RESEARCH COMMUNICATION

ADP-ribosylation-factor-regulated phospholipase D activity localizes to secretory vesicles and mobilizes to the plasma membrane following *N*-formylmethionyl-leucyl-phenylalanine stimulation of human neutrophils

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Phospholipase D (PLD) is responsible for the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline. Human neutrophils contain PLD activity which is regulated by the small GTPases, ADP-ribosylation factor (ARF) and Rho proteins. In this study we have examined the subcellular localization of the ARF-regulated PLD activity in non-activated neutrophils and cells 'primed' with *N*-formylmethionyl-leucyl-phenylalanine (fMetLeuPhe). We report that PLD activity is localized at the secretory vesicles in control cells and is mobilized to the plasma membrane upon stimulation with fMetLeuPhe. We conclude that the ARF-regulated PLD activity is translocated to the plasma membrane by secretory vesicles upon stimulation of neutrophils with fMetLeuPhe in inflammatory/priming doses. We propose that this relocalization of PLD is important for the subsequent events occurring during neutrophil activation.

INTRODUCTION

Neutrophils are terminally differentiated haematopoietic cells which constitute the primary mobile cellular defence against intruding micro-organisms. Essential components of their defence mechanisms are stored in membranes of intracellular mobilizable granules and vesicles, which are recruited to the plasma membrane upon stimulation. Neutrophils possess azurophilic, specific and gelatinase-containing granules and secretory vesicles. The secretory vesicle is the first mobilizable intracellular compartment which fully translocates to the plasma membrane after stimulation with nanomolar concentrations of inflammatory mediators such as *N*-formylmethionyl-leucyl-phenylalanine (fMetLeuPhe), leukotriene B_4, platelet-activating factor or interleukin-8. The membranes of secretory vesicles contain alkaline phosphatase on their lumenal surface [1], cytochrome b_{558} [2], complement receptor 1 (CD35) [3], 25% of the fMetLeuPhe receptor [4], FcγR-III (CD16) and Mac-1 (CD11b/CD18, $\alpha_m\beta_2$, C3) [5]. The translocation of these signalling molecules increases the responsiveness of the cells when triggered subsequently, a phenomenon commonly referred to as 'priming'.

fMetLeuPhe stimulation of 'primed cells' leads to secretion of the different granules in a regulated fashion, with azurophilic, specific and gelatinase granules being mobilized to increasing extents respectively. fMetLeuPhe stimulates phospholipase D (PLD), the activity of which is also enhanced by 'priming'. PLD hydrolyses phosphatidylcholine (PC) to release choline and the membrane-associated phosphatidate (PA). PA is considered to function as a second messenger that regulates neutrophil function, including exocytosis and NADPH oxidase. PLD activity in human neutrophils is regulated by ADP-ribosylation factor (ARF), Rho and the conventional isoforms of protein kinase C [6–11]. Of the three regulators identified, ARF is the most potent [9,10,12]. The human PLD (hPLD1), which has been cloned recently from the HeLa cDNA library, is also stimulated by ARF, Rho proteins and protein kinase C [12,13], suggesting that this entity is most likely responsible for the activity found in neutrophils.

Both the mobilization of azurophilic granules and PLD activity can be stimulated by addition of Ca^{2+} and guanine nucleotides to permeabilized neutrophils and to the related cell-line, HL60 cells. Cytosol-depletion by extensive permeabilization abolishes both secretory function and PLD activity, and both responses can be restored upon re-addition of ARF1 [14]. Secretion from intact neutrophils can also be blocked by ethanol, a reagent that interferes with PA production resulting from PLD activity [15–17]. All these data indicate that PLD activity is required for the secretion of azurophilic granules. A possible mechanism of how ARF-regulated PLD participates in exocytosis comes from the observation that ARF also stimulates the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in permeabilized cells via a PA-regulated PIP 5-kinase [14]. The function of PIP_2 in exocytosis is not fully understood.

In this study we have examined the subcellular localization of the ARF-regulated PLD activity in non-activated neutrophils and cells 'primed' with fMetLeuPhe. We report that PLD activity is localized at the secretory vesicles in control cells and is mobilized to the plasma membrane upon stimulation with fMetLeuPhe. We conclude that the ARF-regulated PLD activity is translocated to the plasma membrane by secretory vesicles upon stimulation of neutrophils with fMetLeuPhe in inflammatory/priming doses. We propose that this relocalization of PLD is important for the subsequent events occurring during neutrophil activation.

Abbreviations used: ARF, ADP-ribosylation factor; PA, phosphatidate; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; fMetLeuPhe, *N*-formylmethionyl-leucyl-phenylalanine; GTP[S], guanosine 5′-[γ-thio]triphosphate; HLA, human leucocyte antigen; HSA, human serum albumin.

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METHODS

Subcellular fractionation

Human neutrophils were isolated from blood and fractionated on a three-layer Percoll gradient as described [2]. Free-flow electrophoresis was carried out exactly as described previously [18]. Before fractionation, where indicated, neutrophils were stimulated with 10 nM fMetLeuPhe for 15 min at 37 °C exactly as described previously [5].

Marker assays

For analysis of the subcellular fractions, the following assays were used: latent alkaline phosphatase [1] and human serum albumin (HSA) (secretory vesicles) [19], human leucocytes antigen (HLA) class I [20] and alkaline phosphatase (plasma membranes), myeloperoxidase (azurophilic granules) [19], lactoferrin (specific granules) [19] and gelatinase (gelatinase granules) [2]. Galactosyltransferase activity was used as a Golgi marker [21]. Briefly, 50 μl of each fraction was incubated with 50 μl of reaction mixture containing 3.125 mg of trypsin inhibitor, uridine diphospho-D-[6-^{3}H]galactose (Amersham) (130000 d.p.m./assay), in 62.5 mM Mes-Na (pH 7.5), 37.5 mM $MnCl_2$, 2.5 mM Mg-ATP, 1.25 mg/ml BSA and 0.25% Triton X-100. Reaction mixtures were incubated for 1 h at 37 °C, then quenched with ice-cold 1% phosphotungstic acid in 10% trichloroacetic acid. Precipitates were washed twice and transfer activity was monitored by liquid-scintillation analysis.

In vitro assay for PLD activity

Phospholipid vesicles were used to assay the gradient fractions for PLD activity essentially as described [7,21]. Briefly, vesicles were composed of phosphatidylethanolamine, PIP_2 and dipalmitoyl-PC in the molar ratio of 10:0.3:1, with the inclusion of [*methyl*-^{3}H]choline-radiolabelled dipalmitoyl-PC. PC was used at a final concentration of 8.6 μM. The assay was performed in a buffer consisting of 50 mM Na-Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 1 mM dithiothreitol, 0.5 mM $MgCl_2$ and 2 mM $CaCl_2$. Percoll gradient fractions/free-flow fractions composed one-sixth of the total assay volume, guanosine 5′-[γ-thio]-triphosphate (GTP[S]) was included at 30 μM and recombinant ARF1 at 40 μM. Recombinant ARF1 was expressed in *Escherichia coli* and purified as described [8]. NaCl was also included at 500 mM [22]. The reaction mixtures were incubated at 37 °C for 1 h, after which labelled choline release was determined by liquid-scintillation analysis.

For analysis of the free-flow fractions, the above assay proved to be insensitive and was therefore modified. Essentially, the assay conditions are the same as above, except that the PC (dipalmitoyl, C_{16}) was substituted for a shorter chain-length PC (didecanoyl, C_{10}) [22]. Radiolabelled didecanoyl-PC was obtained from Amersham, U.K.

Western blot analysis

Percoll gradient fractions were analysed for ARF and RhoA immunoreactivity using monoclonal antibodies. After SDS/PAGE, fractions were Western-blotted onto PVDF membranes and probed for ARF (rat monoclonal) [21] or RhoA (mouse monoclonal, Santa Cruz Biotechnology) using established procedures. Detection was by enhanced chemiluminescence.

RESULTS

Subcellular fractionation of human neutrophils

To identify the subcellular localization of PLD activity, human neutrophils were fractionated on a three-layer Percoll gradient that allowed separation of the different granules and secretory vesicles. Analysis of markers indicated that azurophilic, specific and gelatinase granules were well separated, whereas plasma membrane and secretory vesicles partially overlapped (compare Figures 1b and 1c). Fractions were additionally assayed for the Golgi enzyme marker galactosyltransferase (Figure 1b). A large portion of the galactosyltransferase activity coincided with the

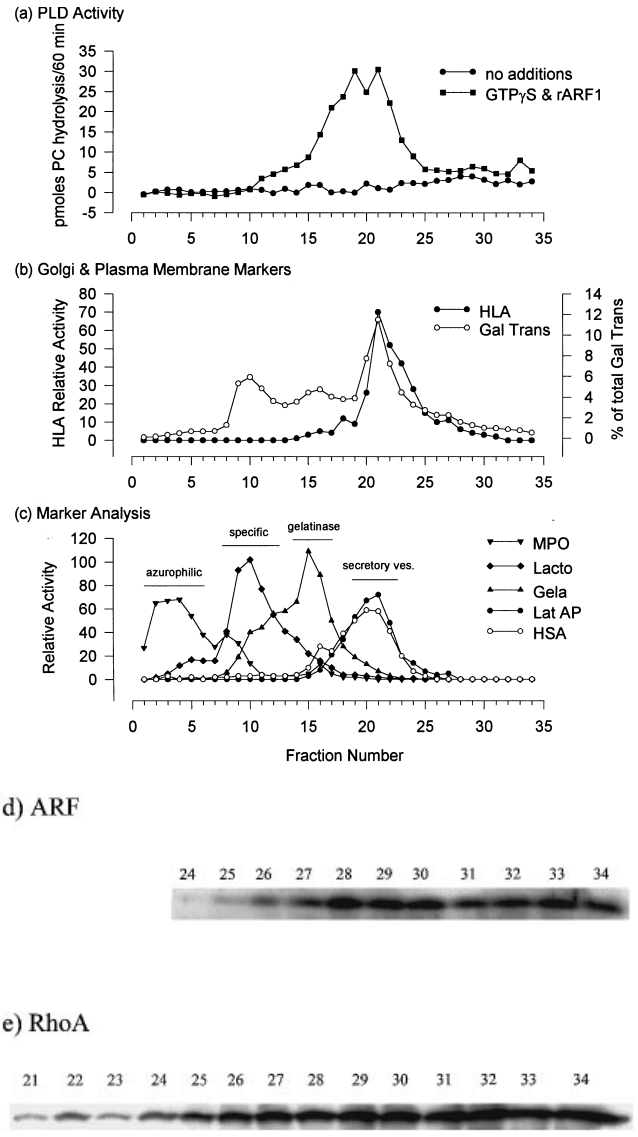

Figure 1 Fractionation of neutrophils on a Percoll gradient

Human neutrophils were fractionated on a three-step Percoll gradient and the fractions were assayed for: (**a**) PLD activity using exogenous dipalmitoyl-PC in the presence of substrate alone, or with the inclusion of 30 μM GTP[S] (GTPγS) and 40 μM recombinant ARF1 (rARF1); (**b**) galactosyltransferase (Gal Trans) (Golgi marker) and HLA (plasma membrane marker); (**c**) myeloperoxidase (MPO) (azurophilic granules), lactoferrin (Lacto) (specific granules), gelatinase (Gela), latent alkaline phosphatase (Lat AP), and HSA; (**d**) ARF immunoreactivity; (**e**) RhoA immunoreactivity. The data are obtained from a single fractionation run that is representative of three separate fractionations.

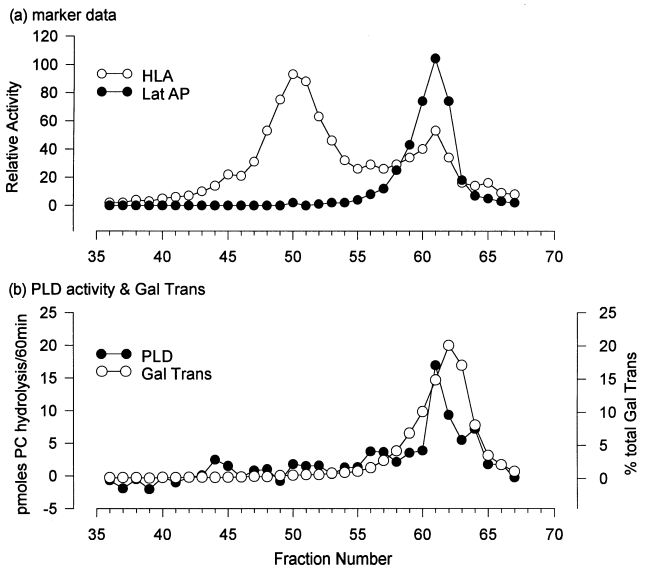

Figure 2 Separation of the γ-band by free-flow electrophoresis

PLD activity was concentrated in the region containing plasma membrane, secretory vesicles and Golgi. This region (γ-band) was further fractionated using free-flow electrophoresis. (**a**) HLA (plasma membrane marker) and latent alkaline phosphatase (Lat AP) (secretory vesicles marker); (**b**) ARF-stimulated PLD activity assayed in the presence of 30 μM GTP[S] and 40 μM recombinant ARF1 using didecanoyl-PC as substrate and galactosyl transferase (Gal Trans; Golgi marker). The data were obtained from a single fractionation that is representative of three separate fractionations.

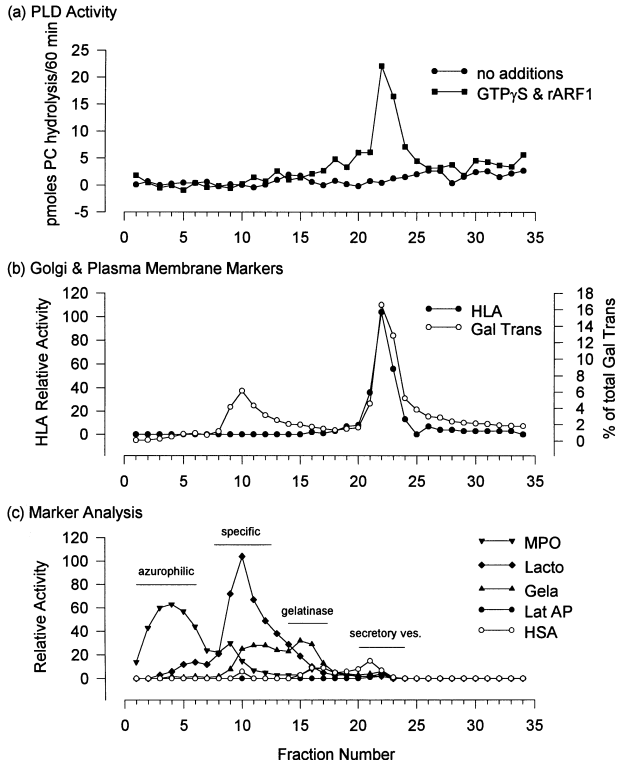

Figure 3 Fractionation of fMetLeuPhe-stimulated neutrophils on a Percoll gradient

fMetLeuPhe-stimulated human neutrophils were fractionated on a three-step Percoll gradient and the fractions were assayed for (**a**) PLD activity using exogenous dipalmitoyl-PC in the presence of substrate alone, or with the inclusion of 30 μM GTP[S] (GTPγS) and 40 μM recombinant ARF1, (**b**) galactosyl transferase (Gal Trans) (Golgi marker) and HLA (plasma membrane marker); (**c**) myeloperoxidase (MPO) (azurophilic granules), lactoferrin (Lacto) (specific granules), gelatinase (Gela) (gelatinase-containing granules), latent alkaline phosphatase (Lat AP) and HSA (secretory vesicles marker). Neutrophils were stimulated with 10 nM fMetLeuPhe for 15 min at 37 °C. The data were obtained from a single fractionation that is representative of two separate fractionations.

markers for plasma membrane and partially overlapped with secretory vesicles. Two smaller peaks of galactosyltransferase activity were also found which coincided with the enzyme markers for specific and gelatinase granules.

The fractions were analysed using exogenous [^{3}H]PC as substrate in the presence of recombinant ARF1 and GTP[S] (Figure 1a). The majority of the PLD activity was found in a broad peak where the plasma membrane, Golgi and secretory vesicle markers were concentrated. There was a further low activity in fractions 10–16 which matched with the gelatinase granules. In the absence of added ARF1 and GTP[S], no PLD activity could be detected. Endogenous ARF and Rho proteins in the gradient fractions were examined by Western blot analysis with specific antibodies. ARF (Figure 1d) and RhoA (Figure 1e) were both detected mainly in fractions 25–34, which is a cytosolic region of the gradient. In addition some RhoA was also detected in fraction 22.

Free-flow electrophoresis of γ-band

PLD activity was primarily found in a region coinciding with markers for plasma membrane, secretory vesicles and Golgi markers (referred to as the γ-band). To resolve the activity further, the γ-band was subjected to free-flow electrophoresis to separate the plasma membrane and secretory vesicles (Figure 2a) [18]. Analysis of the Golgi marker indicated that the majority of galactosyltransferase activity co-migrated with the secretory vesicles. Free-flow electrophoresis resolved the plasma membrane such that there was no contamination from the secretory vesicle or Golgi markers (Figures 2a and 2b). However, the fractions enriched in secretory vesicles and Golgi were contaminated with some plasma membranes (Figure 2a). The individual fractions were analysed for ARF1-dependent PLD activity using didecanoyl-PC to enhance the sensitivity of the assay [22] (Figure 2b). The main peak of activity coincided with the region enriched in secretory vesicles and Golgi.

These data did not allow us to distinguish unambiguously whether PLD activity was localized at the Golgi or secretory vesicles. Previous studies have indicated that ARF is localized at the Golgi, and ARF-regulated PLD activity has been found in this location as well [23,24]. To examine the precise location of the PLD activity, we took advantage of the fact that upon stimulation with fMetLeuPhe, the secretory vesicles fuse completely with the plasma membrane.

Fractionation of fMetLeuPhe-stimulated neutrophils

Human neutrophils when stimulated with fMetLeuPhe mobilize completely their secretory vesicles and partially the gelatinase-containing granules [18]. fMetLeuPhe-stimulated neutrophils were fractionated initially on the three-step Percoll gradient. Marker enzyme data, shown in Figure 3(c), reveal the complete disappearance of HSA and of latent alkaline phosphatase, confirming the complete mobilization of the secretory vesicles. In addition, the peak containing gelatinase is diminished, indicating the partial disappearance of the gelatinase-containing granules (compare Figure 1c with Figure 3c).

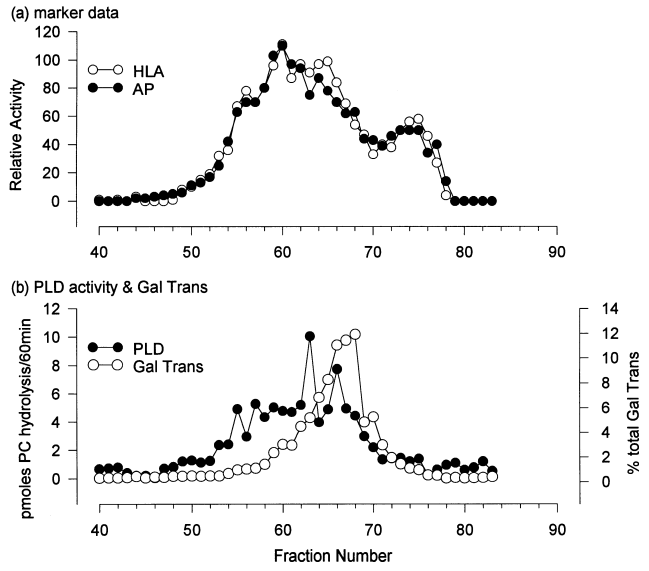

Figure 4 Separation of the γ-band obtained from fMetLeuPhe-stimulated neutrophils by free-flow electrophoresis

The γ-band from fMetLeuPhe-stimulated cells was further fractionated using free-flow electrophoresis. (**a**) HLA (plasma membrane marker) and alkaline phosphatase (AP) (secretory vesicles marker); (**b**) ARF-stimulated PLD activity assayed in the presence of 30 μM GTP[S] and 40 μM recombinant ARF1 using didecanoyl-PC as substrate and galactosyl transferase (Gal Trans) (Golgi marker). The data were obtained from a single fractionation that is representative of two separate fractionations.

In fMetLeuPhe-stimulated neutrophils, ARF-regulated PLD activity is localized in a sharp peak which coincides with the plasma membrane and Golgi markers. The PLD activity in fractions 10–16 is considerably reduced when compared with unstimulated cells, whereas the distribution of the galactosyltransferase activity is similar to that seen in unstimulated cells (Figure 3b). However, the PLD activity associated with the gelatinase granule markers was diminished.

The γ-band from stimulated neutrophils was also subjected to free-flow electrophoresis separation. Under these conditions the plasma membrane fractionates as a broad band which is enriched in HLA and alkaline phosphatase, confirming the fusion of the secretory vesicles with plasma membrane (Figure 4a). PLD activity was now found in one broad peak (Figure 4b) similar to that of the plasma membrane. Galactosyltransferase was mainly found in a small number of fractions (Figure 4b) whose location was not altered significantly by stimulation.

DISCUSSION

The observation that ARF is a major regulator of PLD activity has lead to the concept that PLD activity would be localized at the Golgi membranes. In a number of cells lines, Ktistakis et al. [24,25] have shown the presence of PLD activity at the Golgi membranes. In HL60 cells, ARF-regulated PLD activity was identified at the plasma membrane as well as at endomembranes [21]. Stimulation of intact HL60 cells with fMetLeuPhe, followed by subcellular fractionation, confirmed the activation of PLD at the plasma membrane as well [21]. The function of ARF-regulated PLD activity at the Golgi is to participate in the budding of vesicles [25]. However, the human neutrophil is fully differentiated and therefore the secretory granules and vesicles are pre-formed and await the arrival of a transmembrane signal by an agonist–receptor interaction. PLD activity is triggered within seconds upon occupation of receptors by appropriate ligands, and our results indicate that it participates in the secretion of pre-formed granules [14]. ARF can partially restore secretory competence in HL60 cells which are depleted of cytosolic proteins. To understand how ARF-regulated PLD activity participates in the mobilization of granules in neutrophils, we have examined the localization of the activity and whether its location changes on 'priming'.

Subcellular localization of the ARF-regulated PLD activity clearly demonstrates that the azurophilic and specific granules are totally devoid of this activity. The main peak of activity coincided with a fraction that was enriched in plasma membranes, Golgi and secretory vesicles. Free-flow electrophoresis had to be used to further fractionate this region, and from these results it was clear that no ARF-regulated PLD activity was detectable at the plasma membrane. It is possible that there is some activity present which is too low to be detected with the assay used here. Alternatively, the plasma membrane may possess a PLD activity that is regulated by other unidentified regulators. Our results clearly demonstrate that a large proportion of the ARF-dependent PLD activity found in neutrophils co-fractionated with secretory vesicle/Golgi-enriched fractions.

Secretory vesicles are rapidly and completely mobilized during 'priming' with fMetLeuPhe. We therefore stimulated the cells with fMetLeuPhe before fractionation. On a Percoll gradient, the PLD activity again coincided with the plasma membrane/Golgi markers. The secretory vesicles had fused with the plasma membrane and were no longer detected. When the region enriched in ARF-regulated PLD activity was fractionated further on free-flow electrophoresis, the PLD activity was now coincident with plasma membrane markers. The Golgi markers remain in a discrete band which partly overlaps with the plasma membrane markers.

The stimulus-induced mobilization of the PLD activity to the plasma membrane implies that the activity resides in the secretory vesicles. This would suggest that the initial step in neutrophil degranulation is the relocalization of PLD residing on the membrane of secretory vesicles, to the plasma membrane. Ethanol does not block the mobilization of secretory vesicles, suggesting that PLD is not involved in the fusion of secretory vesicles with the plasma membrane (H. Sengelov, L. Kjeldson and N. Borregaard, unpublished work). However, ethanol does block mobilization of the remaining granules and therefore the mobilization of granules in stimulated 'primed' neutrophils must involve PLD activity at the plasma membrane [15–17].

The function of PLD is not understood, but it has been suggested that PA functions as a second messenger. One recent study has speculated that PLD-derived PA stimulates PIP 5-kinase, which would lead to the production of PIP_2 [14]. PIP_2 may function either as a substrate for PI 3-kinase, or alternatively, may participate in the recruitment of proteins required for the secretory event. Although many of the proteins of the fusion apparatus have not been identified in human neutrophils specifically, proteins such as dynamin or synaptotagmin all bind to PIP_2.

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