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Cleavage of annexin I in human neutrophils is mediated by a membrane-localized metalloprotease

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Abstract

A truncated form of annexin I, formed during Ca^{2+}-induced translocation to neutrophil specific granules and secretory vesicles/plasma membranes, is generated through the action of an endogenous membrane protease. The cleavage of annexin I is inhibited by the metalloprotease inhibitor 1,10-phenanthroline as well as by Triton X-100 and dithiothreitol, classifying the protease as a membrane-bound, thiol-dependent metalloprotease. The cleavage site is located close to the N-terminal of annexin I, leaving a truncated form of the molecule, des_{1-8} annexin I, that contains the Ca^{2+}-binding sites, as well as a number of phosphorylation sites of importance for the function of the protein. When assessing binding capacity to different neutrophil organelles, full-length annexin I bound to azurophil granules, specific granules, and secretory vesicles/plasma membranes, while des_{1-8} annexin I only bound to specific granules and secretory vesicles/plasma membranes, but not to azurophil granules (C. Sjölin, C. Dahlgren, Biochim. Biophys. Acta 1281 (1996) 227–234). This implies that there are different mechanisms of binding to neutrophil organelles of full-length annexin I and the truncated form, and that cleavage of annexin I might be of regulatory importance for the degranulation process.

Keywords: Annexin I; Neutrophil; Cleavage site; Degranulation; Membrane localized metalloprotease

1. Introduction

Annexin I is a member of the annexin family of calcium-dependent, phospholipid-binding proteins [1–4]. Much structural and biochemical data on annexin I (as well as on other members of the family) have been published [5–9], yet our understanding of the precise biological function(s) of this protein is vague. The fact that annexin I is a fusogenic protein [10–13] suggests that it may be part of the secretory machinery present in many different cell types [2,14–17]. The core of annexin I is made of four repeats of a consensus sequence of 70 amino acids carrying the biochemical properties of Ca^{2+} dependency and phospholipid binding. The N-terminal domain of annexin I is essential for the biological function(s) of the molecule. The membrane-binding and vesicle aggregation characteristics of the protein are regulated by phosphorylation of the N-terminal domain [18–21] and the calcium sensitivity for membrane binding is altered in N-terminal truncated forms of the molecule [21], possibly due to conformational changes of the protein induced by such truncations.

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Annexin I present in human neutrophils [11,15,22] binds without selectivity to all the different granule membranes (azurophil granules, specific granules as well as secretory vesicles/plasma membranes). However, the neutrophils possess a protease that can cleave the annexin I molecule during translocation of the protein to the specific granules and the secretory vesicles/plasma membranes. The truncated form of annexin I shows a selectivity with respect to binding that the full-length protein lacks [23]. The truncated form binds Ca^{2+}-dependently to the specific granules and the secretory vesicles/plasma membranes, but not to the azurophil granules. These data support the idea that annexin I together with its truncated derivative may have roles in the hierarchical mobilization of the different granule populations present in neutrophils. Very little is known concerning the mechanism responsible for cleavage of annexin I during calcium-induced translocation. In the present study we show that an N-terminal truncated form of annexin I, des_{1-8} annexin I, is generated through the action of a membrane-bound metalloprotease.

2. Materials and methods

2.1. Materials

Reagents used in this work were obtained from the following sources: Percoll and Ficoll-Paque were purchased from Pharmacia, Uppsala, Sweden. ATP, 5-bromo-4-chloro-3-indolyl phosphate, EGTA, Hepes, NBT, PMSF, Triton X-100 and histidine were products of Sigma Chemical Co., St. Louis, MO. 3,4-Dichloro-isocoumarin, DFP, and 1,10-phenanthroline were obtained from Aldrich, Steinhem, Germany. Yeast Nitrogen Base without amino acids was obtained from Difco Laboratories, Detroit, MI. Aprotinin, E-64, pepstatin, leupeptin and Pefabloc were obtained from Boehringer Mannheim, Mannheim, Germany. The PVDF membrane was a product of Millipore, Bedford, MA. Goat anti-mouse IgG was obtained from Jackson Immuno-Research Laboratories, West Grove, PA. Monoclonal mouse anti-annexin I was from Zymed Laboratories, San Francisco, CA.

2.2. Purification of recombinant human annexin I

Human annexin I cDNA (subcloned into plasmid YEpDB60) was kindly provided by Dr. C. Creutz, Department of Pharmacology, University of Virginia, Charlottesville, VA. Recombinant annexin I was expressed in *Saccharomyces cerevisiae*, strain C2Y6, as described [24]. The vector incorporates the promoter region of the GAL10 gene and yeast cells grown on galactose medium provide a high expression level of annexin I. The recombinant protein was isolated from yeast extract by calcium-dependent lipid binding, using endogenous yeast membranes as the lipid source [25].

Briefly, a yeast culture was grown to a density of 1×10^8 cells/ml in synthetic minimal galactose medium supplemented with histidine (2% galactose, 1.6 g/l yeast nitrogen base without amino acids, 11.1 g/l succinic acid, 0.5% (w/v) ammonium sulfate, 20 mg/l histidine, and ~7 g/l NaOH, pH 7.3). Cells were harvested by centrifugation and washed once in buffer H (20 mM Hepes (pH 7.4), at 4°C, 150 mM KCl, 2 mM $MgCl_2$, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM Pefabloc). The pellet was resuspended in buffer H and lysed at −20°C using an X-press [26]. The homogenate was centrifuged at $14\,400\times g$ (10 min, 4°C), and Ca^{2+} (4 mM) was added to the supernatant which was left on ice for 15 min, and centrifuged at $100\,000\times g$ (30 min, 4°C). The pellet was washed twice in buffer H. The final pellet was resuspended in buffer H with EGTA (5 mM), left on ice for 15 min and centrifuged at $200\,000\times g$ (30 min, 4°C). Finally, the EGTA-containing buffer was exchanged for relaxation (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.4) buffer supplemented with 1 mM Pefabloc using a PD-10 Sephadex G-25 M column (Pharmacia Biotech, Uppsala, Sweden).

2.3. Isolation of granulocytes

Human polymorphonuclear leukocytes (neutrophil granulocytes) were isolated from buffy coats [27]. Briefly, erythrocytes were sedimented in dextran and leukocytes were collected from the supernatant by centrifugation and purified from remaining erythrocytes by hypotonic lysis. Mononuclear leukocytes

were removed by centrifugation on Ficoll-Paque. The purified granulocytes were washed twice in Krebs–Ringer medium without Ca^{2+} (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, pH 7.3) and resuspended in cold relaxation buffer supplemented with 1 mM ATP$(Na)_2$, 0.5 mM PMSF, and 1 mM Pefabloc.

2.4. Disruption of cells and isolation of cytosol and subcellular organelles

Neutrophil cytosol and subcellular organelles were isolated according to the method described by Borregaard et al. [28]. Neutrophils in relaxation buffer were disrupted by nitrogen cavitation (2500 kPa, 5 min; Parr, Moline, IL), and EGTA (1.5 mM) was added to the homogenate to avoid aggregation of granules. The homogenate was centrifuged at $400\times g$ (15 min, 4°C) and the post-nuclear supernatant was layered onto discontinuous Percoll gradients. Percoll, EGTA (15 mM), relaxation buffer (10× concentrated), and distilled water were mixed to give solutions of densities 1.05 g/ml and 1.12 g/ml. A portion of 14 ml of the light solution was underlaid with 14 ml of the heavier solution and the post-nuclear supernatant was layered on top of the gradient. After centrifugation at $32\,800\times g$ (35 min, 4°C) using a fixed-angle Beckman JA20 rotor, three bands were visible and collected with a Pasteur pipette. The bands characterized by marker protein analysis are denoted α (azurophil granules, myeloperoxidase as marker), β (specific granules, vitamin B_{12} binding protein as marker) and γ (secretory vesicles/plasma membranes, alkaline phosphatase as marker) in order of decreasing density [22,28]. The cytosol was prepared from the post-nuclear supernatant without addition of EGTA. The supernatant was centrifuged for 60 min ($100\,000\times g$, 4°C) to remove the organelles.

In order to remove proteins loosely associated with membranes, the γ-fraction was first washed in relaxation buffer (centrifuged at $100\,000\times g$, 30 min, 4°C), then with the same buffer supplemented with 1 M NaCl, and finally in relaxation buffer once again before use in translocation experiments with recombinant annexin I. This procedure has been shown to remove proteins loosely associated with the plasma membrane and leaves integral membrane proteins such as the *b*-cytochrome of NADPH-oxidase [29].

2.5. Calcium-induced translocation of annexin I

Purified recombinant annexin I (1×10^8 yeast cell equivalents) or neutrophil cytosol (1×10^8 cell equivalents) in relaxation buffer (1 mM Pefabloc) was mixed with secretory vesicles/plasma membranes (3×10^8 cell equivalents) in relaxation buffer (containing 1 mM Pefabloc) and either EGTA (1 mM) or Ca^{2+} (1 mM) was added. The mixtures were incubated for 15 min at 37°C and centrifuged at $65\,000\times g$ for 15 min. The collected membranes were washed once, resuspended in relaxation buffer (1 mM Pefabloc, 2 mM EGTA; 300 μl), incubated for 15 min, and centrifuged at 22 000 psi (20 min, 4°C) in an airfuge (Beckman Instruments, Fullerton, CA). The supernatants, containing calcium-dependent, phospholipid-binding proteins, were stored in sample buffer (reduced) at −20°C until analyzed.

2.6. Proteolytic cleavage of annexin I

Granules (5×10^6 cell equivalents) were mixed with recombinant annexin I (1×10^7 yeast cell equivalents) and Ca^{2+} (2 mM) or EGTA (2 mM) was added. The samples were incubated for 30 min at 37°C in the absence or presence of one of the protease inhibitors or dithiothreitol and Triton X-100. The mixtures were stored in sample buffer at −20°C until analysis.

2.7. Amino acid sequencing of the N-terminally truncated form of annexin I

Neutrophils were isolated from 20 buffy coats. Cells in relaxation buffer were disrupted by nitrogen cavitation. Cytosol and secretory vesicles/plasma membranes were isolated and mixed in a ratio of 1:1. Ca^{2+} (2 mM) was added to induce translocation of annexin I to secretory vesicles/plasma membranes (as described in Section 2.5). The final EGTA extract was mixed with sample buffer, electrophoretically separated, and transferred to a PVDF-membrane. Proteins were visualized by staining with Coomassie Brilliant blue. The truncated form of annexin I was excised from the membrane and subjected to auto-

mated Edman degradation and the amino acid sequence was scanned against the Swissprot databank (P04083) for determination of the cleavage site.

2.8. SDS–PAGE and immunoblotting

Proteins from equal amount of cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% polyacrylamide gels [30] and either transferred electrophoretically to PVDF membranes [31] or silver-stained. The PVDF membranes were incubated in blocking buffer (1% BSA, 1% skimmed milk, 0.05% Tween-20 in PBS) for 1 h in room temperature and then for at least 1 h with monoclonal mouse anti-annexin I antibodies (1:5000 dilution). The membranes were finally incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:2000 dilution). The blots were developed with bromochloroindolyl phosphate (BCIP; 0.15 mg/ml) and Nitro Blue tetrazolium (NBT; 0.3 mg/ml) in 0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8.

3. Results

3.1. Translocation of cytosolic proteins to neutrophil organelles

Previous work has shown that annexin I (as well as several other members of the annexin family) is able to bind Ca^{2+}-dependently to all the different types of neutrophil organelles which can be isolated on Percoll gradients (i.e., azurophil granules, specific granules, and secretory vesicles/plasma membranes) [22,23]. In contrast to the full-length annexin I molecule (40 kDa), a truncated form of the protein (39 kDa) binds only to the specific granules and secretory vesicles/plasma membranes but not to the azurophil granules [23] (Fig. 1). The fact that only the full-length annexin I was present in the cytosol, indicates that the molecule is cleaved during the translocation process. Our earlier results imply that the annexin I-cleaving protease is present in the specific granules and the secretory vesicles/plasma membranes but not in the azurophil granules. From these experiments we could not determine whether the protease is cytosolic and co-translocates with annexin I to the organelles or exerts its action primarily on the membrane-bound annexin I molecule.

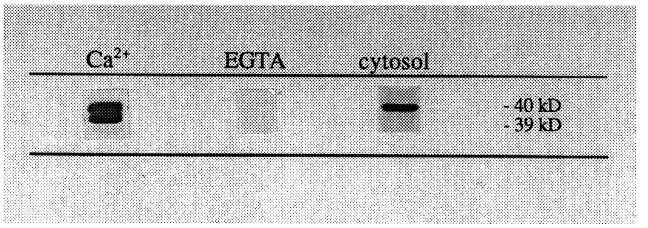

Fig. 1. Translocation of annexin I in neutrophil cytosol to secretory vesicles/plasma membranes. Isolated secretory vesicles/plasma membranes were mixed with cytosol in the presence of Ca^{2+} (2 mM) or EGTA (2 mM). The membranes were collected and washed. Annexin I was released from the membranes by extraction in an EGTA-containing buffer. The organelles were removed and the supernatants were electrophoresed under reducing conditions, electroblotted onto PVDF membranes, and detected with mouse anti-annexin I antibodies. The cytosol contained only full-length annexin I (40 kDa, right). The 40 kDa annexin I and a 39 kDa truncated form were detected following translocation in the presence of Ca^{2+} (left), but not in the presence of EGTA (middle).

3.2. Translocation of recombinant annexin I

To determine the localization of the protease responsible for cleavage of the annexin I molecule we depleted the translocation system from other cytosolic proteins through the use of recombinant human annexin I.

The recombinant annexin I was cleaved Ca^{2+}-dependently (Fig. 2) also in the absence of cytosolic proteins and both full-length annexin I and the truncated form of the molecule were also bound Ca^{2+}-dependently (no cleavage or binding at concentrations lower than 0.1 mM) to the neutrophil specific granules and secretory vesicles/plasma membranes (shown for the secretory vesicles/plasma membranes in Fig. 3). Loosely associated membrane proteins were removed by high salt washing prior to the translocation experiments. However, the ability to cleave annexin I was retained also in the washed membranes (shown for the secretory vesicles/plasma membranes in Fig. 4). Taken together, these results show that the protease responsible for truncation of annexin I during translocation to the specific granules and the secretory vesicles/plasma membranes is not cytosolic, but is integrated in the membrane of these neutrophil organelles.

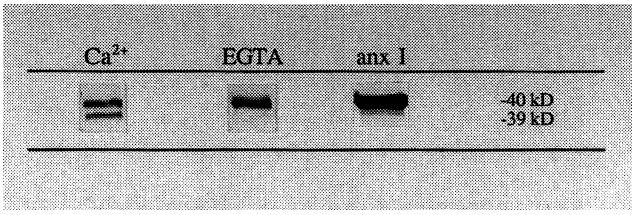

Fig. 2. Cleavage of recombinant annexin I by secretory vesicles/plasma membranes. Recombinant annexin I (anx I) was incubated with secretory vesicles/plasma membranes in the presence of Ca^{2+} (2 mM) or EGTA (2 mM) for 30 min at 37°C. Without further processing, the samples were electrophoresed under reducing conditions and electroblotted onto PVDF membranes and detected with mouse anti-annexin I antibodies. The full-length recombinant annexin I (40 kDa, right) was cleaved Ca^{2+}-dependently to generate a 39 kDa truncated form (left). The cleavage of annexin I was inhibited by EGTA (middle).

3.3. Determination of the cleavage site on annexin I

The truncated form of annexin I was excised from a PVDF membrane after separation by SDS–PAGE and subsequent transfer. The protein was subjected to automated Edman degradation to determine the N-terminal sequence of the protein. We found that the N-terminus was composed of the amino acids K–Q–A–W–F–I, a sequence that only matched the human annexin I sequence [32], starting at amino acid number nine from the N-terminus of the full-length protein (Table 1). Subsequently, the 39 kDa truncated annexin I molecule lacks the eight first amino acids (hereafter called des_{1-8} annexin I) of the full-length protein, and the protease thus cleaves the protein between the amino acids leucine and lysine in position 8 and 9, respectively, of the annexin I molecule.

3.4. Classification of the annexin I-cleaving protease

To classify the protease, inhibitor studies were performed and the results are shown in Table 2. We found that the serine protease inhibitors phenylmethylsulfonylfluoride, Pefabloc, diisopropylfluorophosphate, and leupeptin had no effect on the cleavage of annexin I, and this was true also for the aspartic proteinase inhibitor pepstatin and the cysteine proteinase inhibitor E-64. The cleavage of annexin I was, however, totally inhibited by 1,10-phenanthroline, indicating that the enzyme is a metalloprotease, of which the very great majority are zinc-containing enzymes [33].

The importance of thiol groups were studied by the inclusion of dithiothreitol (5 mM) to the reaction mixture which completely inhibited the cleavage of the annexin I molecule, suggesting that the metalloprotease requires intact disulfide bonds for proteolytic activity. A lower concentration of dithiothreitol partially inhibited the protease. The cleavage of annexin I was also partly inhibited by Triton X-100,

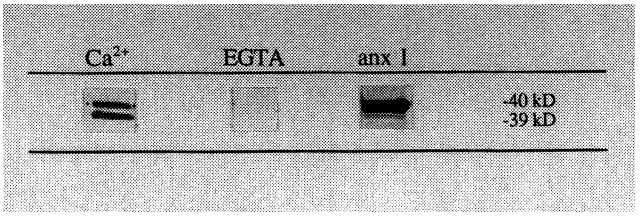

Fig. 3. Translocation of recombinant annexin I to secretory vesicles/plasma membranes. Recombinant annexin I (anx I) was mixed with isolated secretory vesicles/plasma membranes in the presence of Ca^{2+} (1 mM) or EGTA (1 mM). The membranes were collected and washed. Annexin I was released from the membranes by extraction in an EGTA-containing buffer. The organelles were removed, the supernatants were electrophoresed under reducing conditions and electroblotted onto PVDF membranes, and detected with mouse anti-annexin I monoclonal antibodies. The full-length recombinant annexin I (40 kDa, right), as well as a 39 kDa truncated form was detected following calcium-induced translocation (left). In the presence of EGTA, translocation was inhibited (middle).

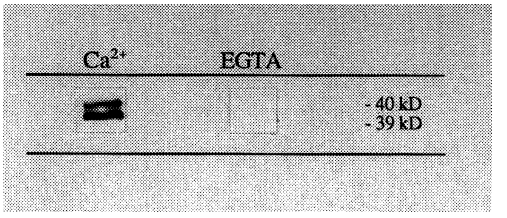

Fig. 4. Translocation of recombinant annexin I to secretory vesicles/plasma membranes depleted of peripheral membrane proteins. These secretory vesicles/plasma membranes were mixed with recombinant annexin I in the presence of Ca^{2+} (1 mM) or EGTA (1 mM). The secretory vesicles/plasma membranes were collected and washed. The annexin I molecules were released from the organelles by extraction in an EGTA-containing buffer. The organelles were removed, the supernatants were electrophoresed under reducing conditions and electroblotted onto a PVDF membrane, and detected with mouse anti-annexin I monoclonal antibodies. The full-length recombinant annexin I (40 kDa, right), as well as a 39 kDa truncated form was detected following calcium-induced translocation (left). In the presence of EGTA, translocation was inhibited (middle).

suggesting that the protease works more efficiently in an intact membrane.

4. Discussion

The truncated form of annexin I that is formed during Ca^{2+}-regulated in vitro translocation and binding of the protein to neutrophil secretory vesicles/plasma membranes is generated through the action of an endogenous membrane protease. Such protease activities have been described also in other cells [34], but the responsible protease(s) has so far not been identified through isolation. There are many similarities between the neutrophil annexin I-cleaving protease and that found in other cells [34]: (i) it is membrane bound; (ii) annexin I but not other members of the annexin family of Ca^{2+}-regulated membrane binding proteins are cleaved by the proteases, indicating that these membrane proteases recognize specific sites within the annexin I molecule; (iii) the activity is inhibited by Triton X-100 and dithiothreitol. Based on the inhibitory activity of 1,10-phenanthroline and dithiothreitol, we classify the neutrophil annexin I cleaving protease as a membrane-bound, thiol-dependent metalloprotease. Membrane-bound metalloproteases and metallopeptidases have been described in other cell types [35–37], and it is interesting to notice that it has been suggested that their function may involve regulation of intracellular signaling achieved through cleavage of cytoplasmic messenger molecules [35].

Table 1
Amino acid sequence of the truncated annexin I molecule, recovered after translocation of full-length annexin I to secretory vesicles/plasma membranes[a]

Amino acid residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Truncated annexin I									K	Q	A	W	F	I
Full-length annexin I	M	A	M	V	S	E	F	L	K	Q	A	W	F	I

[a]The protein was transferred to a PVDF-membrane from an SDS–PAGE gel. The truncated form of annexin I was excised from the membrane and subjected to automated Edman degradation. The sequence of human full-length annexin I was compared with the N-terminal sequence of the truncated annexin I molecule.

Table 2
Effect of different inhibitors on the cleavage of annexin I mediated by a neutrophil protease present in the secretory vesicles/plasma membranes

Inhibitor used	Concentration	Effect
DTT	1 mM	+[a]
DTT	5 mM	++[b]
Triton X-100	1.0%	+
Metalloprotease inhibitor		
1,10-Phenanthroline	1 mM	++
Serine proteinase inhibitors		
3,4-DCl	1 mM	–[c]
Pefabloc	1 mM	–
PMSF	1 mM	–
DFP	0.2 μg/ml	–
Leupeptin	10 μM	–
Aspartic proteinase inhibitors		
Pepstatin	10 μM	–
Cysteine proteinase inhibitors		
E-64	4 μg/ml	–

[a]Cleavage was partially inhibited.
[b]Cleavage was completely inhibited.
[c]No inhibition of cleavage.

The annexin I molecule is cleaved also during Ca^{2+}-regulated translocation to the specific granules (but not to the azurophil granules). Although we have not characterized this cleavage in detail our results strongly suggest that the same protease is present in the specific granules.

Cleavage of annexin I has been reported to occur at many different sites, more or less close to the N-terminus of the molecule (e.g., cleaved at lysine[9], at tryptophan[12], at lysine[26] and lysine[29]) [20,21,38,39]. We found that cleavage of annexin I by the neutrophil annexin I-cleaving metalloprotease, occurs close to the N-terminal of the molecule, leaving a truncated form of the molecule, des_{1-8} annexin I, that contains the Ca^{2+}-binding consensus sequence, as well as a number of phosphorylation sites (i.e., tyrosine[21], threonine[24], serine[27] and serine[28]) of importance for protein function. This cleavage site is unique for the neutrophil annexin I cleaving proteinase.

As mentioned, members of the annexin family possess a structurally homologous core region. This part of the annexins is responsible for Ca^{2+}- and phos-

pholipid-binding and interaction with cytoskeletal components. In contrast, the N-terminal regions are structurally diverse and confer the specific functions of the different members of the annexin family. With respect to the function of annexin I, many studies have shown its capacity to promote granule aggregation and membrane fusion, indicating that the molecule may constitute part of a secretory apparatus [10–13]. Modulation of the N-terminus of the molecule has thus been suggested to be of importance as a basis for regulation of secretory and fusogenic events [7,11,19,20,38,39]. In contrast to the full-length annexin I, des_{1-8} annexin I binds only to specific granules and secretory vesicles/plasma membranes and not to azurophil granules [23], implying different mechanisms for binding to neutrophil organelles of full-length annexin I and des_{1-8} annexin I. The inability of des_{1-8} annexin I to bind azurophil granules is probably not an effect of an altered calcium sensitivity (which has been described for truncated annexin I molecules in other biological systems) as the truncated forms of annexin I usually require less calcium for membrane binding or vesicle aggregation [14,20,21]. All in all, our data provide support for the idea that the presence of both full-length annexin I and des_{1-8} annexin I in neutrophils may be of importance in differential exocytosis. It is also interesting to note that the selectivity in annexin I binding has been described in other cells, and it has been suggested that the protein and its derivatives are involved in endocytic processing [38]. This suggestion originates from the observation that translocation of annexin I to early endosomes was found to critically depend on the presence of an intact N-terminal domain of the molecule whereas association with late endosomes requires an N-terminal truncation of the molecule.

The cleavage of annexin I may be of regulatory importance for the neutrophil degranulation process, since the truncated form of annexin I shows differential binding capacity to the neutrophil granules. It may also be of importance for the processing and maturation of endocytic vesicles formed during maturation of the cells in the bone marrow (i.e., the secretory vesicles formed through an endocytic process during the later stages of myelopoiesis [40]) or during uptake and killing of foreign microorganisms (phagosome formation and phagolysosomal maturation following endocytic uptake of particles) [41]. Furthermore, annexin I-binding to neutrophil organelles may be a good model to disclose the molecular background to the phenomenon of cytosolic proteins which bind selectively to different subcellular organelles.

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