Purification and Characterization of an Abundant Cytosolic Protein from Human Neutrophils That Promotes Ca^{2+}-dependent Aggregation of Isolated Specific Granules

Joel D. Ernst,*‡ Elaine Hoye,* R. Alexander Blackwood,* and David Jaye*

*Department of Medicine, *Rosalind Russell Arthritis Research Laboratory, and ‡Division of Infectious Diseases, San Francisco General Hospital and the University of California, San Francisco, San Francisco, California 94143-0868*

Abstract

Intracellular ionized calcium has been strongly implicated in mediating several responses of human neutrophils to stimulation. However, proteins that serve as effectors of these responses have not been well characterized. To identify proteins that might serve as mediators of the effects of Ca^{2+} in human neutrophils, we isolated proteins that bind to membrane phospholipids in a Ca^{2+}-dependent manner. The most abundant of these, a protein of 33 kD, was readily purified to homogeneity, and was found to bind to phosphatidylserine vesicles in the presence of 2 μM ionized Ca^{2+}. In addition, this purified protein promoted Ca^{2+}-dependent aggregation of isolated specific granules from human neutrophils, indicating that it might mediate membrane–membrane contact during processes such as phagosome–lysosome fusion or degranulation. This protein was localized to the cytoplasm of unstimulated neutrophils and found to account for ~ 1% of the cytosol protein. Amino acid sequence of several peptides derived from the purified protein revealed that it is identical to lipocortin III, a recently described member of the annexin family that is scarce in other cells and tissues. The abundance of this protein, together with its Ca^{2+}-dependent membrane effects, suggest that it mediates membrane-localized events in stimulated neutrophils, such as phagosome–lysosome fusion or degranulation. (*J. Clin. Invest.* 1990. 85:1065–1071.) lipocortin • annexins • calcium-binding proteins • phospholipid-binding proteins • neutrophil degranulation

Introduction

The role of ionized Ca^{2+} as a second messenger in a wide variety of cells, including human PMN, is well established. In human PMN transient increases in intracellular ionized calcium concentration ($[Ca^{2+}]_i$)[1] occur rapidly in response to activation by a variety of soluble as well as particulate stimuli (1, 2). In response to some stimuli, increased $[Ca^{2+}]_i$ appears to be essential, as inhibition of increases in $[Ca^{2+}]_i$ blocks superoxide anion production and exocytosis (1). Furthermore, secretion of granule contents as well as production of superoxide anion can be induced using Ca^{2+} ionophores (3). Finally, a role for Ca^{2+} in phagosome–lysosome fusion in PMN is suggested by the finding that when these cells ingest opsonized particles, the highest ionized Ca^{2+} concentration is in the periphagosomal region (2), where granule membranes fuse with the phagosome membrane.

Despite the evidence that Ca^{2+} is necessary for certain responses in PMN, the proteins that serve as Ca^{2+}-modulated cellular effectors have not been clearly identified. In other systems, recent efforts to identify intracellular proteins that mediate the effects of Ca^{2+} have led to the identification of a family of proteins, the annexins (4). All of the annexins bind to membrane phospholipids in the presence of Ca^{2+}, and are structurally related: all contain four or eight repeats of a 70–amino acid sequence that is believed to represent the Ca^{2+}- and phospholipid-binding domain. While all nucleated mammalian cells appear to contain at least one annexin protein, the number present and the abundance of individual annexins varies widely in different cell types.

In a first step toward understanding the mechanism of Ca^{2+}-modulated events in human PMN, we examined these cells for proteins that bound reversibly to membrane phospholipids in a Ca^{2+}-dependent manner. This technique yielded several proteins, including small quantities of synexin (5), as well as an abundant protein with a molecular mass of 33 kD. We describe purification of this protein and characterization of it as a member of the annexin family. This protein is especially abundant in PMN, in contrast to other cells and tissues that have been examined.

Methods

Preparation of human PMN. PMN were isolated and purified (> 97%) from venous blood of normal human volunteers by the method of Böyum (6), using Hypaque-Ficoll gradients and dextran sedimentation. Representative cell suspensions contained < 1 platelet per 10 PMN. Human PMN cytosol and specific granules were prepared exactly as described previously (5), after sonicating cells in the presence of diisopropylfluorophosphate, leupeptin, pepstatin, and EDTA.

Purification of Ca^{2+}-dependent, phospholipid-binding proteins. Soluble proteins in PMN cytosol were subjected first to precipitation with ammonium sulfate added to 25% saturation (to precipitate synexin) (5). Proteins in the supernatant were then precipitated by the further addition of solid ammonium sulfate to 95% saturation. Precipitated proteins were suspended in, and dialyzed against, 0.3 M NaCl, 0.1 M TES (*N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), pH 7.4, containing 0.1 mM EDTA. After dialysis $CaCl_2$ was added to 1 mM, and proteins were applied to a column of liposomes prepared with phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (2:2:1) covalently coupled to Sepharose as described previously (5). The column was washed extensively with buffer containing 1 mM Ca^{2+} before eluting bound proteins by reducing the concentration of Ca^{2+}, first with the same buffer containing 0.1

Address correspondence to Dr. Joel D. Ernst, University of California, Box 0868, San Francisco, CA 94143-0868.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium concentration; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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mM Ca^{2+}, then with the same buffer containing 2.5 mM EGTA (instead of Ca^{2+}). Eluted proteins were dialyzed against either 20 mM sodium acetate, pH 5.0, containing 0.5 mM EDTA (for ion-exchange chromatography on Mono S) or 20 mM bis-tris-propane, pH 6.8 (for chromatography on Mono Q). Samples were applied to the designated column in an FPLC system (Pharmacia Fine Chemicals, Piscataway, NJ) operated at room temperature, and were eluted with a linear gradient of NaCl in the appropriate buffer.

Lipocortins I and III, calpactin I, endonexin II, p68, and protein II were purified from human placenta using a modification of the procedure of Haigler et al. (7, 8).

Electrophoresis. One-dimensional SDS-PAGE was performed using the Laemmli buffer system (9), and two-dimensional PAGE was performed by the method of O'Farrell (10).

Calcium-dependent binding to phospholipid vesicles. All components in the assay were prepared in 0.1 M KCl, 50 mM Hepes, pH 7.0, containing 1 mM EGTA. Stock solutions of EGTA/Ca^{2+} were prepared at four times the desired final concentrations of Ca^{2+} using the method of Bers (11) to predict the free [Ca^{2+}] in the presence of EGTA. After dilution to the working concentration, ionized [Ca^{2+}] was measured using a Ca^{2+}-selective electrode (Orion Research Inc., Cambridge, MA). Phospholipids were dried from chloroform under a stream of N_2, and were dispersed at 4 mg/ml in the buffer described above by two cycles of sonication with a probe sonicator. Binding of proteins to phospholipid vesicles was assayed in a total volume of 100 μl containing 2.5 μg of purified protein, 100 μg phospholipid, and 25 μl of the desired stock solution of Ca^{2+}, which was added last. The mixtures were incubated at room temperature for 10 min, then centrifuged for 10 min in an Eppendorf microfuge (15,600 *g*; Brinkman Instruments, Inc., Westbury, NY). Supernatants were aspirated from the pelleted phospholipid, and appropriate volumes of Laemmli sample buffer were added to the supernatants and pellets. After boiling, the entire volume of each supernatant and pellet was subjected to SDS-PAGE on gels containing 10% acrylamide. After electrophoresis, gels were stained with Coomassie blue, destained, and the relative quantities of protein in each pellet and supernatant determined using a Quickscan Jr. densitometer (Helena Laboratories, Beaumont, TX). The fraction of protein bound to phospholipid (percent) was calculated using integrated areas under the densitometer peaks and the following equation: pellet/(pellet + supernatant) ×100. Controls included reaction mixtures lacking either Ca^{2+} or phospholipid. Negligible amounts of protein were pelleted when either of these components was omitted. However, as it was necessary to leave some supernatant on the pellet to avoid disrupting the pellet, ~ 10% of the added protein appeared in the pellet fraction, even in control experiments. To correct for this and to avoid overestimating the fraction of protein bound, 10% was subtracted from the bound fraction in displaying the results of all experiments.

Antibodies. Antibodies directed against a synthetic peptide representing a consensus sequence present in annexins (with the sequence K-G-A-G-T-D-E-D-S-L-I-E-I-L-A-Y-R) were prepared and characterized as described in detail elsewhere (8). Antisera directed against the 33-kD protein from human PMN, as well as against human endonexin II and human protein II, were prepared by immunizing rabbits with individual proteins purified to homogeneity from human PMN or placenta. The identity of the purified proteins was confirmed by amino acid sequencing and/or by recognition by specific antibodies provided by Drs. Harry Haigler (endonexin II) and Volker Gerke and Klaus Weber (porcine protein II). Initial doses of 50 μg of each protein were administered subcutaneously in complete Freund's adjuvant, and four booster doses of 50 μg each were administered monthly in incomplete Freund's adjuvant. Rabbits were anesthetized and exsanguinated when antibody titers (as indicated by ELISA using purified proteins) reached a plateau. Specificity of the rabbit antisera for the proteins used as immunogens, compared with other annexins, was determined both by ELISA and by Western blotting. Specific proteins were quantified in human PMN by a solid-phase Western blot immunoassay. PMN (> 97%) were lysed in PBS (6.25×10^7/ml) containing 2 mM diisopropylfluorophosphate, 1 mM PMSF, 5 mM EDTA, and 1% Triton X-100. After centrifugation (1,000 *g*, 5 min) to pellet nuclei, the lysate supernatants were diluted with 4× Laemmli sample buffer to 5×10^7 cell equivalents/ml. Serial twofold dilutions were prepared in sample buffer such that 20 μl represented from 2.5×10^5 to 7.8×10^3 cell equivalents, and these dilutions from the cells of two donors were subjected to SDS-PAGE and Western blotting.

Western blotting. Western blotting was performed essentially as described by Burnette (12). Bound antibodies were detected using biotinylated goat anti-rabbit Ig followed by streptavidin-alkaline phosphatase, with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates. For quantitative Western blotting, membranes were subsequently treated with affinity-purified rabbit anti-goat Ig (0.5 μg/ml) (Zymed Laboratories, Inc., South San Francisco, CA). After washing, bound IgG was detected using staphylococcal protein A labeled with ^{125}I using Bolton-Hunter reagent (0.5 μCi/ml) (Amersham Corp., Arlington Heights, IL). After washing to remove unbound ^{125}I, equal portions of blots containing the relevant proteins were excised and subjected to gamma counting. Standard curves were prepared using purified protein in fourfold dilutions ranging from 100 to 0.39 ng applied to adjacent lanes of a gel, and treated in a manner identical to samples of PMN lysates. Blank values were determined by excising a portion of each nitrocellulose membrane that was not in contact with the original gel (but that was exposed to all incubation solutions). Blank values for each membrane were subtracted from the values determined for samples on that membrane before using the sample values for either standard curve construction or for experimental determinations. Results were evaluated by linear regression analysis of the values for the standard curve, followed by interpolation of experimental values, using only those values that corresponded to the linear portion of the standard curve.

Immunofluorescence. For immunolocalization, peripheral blood leukocytes (prepared by dextran sedimentation and hypotonic lysis of red blood cells) were allowed to adhere to uncoated glass coverslips in HBSS containing 0.15% BSA for 30 min at 37°C. Cells were fixed in 3.7% paraformaldehyde, then permeabilized with 0.04% saponin. The coverslips were then incubated in the primary antiserum diluted in PBS containing 0.01% Triton X-100, 0.02% SDS, and 5% normal goat serum. After rinsing, the coverslips were incubated in FITC-conjugated, affinity-purified goat anti-rabbit IgG (Zymed; 37.5 μg/ml) in the same buffer as for the primary antibody. After extensive rinsing, cells were stained with Evan's blue 0.1 mg/ml in water and the coverslips were air-dried and mounted for viewing and photography in 10% polyvinyl alcohol, 25% glycerol, 0.1 M Tris-HCl (pH 8.5) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane to reduce quenching.

Miscellaneous experimental procedures. All protein assays were performed by the method of Bradford (13) using the kit provided by Bio-Rad Laboratories (Richmond, CA) and standard curves constructed with BSA. Subcellular fractionation and assay of aggregation of isolated PMN specific granules were performed exactly as previously described (5). Peptide fragments generated from the purified protein by cleavage with either CNBr or lysyl-endopeptidase were separated by reverse-phase chromatography, and amino acid sequence was determined using a gas-phase sequenator (model 470A; Applied Biosystems Inc., Foster City, CA) equipped with an on-line PTH analyzer.

Results

SDS-PAGE analysis of human PMN cytosol proteins that eluted from the column of immobilized liposomes by reducing the Ca^{2+} concentration to 0.1 mM revealed two predominant bands that migrated with apparent molecular masses of ~ 36 and 33 kD (Fig. 1 *A*). Two-dimensional PAGE on the same samples revealed the presence of one predominant protein spot at each of these molecular masses, as well as one (36 kD) or two (33 kD) minor protein spots (Fig. 1 *B*). Similar analysis of the proteins eluted from the column with EGTA revealed

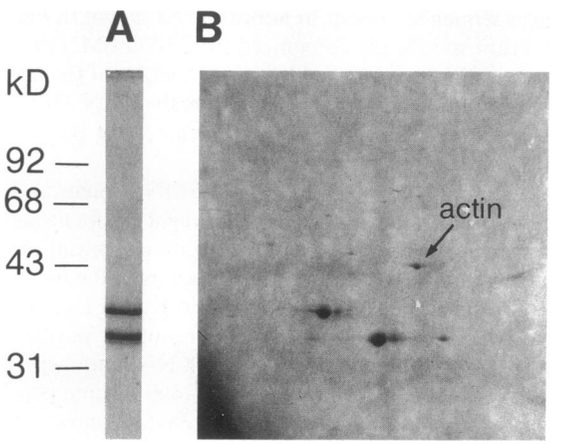

Figure 1. A, SDS-PAGE of PMN cytosol proteins that bound reversibly to immobilized PS/PC/PE in the presence of 1 mM Ca^{2+} and eluted with reduction of the Ca^{2+} concentration to 0.1 mM. Approximately 10 μg of protein was loaded on the gel, which was stained with Coomassie blue. *B,* Two-dimensional PAGE of the same fraction as in *A.* Isoelectric focusing was performed in the first dimension, and SDS-PAGE in the second. Approximately 10 μg of protein was loaded on the gel, which was stained with Coomassie blue. For orientation, the minor spot (*actin*) corresponds to the electrophoretic mobility of PMN actin. Results are representative of those obtained on multiple occasions, with the exception that actin is infrequently detected.

that there was a prominent protein spot at 68 kD and several minor spots at 33 kD. Amino acid sequence determinations and immunoblotting studies revealed that the less abundant 33-kD proteins that eluted with 0.1 mM Ca^{2+} were endonexin I (also known as protein II) and endonexin II. Peptide mapping established that the 33-kD proteins eluted with EGTA represented the same three proteins that eluted with 0.1 mM Ca^{2+}. Insufficient quantities of these were available for more detailed characterization. This report focuses mainly on the abundant 33-kD protein that was obtained by elution with 0.1 mM Ca^{2+}.

The major 33-kD protein was purified to apparent homogeneity by subsequent ion-exchange chromatography (Fig. 2). Since no unique activity could be attributed to this protein during purification, recovery of the protein was not calculated. However, as detailed in Table I, the purification procedure described yielded 35 μg of electrophoretically pure 33-kD protein, beginning with cytosol from ~ 10^9 PMN.

To determine whether the protein we purified bound directly to phospholipids in a Ca^{2+}-dependent manner (rather than to another protein that bound to phospholipids on the column), we examined whether the purified 33-kD PMN protein bound to PS, using a simple centrifugation assay. As shown in Fig. 3, the purified protein bound to PS vesicles in a strictly Ca^{2+}-dependent manner under conditions of phospholipid excess. Detectable binding under the conditions of this

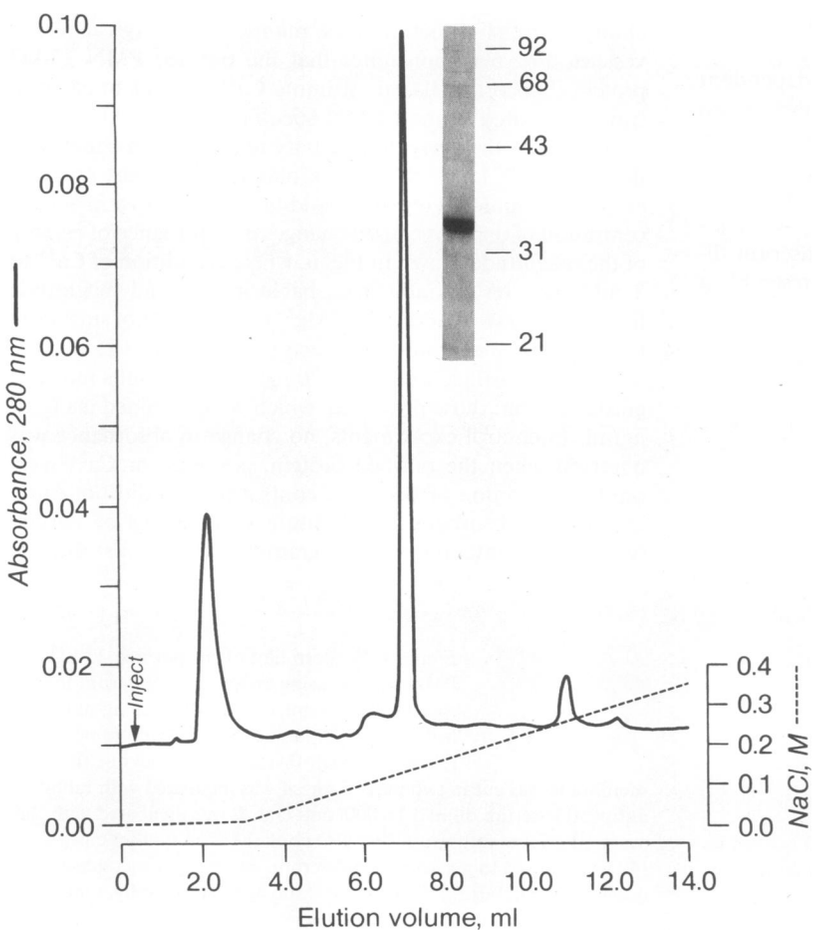

Figure 2. Mono Q (anion-exchange) chromatography of a sample corresponding to that shown in Fig. 1. SDS-PAGE of a portion of the peak that eluted at ~ 0.12 M NaCl is shown in the inset. Two-dimensional PAGE revealed that the band was composed of only one spot. The smaller peak that did not bind to the column ran with an electrophoretic mobility corresponding to 36 kD, and the small peak that eluted with 0.26 M NaCl ran as a 32–33-kD band. Results are typical of those obtained from > 20 separate preparations of PMN cytosol.

Table I. Yield of 33-kD Protein from Human PMN

Sample	Total protein
PMN cytosol (1×10^9 cells)	27 mg
Liposome column flow-through and Ca^{2+} wash	26 mg
0.1 mM Ca^{2+} eluate from liposome column	330 μg
EGTA eluate from liposome column	77.5 μg
Mono Q peak fraction	35 μg

Purification procedures were performed as described in text. Protein content of fractions was determined using the method of Bradford (13). Purity was determined by SDS-PAGE. Data shown are representative of those obtained from > 20 preparations of PMN cytosol.

assay occurred at 2 μM Ca^{2+}, while maximal binding required 8 μM Ca^{2+}. Half-maximal binding required 3.5 μM ionized Ca^{2+}. The 33-kD PMN protein did not bind to either PC or PE at ionized Ca^{2+} concentrations up to 450 μM. The finding that the purified protein bound to pure PS vesicles in the presence of micromolar concentrations of Ca^{2+}, while it eluted from the liposome column at 0.1 mM Ca^{2+}, initially appeared contradictory. However, we have subsequently determined that the presence of PC significantly increases the Ca^{2+} requirement for binding of the 33-kD PMN protein (and other annexins) to PS (Blackwood, R. A., and J. D. Ernst, manuscript in preparation). Therefore, the purified 33-kD PMN protein binds directly to pure PS in the presence of low micromolar concentrations of Ca^{2+}, while the presence of PC in the liposomes used for affinity purification facilitates elution of the protein from the affinity matrix (at 0.1 mM Ca^{2+}).

In addition to sharing the property of Ca^{2+}-dependent binding to phospholipids, members of the annexin family also share structural similarities. The most prominent of these is a highly conserved 17–amino acid sequence that is present in at least four copies per protein. To determine whether this sequence motif is present in the 33-kD PMN protein, we examined it by Western blotting, using polyclonal antiserum directed against a 17–amino acid synthetic peptide resembling the consensus sequence present in annexins. As shown in Fig. 4, this antiserum specifically recognized the 33-kD PMN protein, and recognition was blocked by preincubation of the antiserum with the synthetic peptide. Therefore, the PMN 33-kD protein is similar antigenically to other members of the annexin family.

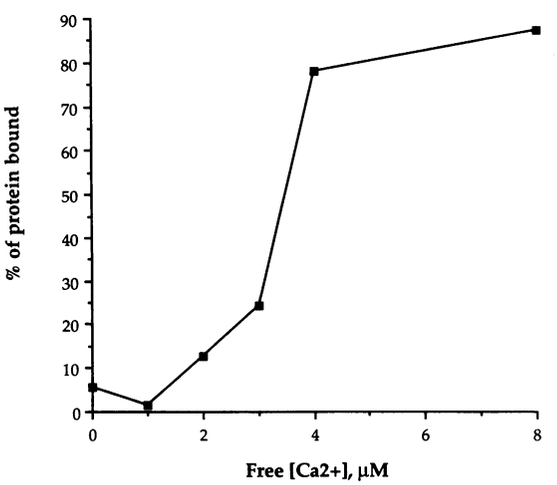

Figure 3. Ca^{2+} dependence of binding of the PMN 33-kD protein to pure PS vesicles under conditions of lipid excess. Results shown are representative of three determinations of binding at each calcium concentration.

Further similarity between the 33-kD PMN protein and other members of the annexin family was sought by obtaining amino acid sequence from the purified protein. Initial attempts at determining NH_2-terminal sequence from the intact protein were unsuccessful, as it was found to have a blocked amino terminus. Therefore, 100 μg (~ 3 nmol) of purified protein was cleaved either with CNBr or with lysyl-endopeptidase, and sequence was determined from a total of nine peptides purified by reverse-phase chromatography. As shown in Fig. 5, unambiguous sequence was determined for a total of 115 amino acids: over one-third of the predicted total, based on the electrophoretic mobility of the protein. Also shown in Fig. 5 is comparison of the determined amino acid sequence of the fragments from the purified PMN 33-kD protein with the sequence predicted from the cDNA sequence of human lipocortin III (14). All of the residues determined from fragments of the purified 33-kD protein from human PMN correspond to amino acids predicted from the lipocortin III cDNA. Therefore, the protein we have purified is likely to be identical to lipocortin III, which was recently described as an apparent inhibitor of phospholipase A_2.

One property predicted for a membrane-binding protein that might mediate fusion of membranes of phagocytic vesicles and neutrophil granules is that of synexin-like activity, i.e., the ability to crosslink membranes, manifested as aggregation of vesicles. Fig. 6 demonstrates that the purified PMN 33-kD protein (lipocortin III) can promote Ca^{2+}-dependent aggregation of isolated human PMN specific granules. Under the conditions of the assay, this activity required high concentrations of Ca^{2+}: 1 mM led to the maximal apparent rate and extent of granule aggregation. Addition of Ca^{2+} to a final concentration of 0.5 mM caused changes in absorbance of ~ 50% of the magnitude shown in Fig. 6, whereas addition of Ca^{2+} to 2 mM gave results indistinguishable in rate and magnitude from those shown. Addition of Mg^{2+} (1 mM) did not substitute for, potentiate, or inhibit the effects of 500 μM or 1 mM Ca^{2+}. Addition of purified protein to 20 μg/ml gave results indistinguishable from those presented, which were obtained using 10 μg/ml. In control experiments, no change in absorbance was observed when the purified protein, granules, or Ca^{2+} were omitted. Addition of BSA as a control protein did not cause changes in absorbance, and dilute suspensions of Percoll (which can contaminate these granule preparations) did not

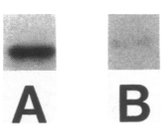

Figure 4. Western blot of the purified 33-kD PMN protein using an antipeptide serum that specifically recognizes annexins. 0.5 μg of purified protein per lane was transferred to nitrocellulose after SDS-PAGE. After blocking, the membrane was cut in two parts. Lane *A* was incubated with rabbit antipeptide serum diluted 1:1000, and lane *B* was incubated with the same dilution of antiserum that also contained the synthetic peptide (40 μg/ml) used to prepare the antiserum. Bound IgG was subsequently detected using ^{125}I-protein A and autoradiography. Only the relevant portion of the blots is shown.

PEPTIDE	SEQUENCE	AMINO ACIDS IN LIPOCORTIN III
C-1:	ISILTERSNAQRQL	42-55
L-8:	GDLSGHFEH	74-82
L-3:	GAGTNEDALIEILTTRTSRQMK	105-126
L-6:	ALLTLADGRRDESLK	155-169
L-7+2+4:	QDAQILYKAGENRWGTDEDKFTEILCLRLFPQL	177-209
L-1:	GIGTDEFTLNR	264-274
L-5:	SDTSGDYEITL	305-315

Figure 5. Amino acid sequence determined from nine peptide fragments of the purified 33-kD PMN protein after cleavage with either CNBr (*C-1*) or lysyl-endopeptidase (*L-1–8*). The column on the right shows the position of the corresponding sequence predicted from a cDNA clone encoding human lipocortin III (14). The peptide designated L-7+2+4 is a composite of three peptides sequenced to their COOH-termini, which proved to be contiguous when compared with the deduced sequence of lipocortin III.

change absorbance when incubated in the presence of the purified protein and Ca^{2+}.

A polyclonal antiserum was prepared by immunizing rabbits with human lipocortin III purified from placenta. This antiserum, when used at a 1:10,000 dilution, recognized as little as 1.6 ng of purified lipocortin III on Western blots. Furthermore, it recognized a single protein band (with the expected electrophoretic mobility) in detergent lysates of human PMN (Fig. 7). Preimmune serum from the same rabbit did not give any detectable reaction with blotted PMN proteins. Since two other annexins with similar molecular weights have been described, we examined the ability of this antiserum to recognize human endonexin II and human protein II (also known as human placental anticoagulant protein IV [15] or PP4-X [16]) purified from human placenta. When these proteins (up to 100 ng/lane) were examined on Western blots with this antiserum at a 1:10,000 dilution, no reactivity above background was detected. Therefore, the 33-kD band detected in lysates of PMN clearly represents the protein used to raise this antiserum.

This specific antiserum was used to determine that lipocortin III is a highly abundant protein in human PMN. Using a solid-phase Western blot immunoassay, we found that PMN contain 328±56 ng lipocortin III/10^6 cells (mean±SD; $n = 12$). As human PMN contain ~ 30 μg of cytosol protein/10^6 cells (J. D. Ernst, unpublished observations), this represents ~ 1% of their cytosolic protein.

Finally, we used this antiserum to examine the distribution of the 33-kD PMN protein by indirect immunofluorescence (Fig. 8). The intracellular location of the protein was established by the lack of fluorescence of cells that were not permeabilized (not shown), and a cytosolic distribution of the protein was indicated by a reticular pattern of fluorescence that did not correspond to any obvious intracellular structure. This finding is consistent with our finding that the 33-kD protein is found in the cytosol fraction after disruption of PMN by sonication.

Discussion

In an effort to further define the pathways determining responses of human PMN to stimuli that increase $[Ca^{2+}]_i$, we examined the cytosol of these cells for proteins that bound to membrane phospholipids in a Ca^{2+}-dependent manner. We found that these cells possess several proteins that exhibit this property. We characterized the most abundant of these, and found that it is identical to lipocortin III, a member of the annexin family that is scarce in most cells and tissues examined to date. We have determined that this protein represents ~ 1% of the total cytosol protein in human PMN, and found

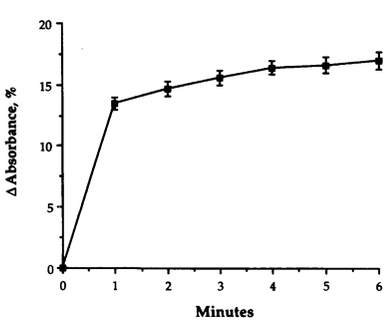

Figure 6. Aggregation of isolated PMN specific granules by the purified 33-kD PMN protein (10 μg/ml) in the presence of 1 mM Ca^{2+}. Values are means±SD of four separate experimental determinations for each time point. Phase-contrast microscopy confirmed that the change in absorbance corresponded to aggregation of granules.

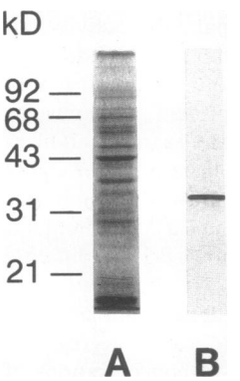

Figure 7. Specificity of rabbit anti–lipocortin III antiserum: Western blot analysis of human PMN detergent lysate. *A*, Coomassie blue–stained proteins in a lysate prepared from human PMN (125,000 cell equivalents; ~ 4 μg total protein) resolved by SDS-PAGE on a minigel containing 12.5% acrylamide. *B*, Western blot of the same quantity of the same lysate reacted with polyclonal antiserum specific for human lipocortin III. Positions of molecular mass markers (in kilodaltons) for both portions of the figure are shown on the left.

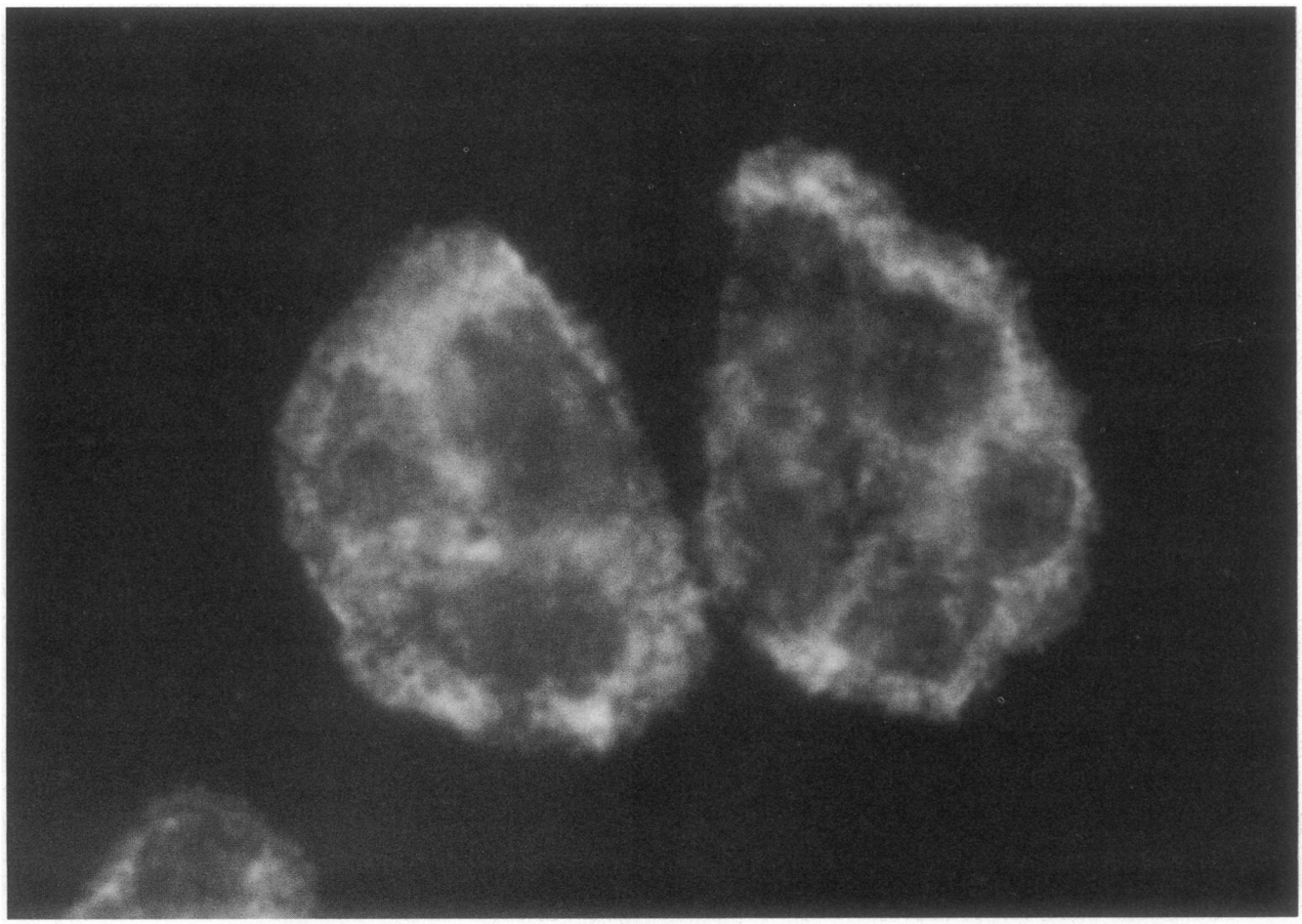

Figure 8. Indirect immunofluorescence using the polyclonal anti–lipocortin III antiserum (as shown in Fig. 7) to stain permeabilized human neutrophils (original magnification ×1000). Rabbit anti–lipocortin III immune serum was used at a dilution of 1:200. When preimmune serum from the same rabbit was used at the same concentration, no fluorescence was observed.

that it has a coarse reticular distribution in the cytosol of unstimulated cells.

This protein not only binds to phospholipids in the presence of Ca^{2+}, but also promotes Ca^{2+}-dependent vesicle–vesicle aggregation, a property previously suggested to imply a potential role in mediating exocytosis (17). While a role in exocytosis has not been proven, all of the proteins that have been found to exhibit this property are in the family of structurally related proteins, termed annexins. In our studies, as well as in others, this activity requires Ca^{2+} concentrations far in excess of those found within stimulated cells. While this high Ca^{2+} requirement might seem to make this activity unlikely to be expressed in vivo, the conditions of the assay also differ greatly from those in intact cells in other ways. The assay is performed with a dilute solution of protein, and must be performed with a dilute suspension of granules to permit measurements of light scattering. While we have not thoroughly examined the effect of these deviations from the conditions that exist in intact cells with regard to the Ca^{2+} requirement, the conditions of this assay must be acknowledged as differing substantially from those in intact cells.

A recent report (18) presents evidence that another member of the annexin family, calpactin I, causes aggregation of isolated adrenal chromaffin granules at concentrations of Ca^{2+} that are more nearly physiologic than those used in this study. However, there are many differences between the results in that report and those reported here. First, chromaffin granules are much more uniform in size, shape, and light scattering properties than PMN specific granules. This presents a more favorable signal-to-noise relationship in the assay, which permits the detection of very small changes in absorbance. Second, that study showed that while detectable aggregation of chromaffin granules occurred in the presence of micromolar concentrations of ionized calcium, aggregation was greater in both rate and extent when higher calcium concentrations (1 mM) were used. Moreover, the assay used in that study is performed at lower ionic strength (30 mM KCl, 40 mM Hepes, remainder of osmoles as sucrose). For these reasons it is difficult to directly compare the Ca^{2+} concentrations required in these two systems; however, it is possible that these two related proteins require different concentrations of Ca^{2+} to exhibit granule-aggregating activity.

The same protein (lipocortin III) that we describe from human PMN has recently been described as possessing two other activities. It has been characterized as a lipocortin, in that it can inhibit the activity of phospholipase A_2 in an in vitro assay (14), and it has also been recently characterized as an inhibitor of blood coagulation (15). Both of these activities

appear to be a consequence of Ca^{2+}-dependent phospholipid binding (7, 19, 20), as these reactions require access of the active enzymes to phospholipids, which act as substrates (for phospholipase A_2) or cofactors (for clotting Factors Xa and Va). Our finding that lipocortin III (also termed placental anticoagulant protein III [15]) can promote aggregation of isolated PMN specific granules suggests a positive rather than negative regulatory role for this protein. However, further work is necessary to firmly establish a clear intracellular function for this and other annexins.

The predominance of lipocortin III among the 32–33-kD annexins in human PMN is in contrast to its scarcity (compared with protein II and endonexin II) in human placenta (7, 15) and spleen, and its apparent absence from human liver (Ernst, J. D., and E. Hoye, unpublished observations). Indeed, the relative scarcity and/or restricted distribution of this protein is reflected in the fact that it is the most recent of the annexins to be described (14, 15). Further studies of the cellular location of this protein in the tissues in which it is found will be necessary before firm statements can be made regarding the nature of its distribution; nevertheless, we find that it is especially abundant in human PMN.

The abundance of this protein, together with its ability to reversibly associate with membranes in a Ca^{2+}-dependent manner as well as to promote granule–granule aggregation, makes it a candidate modulator of membrane-localized, Ca^{2+}-modulated events such as phagosome–lysosome fusion in human PMN. Further work to define the potential role of this protein in either exocytosis or phagosome–lysosome fusion in PMN is currently in progress.

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