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Translocation of annexin XI to neutrophil subcellular organelles

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Abstract

In an earlier study, annexin XI was found to be present in the cytosol of neutrophil granulocytes (Blood (1996) 87, 4817). The protein was isolated by calcium-dependent translocation to specific granules and was found to be a 42-kDa truncated form of annexin XI. Using human autoantibodies directed against annexin XI we have now reinvestigated the ability of full size annexin XI to translocate to different neutrophil organelles isolated by subcellular fractionation. The autoantisera used recognised a protein of 55-kDa in neutrophil cytosol and comparison with a whole cell lysate indicated that the larger portion of the cellular content of this protein is localised to the cytosol. Azurophil granules, specific granules and secretory vesicles/plasma membrane were isolated by subcellular fractionation on Percoll gradients, mixed respectively with neutrophil cytosol and the calcium concentration was raised. Immunoblotting showed that annexin XI translocated to specific granules and secretory vesicles/plasma membrane at 100 μmol/l calcium. When raising the concentration of calcium to 1 mmol/l, annexin XI translocated to the azurophil granules as well. Periphagosomal translocation of annexin XI occurred during phagocytosis of yeast particles, implying that this protein plays a role in the events associated with the phagocytic process.

Keywords: Annexin XI; Neutrophil; Phagocytosis

1. Introduction

The major task of neutrophil granulocytes is to protect the body from infection. This is achieved through phagocytosis and subsequent killing of micro-organisms that invade the tissues. Migration of neutrophils to the site of infection, as well as phagocytosis and killing of the microbe, involves mobilisation of different intracellular storage compartments (granules). Four different granules, differing in the content of matrix proteins and membrane-bound receptors, have been characterised in the neutrophil [1]. Regulation of secretion also differs between the granules, as well as their basic biological functions. That different pathways of secretion exist, can be demonstrated by using a variety of activating agents, and by manipulating the concentration of intracellular free calcium [2,3]. The secretory vesicles are the organelles that are most easily mobilised to the cell surface, followed by the gelatinase granules and the specific granules. The azurophil granules are mainly involved in formation of a phagolysosome, i.e. degranulation through granule fusion with a plasma membrane-derived phagosome. Although the molecular mechanisms are not known in detail, there is considerable evidence that granule secretion as well as phagolysosomal fusion is regulated by transient

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increases in the intracellular free calcium concentration and that there may be involvement of cytosolic proteins that bind to membranes in a calcium-dependent manner, e.g. the annexins.

Annexins are characterised by their ability to bind, above all, to negatively charged phospholipids in a calcium-dependent manner. Up to 13 different annexins have been described, which are generally built up of four (eight in the case of annexin VI) repeats of a 70 amino acid sequence, which contain the calcium- and phospholipid-binding sites. The N-terminal part of annexins, on the other hand, is unique for each family member and it often contains sites for phosphorylation, ligand-binding and truncation [4]. Many annexins are present in the human neutrophil, and we have earlier shown that several of these can bind to different organelles isolated by subcellular fractionation [5]. One of the neutrophil annexins, annexin III, translocates to the periphagosomal region during phagocytosis [6], implying that this annexin plays a specialised role in the metabolic and structural events that are associated with phagocytosis and phagolysosome fusion. In the search for yet other proteins that could be involved in the differential regulation of the neutrophil organelles, we identified and purified a neutrophil cytosolic protein that binds to specific granules when the calcium concentration is raised [7]. The protein of 42 kDa was identified as a fragment (truncated form) of annexin XI. This protein is a newly described member of the annexin family. Its N-terminal part harbours a site that binds calcyclin [8], a calcium-binding protein of the S-100 family. Annexin XI has been studied in other systems under the name human autoantigen 56K [9]. Antibodies against this protein can be found in 4–10% of patients with a systemic autoimmune disorder, predominantly in sera from patients with rheumatoid arthritis, systemic lupus erythematosus or Sjögren's syndrome [10]. Antibodies in these sera recognise the N-terminal part of the molecule, which is specific for annexin XI and not shared with other annexins. These antibodies may thus be ideal for studies designed to determine translocation properties of full length annexin XI.

Our aim with the present study was to investigate the binding of annexin XI to different subcellular neutrophil organelles and to determine the subcellular localisation of the protein during phagocytosis.

2. Materials and methods

2.1. Isolation of granulocytes

Human polymorphonuclear granulocytes (neutrophils) were isolated from buffy coats by sedimentation in dextran, hypotonic lysis and centrifugation on Ficoll-Hypaque according to the methods of Bøyum [11]. The neutrophils were washed twice in Krebs-Ringer medium supplemented with 10 mmol/l glucose (KRG), and resuspended in the same buffer or in saline.

2.2. Isolation of cytosol and organelles

Neutrophils suspended in saline were DFP-treated (diisopropyl fluorophosphate, 10 minutes on ice, final concentration 5 mmol/l), washed and thereafter resuspended in relaxation buffer (100 mmol/l KCl, 3 mmol/l NaCl, 3.5 mmol/l $MgCl_2$, 10 mmol/l Pipes, 1 mmol/l $ATP(Na)_2$, 0.5 mmol/l phenylmethanesulfonyl fluoride (PMSF), (pH 7.4)) supplemented with Pefabloc (1 mmol/l), and disintegrated by nitrogen cavitation [12]. Cytosol was prepared by collecting the cavitate without EGTA and removing all organelles by centrifugation at $100\,000 \times g$ for 1.5 h (4°C). The pellet was discarded and the supernatant (cytosol) stored at 4°C until used [5]. For isolation of azurophil granules (α), specific granules (β) and secretory vesicles/plasma membranes (SV/PM) (γ), EGTA was added to the nitrogen cavitate according to the original fractionation protocol [12]. The postnuclear supernatant was centrifuged on a two-step density gradient of Percoll. The α, β, and γ-band were collected and washed free of EGTA and Percoll, and were thereafter resuspended in relaxation buffer. Each organelle fraction was checked by marker molecule analyses [5] and kept at 4°C until used.

2.3. Translocation of cytosolic proteins

Translocation of cytosolic proteins to azurophil granules, specific granules and SV/PM was performed as described earlier [5]. Briefly, cytosol was mixed with the organelle preparation in ratio 1/5 (i.e. the mixture contained 4.4×10^7 cell equivalents of cytosol and 2.2×10^8 cell equivalents of organelle in a final volume of 1.6 ml). The mixtures were incubated at 37°C for 15 min in the presence of

$CaCl_2$ (concentration indicated in the figure legends). The organelles were collected by centrifugation and the resulting supernatant (denoted 'remaining cytosol') was kept frozen until analysed. The organelles were washed once and the proteins that translocated in a calcium-dependent manner to the organelle membranes were extracted by resuspending the organelles in relaxation buffer containing 2 mmol/l EGTA. The organelles were removed and the supernatant (EGTA-extract) was kept frozen until analysed by immunoblotting.

2.4. Antisera

Annexin XI was determined by use of three human sera from patients with a systemic autoimmune disorder, and which contained high titres of annexin XI antibodies, as described in detail earlier [10]. The patient antisera were kindly provided by Dr. Walther van Venrooij, University of Nijmegen, The Netherlands. A rabbit antiserum against human neutrophil cytosol was prepared as described earlier [7].

2.5. SDS / PAGE and immunoblotting

SDS/PAGE was performed according to Laemmli [13] using 9 or 10% homogenous polyacrylamide gels which were electroblotted according to Towbin [14] onto PVDF membranes. Membranes were blocked in 1% BSA, 1% dry milk, 0.05% Tween-20 in PBS (blocking buffer), then incubated with patient sera (1/500) or anti cytosol serum (1/500) in blocking buffer. Binding of antibodies was detected by incubation with alkaline phosphatase conjugated rabbit-anti-human Ig or swine-anti-rabbit Ig. Blots were developed in nitroblue tetrazolium/bromo-chloro-indolyl-phosphate (NBT/BCIP) substrate. SDS-PAGE samples of whole cells were prepared by adding Laemmli reduced sample buffer to isolated neutrophils. The sample was heat treated and then centrifuged in a microfuge (Beckman Instruments) for 5 minutes. The supernatant was loaded on the gel.

2.6. Phagocytosis assay and indirect immunofluorescence

Neutrophils resuspended in KRG supplemented with 1 mmol/l $CaCl_2$ (2×10^6 cells/ml), were allowed to adhere to glass slides during 15 minutes at 37°C in a moist chamber. The glass slides were washed twice in KRG (37°C) and non-opsonized yeast particles (*Saccharomyces cerevisiae*; 10 yeast particles/neutrophil) were added to them [15]. The slides were incubated for 25 minutes and thereafter washed to remove non-phagocytosed yeast particles. The slides were fixed in paraformaldehyde (4%, ice-cold). The cells were permeabilized for one minute with Triton X-100 (1% v/v in PBS, pH 7.3) and immunostaining was done with human anti annexin XI antibodies. In order to reduce non-specific binding, the slides were blocked with 20% normal rabbit serum (NRS) and then incubated for 30 minutes with the human anti-annexin XI antisera, pooled from three donors (dilution 1/10 in 1% BSA; 20% NRS in PBS). After another incubation in 20% NRS, FITC-labelled rabbit-anti-human IgG (Dako F0123), diluted 1/80 in 1% BSA; 20% NRS in PBS was used as secondary antibody. Glass slides were mounted in gelvatol and the slides were analysed by fluorescence- and light microscopy (Axiovert, Zeiss A.G., Germany). Controls consisted of (1) samples incubated with normal human serum instead of the annexin XI antisera, and (2) neutrophils adherent to glass slides and fixed without having been fed with yeast particles, but otherwise treated exactly as described above.

2.7. Chemicals

ATP, nitroblue tetrazolium, bromo-chloro-indolyl-phosphate and PMSF were bought from Sigma Chemical Co., IL, USA. Percoll was from Pharmacia, Uppsala, Sweden. Pefabloc and BSA was from Boeringer-Mannheim, Germany. The PVDF-membrane was purchased from Millipore, Bedford, MA, USA. The antibody conjugates used were from Dakopatts AS (F0123, D336, D306), Glostrup, Denmark, and DFP was from Aldrich, Germany.

3. Results

3.1. Detection of annexin XI in neutrophil cytosol

In an earlier study, performed to identify neutrophil cytosolic proteins with calcium-dependent

membrane-binding characteristics, we isolated a 42-kDa fragment (truncated form) of annexin XI [7]. Its identification was accomplished by use of an antiserum against a multitude of neutrophil cytosolic proteins in combination with a purification procedure involving translocation to specific granules. In order to allow specific studies of full length annexin XI in the neutrophil, three human autoantisera containing annexin XI-antibodies were used in immunoblotting of cytosolic proteins from neutrophils (Fig. 1A). The antisera reacted mainly with a protein of molecular weight around 55 kDa. By comparison with a preparation of purified recombinant annexin XI (M_r 54), it was concluded that the 55 kDa protein corresponds to the neutrophil form of annexin XI (not shown). In contrast to the autoantisera, the cytosol antiserum originally used to isolate neutrophil annexin XI recognises a multitude of proteins in neutrophils. The presence of annexin XI antibodies in the cytosol antiserum was confirmed using a preparation of purified annexin XI.

3.2. Calcium-dependent translocation of annexin XI

In the previous study [7], we observed that the truncated form of annexin XI (42 kDa) selectively interacts with membranes of specific granules, while no binding to either azurophil granules or SV/PM was detected. Using the patient sera, recognising full length annexin XI, we wanted to find out whether the intact annexin XI molecules interact with the different neutrophil organelles in the same manner. SV/PM was mixed with cytosol and incubated either with EGTA or with different amounts of calcium. A number of cytosolic proteins translocated to the SV/PM in a calcium-dependent manner as is detected by the anti cytosol antiserum (Fig. 1B). Using the patient autoantisera instead (Fig. 1C), we found a protein of molecular mass around 55 kDa that was translocated to the SV/PM in a calcium-dependent manner. We therefore conclude that annexin XI is one of the proteins that translocates to these membranes and that the specific translocation of the truncated form of

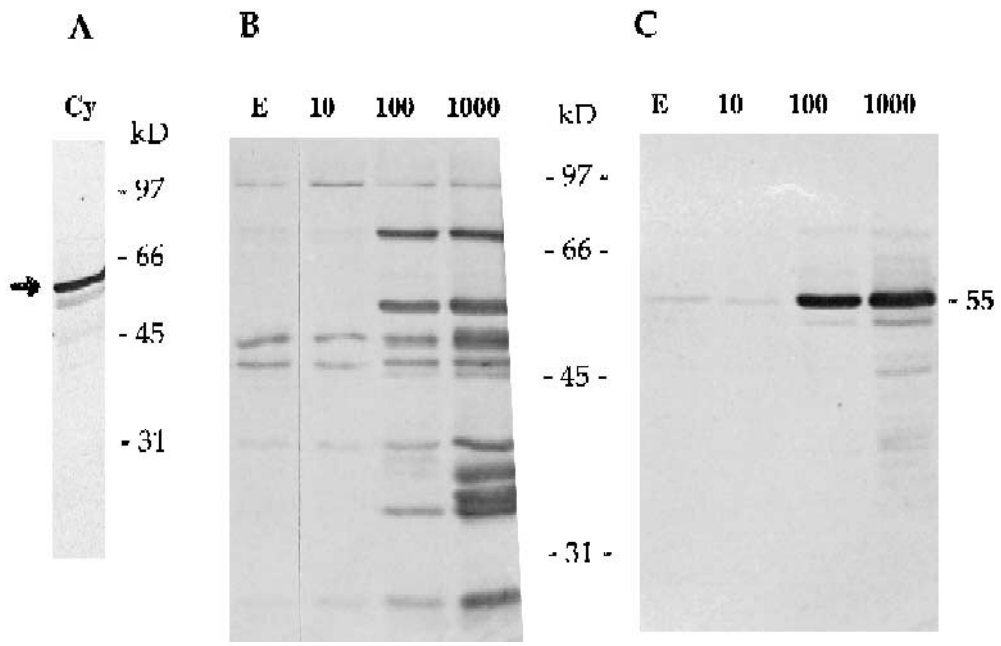

Fig. 1. Dependence on calcium of the translocation to secretory vesicles/plasma membrane of neutrophil cytosolic proteins and annexin XI. Isolated secretory vesicles/plasma membrane (SV/PM) was mixed with cytosol (Cy) in the presence of: 2 mmol/l EGTA (E); 10, 100, or 1000 μmol/l $CaCl_2$. The organelles were collected and washed, and the proteins translocated in a calcium dependent manner were released from the organelles by extraction in an EGTA containing buffer. The organelles were removed and the supernatants were subject to electrophoresis under reducing conditions and electroblotted onto PVDF membrane. The blots were probed with (A and C) anti annexin XI autoantisera, pooled from three patients, or (B) a neutrophil cytosol antiserum. The arrow in A indicates the position of 55 kDa annexin XI present in cytosol.

annexin XI is not valid for annexin XI of full length. A calcium concentration of 100 μmol/l seemed to be close to, or exceed, the value for half maximal binding, since an increase to 1 mmol/l did not dramatically increase the amount of annexin XI translocated.

3.3. Binding of annexin XI to different neutrophil organelles

To investigate whether the three granule subsets differ regarding the interaction with annexin XI, translocations to the different organelles were made at 100 μmol/l calcium. At this concentration, a protein of 66 kDa, (earlier identified as annexin VI [5]), translocates equally well to azurophil granules, specific granules and SV/PM (Fig. 2A, samples 1–3). Other proteins in the range between 46–55 kDa translocated mainly to the specific granules and SV/PM. However, on raising the calcium concentration to 1 mmol/l, translocation of these proteins occurred also to the azurophil granules (not shown). Use of the annexin XI specific antisera to probe the same samples showed that a protein of 55 kDa, as well as one of 52 kDa, translocated to the specific granules and the SV/PM but not to the azurophil granules (Fig. 2B, samples 1–3). Most of the content of annexin XI in the cytosol was translocated to SV/PM, as little annexin XI remained in the cytosol (Fig. 2B, sample 7). The annexin XI-binding capacity of azurophil granules was lower than that of the other organelles, (both at 100 μmol/l and at 1 mmol/l concentration of calcium). This conclusion was based on the fact that only a fraction of the annexin XI available in the cytosol associated with the granules, even though there was a 5-fold excess of them (calculated in cell equivalents). Following translocation to azurophil granules, most of the annexin XI was recovered in the cytosol, indicating that the lack of membrane-bound annexin XI is not due to a degradation of the protein at azurophil granules, but really that these granules bind annexin XI less efficiently than the specific granules and the secretory vesicles/plasma membrane. The fact that the annexin XI antibodies (which are known to recognise the N-terminal part of the protein) do not recognise the

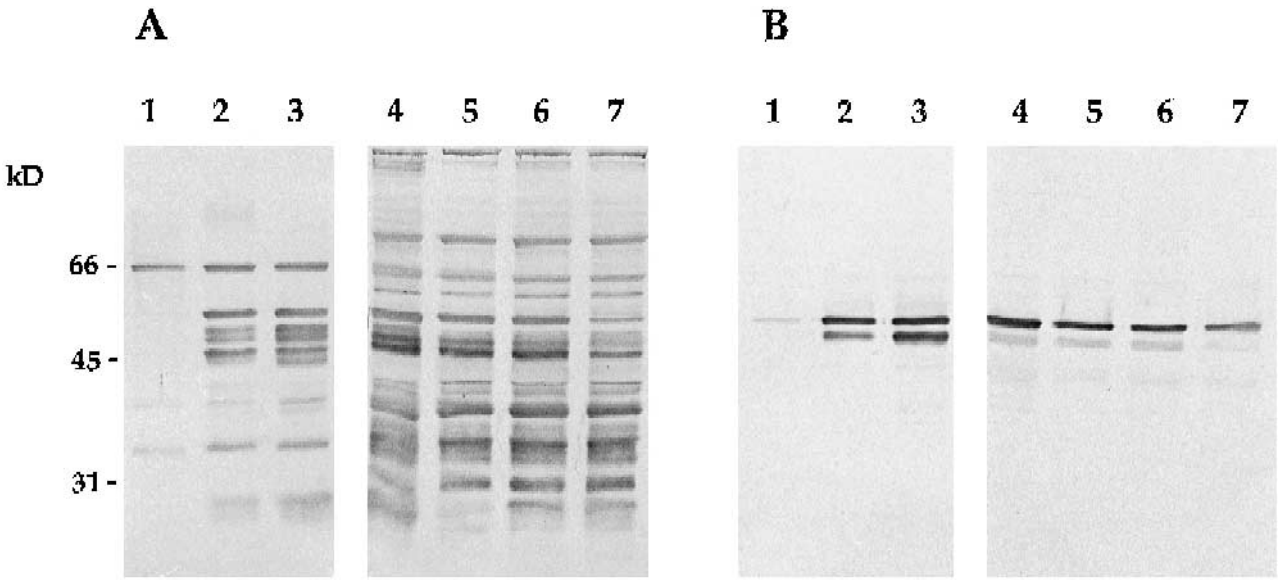

Fig. 2. Capacity of neutrophil organelles to bind annexin XI. Neutrophil cytosol (4) was mixed and incubated with azurophil granules, specific granules or secretory vesicles/plasma membrane (SV/PM), respectively, in the presence of 100 μmol/l calcium. The mixtures were separated into 'remaining cytosols' (5–7) and organelles (1–3) by centrifugation. The organelles were washed and the proteins that were associated with the membranes were extracted by EGTA. EGTA-extracts and 'remaining cytosols' from azurophil granules (1, 5), specific granules (2, 6) and secretory vesicles/plasma membrane (3, 7) respectively, as well as the cytosol preparation (4) were subjected to electrophoresis and immunoblotting using (A) anti neutrophil cytosol antisera or (B) annexin XI autoantisera.

42-kDa truncated form of annexin XI that we earlier isolated from neutrophils, confirms our conclusion that it is the N-terminal part of the molecule that is missing in the truncated form of the molecule.

3.4. Accumulation around the phagosome of annexin XI

In order to examine whether the subcellular localisation of annexin XI is altered also in intact cells upon activation, neutrophils were allowed to phagocytose yeast particles. They were then probed with the annexin XI autoantisera and visually inspected by immunofluorescence microscopy. In resting (non-phagocytosing) neutrophils the fluorescence was diffuse over the whole cell, in accordance with a cytoplasmic localisation of annexin XI (as shown in Fig. 3A by those cells that have not phagocytosed yeast). In cells containing yeast particles, an accumulation of fluorescence could be detected around the ingested particles (Fig. 3A), indicating that annexin XI accumulates in the periphagosomal region. It should be noted that, in a whole cell lysate of neutrophils, the antisera not only reacts with the 55 kDa-protein, but also with a 48 kDa protein as well as with a few other proteins of lower molecular weight (Fig. 3C).

4. Discussion

Annexin XI is a recently identified member of the annexin family. The protein was originally called calcyclin-associated-protein (CAP-50), and it was identified through its interaction with calcyclin [16]. We recently isolated a cytosolic protein in human neutrophils by calcium-dependent translocation to specific granules. The protein turned out to be identical with annexin XI, just as the 56 K autoantigen [9], which was isolated by immunoprecipitation using sera from patients suffering from different vasculitic autoimmune disorders. The autoantibodies of patient sera are specific for annexin XI and directed towards the N-terminal part of the molecule [10], which is the part found to be missing in the truncated form of the protein that we isolated in neutrophils [7]. That result is also confirmed in the present study, as the 42 kDa protein was not recognised by the N-terminal-specific antibodies in patient sera (not shown). Lacking specific antibodies against annexin XI, we used patient sera in order to study the translocation properties of neutrophil annexin XI in its full length. The reactivity of the antibodies with the N-terminal part of the molecule thus ensured that the intact molecule rather than the truncated form was studied.

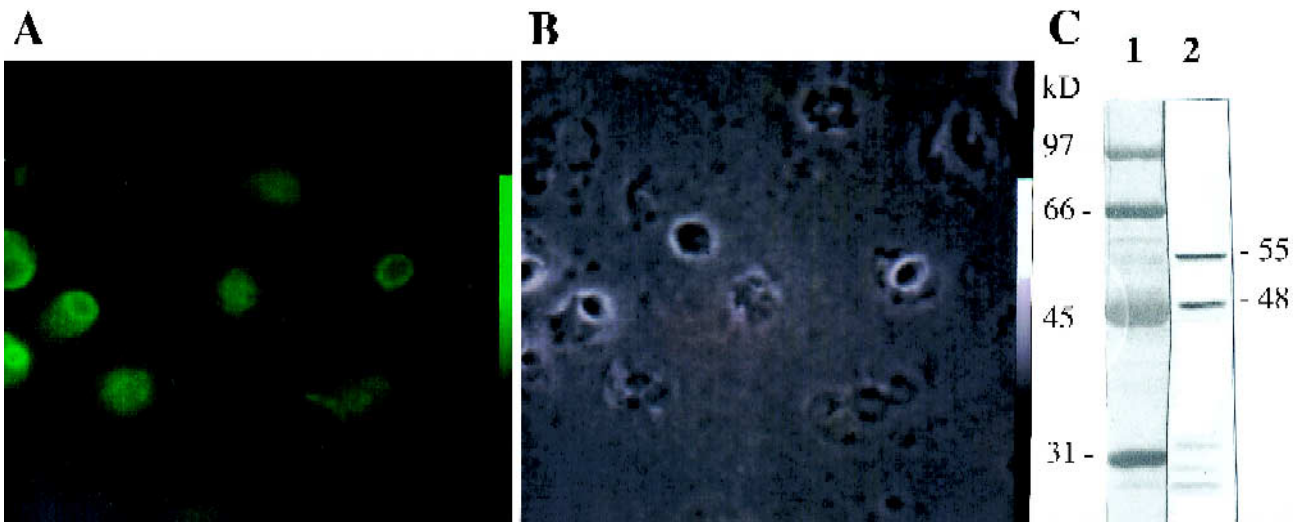

Fig. 3. Indirect immunofluorescence showing the localisation of annexin XI in neutrophils. Phagocytosing neutrophils, prepared as described in materials and methods, were probed with annexin XI antisera and visualised by (A) fluorescence- or (B) light microscopy. (C) shows a blot of (1) Coomassie stained standard molecular weight markers and (2) a whole cell lysate of $1 \cdot 10^6$ neutrophils, probed with anti annexin XI autoantisera.

The results show that in vitro translocation of annexin XI to the various organelle membranes in the neutrophil differs. Azurophil granules were not as efficient in binding annexin XI as were the specific granules and the SV/PM. This is a translocation behaviour that annexin XI shares with annexin II and a 66-kDa annexin IV-related protein [5]. All these three annexins require roughly the same calcium concentration to translocate to the respective type of granule. A calcium concentration of 100 μmol/l seems to exceed the value for half-maximal translocation to specific granules and SV/PM, whereas translocation to the azurophil granules requires higher calcium concentrations. In contrast, other annexins have the property of translocating in the same way (regarding calcium-requirement and amounts of annexin translocated) to the different neutrophil granule-membranes. One of these is annexin VI, which binds equally well to all three granule subsets and binding seems to be maximal already at 100 μmol/l calcium according to this and an earlier [5] study. Also full length annexin I does not discriminate between different granules [5,17].

In the neutrophil, annexin XI was first identified as a 42 kDa protein that was specifically present in translocation extracts from specific granules. The present results reveal that there is no specificity among the organelle-membranes in binding of the full length annexin XI, even though azurophil granules are not as capable in binding annexin XI as are the specific granules and SV/PM. The most probable explanation why the 42 kDa truncated form of annexin XI was found only in association with specific granules is that full length annexin XI was degraded by enzymes present in these granules during the course of the translocation process. However, further studies are needed before the occurrence of the 42 kDa protein can be dismissed as being merely an artefact. In fact the possibility that it has biological relevance is not as farfetched as it may appear. In the case of annexin I, evidence in vitro speaks for the existence of a cleaving factor on membranes of specific granules and SV/PM that cleaves annexin I (40 kDa), leaving a fragment of 38 kDa with altered biological properties [17,18].

In a resting neutrophil, the intracellular free calcium concentration is around 100 nmol/l. During maximal activation of the cell, the subsequent rise in intracellular calcium has been reported to reach micro-molar levels [19]. It may then be argued, that the levels of calcium required for translocation of annexins in vitro, is too high for the phenomenon to have any relevance in the in situ situation. However, measurements of intracellular calcium involves methods that record the calcium concentration on average in cells and which are sensitive only in the nano-molar range. It has been reported that the intracellular calcium concentration in fact can rise up to the millimolar range locally in an activated cell [20,21]. Our data showing that annexin XI accumulates around the phagosome, give support for the relevance of the translocation phenomenon, as does the fact that annexin III has been reported to accumulate in the region around the phagosome in neutrophils [6], and around intracellular inclusions of *Chlamydiae* [22]. Annexin III requires the same high calcium concentration for translocation in vitro (unpublished observation) as does other annexins [5].

The autoantisera reacted mainly with a 55 kDa protein, but in isolated cytosol and in EGTA-extracts, also a 52 kDa band was seen. The fact that this band not only is recognised by the anti annexin XI sera, but also by the anti cytosol serum, suggests that it is a degradation product of annexin XI, as does the fact that a 52 kDa band co-purifies with 55 kDa annexin XI when annexin XI is isolated by ion-exchange chromatography. It is also interesting to note that EGTA-extracts of specific granules and SV/PM contain relatively high amounts of 52 kDa band than does the cytosol, which is an indication that degradation of annexin XI occurs to some extent during the translocation process. In the whole-neutrophil extract, the autoantisera reacted mainly with the protein of 55 kDa, but a protein of 48 kDa was detected as well. This protein species is lacking in the low-speed supernatant of cavitated cells; a preparation that is devoid of nuclei and unbroken cells. The 48 kDa species might thus be a protein associated with nuclei, but since no nuclear staining was seen in the indirect immunofluorescence studies, we believe the immunofluorescence obtained in neutrophils using the patient sera is due to the antibodies reacting with the 55 kDa protein on immunoblots. This observation, together with the fact that the 55 kDa protein translocates in vitro to neutrophil organelles, strengthens the assumption that it is indeed annexin

XI that translocates to the phagosomal region upon phagocytosis, even though the specificity, with respect to translocation, has not been confirmed by inhibition studies using purified annexin XI.

In other cells, like the fibroblast, annexin XI has been reported to be localised primarily in the nucleus [23]. As no nuclear staining was seen in neutrophils, it seems that annexin XI is localised primarily in the cytosol in this cell. Our fractionation data also indicate this, as the major part of annexin XI is recovered in the cytosol upon subcellular fractionation. The present study shows that an in vitro rise in calcium changes the localisation of annexin XI from being cytoplasmic to being organelle-associated. The subcellular localisation of annexin XI (as well as other annexins) is, however, most likely determined also by other factors. The N-terminal tail of annexin XI has been reported to be necessary for the nuclear localisation [24] and in the same study annexin XI lost its phospholipid-vesicle binding capacity upon phosphorylation. The importance of the amino acid composition of the N-terminal for the subcellular localisation has recently been demonstrated in human COS7 cells transfected with recombinant annexin XI. A granule-like/aggregating subcellular localisation was obtained with annexin XI-A while transfection with annexin XI-B (which differs from the A-form in about 40 amino acids in the N-terminal tail) gave a cytoplasmic immunofluorescence pattern [25]. Even though the factors that determine the subcellular localisation of annexin XI remain to be identified, the periphagosomal translocation of annexin XI during phagocytosis of yeast particles implies, that this protein plays a role in the events associated with the phagocytic process.

Acknowledgements

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