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Ca^{2+}-dependent translocation of cytosolic proteins to isolated granule subpopulations and plasma membrane from human neutrophils

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In order to identify cytosolic proteins involved in control of granule exocytosis in human neutrophils, subcellular fractions enriched in each of the 3 major granule subsets were incubated with cytosol from neutrophils in the presence or absence of Ca^{2+}. After washing, proteins were eluted from the organelles by EGTA. Annexins I, II, IV and VI were found to bind to all organelles studied. In addition, a 28-kDa protein was found to bind exclusively to plasma membranes and secretory vesicles, the most readily exocytosed organelle of neutrophils. Ca^{2+}-dependent association of cytosolic proteins to different granule subsets may control differential exocytosis of granules.

Annexin; Exocytosis; Azurophil granule; Specific granule; Secretory vesicle

1. INTRODUCTION

Granule exocytosis, a key phenomenon in biology, controlling neurotransmission, hormone secretion and essential neutrophil functions, is generally believed to be governed by the intracellular concentration of Ca^{2+} [1]. The mechanism by which rises in intracellular Ca^{2+} induce exocytosis is not known. Annexins, a ubiquitous family of cytosolic proteins of MW 33–68 kDa which bind to phospholipids in the presence of Ca^{2+}, have been shown to mediate aggregation and fusion of isolated chromaffine bovine granules and of human neutrophil specific granules [2,3]. This does not explain the selectivity and hierarchy of granule exocytosis characteristic of cells containing more than one mobilizable granule subset.

Human neutrophils contain 4 distinct 'granule' subsets [4], all mobilized, but to different degrees, by rises in intracellular Ca^{2+}; azurophil granules, specific granules, gelatinase containing granules, and secretory vesicles, in the order of increasing degrees of mobilization (Sengeløv, H., Kjeldsen, L. and Borregaard, N., submitted). To provide a molecular basis for the selectivity and hierarchy of neutrophil granule exocytosis, a systematic search for cytosolic proteins that bind to granules in a Ca^{2+}-dependent way was conducted in a cell-free system consisting of isolated cytosol and isolated granule subsets.

Abbreviations: HLA: human histocompatibility leukocyte antigens; PIPES: piperazine-*N*,*N*'-bis[2-ethane-sulfonic acid].

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2. MATERIALS AND METHODS

Human neutrophils were isolated from fresh buffy coats from 2–6 units, resuspended at 2×10^8/ml in 100 mM KCl, 3.0 mM NaCl, 10 mM PIPES pH 7.2 (buffer A), disrupted by nitrogen cavitation without EGTA and centrifuged 100,000 × *g* for 45 min. The supernatant was used as source of cytosolic proteins. For isolation of granules, neutrophils were isolated from 450 ml freshly drawn blood and cavitated as described [5]. The post-nuclear supernatant was applied on a 2-layer Percoll (Pharmacia, Uppsala) density gradient [5]. The gradients were fractionated by aspiration from the bottom of the centrifuge tubes and the fractions combined. Marker proteins were quantitated by enzyme-linked immunosorbent assays or enzyme activity [5–7]. Percoll was removed by centrifugation, and isolated material from the α, β and γ bands [5] was separately resuspended in 1.5 ml buffer A. Secretory vesicles and plasma membranes were separated by high-voltage free-flow electrophoresis using an Elphor Vap 22 (Bender & Hobein, Munich, Germany) [7]. Ca^{2+}-dependent binding of cytosolic proteins to granules and plasma membranes was measured by incubating 500 μl of α, β or γ bands with 500 μl cytosol in the presence or absence of 0.5 mM $CaCl_2$ for 15 min at 37°C, followed by centrifugation 20,000 × *g* for 5 min to sediment granules and membranes. The supernatants were removed and the pellets resuspended in 1 ml buffer A containing 0.5 mM $CaCl_2$ to wash off non-bound proteins from the membranes. After recentrifugation, the supernatants were removed and the pellets resuspended in 400 μl buffer A containing 1 mM EGTA. After incubation at 37°C for 10 min, membranes and granules were sedimented by centrifugation for 10 min at 100,000 × *g* in an Airfuge (Beckman). The supernatants which contain proteins eluted from membranes by EGTA, were aspirated and the proteins visualized by SDS-PAGE [8] and identified by immunoblotting [9] with monoclonal antibodies from Zymed Lab. Inc. San Francisco, CA.

3. RESULTS AND DISCUSSION

Fig. 1 shows a typical separation profile of human neutrophils on a 2-layer Percoll gradient. The α band contains 85% of the azurophil granule marker myeloperoxidase. The β band contains 97% of the specific

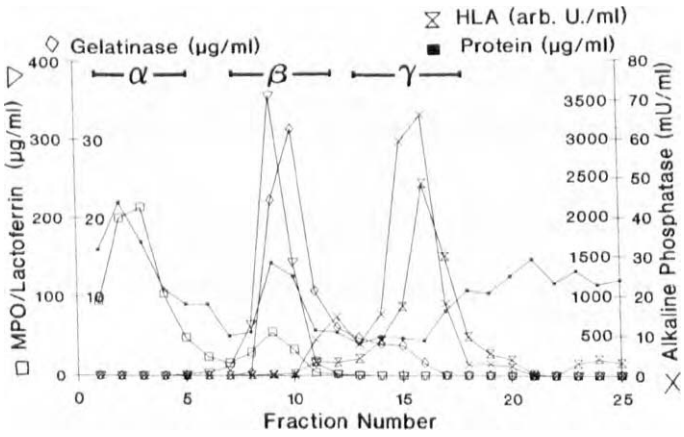

Fig. 1. Subcellular fractionation of post-nuclear supernatant from 3 × 10^8 neutrophils on a 2-layer Percoll density gradient. The fractions comprising α, β and γ bands were pooled.

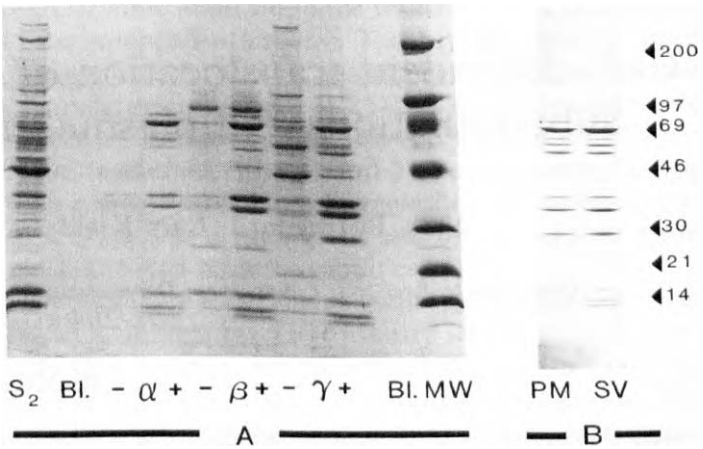

Fig. 2. SDS-PAGE of material eluted by 1 mM EGTA from α, β and γ bands incubated with cytosol in the presence (+) or absence (−) of 0.5 mM $CaCl_2$, 25 μl out of 300 μl eluted material was applied. 5 μl of untreated cytosol was applied. A 5–20% gradient gel was run under reducing conditions [8] (A). In B, plasma membranes (PM) containing 80% of the HLA and secretory vesicles (SV) containing 95% of the latent alkaline phosphatase were separated by subjecting the γ band from 10^8 cells to free-flow electrophoresis [7] and incubated with cytosol in the presence of 0.5 mM $CaCl_2$ and eluted with EGTA, 100 μl of 300 μl eluted was applied.

granule marker, lactoferrin and gelatinase in addition to 15% of myeloperoxidase. The γ band contains 90% of the plasma membrane marker HLA-class I and 85% of the marker for secretory vesicles, latent alkaline phosphatase. The cytosol (S_2) is located on top of the gradient. The reproducibility of this technique and the purity of fractions assessed by electron microscopy have been validated in previous publications [4–7].

When S_2 is incubated with isolated granules and plasma membrane vesicles in the presence of 0.5 mM $CaCl_2$, several proteins can be eluted by incubation with 1 mM EGTA (Fig. 2A). Three distinct proteins at 33, 36 and 68 kDa are observed. These were identified as annexin VI (68 kDa), annexin I and II (36 kDa) and as annexin IV (33 kDa) (Fig. 3). The presence of other annexins cannot be excluded. Although binding to α granules was less intense than binding to other organelles as judged from SDS-PAGE profile, no qualitative difference was observed in the binding of annexins to material from the α, β or γ band. Thus, annexins are not likely to control the selectivity of neutrophil granule exocytosis by preferential binding to granule subsets. In contrast, a prominent 28-kDa protein was observed to selectively bind to the material from the γ band containing plasma membranes and secretory vesicles. These light membranes of the γ band were separated by high-voltage free-flow electrophoresis [7]. No qualitative difference was observed in the binding of cytosolic proteins to isolated plasma membranes and isolated secretory vesicles (Fig. 2B). This is not surprising since secretory vesicles, which in neutrophils comprise the majority of light membranes [7], are endocytic in origin and therefore likely to have a high degree of homology with the plasma membrane [6]. Annexins are known to bind to acid phospholipids. Whether the 28-kDa protein, which displays selective binding to highly mobilizable vesicles, binds to phospholipids or other membrane structures, is not known. Washing the γ band in 2 M urea, 50 mM glycine pH 7.0 to deplete membranes of adsorbed proteins before incubation with cytosol did not change the binding of annexins or of the 28-kDa protein (not shown). Although the Ca^{2+} concentration used here to demonstrate membrane binding is clearly non-physiological, and lowering the $CaCl_2$ concentration to 50 μM resulted in minimal binding of any cytosolic protein to membranes (not shown), this does not rule out that binding can occur at levels of intracellular Ca^{2+} (1 μM)

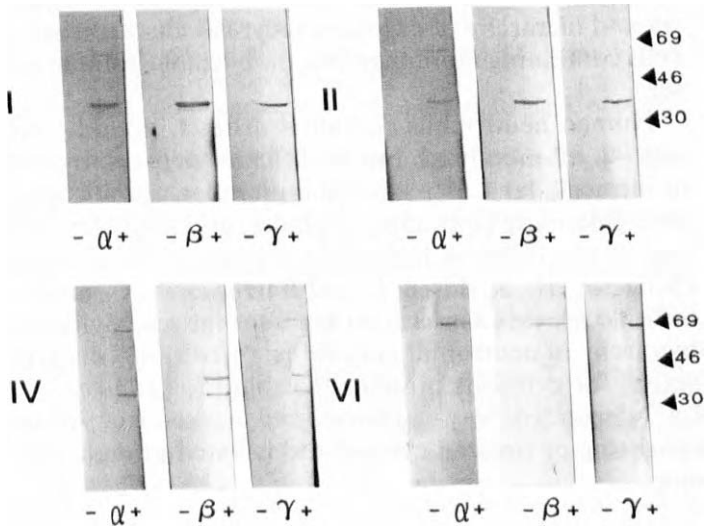

Fig. 3. Immunoblot of material eluted by 1 mM EGTA from α, β and γ bands after incubation with cytosol (Fig. 2A). 5 μl of eluted material was applied to electrophoresis on 10% SDS-polyacrylamide under reducing conditions [8] and transferred to nitrocellulose membranes [9]. Strips were cut and probed with monoclonal antibodies to annexin I, II, IV and VI. Detection was performed with peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark).

reached during neutrophil granule exocytosis, since the Ca^{2+} concentration may be much lower than added $CaCl_2$ in the assay system.

Defining the role that the 28-kDa protein may play during granule exocytosis must await its purification and the generation of antibodies that may be introduced in permeabilized cells or used in cell-free systems. The demonstration of its selective binding to fractions containing highly mobilizable vesicles makes this protein of potential interest.

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