Annexin 3 is associated with cytoplasmic granules in neutrophils and monocytes and translocates to the plasma membrane in activated cells

Véronique LE CABEC and Isabelle MARIDONNEAU-PARINI*
INSERM U332, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, Paris, France

Annexins are soluble proteins capable of binding to phospholipid membranes in a calcium-dependent manner. Annexin 3, a 33 kDa protein mainly expressed in neutrophils, aggregates granules in cell-free assays, and a 36 kDa variant of this protein, specifically expressed in monocytes, has recently been identified. To obtain further information on these proteins, we defined their subcellular localization in resting and activated cells by immunofluorescence microscopy. Both proteins were associated with cytoplasmic granules in resting cells. We obtained evidence to indicate that, in neutrophils which possess a heterogenous granule population, annexin 3 was more likely to be associated with the specific granules. In cells activated with phorbol 12-myristate 13-acetate or opsonized zymosan, the 33 kDa and 36 kDa proteins translocated to the plasma or the phagosome membrane. Upon stimulation with A23187, annexin 3 translocated to the plasma membrane only in neutrophils. We also report that while annexin 3 was associated with restricted membranes in intact cells, it binds indiscriminately to every membrane fraction in cell-free assay. In conclusion, association of both forms of annexin 3 with granules suggests that these proteins could be implicated in processes of granule fusion.

INTRODUCTION

Phagocytic cells accomplish their task of host defense by destroying microorganisms through a combination of phagocytosis, generation of reactive oxygen metabolites and release of granule contents. When neutrophils are activated, they degranulate by fusion of specific and azurophil granules with the phagosome and/or the plasma membrane. Calcium is necessary for various responses in neutrophils, and there is evidence that translocation and fusion of granules with the plasma membrane is regulated by transient increases in intracellular calcium concentrations.

Among the proteins potentially involved in regulating the association and fusion of granules with the acceptor membrane, annexins appear to be good candidates. Indeed, proteins from the lipocortin/annexin family are able to bind to membrane phospholipids in the presence of calcium [1–5]. These proteins are structurally related; they all share four or eight repeated sequences of 70 amino acids that contains the calcium- and phospholipid-binding domains (for reviews see [6–8]). The physiological roles of these proteins remain unclear, but several functions have been proposed: membrane-cytoskeleton linkage, inhibition of phospholipase A2, mediation of intracellular calcium signals, transmembrane calcium transport, fusion of vesicles in secretory cells and involvement in the exocytic machinery [2,7–9]. Several members of the annexin family have been implicated in intracellular membrane fusion and granule aggregation [6]: annexin 7 (synexin) mediates aggregation and apparent fusion of chromaffin granules [10–13]; a similar function has been assigned to annexin 2 (calpactin) [9,14–16] and annexin 1 promotes calcium-dependent fusion of liposomes [17] as well as fusion of phospholipid vesicles with plasma membranes isolated from neutrophils [18,19]. In human neutrophils, annexin 3 represents 1% of cytosolic proteins [20], whereas it is expressed at low levels and often undetectable in many cell types [1,21]. When cells are engulfing yeast particles, annexin 3 accumulates at the periphagosomal area [22]. In addition, it promotes aggregation *in vitro* of isolated specific granules in the presence of 1 mM calcium [20] but its effect on isolated azurophil granules has not been investigated. A 36 kDa annexin 3 variant has recently been identified in human monocytes [23]. This protein is not expressed in human neutrophils, which only contain the originally described 33 kDa form. While both forms are expressed in undifferentiated HL60 cells to a similar level, differentiation of these cells along the neutrophilic or monocytic pathway is accompanied by progressive accumulation of the 33 kDa or the 36 kDa protein, respectively [23]. This suggests that the 33 kDa and 36 kDa proteins could play specific roles in neutrophils and monocytes.

In an attempt to study further the 33 kDa and 36 kDa forms of annexin 3 in phagocytes, we decided to investigate their subcellular localization in resting and activated cells. Using immunofluorescence microscopy, we report that both forms of annexin 3 were associated with cytoplasmic granules in resting cells, and translocate to the plasma and/or the phagosome membrane under particular conditions of stimulation. In addition, we observed that annexin 3 associates indiscriminately to every cell membrane in cell-free assay while its membrane association is restricted to specific compartments in intact cells.

EXPERIMENTAL

Antibodies

The specificities of rabbit anti-(annexin 3) antibodies and anti-(rap2) antibodies have been described elsewhere [23,24]. Polyclonal antibodies directed against intrinsic membrane proteins of azurophil granules were obtained by immunizing rabbits with azurophil granule membranes extracted as described previously [24]. The immune serum did not cross-react with other neutrophil subcellular fractions. The antisera were affinity purified on their corresponding nitrocellulose-bound antigen as described previously [24,25].

Abbreviations used: OZ, opsonized zymosan; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; MEM, minimum essential medium; DMSO, dimethyl sulphoxide.

* To whom correspondence should be addressed at, LPTF, CNRS UPR 8221, 205 Route de Narbonne, 31077 Toulouse Cedex, France.

Cell isolation and cell culture

Human leukocytes

Neutrophils and mixed monocytes/lymphocytes were isolated from blood collected from healthy donors, after Dextran T-500 sedimentation and centrifugation through Ficoll separating solution as previously described [26], and resuspended in Hepes-buffered minimum essential medium. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (stock solution 1 mg/ml dimethyl sulphoxide (DMSO) stored at −80 °C, Sigma Chemical Co.), A23187 (stock solution 10^{-2} M in DMSO, Sigma Chemical Co.) or zymosan opsonized in human serum as previously described [26].

HL60 cells

Cells were grown in suspension in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.), 1% L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a 5% CO_2-humidified atmosphere, and differentiated as described previously [27]. Briefly, differentiation along the monocytic pathway was induced by exposing HL60 [$(2–4) \times 10^5$ cells/ml] to 20 ng/ml of PMA for 4 days in large-culture Petri dishes, and neutrophil differentiation was obtained by exposing the HL60 cells to 1.3% DMSO (v/v) for 6 days.

Subcellular fractionation

Subcellular fractionation of neutrophils on discontinuous Percoll gradient was performed as previously described [27,28]. Briefly, neutrophils resuspended in relaxation buffer [27] (1×10^9 cells/10 ml) were cavitated in a nitrogen bomb at 375 p.s.i., collected in a tube containing EGTA (6 mM, final concentration) and centrifuged in order to remove debris, nuclei and intact cells. The supernatant was subjected to centrifugation at 48000 *g* for 20 min at 4 °C on a discontinuous isotonic Percoll gradient; four fractions corresponding to the cytosol, the plasma membrane-enriched fraction, the specific and the azurophil granules were collected and washed to remove the Percoll by centrifugation at 245000 *g* for 90 min at 4 °C. The granule and membrane fractions were resuspended in 0.34 M sucrose, 10 mM Pipes, pH 7.2 (5×10^8 cell equivalents/ml). Markers (alkaline phosphatase for the plasma membrane, lactoferrin for the specific granules and β-glucuronidase for the azurophil granules) were assayed in each subcellular fraction as previously described [29–32].

In some experiments, neutrophil subcellular membrane fractions (10^7 cell equivalents) were incubated in the presence of cytosol run on a G25 Sephadex column (column PD-10, Pharmacia) and supplemented by 1 mM EGTA, 1 μM or 100 μM calcium for 15 min at 37 °C. To remove the cytosol, the membrane fractions were centrifuged, the pellets were washed in PBS with or without 1 mM calcium and resuspended in SDS sample buffer at 100 °C.

HL60 cells were sonicated (ten 3 s pulses) at 4 °C with a Branson sonifier 450 set on 1 in PBS supplemented with 1 mM EDTA and proteinase inhibitors (aprotinin, 1000 units/ml; pepstatin, 1 μg/ml; leupeptin, 3 μg/ml; and PMSF, 1 mM). After centrifugation at 150 *g* for 8 min at 4 °C, the supernatant was centrifuged at 200000 *g* for 45 min at 4 °C, in order to separate the cytosol from the cell membranes.

SDS/PAGE and immunoblot analysis

Proteins solubilized by boiling in SDS–sample buffer for 10 min [33] were separated on SDS/PAGE (12% polyacrylamide gel) using the Mini-Protean II cell (Bio-Rad Laboratories, France) and transfered to nitrocellulose. The nitrocellulose sheets were subsequently treated as previously described [27].

Indirect immunofluorescence

Human neutrophils or monocytes were allowed to adhere for 5 or 30 min, respectively, onto uncoated sterilized glass coverslips in a 24-well tissue-culture plate as previously described [24]. For immunolocalization of annexin 3 in DMSO-differentiated HL60 cells, the glass coverslips were coated with poly-L-lysine (molecular mass 70–150 kDa, Sigma) and the cells suspended in RPMI were centrifuged onto the coverslips at 300 *g* for 10 min at 37 °C (1 ml/well, containing 4×10^5 cells) as described previously [34].

Cells were fixed and permeabilized in methanol at −20 °C for 6 min, incubated in 0.1% PBS–Tween for 5 min and exposed to primary and secondary antibodies [affinity purified fluorescein isothiocyanate (FITC)-conjugated goat anti-(rabbit IgG)] (KPL, Gaithersburg, MD, U.S.A.) [24]. In a few experiments, cells were fixed with 3.7% paraformaldehyde, in PBS supplemented with 0.03 M sucrose for 15 min at 4 °C, rinsed in 0.1% PBS–BSA and permeabilized with 0.05% saponin in PBS–BSA for 15 min [34]. The subsequent steps were performed as described above.

May–Grünwald Giemsa staining

After cell fixation and permeabilization, neutrophils were stained as previously described [35].

Measurement of superoxide production and lactoferrin release

The rate of superoxide generation by intact neutrophils was determined as previously described [26] using the superoxide dismutase-inhibitable cytochrome *c* reduction. Degranulation of specific granule was assayed by measuring lactoferrin release as previously described [30].

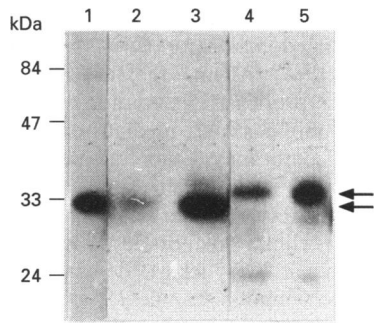

Figure 1 Distribution of annexin 3 between the cytosol and cell membranes in HL60 cells

Equal amounts of proteins (25 μg/well) from membrane (lanes 2 and 4) or cytosolic fractions (lanes 3 and 5) of DMSO-differentiated HL60 cells (lanes 2 and 3) and PMA-differentiated HL60 cells (lanes 4 and 5) fractionated in the presence of 1 mM EGTA were analysed by immunoblotting. The proteins were run on a 12% SDS/PAGE gel and transfered to nitrocellulose filters which were incubated with anti-annexin 3 antibodies, and the bands were visualized using ^{125}I-protein A. 50 ng of purified 33 kDa annexin 3 were loaded in lane 1. Molecular mass standards are shown on the left. Arrows indicate the position of the 33 and 36 kDa forms of annexin 3.

RESULTS

As a first step for determining the subcellular localization of the 33 kDa and 36 kDa annexin 3, we investigated whether the 36 kDa variant protein behaved as the other members of the annexin family, i.e. is cytosolic rather than membrane-associated.

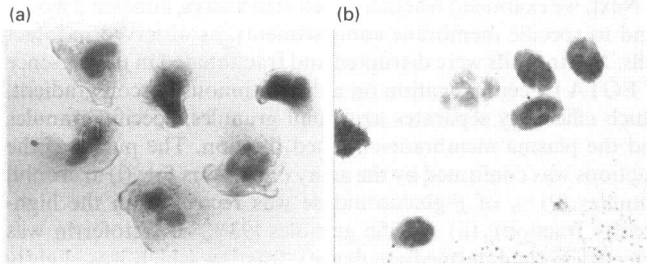

Figure 2 May–Grünwald Giemsa staining

Adherent resting neutrophils were fixed and permeabilized as described in the Methods section with methanol at −20 °C (**a**) or with paraformaldehyde and saponin (**b**) and subjected to May–Grünwald Giemsa staining.

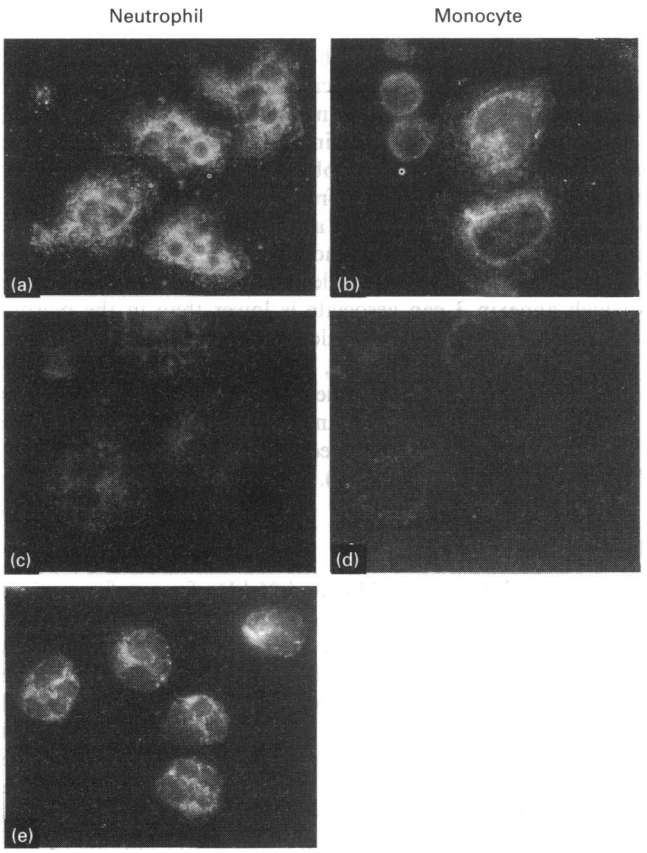

Figure 3 Subcellular localization of annexin 3 in resting neutrophils and monocytes by indirect immunofluorescence

Adherent resting neutrophils (a, c, e) or monocytes (b, d) were fixed and permeabilized, as described in the Methods section, with methanol at −20 °C (a–d) or with paraformaldehyde and saponin (e), and subjected to indirect immunofluorescence with affinity purified anti-annexin 3 antibodies. Antibodies were revealed by fluorescein-conjugated affinity purified goat anti-(rabbit IgG) (magnification × 1050). When the fluorescein-conjugated antibodies were used alone, no fluorescence was observed in neutrophils (c) or in monocytes (d).

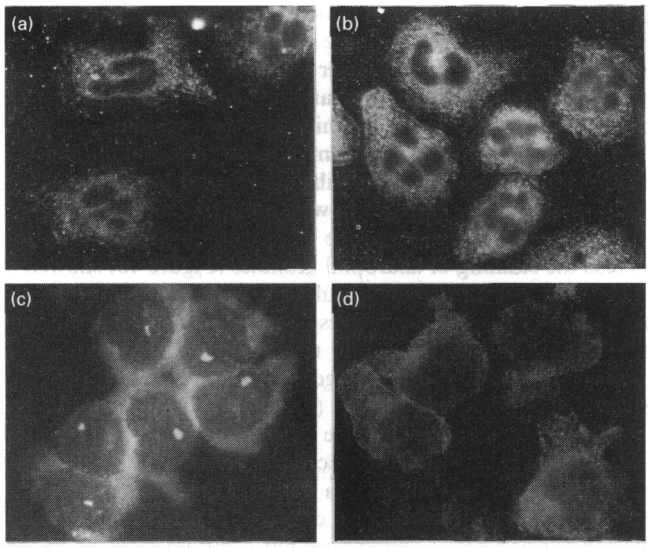

Figure 4 Staining of specific and azurophil granules in resting neutrophils, and immunolocalization of annexin 3 in DMSO-differentiated HL60 cells

Adherent resting neutrophils (a, b) were stained with affinity purified anti-rap 2 (a) or anti-azurophil granule membrane antibodies (b) as described in the legend to Figure 3 (magnification × 1050). HL60 cells differentiated in neutrophils with DMSO were centrifuged on poly-L-lysine-coated glass coverslips, fixed with methanol and stained with anti-annexin 3 antibodies (c) or anti-azurophil granule membrane antibodies (d).

Membrane and cytosolic fractions were prepared from HL60 cells, differentiated into monocytes with PMA, which express the 36 kDa form, and compared with HL60 cells differentiated into neutrophils with DMSO, which express the 33 kDa form, as previously described [23]. The original 33 kDa form of annexin 3 was located mainly in the cytosol of DMSO-differentiated cells. Similarly, the 36 kDa variant protein behaved as a soluble protein in PMA-differentiated HL60 cells (Figure 1). However, a small fraction of annexin 3 remained membrane-bound independently of the presence of EDTA in the fractionation medium, and this was not due to contaminating cytosolic annexin 3 since the membranes were washed-free of cytosol.

In activated neutrophils, regional increments in intracellular calcium concentration have been visualized [36]. These variations cannot be mimicked in fractionation media, and since proteins from the annexin family can associate with membranes or dissociate in a calcium-dependent manner, subcellular fractionation appeared to be inappropriate. Consequently, we investigated the subcellular localization of annexin 3 by indirect immunofluorescence. A rapid cell-fixation technique must be used to minimize modifications of intracellular calcium concentrations and consequent redistribution of annexin 3. Therefore, cell fixation using paraformaldehyde or cold methanol were compared on neutrophils. Before fixation, neutrophils are allowed to adhere briefly to glass coverslips to facilitate the localization of cytoplasmic proteins [24] when compared with round cells fixed in suspension. Very different cell morphology was obtained with methanol (Figures 2a and 3a) or paraformaldehyde (Figures 2b and 3e), the cells being retracted after paraformaldehyde fixation. We also observed that microtubules were present in the cytoplasm of methanol-fixed neutrophils, while they were mostly depolymerized in neutrophils fixed with paraformaldehyde (not shown). In addition, methanol at −20 °C provides a rapid cell fixation, avoiding redistribution of cytoplasmic proteins, in contrast to paraformaldehyde [37]. Conse-

quently, we considered that, in the present study, methanol was more appropriate to maintain the neutrophil morphology and to localize soluble proteins. Under these conditions, annexin 3 appeared as cytoplasmic granular structures in resting neutrophils (Figure 3a). As neutrophils contain an heterogeneous population of granules, we attempted to identify the granules stained with anti-annexin 3 antibodies. Annexin 3 (Figure 3a) and rap2, a protein associated with the membrane of specific granules [24] (Figure 4a), gave similar fluorescence profiles, whereas the staining of azurophil granules (Figure 4b) showed a distinct partitioning of the granules; they appeared more widespread in the cytoplasm than specific granules. In addition, although we have to consider the resolution limits of light microscopy, it seems that larger dots were obtained when azurophil granules were stained (Figure 4b). Differentiation of HL60 cells with DMSO along the neutrophilic pathway does not induce the formation of specific granules, whereas azurophil granules are present [38] and can be stained with anti-azurophil antibodies (Figure 4d). In these cells, no granular staining was detected for annexin 3 (Figure 4c) but a diffuse staining in the cytoplasm and large patches in nuclei were visualized. Next, the subcellular localization of the 36 kDa variant form of annexin 3 was studied in resting human monocytes. As observed in neutrophils, cytoplasmic granules were stained (Figure 3b). No fluorescence was observed when the FITC-conjugated second antibody was used alone (Figure 3c and d).

Next, the localization of annexin 3 was examined in neutrophils and monocytes activated with the phorbol ester PMA, the calcium ionophore A23187, or opsonized zymosan (OZ). In neutrophils stimulated with PMA (50 ng/ml), annexin 3 was clearly detected at the plasma membrane (Figure 5a). As expected for activated cells involved in granule exocytosis, fewer granules were stained in the cytoplasm when compared with resting cells. A similar pattern of fluorescence was observed in PMA-stimulated monocytes (Figure 5a). When the cells were stimulated with A23187 (2.5×10^{-6} M) for 1 min, we detected a staining of the plasma membrane in neutrophils, whereas in monocytes, the plasma membrane was not labelled (Figure 5a), even in cells stimulated for a longer period of time (data not shown). Under these conditions, both neutrophils and monocytes were efficiently activated by A23187 as determined by the release of O_2^- (neutrophils: 3.1 nmol/10^6 cells per min; monocytes: 0.55 nmol/10^6 cells per min). Stimulation of monocytes and neutrophils with A23187 for 10 min provided in both cases the apparition of large fluorescent patches in the cytoplasm (Figure 5a). This might correspond to calcium-induced annexin 3 self-association as observed for other members of the annexin family [39]. Neutrophil incubation for 15 min with OZ (1.5 mg/ml) produced a high apparent fluorescence intensity in the periphagosomal region (Figure 5a), confirming a previous observation that annexin 3 associates with the phagosome membrane in neutrophils [22]. Periphagosomal staining was also observed in monocytes that ingested OZ (Figure 5a).

We attempted to determine whether translocation of annexin 3 to the plasma membrane occurred with the same time dependence as exocytosis of specific granules and activation of NADPH oxidase. Cells were stimulated with PMA and submitted in parallel to (i) immunofluorescence analysis with anti-annexin 3 antibodies, (ii) measurement of O_2^- production and (iii) measurement of lactoferrin release. Generation of O_2^- and release of lactoferrin increased with parallel kinetics. Similarly, association of annexin 3 with the plasma membrane was progressive, as anti-annexin 3 antibodies stained the plasma membrane more intensely at 15 min than at 5 min of stimulation (Figure 5b).

Next, we examined whether in cell-free assays, annexin 3 would bind to specific membrane compartments, as observed in intact cells. Neutrophils were disrupted and fractionated in the presence of EGTA by centrifugation on a discontinuous Percoll gradient, which efficiently separates azurophil granules, specific granules and the plasma membrane-enriched fraction. The purity of the fractions was confirmed by the assay of markers for: (i) azurophil granules (91% of β-glucuronidase was recovered in the high-density fraction), (ii) specific granules [93% of lactoferrin was detected in the intermediate density fraction which was slightly contaminated by azurophil granules (9%)] and (iii) plasma membrane (98% of alkaline phosphatase was present in the plasma membrane-enriched fraction which was contaminated by 6% of the specific granule marker). The cytosol was run on a G-25 size-exclusion-chromatography column to remove calcium and EGTA, and incubated with granule or membrane fractions in the presence of 1 mM EGTA, 1 μM or 100 μM calcium. As shown in Figure 6a, in the absence of calcium, annexin 3 was mainly detected in the cytosol; only a small fraction of the protein was associated with the plasma membrane-enriched fraction. In the presence of calcium, annexin 3 associated indiscriminately with the three membrane fractions, indicating that, in cell-free assay, annexin 3 did not bind to specific membrane compartments, as observed in intact cells. Equal amounts of proteins from each fraction were loaded on the gels. In granules, most of the proteins assayed are soluble, while in the plasma membrane-enriched fraction, the proteins are membranous. Therefore, in the granules, the percentage of membrane to which annexin 3 can associate is lower than in the plasma membrane. As cell activation could modify the binding of annexin 3 with intracellular membranes, similar experiments were performed using PMA-stimulated neutrophils. As shown on Figure 6, similar binding properties and calcium dependencies were observed with annexin 3 isolated from resting (Figure 6a) or PMA-activated cells (Figure 6b).

DISCUSSION

Here we report that the 33 kDa and 36 kDa forms of annexin 3 are associated with cytoplasmic granules in resting neutrophils and monocytes, respectively. When neutrophils and monocytes were activated with soluble stimuli, annexin 3 translocated to the plasma membrane, while, upon phagocytosis, it was delocalized to the phagosomal membrane. Since granules can fuse with distinct membrane sites in activated cells (i.e. with the phagosome in response to particulate stimuli or with the plasma membrane with soluble stimuli), it can be proposed that annexin 3 associates with an acceptor membrane which is the site of granule fusion. We also report that, in cell-free assay, annexin 3 can bind

Figure 5 Subcellular localization of annexin 3 in activated human leukocytes

(**a**) Adherent human neutrophils or monocytes were stimulated with PMA (50 ng/ml) for 15 min, with the calcium ionophore A23187 (2.5×10^{-6} M) for 1 min or 10 min, or with OZ (1.5 mg/ml) for 15 min, and subjected to indirect immunofluorescence with affinity-purified anti-annexin 3 antibodies as described in the legend to Figure 3 ($\times$ 900). Small and large arrows show the plasma membrane and the periphagosomal labelling, respectively. (**b**) Human neutrophils were stimulated with PMA and submitted in parallel to immunofluorescence analysis with anti-annexin 3 antibodies (right panels), measurement of O_2^- production (left panel, ■) and measurement of lactoferrin release (left panel, ○) as described in the Methods section. Release of lactoferrin is expressed as a percentage of the total lactoferrin content.

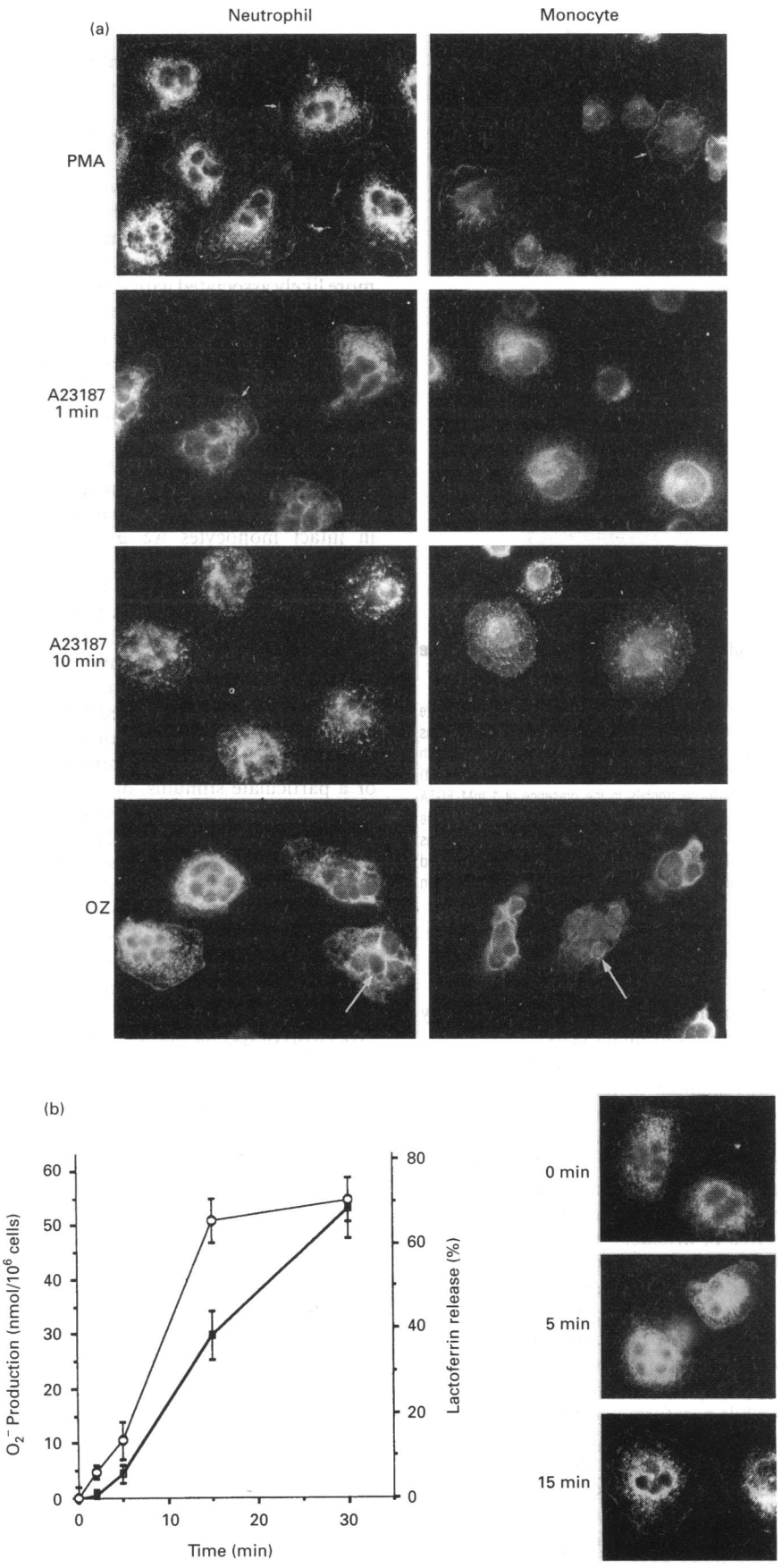

Figure 5 For caption see facing page.

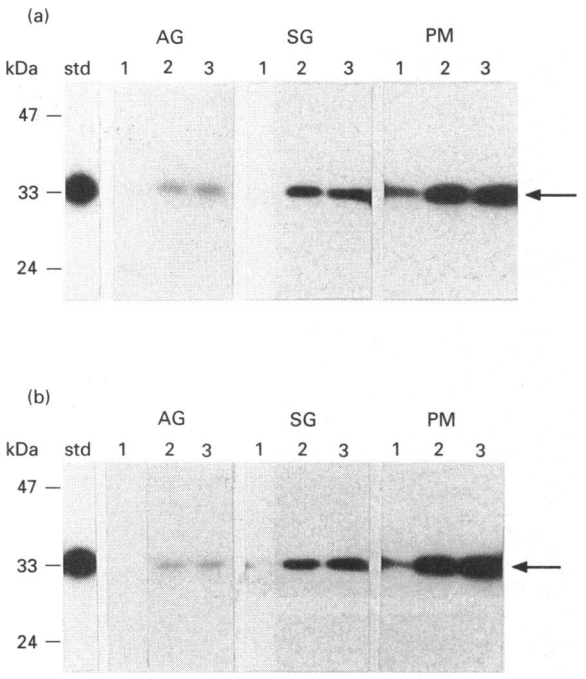

Figure 6 Association, *in vitro*, of annexin 3 with neutrophil membrane fractions

Subcellular fractions from resting neutrophils or cells activated with PMA for 15 min were prepared in the presence of EGTA on discontinuous Percoll gradient. The cytosolic fractions were run on a G25 size-exclusion-chromatography column and mixed, for 15 min at 37 °C, with azurophil granules (AG), specific granules (SG), or plasma membrane-enriched fraction (PM) isolated from resting (**a**) or PMA-stimulated (**b**) neutrophils in the presence of 1 mM EGTA (lanes 1) or in the presence of 1 μM calcium (lanes 2) or 100 μM calcium (lanes 3). The particulate fractions were washed free of cytosol and equal amounts (25 μg/lane) of proteins were analysed by Western-blotting as described in the legend of Figure 1. 100 ng of purified 33 kDa annexin 3 was loaded in the first lane (std). Molecular mass markers are shown on the left. Arrows indicate the position of the 33 kDa form of annexin 3.

indiscriminately to various membrane types while it is specifically associated with granule membranes in intact cells.

Most of our results are based on the use of immunofluorescence as cell fractionation was not adapted to localize proteins that bind to membranes or dissociate, depending on the calcium concentration. Immunofluorescence has been widely used to determine the distribution of proteins within cells. Although this technique can provide novel information on the location, and hence, function of proteins whose properties are poorly understood, it can also be a source of artefacts, especially when the protein studied is soluble. Unless the fixation procedure immobilizes all soluble proteins completely and rapidly, redistribution could lead to wrong interpretations. Indeed, Melan and Sluder [37] have introduced soluble FITC-conjugated proteins into living cells and showed that cell fixation/permeabilization with −20 °C methanol largely preserves the cytoplasmic distribution of these proteins while paraformaldehyde fixation prior to saponin permeabilization led to misleading protein distribution. In addition to soluble proteins, distribution of membrane-associated proteins has also been maintained by fixation with cold methanol [24,34]. We also report here that while the morphology of adherent neutrophils was maintained after methanol fixation, the cells shrank upon paraformaldehyde fixation. Therefore, we concluded that, in this particular study, methanol fixation was more adapted to study the subcellular localization of annexin 3. We have been able to demonstrate that annexin 3 is associated with cytoplasmic granules in human neutrophils and monocytes. Neutrophils contain heterogeneous populations of granules. We show that annexin 3 and rap 2, a protein associated with the specific granules [24], exhibited a similar granular staining concentrated in the perinuclear area. Azurophil granules appeared under apparently larger dots and spread throughout the cytosol (Figure 4) as previously observed in adherent neutrophils [40]. The absence of granular staining in the cytoplasm of HL60 cells which do not contain specific granules, while they do possess azurophil granules, further supports that annexin 3 is more likely associated with specific than with azurophilic granules in human neutrophils. Interestingly, we have previously reported that differentiation of HL60 cells into neutrophil-like cells does not result in a hyperexpression of annexin 3 quantitatively similar to that in neutrophils [23]. This fact might be related to the absence of specific granule formation during HL60 cell differentiation.

Like the other members of the annexin family, the 36 kDa variant of annexin 3 was mainly detected in the cytosol when monocytic cells were fractionated in the presence of EGTA, while in intact monocytes we report that it was associated with cytoplasmic granules.

Although proteins from the annexin family are generally assumed to be soluble at low calcium concentrations, it appears from our data that the 33 kDa and 36 kDa annexin 3 are associated with granule membranes in resting cells, in which the intracellular calcium concentration is about 75 nM [36]. A similar observation has been reported for annexin 2 which is associated with the plasma membrane of resting chromaffin cells [41,42].

When monocytes or neutrophils were stimulated with a soluble or a particulate stimulus, distinct patterns of fluorescence were obtained. Indeed, in PMA-stimulated cells, the plasma membrane was stained and the cytoplasmic granular staining was less intense than in resting cells. This probably results from the decreased number of granules in the cytoplasm as they are involved in the process of exocytosis. Fusion of granules with the plasma membrane is probably accompanied by the transfer of annexin 3 to the acceptor membrane. Furthermore, we observed that translocation of annexin 3 to the plasma membrane upon neutrophil activation seems to follow similar time courses as exocytosis of specific granules and activation of NADPH oxidase.

In cells phagocytosing OZ, annexin 3 appeared at the phagosome membrane, suggesting that granules bearing annexin 3 are fusing preferentially with the phagosomal membrane rather than the plasma membrane [43].

In neutrophils, an increase in cytosolic free calcium triggered upon contact with opsonized particles is necessary to control the subsequent fusion of specific granules with the plasma membrane, while the ingestion step of phagocytosis is calcium-independent [44,45]. Therefore, one can hypothesize that calcium-dependent proteins associated with this granule population, such as annexin 3, might play a critical role in the phagosome–granule fusion and, by extension, in the fusion of the granules with the plasma membrane when neutrophils are stimulated with soluble stimuli. The presence of a calcium-binding protein such as annexin 3 on, presumably, the specific granules of neutrophils could account for the lower calcium concentration capable of mediating the fusion of this granule population when compared with the azurophils [45,46].

Owing to the similar behaviour of the 33 kDa and 36 kDa annexin 3 in resting cells and upon activation with OZ and PMA, it seems likely that these proteins are involved in a role common to these two cell types. A single difference between neutrophils and monocytes consists in the absence of annexin 3 at the plasma

membrane in monocytes stimulated with A23187, whereas the 33 kDa protein appeared very rapidly at the plasma membrane of neutrophils. At present, we cannot explain this discrepancy as we cannot exclude differences in behaviour of the granules in the two cell populations in response to a calcium ionophore.

It is interesting to note that proteins which bind indiscriminately to every type of membrane in cell-free assays present different behaviours in intact cells. Indeed, we have observed that annexin 3 isolated from either resting or PMA-stimulated neutrophils binds to the membrane of specific or azurophil granules and plasma membrane-enriched fraction, whereas its association is restricted to specific compartments in intact resting and activated cells. These observations indicate that results obtained in cell-free assays with annexin 3 have to be interpreted with caution, as they cannot predict its localization, and therefore its function, in cells.

Several proteins of the annexin family (annexins 1, 2 and 7) have been implicated in processes of vesicle aggregation and fusion [9,10,12–15,17,18,41,42,47]. In addition to previous results showing that annexin 3 aggregated isolated specific granules [20], our observation that annexin 3 is associated with granules and to the plasma membrane or the phagosomes, suggests that it could also be involved in fusion processes. Alternatively, some of the other putative functions of annexins, which include inhibition of phospholipase A2 [48–51], modulation of the intracellular levels of inositol cyclic 1,2-phosphate [52] and interactions with the cytoskeleton [53,54], may be exerted by annexin 3. The limiting membrane of the specific granules serves as a reservoir of proteins which play their functional role after they translocate to the plasma membrane upon degranulation. Therefore, we cannot exclude that, in addition to its potential role in the fusion process, annexin 3 may regulate other functions in neutrophils and monocytes.

V. le C. is the recipient of a fellowship from the Ministère de la Recherche et de la Technologie.

REFERENCES

1 Pepinsky, R. B., Tizard, R., Mataliano, R. J., Sinclair, J., Miller, G. T., Browning, J. L., Pingchang Chow, E., Huang, K. S., Pratt, D., Wachter, L., Hession, C. and Wallner, B. P. (1988) J. Biol. Chem. **263**, 10799–10811
2 Burns, A. L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M. R. and Pollard, H. B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3798–3802
3 Hauptmann, R., Maurer-Fogy, I., Krystek, E., Bodo, G., Andree, H. A. M. and Reutelingsperger, C. P. M. (1989) Eur. J. Biochem. **185**, 63–71
4 Johnston, P. A., Perin, M. S., Reynolds, G. A., Wasserman, S. A. and Südhof, T. C. (1990) J. Biol. Chem. **265**, 11382–11388
5 Haigler, H. T., Fitch, J. M., Jones, J. M. and Schlaepfer, D. D. (1989) Trends Biochem. Sci. **14**, 48–50
6 Klee, C. B. (1988) Biochemistry **27**, 6645–6653
7 Crompton, M. R., Moss, S. E. and Crumpton, M. J. (1988) Cell **55**, 1–3
8 Burgoyne, R. D. and Geisow, M. J. (1989) Cell Calcium **10**, 1–10
9 Sarafian, T., Pradel, L. A., Henry, J. P., Aunis, D. and Bader, M. F. (1991) J. Cell Biol. **114**, 1135–1147
10 Creutz, C. E. (1981) J. Cell Biol. **91**, 247–256
11 Creutz, C. E., Pazoles, C. J. and Pollard, H. B. (1978) J. Biol. Chem. **253**, 2858–2866
12 Creutz, C. E. and Sterner, D. C. (1983) Biochem. Biophys. Res. Commun. **114**, 355–364
13 Meers, P., Bentz, J., Alford, D., Nir, S., Papahadjopoulos, D. and Hong, K. (1988) Biochemistry **27**, 4430–4439
14 Drust, D. S. and Creutz, C. E. (1988) Nature (London) **331**, 88–91
15 Ali, S. M., Geisow, M. J. and Burgoyne, R. D. (1989) Nature (London) **340**, 313–315
16 Ali, M. S. and Burgoyne, R. D. (1990) Cell. Signalling **2**, 265–276
17 Blackwood, R. A. and Ernst, J. D. (1990) Biochem. J. **266**, 195–200
18 Oshry, L., Meers, P., Mealy, T. and Tauber, A. I. (1991) Biochim. Biophys. Acta **1066**, 239–244
19 Meers, P., Mealy, T. and Tauber, A. I. (1993) Biochim. Biophys. Acta **1147**, 177–184
20 Ernst, J. D., Hoye, E., Blackwood, R. A. and Jaye, D. (1990) J. Clin. Invest. **85**, 1065–1071
21 Comera, C., Rothhut, B., Cavadore, J. C., Vilgrain, I., Cochet, C., Chambaz, E. and Russo-Marie, F. (1989) J. Cell. Biochem. **40**, 361–370
22 Ernst, J. D. (1991) J. Immunol. **146**, 3110–3114
23 Le Cabec, V., Russo-Marie, F. and Maridonneau-Parini, I. (1992) Biochem. Biophys. Res. Commun. **189**, 1471–1476
24 Maridonneau-Parini, I. and de Gunzburg, J. (1992) J. Biol. Chem. **267**, 6396–6402
25 Goud, B., Zahraoui, A., Tavitian, A. and Saraste, J. (1990) Nature (London) **345**, 553–556
26 Maridonneau-Parini, I., Tringale, S. M. and Tauber, A. I. (1986) J. Immunol. **137**, 2925–2929
27 Maridonneau-Parini, I., Yang, C. Z., Bornens, M. and Goud, B. (1991) J. Clin. Invest. **87**, 901–907
28 Borregaard, N., Heiple, J. M., Simons, E. R. and Clark, R. A. (1983) J. Cell Biol. **97**, 52–61
29 De Chatelet, L. R. and Cooper, M. R. (1970) Biochem. Med. **4**, 61–68
30 Bjerrum, O. W. and Borregaard, N. (1990) Scand. J. Immunol. **31**, 305–313
31 Maridonneau-Parini, I., Malawista, S. E., Stubbe, H., Russo-Marie, F. and Polla, B. S. (1993) J. Cell. Physiol. **156**, 204–211
32 Brittinger, G., Hirshorn, R., Douglas, S. D. and Weissman, G. (1968) J. Cell Biol. **37**, 394–411
33 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
34 Marie-Cardine, A., Maridonneau-Parini, I., Ferrer, M., Danielian, S., Rothhut, B., Fagard, R., Dautry-Varsat, A. and Fischer, S. (1992) J. Immunol. **148**, 3879–3884
35 Pappenheim, A. (1908) Med. Klin. **4**, 1244–1247
36 Sawyer, D. W., Sullivan, J. A. and Mandell, G. L. (1985) Science **230**, 663–666
37 Melan, M. A. and Sluder, G. (1992) J. Cell Sci. **101**, 731–743
38 Bainton, D. F. (1992) in Inflammation. Basic Principles and Clinical Correlates (Gallin, J. I., Goldstein, I. M. and Snyderman, R., eds.), pp. 303–324, Raven Press, New York
39 Zaks, W. J. and Creutz, C. E. (1991) Biochemistry **30**, 9607–9615
40 Pryzwansky, K. B. and Breton-Gorius, J. (1985) J. Lab. Invest. **53**, 664–671
41 Burgoyne, R. D. (1988) Nature **331**, 20
42 Nakata, T., Sobue, K. and Hirokawa, N. (1990) J. Cell Biol. **110**, 13–25
43 Joiner, K. A., Ganz, T., Albert, J. and Rotrosen, D. (1989) J. Cell Biol. **109**, 2771–2782
44 Jaconi, M. E. E., Lew, D. P., Carpentier, J. L., Magnusson, K. E., Sjögren, M. and Stendahl, O. (1990) J. Cell Biol. **110**, 1555–1564
45 Lew, P. D., Monod, A., Waldvogel, F. A. Dewald, B., Baggiolini, M. and Pozan, T. (1986) J. Cell Biol. **102**, 2197–2204
46 Sengelov, H., Kjeldsen, L. and Borregaard, N. (1993) J. Immunol. **150**, 1535–1543
47 Meers, P., Mealy, T., Pavlotsky, N. and Tauber, A. I. (1992) Biochemistry **31**, 6372–6382
48 Comera, C., Rothhut, B. and Russo-Marie, F. (1989) Eur. J. Biochem. **188**, 139–146
49 Maridonneau-Parini, I., Errasfa, M. and Russo-Marie, F. (1989) J. Clin. Invest. **83**, 1936–1940
50 Huang, K. S., Wallner, B. P., Mattaliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L. K., Chow, E. P., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E. and Pepinsky, R. B. (1986) Cell **46**, 191–199
51 Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R. and Hunter, T. (1986) Cell **46**, 201–212
52 Ross, T. S., Tait, J. F. and Majerus, P. W. (1990) Science **248**, 605–607
53 Sekimoto, S., Tashiro, T. and Komiya, Y. (1991) J. Neurochem. **56**, 1774–1782
54 Kwiatkowska, K., Kerhbtukova, I. A., Gudkova, D. A., Pinaev, G. P. and Sobota, A. (1991) Exp. Cell Res. **196**, 255–263

Received 31 December 1993/9 May 1994; accepted 23 May 1994