaded on SDS-10% polyacrylamide gels and immunoblotted with anti-Rab27a mAb (upper panel) or anti-Rab27b specific polyclonal Ab (lower panel). The migration positions of Rab27a and Rab27b are denoted (arrowheads). Lane 3 shows a long exposure autoradiogram. The positions of the molecular mass markers (in kDa) are shown on the left. (B) Recombinant FLAG-Rab27a/b were used as positive controls in the top two panels. Similar amounts of the FLAG-tagged proteins were loaded into each lane.

FIGURE 3. Expression of Rab27a in distinct cell types and during HL-60 cell differentiation. (A) Presence of Rab27a protein in cell extracts (30 μg protein) derived from the NK-like cell line YT-Indy, NB4 cells and human peripheral blood neutrophils (PMN), assessed by Western blot using an anti-Rab27a polyclonal Ab. (B) Expression of Rab27a protein during DMSO-induced differentiation of HL-60 cells towards the neutrophil lineage, assessed by Western blot (30 μg protein) using an anti-Rab27a polyclonal Ab. The position of Rab27a is indicated (arrowhead). Data shown are representative of three separate experiments.

FIGURE 4. Subcellular distribution of Rab27a in human neutrophils. (A) Equal amounts (40 μg protein) of postnuclear extract (E), soluble (S) and membrane (M) proteins from resting human peripheral blood neutrophils were run on SDS-polyacrylamide gels and analyzed by immunoblotting using an anti-Rab27a polyclonal Ab. The molecular masses (kDa) of protein markers are indicated on the left. (B) Resting human neutrophils were gently disrupted and subjected to subcellular fractionation as described in Materials and Methods. Fractions were collected and analyzed for the activity of specific organelle markers, which are plotted normalized to the fraction with maximal activity. The following markers were assayed. Cytosol (CYT): lactate dehydrogenase (open squares); plasma membrane (PM): HLA (closed circles); tertiary granules (TG): gelatinase (open triangles); specific granules (SG): lactoferrin (closed inverted triangles); azurophil granules (AG): peroxidase (open diamonds). (C) Membrane proteins (30 μg) from the subcellular fractions 2-8 of resting (Rest.) and PMA-activated (Act.) human neutrophils were assayed for Rab27a by immunoblotting. The migration position of Rab27a is indicated (arrowheads). All data shown are representative of three separate experiments.

FIGURE 5. Rab27a is localized at the membranes of both intracellular granules and vesicles of human neutrophils. (A) Cryosections of neutrophils were immunogold labeled with anti-Rab27a (10-nm gold). Rab27a localized (arrows) at the membrane of granules and vesicles (v). (B) To characterize the Rab27a-positive vesicles, neutrophils were double labeled with anti-Rab27a (10 nm-gold) and anti-CD35 (15 nm-gold). No CD35-positive vesicles were positive for Rab27a. Arrows pointed to Rab27a-positive granules. Scale bar, 200 nm.

FIGURE 6. Characterization of Rab27a-positive granules. Neutrophils were double labeled with Rab27a and respectively, gelatinase (Gel, A), lactoferrin (Lf, B) or myeloperoxidase (MPO, C). In *A* and *B*, many of the Rab27a-positive granules are also labeled for gelatinase and lactoferrin respectively (arrows); whereas the majority of Rab27a-positive granules (arrows) were negative for MPO. Scale bar, 200 nm. (D) Ultrathin cryosections were double immunogold-labeled for Rab27a and either gelatinase (marker for tertiary granules), lactoferrin (marker for specific granules) or myeloperoxidase (marker for azurophil granules). Histograms indicate the percentage of Rab27-positive granules displaying colocalization with the marker of these granules. For each experiment at least 300 positive granules were analyzed.

FIGURE 7. Involvement of Rab27a in the secretion of neutrophil granules. (A) Electropermeabilized neutrophils (PMN) were incubated in the absence (Control), or in the presence of P3X63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), rabbit preimmune serum (20 μg/ml), or of increasing concentrations of anti-Rab27a polyclonal Ab, and then activated with Ca^{2+} + GTP-γ-S, and assayed for CD63 and CD66b antigen expression by flow cytometry. Data are expressed as the percentage of CD63 and CD66b cell surface increase upon electropermeabilized neutrophil activation compared with the CD63 and CD66b cell surface upregulation detected in control Ca^{2+} + GTP-γ-S-stimulated electropermeabilized neutrophils in the absence of any Ab (Control). (B) Electropermeabilized neutrophils (PMN) were incubated in the absence (Control), or in the presence of P3X63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), rabbit preimmune serum (20 μg/ml), or of increasing concentrations of anti-Rab27a polyclonal Ab, and then activated with PMA, and assayed for CD66b antigen expression by flow cytometry. Data are expressed as the percentage of CD66b cell surface increase as in *A*.

Mean values ± S.D. of five independent determinations are shown. *Asterisks* indicate values that are significantly different from preimmune serum-treated cells at $p < 0.01$ (**) level by Student's *t*-test.

FIGURE 8. Expression of Rab27 effectors (Slp and Slac2) in human neutrophils. (A) Expression of the Slp and Slac2 family members in human neutrophils. Similar amounts of recombinant T7-tagged Slp1-5 and Slac2-a/c expressed in COS-7 cells (lane 1; see B) and total homogenates of human peripheral blood neutrophils (PMN) (80 μg; lane 2) were loaded on SDS-7.5% polyacrylamide gels and immunoblotted with anti-Slp1, anti-Slp2-a, anti-Slp3-a, anti-Slp4-a, anti-Slp5, anti-Slac2-a, or anti-Slac2-c specific Abs. (B) Recombinant T7-Slp1-5 and T7-Slac2-a/c were used as positive controls in *A*. Similar amounts of the T7-tagged proteins were loaded into each lane. The positions of the molecular mass markers (in kDa) are shown on the left. (C) Expression of *slp1* and *slp3-a* mRNA in human neutrophils and neutrophil-differentiating HL-60 cells. Total RNA was purified from untreated HL-60 cells, HL-60 cells treated with 1.3% (v/v) DMSO for 4 days, and human peripheral blood neutrophils (PMN), and subjected to semiquantitative RT-PCR analysis using specific oligonucleotide primers for each gene. PCR amplification of *β-actin* was used as an internal loading control. The PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. (D) Immunoprecipitation of Slp1 and Slp3-a in human neutrophils. Biotinylated extracts from untreated HL-60 cells, HL-60 cells treated with 1.3% (v/v) DMSO for 4 days, and human peripheral blood neutrophils (PMN), were immunoprecipitated with specific antibodies against Slp1 and Slp3-a or with P3X63 myeloma supernatant, as a negative control, and immunoprecipitates were subjected Western blot. The molecular mass of

the immunoreactive bands are indicated on the right (arrowheads). Data shown are representative of three separate experiments.

Figure 1
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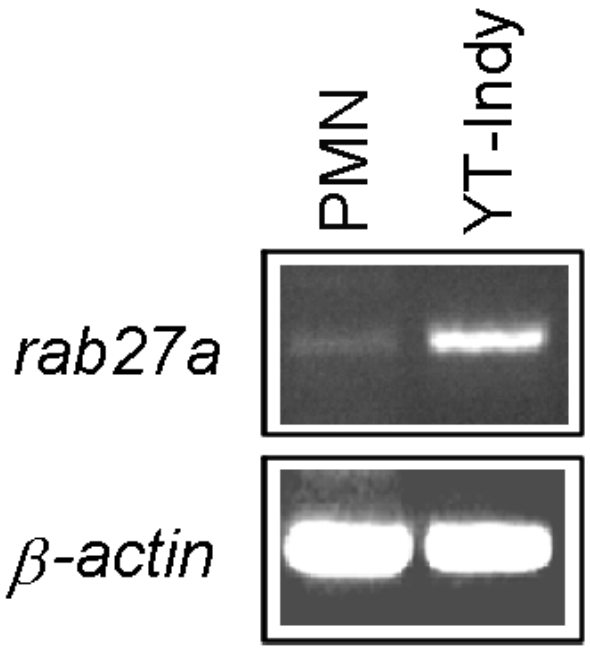

Figure 2
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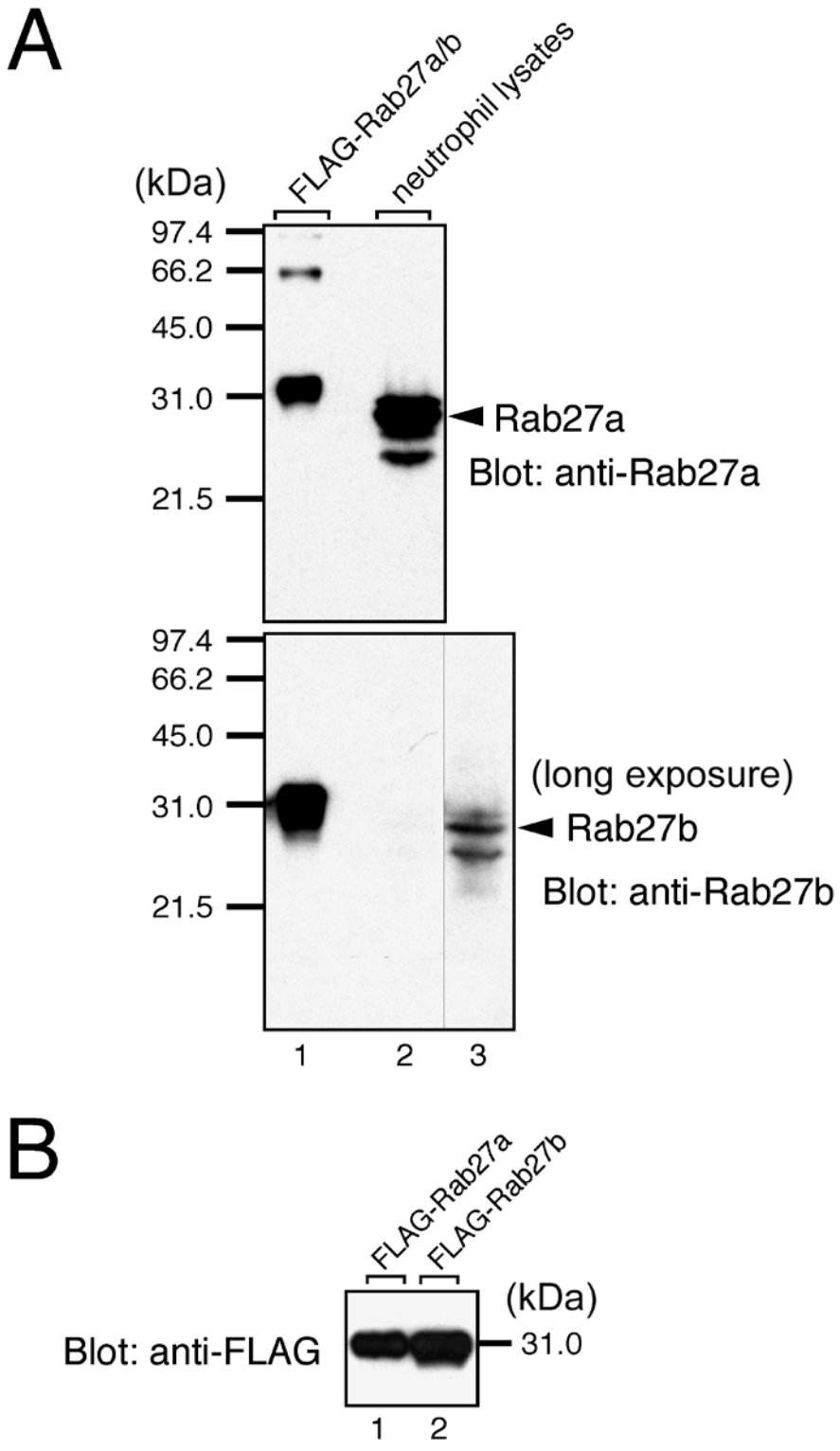

Figure 3
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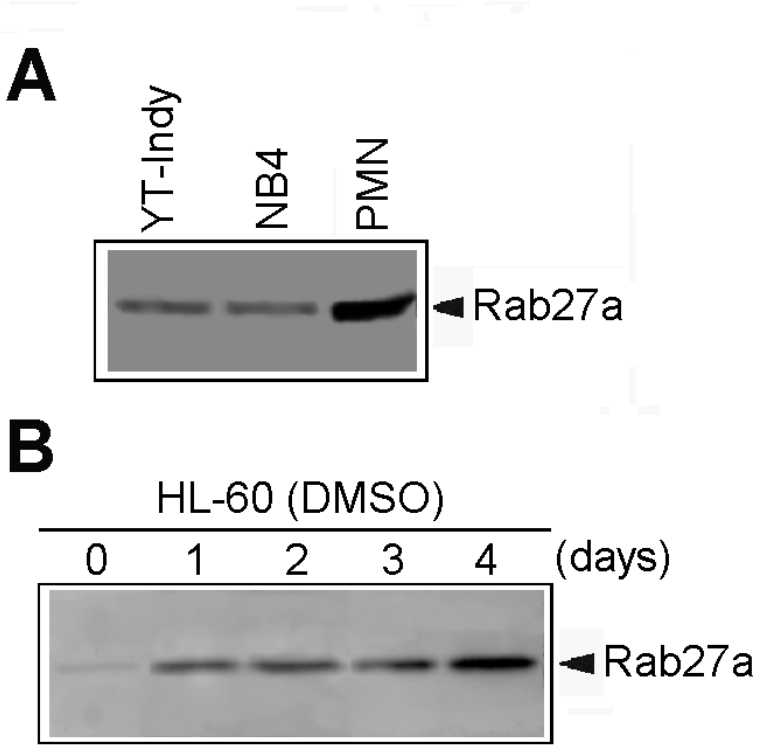

Figure 4
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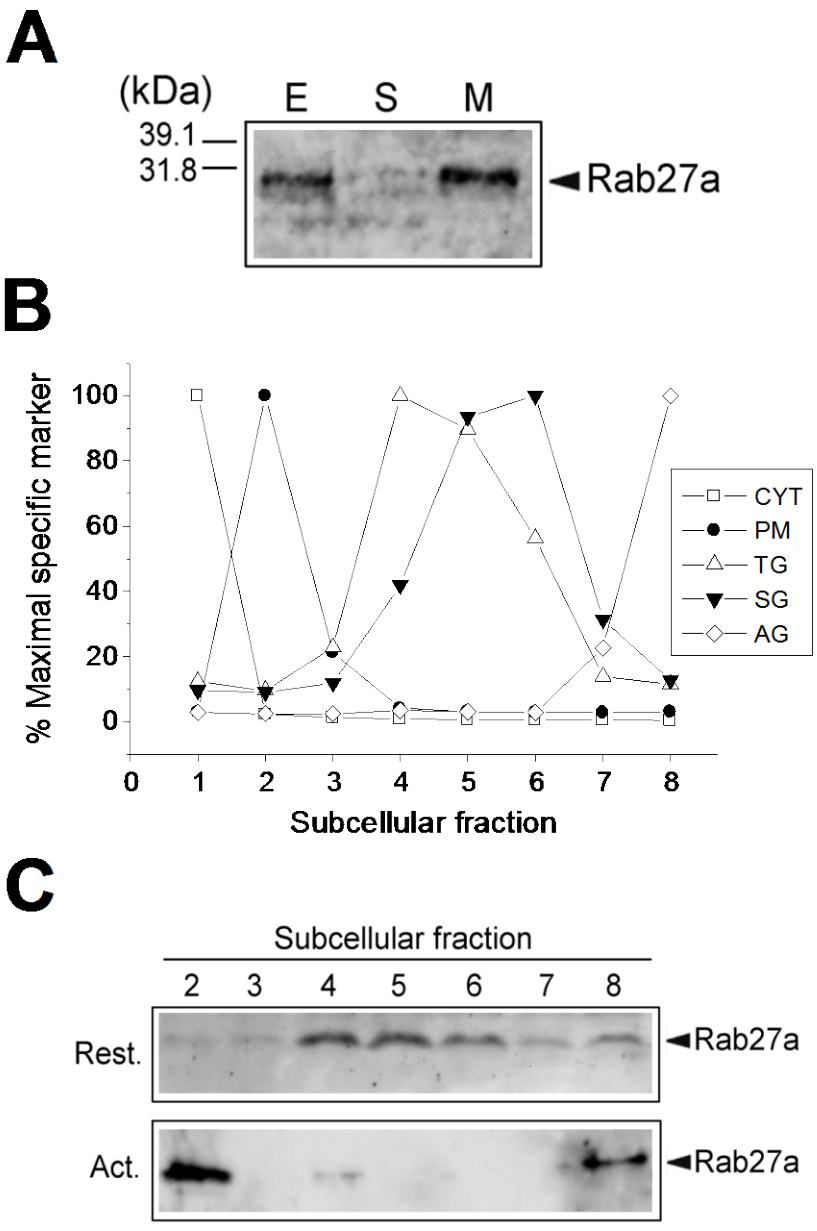

Figure 5
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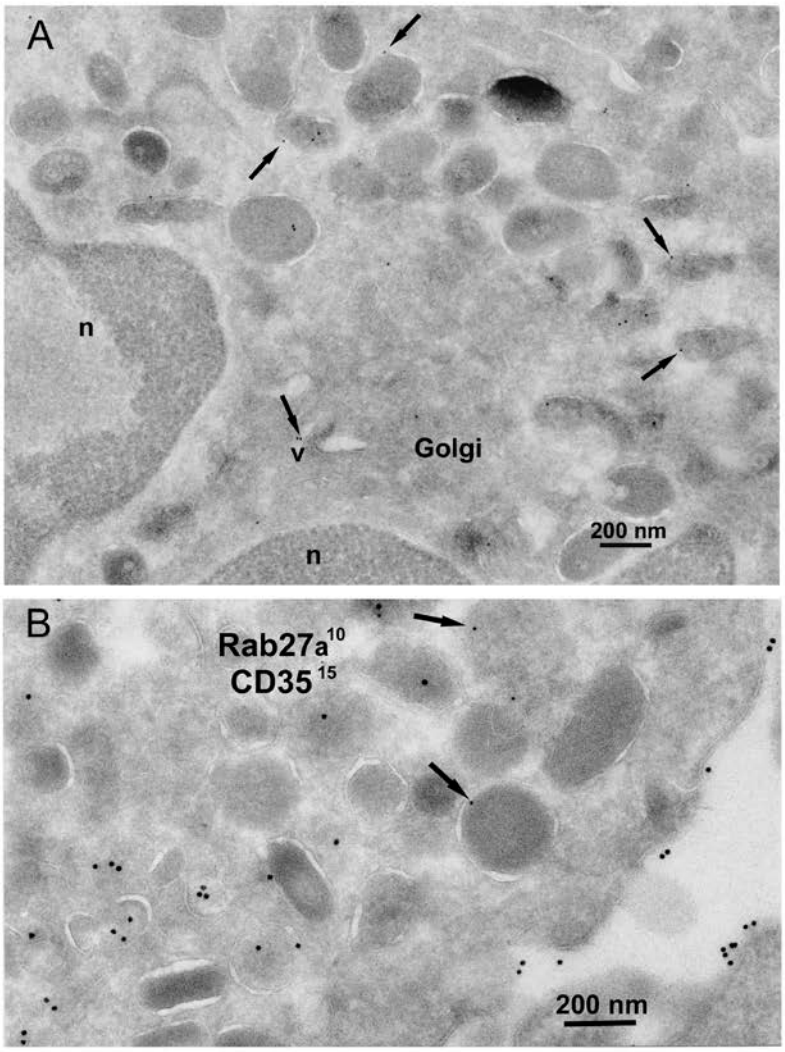

Figure 6
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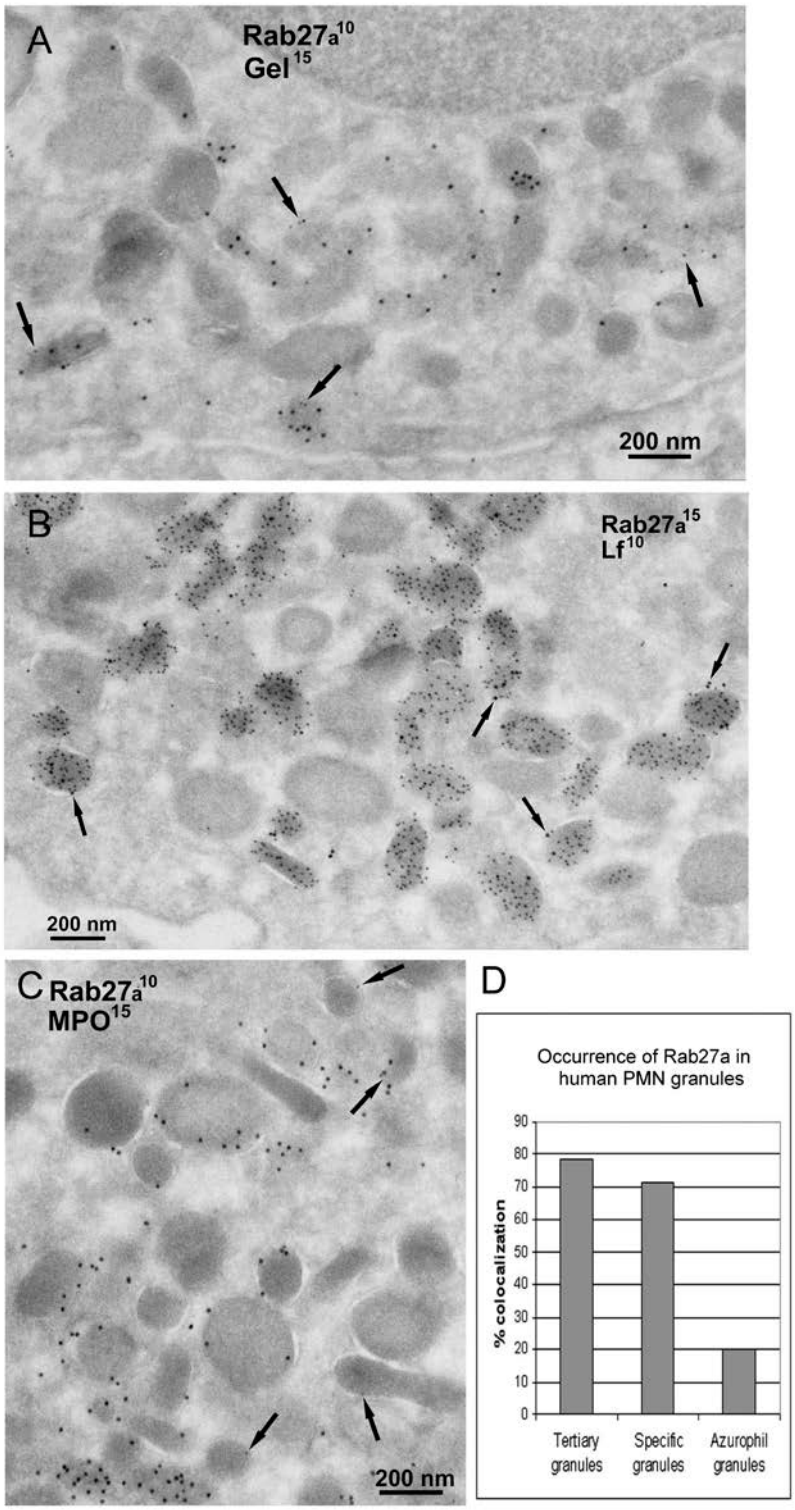

Figure 7
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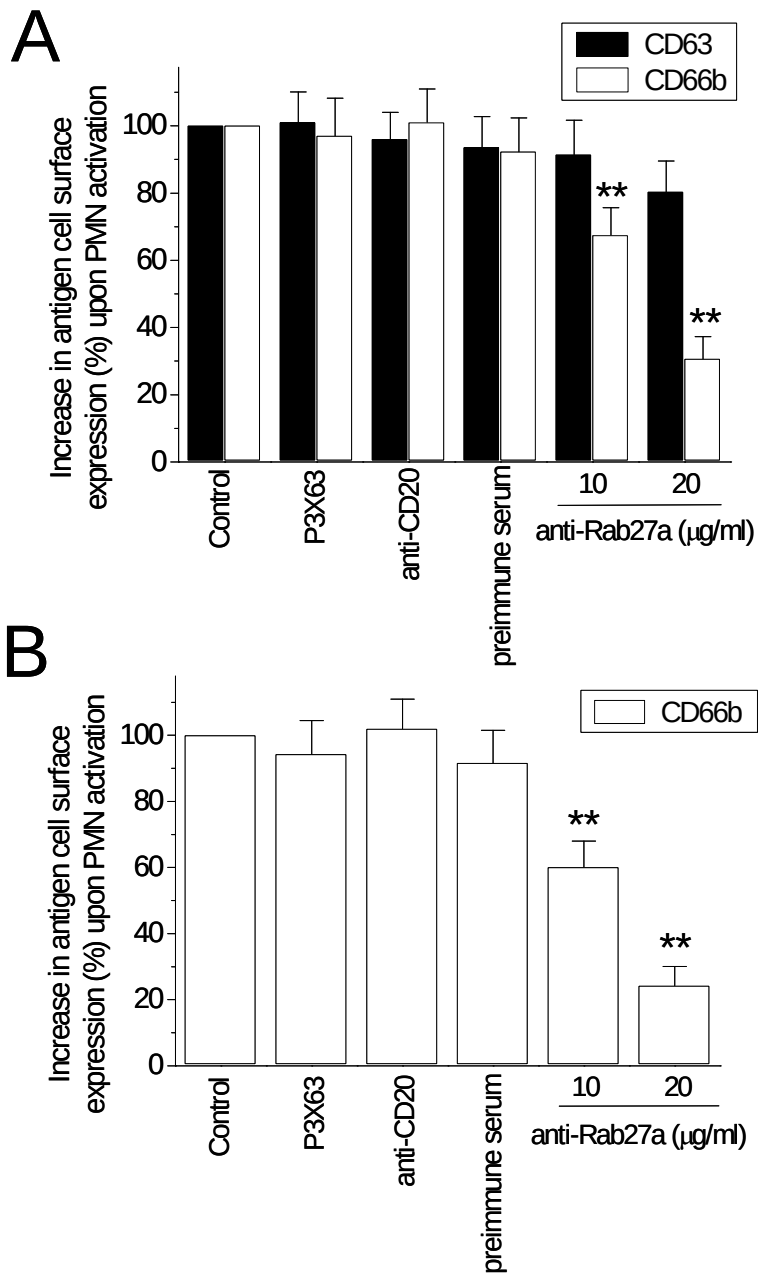

Figure 8

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