Subcellular localization and translocation of the receptor for *N*-formylmethionyl-leucyl-phenylalanine in human neutrophils

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The subcellular localization of *N*-formylmethionyl-leucyl-phenylalanine (fMLP) receptors in human neutrophils was investigated. The fMLP receptor was detected with a high-affinity, photoactivatable, radioiodinated derivative of *N*-formylmethionyl-leucyl-phenylalanyl-lysine (fMLFK). Neutrophils were disrupted by nitrogen cavitation and fractionated on Percoll density gradients. fMLP receptors were located in the β -band containing gelatinase and specific granules, and in the γ -band containing plasma membrane and secretory vesicles. Plasma membranes and secretory vesicles were separated by high-voltage free-flow electrophoresis, and secretory vesicles were demonstrated to be highly enriched in fMLP receptors. The receptors found in secretory vesicles translocated fully to the plasma

membrane upon stimulation with inflammatory mediators. The receptor translocation from the β -band indicated that the receptor present there was mainly located in gelatinase granules. A 25 kDa fMLP-binding protein was found in the β -band. Immunoprecipitation revealed that this protein was identical with NGAL (neutrophil gelatinase-associated lipocalin), a novel protein found in specific granules. In summary, we demonstrate that the compartment in human neutrophils that is mobilized most easily and fastest, the secretory vesicle, is a major reservoir of fMLP receptors. This explains the prompt and extensive up-regulation of fMLP receptors on the neutrophil surface in response to inflammatory stimuli.

INTRODUCTION

Human neutrophils are characterized by their mobility and responsiveness to weak chemotactic stimuli produced at sites of inflammation and cell injury. Identification of the tripeptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP) and related *N*-formylated peptides as potent chemoattractants for human neutrophils (Schiffmann et al., 1975) started intense research in this field, recently highlighted by the molecular cloning of the receptor for fMLP (Boulay et al., 1990b). The nucleotide sequence revealed that the receptor is a member of the G-protein-coupled receptor superfamily with a predicted tertiary structure comprising seven transmembrane α -helices.

Some fMLP receptors are present on the surface of resting neutrophils, but the number of receptors is readily increased after stimulation with chemotactic stimuli, or even after incubating the neutrophils at 37 °C without stimulus (Andersson et al., 1987; Tennenberg et al., 1988). The several-fold and prompt increase in binding sites for fMLP at the neutrophil surface implies that this up-regulation is due to mobilization from an intracellular compartment. Up-regulation of the fMLP receptor after various stimuli has been described in many studies, but only few have focused on the cellular mechanisms underlying this up-regulation, i.e. the subcellular localization of fMLP receptors. The data presented so far on the subcellular localization have shown fMLP receptors to be present in specific granules (Fletcher and Gallin, 1983; Jesaitis et al., 1982) and possibly gelatinase granules (Graves et al., 1992). Although we have documented the existence of gelatinase granules (Kjeldsen et al., 1993a), the kinetics of up-regulation of fMLP-receptors induced by weak stimulation is in conflict with the mobilization

of the aforementioned granules (Sengeløv et al., 1993b). It has been demonstrated that warming the neutrophils to 37 °C for 30 min results in a 2–3-fold surface up-regulation of fMLP receptors. In contrast, we observe only 2–5 % release of gelatinase and no lactoferrin release under these conditions. This discrepancy could be due to a very high concentration of fMLP receptors in the few released gelatinase granules, or to the existence of another, more readily mobilizable, intracellular pool of receptors. This pool of receptors could be located in the secretory vesicles. Secretory vesicles in human neutrophils are intracellular vesicles that contain alkaline phosphatase (Borregaard et al., 1987), cytochrome b_{558} (Bjerrum and Borregaard, 1989; Calafat et al., 1993), and Mac-1 (Sengeløv et al., 1993b; Calafat et al., 1993) in their vesicle membrane and plasma proteins in the matrix (Borregaard et al., 1992). Secretory vesicles have been demonstrated as the most readily mobilizable compartment in neutrophils (Sengeløv et al., 1993a). Translocation of alkaline phosphatase to the plasma membrane is a marker for mobilization of secretory vesicles (Borregaard et al., 1987, 1992). The secretory vesicles from unstimulated neutrophils can be isolated from plasma membrane by combining Percoll-density-gradient centrifugation with high-voltage free-flow electrophoresis (Sengeløv et al., 1992). The aim of the present study was to investigate the localization and mobilization of the fMLP receptor in neutrophils in light of this recently described compartment. The receptor is quantified by covalent photolinking of a 125 I-labelled fMLFK derivative to the receptor (Boulay et al., 1990a). Photolabelling has the advantage that the labelled receptor is identified by its molecular mass on SDS/PAGE, and further allows examination of receptor sidedness in subcellular membranes.

Abbreviations used: fMLP, *N*-formylmethionyl-leucyl-phenylalanine; fMLFK-B-ASD, fMLP N^{ϵ} -[N^{α} -[3-{[2-(*p*-azidosalicylamido)ethyl]dithio}-propionyl]biocytin]-lysine; HBSS, Hanks' Balanced Salt Solution; NOG, *N*-octyl β -glucoside; PAF, platelet-activating factor; NGAL, neutrophil gelatinase-associated lipocalin; PMA, phorbol 12-myristate 13-acetate.

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MATERIALS AND METHODS

Neutrophils

Blood (400 ml) was drawn from healthy volunteers, and $(0.5\text{--}1) \times 10^9$ neutrophils were isolated by dextran sedimentation and Lymphoprep separation, followed by hypotonic lysis as described previously (Boyum, 1968; Sengeløv et al., 1992). If the cells were to be stimulated before cavitation, they were resuspended at 3×10^7 neutrophils/ml in Krebs–Ringer phosphate (119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 0.75 mM CaCl_2 , 15 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 5.5 mM glucose, pH 7.4). Incubation with stimulus was done at 37 °C for 15 min, with a preincubation period of 5 min. Release of granule markers during incubation was measured in a 1 ml sample drawn from the incubation suspension at the end of incubation and determined after centrifugation as the amount of marker present in the supernatant as a percentage of content in supernatant and cell pellet.

Subcellular fractionation

Isolated neutrophils (2×10^7 neutrophils/ml) in saline were treated with 5 mM di-isopropyl fluorophosphate for 10 min at 4 °C. The cells were then centrifuged and resuspended at 3×10^7 neutrophils/ml in relaxation buffer (100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP , 3.5 mM MgCl_2 , 10 mM Pipes, 0.5 mM phenylmethanesulphonyl fluoride, pH 7.3), and cavitated as described by Borregaard et al. (1983). The resulting preparation was collected in EGTA to a final concentration of 1.5 mM, and 10 ml of post-nuclear supernatant was placed on top of a 28 ml two-layer (1.05/1.12 g/ml) Percoll gradient as described by Borregaard et al. (1983). After centrifugation, the α -band (azurophil granules), the β -band (specific and gelatinase granules) (Kjeldsen et al., 1992a) and the γ -band (plasma membrane and secretory vesicles) (Borregaard et al., 1987; Sengeløv et al., 1992), were aspirated by hand, or the gradient was fractionated from the bottom into 1.4 ml fractions through a glass tube connected to a peristaltic pump and a fraction collector. When the γ -band was prepared for free-flow electrophoresis, 25 ml of Percoll of density 1.05 g/ml was used in order to avoid contamination with granules.

High-voltage free-flow electrophoresis

The γ -band was separated into plasma membrane vesicles and secretory vesicles as described by Sengeløv et al. (1992). In short, Percoll was removed from the γ -band by centrifugation at 45000 *g* for 45 min, and the membrane pellet was resuspended in electrophoresis-chamber buffer containing 5 mM acetic acid, 5 mM triethanolamine and 270 mM sucrose, pH 7.4. The membranes were treated with neuraminidase (0.18 unit/ml) for 30 min at 37 °C, and free-flow electrophoresis was conducted at 4 °C with a 110 mA current and 1300 V ($\pm 10\%$) voltage. The fractions obtained were immediately assayed and pooled accordingly into plasma membranes and secretory vesicles. The membranes in the two preparations were re-pelleted by centrifugation as above, and resuspended in buffer for the fMLP-receptor assay.

Preparation of fMLFK- ^{125}I -B-ASD

Synthesis of fMLFK-B-ASD was performed as described by Boulay et al. (1990a), and the product was stored at -80 °C at

a concentration of 1.3 mM in dimethylformamide. Iodination was carried out in the dark at room temperature by the Enzymobead method with 3.25 nmol of fMLFK-B-ASD and 75 MBq of carrier-free Na^{125}I . The iodination mixture (volume 600 μl) was injected into a C-18 Sep-Pak cartridge (Waters, Framingham, MA, U.S.A.) pre-washed with 10 ml of methanol, 10 ml of acetonitrile and 10 ml of water. The cartridge was then flushed with 10 ml of 50 mM KI and 10 ml of water. The column was turned upside down, eluted with 2 ml of 25% acetonitrile/0.1% trifluoroacetic acid and then with 2 ml of 50% acetonitrile/0.1% trifluoroacetic acid. Eluates were collected in four fractions of 1 ml each. Purity of the iodinated probe in the fractions was assessed by t.l.c. on Whatman K6F silica-gel plates run in a solvent of chloroform/methanol/water/acetic acid (72:26:0.7:1.3, by vol.). The purest fractions of fMLFK- ^{125}I -B-ASD (usually fractions 2 and 3) were stored at -20 °C.

fMLP-receptor assay

Percoll was removed from 400 μl subcellular fractions in a Beckman Airfuge at 207 kPa (30 lb/in 2) for 20 min. Membranes/granules were resuspended in 300 μl of chilled Hanks' Balanced Salt Solution (HBSS; CaCl_2 1.0 mM, KCl 5.36 mM, KH_2PO_4 0.43 mM, MgCl_2 0.5 mM, MgSO_4 0.41 mM, NaCl 137.0 mM, NaHCO_3 4.2 mM, Na_2HPO_4 0.5 mM), containing 25 mM Hepes, pH 7.5. For solubilization, 100 μl of this suspension was incubated with 10 mM *N*-octyl β -glucoside (NOG) (Gould et al., 1981) for 15 min, added from a 250 mM stock. Then 2 μl of fMLFK- ^{125}I -B-ASD was added and the mixture was incubated for 30 min on ice in the dark. The amount of fMLFK- ^{125}I -B-ASD in the assay was 3 times the saturating concentration as determined in a binding assay with differentiated HL60 cells (final concn. approx. 5–10 nM). The specific receptor binding was blocked by addition of a 1000-fold excess of unlabelled fMLP when indicated. Covalent binding of fMLFK- ^{125}I -B-ASD was induced by irradiation of the test tubes with 1 J/cm 2 u.v. A light from Phillips TL 09R light tubes. The samples were then mixed 1:1 with sample buffer containing 5 mM dithiothreitol and left at 37 °C for 30 min. The samples were electrophoresed overnight (SDS/PAGE, 10% gel with a 3% stacking gel), 160 μl applied to each lane (Laemmli, 1970). Gels were washed, fixed, dried, and exposed to Kodak X-OMAT AR scientific imaging films at -80 °C for the appropriate time. Quantitative analysis of the autoradiographs was performed by video-scanning densitometry with a commercially available system (CREAM version 4.0; Kem-En-Tec, Copenhagen, Denmark). Receptor saturation was obtained in the assay, as demonstrated by linearity of receptor labelling in the range used (results not shown).

Immunoprecipitation

Anti-NGAL (neutrophil gelatinase-associated lipocalin) antibodies (Kjeldsen et al., 1993b) were coupled to CNBr-activated Sepharose beads (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The antibody-coupled beads (25 or 50 μl of suspension) were incubated with 100 μl of NOG-solubilized, fMLFK- ^{125}I -B-ASD-photolabelled β -band in an Eppendorf tube for 1 h on melting ice and then centrifuged at 7000 rev./min for 15 s in a table centrifuge. The supernatant was aspirated and the pellet washed twice and resuspended in an equal amount of HBSS/Hepes. Supernatant and pellet were electrophoresed and autoradiographed as described above.

Figure 1 Subcellular distribution of fMLP receptors

A total of 3×10^8 unstimulated neutrophils was nitrogen-cavitated, and the post-nuclear supernatant separated on a Percoll gradient and fractionated. Markers were assayed as described in the Materials and methods section. Fraction number is indicated on the abscissa of the graphs; the lower the number, the higher the density. The band definitions are: fractions 1–6, α -band (azurophil granules); fractions 7–11, β -band (specific and gelatinase granules); fractions 12–18, γ -band (secretory vesicles and plasma membrane); and fractions 19–25, cytosol. For fMLP-receptor assay, 400 μ l from each fraction was separated from Percoll, resuspended in 300 μ l of HBSS/Hepes, and 100 μ l was used for assay in the presence of 10 mM NOG. Markers from the same gradient are shown in the two graphs at top, and percentage distribution of densitometry scanning of the autoradiograph in the range 50–70 kDa is depicted in both graphs as a bold line. The fMLP-receptor autoradiograph is shown at the bottom. Symbols: ●, myeloperoxidase (MPO); ▲, lactoferrin; ✱, gelatinase; ■, latent alkaline phosphatase (Lat. Alk. Phos.); ◆, HLA class I.

Marker assays

The alkaline phosphatase activity only measurable in the presence of detergent (0.2% Triton X-100) is marker for secretory vesicles (latent alkaline phosphatase). Alkaline phosphatase was measured as described by deChatelet and Cooper (1970). The other markers used to characterize subcellular fractions and to evaluate granule release were all assayed by an e.l.i.s.a. procedure as described by Borregaard et al. (1992). The antibodies used in the gelatinase e.l.i.s.a. were previously described (Kjeldsen et al., 1992b), and antibodies used for myeloperoxidase (azurophil granules), lactoferrin (specific granules), and HLA class I (plasma membrane) are also previously described (Borregaard et al., 1992).

RESULTS

Receptor localization and orientation in isolated granules and vesicles

Figure 1 shows the fMLP receptor content in solubilized fractions from a Percoll gradient of unstimulated neutrophils. Receptors were found as a smear at 50000–70000 kDa, characteristic of a heavily glycosylated protein, in the β - and γ -band, and a shoulder of receptor labelling was observed in the dense fractions of the γ -band, co-localizing with latent alkaline phosphatase. No receptor labelling was observed in the α -band. Receptor labelling of a sample of β -band mixed with an equal volume of α -band was not decreased more than that due to the dilution itself, suggesting that proteolysis of fMLP receptors in the β -band by proteases from contaminating azurophil granules did not occur. The intense label at 25 kDa in the β -fractions is discussed below. Table 1 shows the average distribution of fMLP receptors in several Percoll gradients.

The luminal side of a granule membrane is exposed on the cell surface when granules fuse with the plasma membrane. This means that the binding site of a granule-stored receptor predestined to be situated on the cell surface must be located on the luminal side of the granule membrane. To illustrate the receptor sidedness, the β - and γ -bands from a Percoll gradient of unstimulated neutrophils were analysed for content of fMLP receptors in the absence and presence of NOG as shown in Figure 2. Nearly all of the receptor labelling in the β -band (lanes 1–3) is 'latent', i.e. only measurable in the presence of detergent,

Table 1 Distribution of markers and fMLP-receptor labelling in Percoll gradients

Results are given as percentage distribution of markers within the bands. Data are means $\pm$ S.D. from four subcellular-fractionation experiments. A 10 ml portion of post-nuclear supernatant from 3×10^8 unstimulated neutrophils was applied on the gradient in each experiment. The gradients were fractionated into 1.4 ml fractions and assayed for markers including fMLP receptor as described in the Materials and methods section. Marker contents of the α , β - and γ -bands were calculated as total content in fractions 1–6, 7–11 and 12–20 respectively.

Band	α	β	γ
Myeloperoxidase	74.2 $\pm$ 3.1	22.6 $\pm$ 2.6	3.2 $\pm$ 1.0
Lactoferrin	10.0 $\pm$ 2.2	85.6 $\pm$ 2.7	4.4 $\pm$ 1.6
Gelatinase	1.5 $\pm$ 1.1	83.9 $\pm$ 0.9	14.6 $\pm$ 1.0
Latent alkaline phosphatase	1.8 $\pm$ 2.1	15.6 $\pm$ 2.2	82.6 $\pm$ 0.8
HLA class I	0.4 $\pm$ 0.5	17.6 $\pm$ 3.6	82.0 $\pm$ 3.7
fMLP receptor	0.0 $\pm$ 0.0	46.9 $\pm$ 7.8	53.1 $\pm$ 7.8

Figure 2 Receptor sidedness and localization in bands from Percoll gradient

SDS/PAGE autoradiographs of fMLP-receptor labelled β -band (lanes 1–3) and γ -band (lanes 4–6) from unstimulated neutrophils and of 'PAF- γ ' (lanes 7–9) from neutrophils stimulated with 10 nM PAF for 20 min at 37 °C before cavitation. A 400 μ l portion of peak fraction from each band was separated from Percoll and resuspended in 300 μ l of HBSS/Hepes. The sample from each band was divided in three (100 μ l each), and assayed for fMLP receptors (a) without detergent, (b) with addition of 10 mM NOG, and (c) with addition of NOG and excess unlabelled fMLP. The whole procedure from drawing of blood to SDS/PAGE was performed on the same day, in order to maintain granule integrity.

in agreement with localization of the receptor binding site on the inside of the granule membrane. In the γ -band (lanes 4–6), the receptors in the plasma membrane can be labelled without detergent, but the labelling is clearly intensified when detergent is present (lane 5 compared with lane 4). The demonstration of a latent receptor pool in the γ -band is analogous to the presence of latent alkaline phosphatase, which identifies secretory vesicles. When the neutrophils are stimulated with inflammatory mediators, secretory vesicles are translocated to the plasma membrane and the latent alkaline phosphatase becomes non-latent. Lanes 7–9 in Figure 2 represent γ -band from neutrophils stimulated with platelet-activating factor (PAF) before cavitation. In line with the disappearance of the alkaline phosphatase latency, no latent fMLP-receptor labelling is found in the γ -band of stimulated neutrophils. These findings suggest that secretory vesicles contain a reservoir of fMLP receptors. To substantiate this, secretory vesicles and plasma membranes from a γ -band from unstimulated neutrophils were separated by high-voltage free-flow electrophoresis (Sengeløv et al., 1992), and pooled fractions containing secretory vesicles or plasma membranes were assayed for receptor (Figure 3). The secretory-vesicle fractions are shown to contain a significant amount of the receptors present in the γ -band (Table 2). After stimulation of the neutrophils with secretagogue, the secretory vesicles are incorporated into the plasma membrane, and separation by free-flow electrophoresis is no longer possible (Sengeløv et al., 1992). The free-flow electrophoresis procedure involves neuraminidase treatment of the plasma membranes. The molecular mass of the desialated receptor was slightly decreased, but agonist binding was not affected (Figure 4). Failure in a previous study to demonstrate desialation of the receptor on SDS/PAGE was probably due to the lower concentration of neuraminidase used in that study (Perez et al., 1987).

Figure 3 Free-flow electrophoresis

The γ -band from 3×10^8 unstimulated neutrophils was subjected to high-voltage electrophoresis as described in the Materials and methods section. Fractions were pooled into plasma membrane (PM) and secretory vesicles (SV) as indicated by horizontal bars, and the membranes were pelleted by ultracentrifugation and resuspended to the same volume in HBSS/Hepes for fMLP-receptor assay. The receptor labelling of 100 μ l of resuspended membranes from each of the two pools is shown below the graph. Symbols: $\blacklozenge$, HLA class I; $\blacksquare$, latent alkaline phosphatase.

Table 2 Distribution of markers and fMLP-receptor labelling in free-flow-electrophoresis (FFE) separations of γ -band

The γ -band from 3×10^8 unstimulated neutrophils fractionated on Percoll (1.05 g/ml) was separated by FFE. The fractions were assayed and pooled into plasma membranes and secretory vesicles according to marker content. The two pools were concentrated (by the same factor) by ultracentrifugation and resuspended in HBSS/Hepes and assayed for fMLP receptor. Results are given as mean percentage distribution ($\pm$ S.D.) in three FFE runs from different Percoll gradients.

	Plasma membrane	Secretory vesicles
HLA class I	72.2 $\pm$ 1.6	27.8 $\pm$ 1.6
Latent alkaline phosphatase	14.9 $\pm$ 5.6	85.1 $\pm$ 5.6
fMLP receptor	51.3 $\pm$ 4.3	48.7 $\pm$ 4.3

Receptor translocation from the β -band

In addition to complete exocytosis of secretory vesicles, stimulation with inflammatory mediators induces partial exocytosis of

Figure 4 fMLP-receptor desialation

The γ -band from PAF (10 nM)-stimulated neutrophils was separated from Percoll and resuspended to its initial volume in HBSS/Hepes. Equal amounts were incubated for 30 min at 37 °C with 0.18 unit/ml neuraminidase or 0.9% NaCl. The membranes were then assayed for fMLP receptors. Lanes: 1, untreated membranes; 2, neuraminidase-treated membranes; 3, neuraminidase-treated membranes assayed in the presence of unlabelled fMLP. The intensity of the label is unchanged, whereas a slight decrease in the molecular mass of the receptor smear from desialated membranes is observed.

gelatinase granules and minor exocytosis of lactoferrin granules (Kjeldsen et al., 1992a). To investigate the translocation of these granules, the receptor distribution within an entire subcellular fractionation of resting and stimulated neutrophils must be analysed. Figure 5 compares the granule markers and receptor distribution in Percoll gradients of resting versus PAF-stimulated neutrophils, and resting versus phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils. After PAF stimulation, only a slight translocation of receptor from the β -fractions to the γ -fractions is observed (Figures 5a and 5b). This indicates that the receptor pool mobilized to the neutrophil surface after weak inflammatory stimuli is mainly derived from secretory vesicles. Release of lactoferrin is 2%, and release of gelatinase is 16% in this experiment. Complete translocation of fMLP receptors to the γ -band is observed after PMA stimulation (Figures 5c and 5d), with a concomitant extensive release of both gelatinase and lactoferrin (84.5% and 55% respectively).

Labelling of the 25 kDa band

In the β -band, heavy labelling of a 25 kDa protein is observed (Figure 1, fractions 8–12). A similar observation was made by Allen et al. (1989). In that study, the protein was partially

sequenced and shown to bind fMLPL with low affinity and high capacity. We have recently identified a 25 kDa protein from human neutrophils which is present in the granules of the β -band and exists in part in complex with gelatinase. This protein was termed NGAL (Kjeldsen et al., 1993b). The total amino acid sequence revealed the protein as a member of the lipocalin family, in which a prominent feature is binding of small lipophilic substances. The 27-amino-acid sequence presented as the N-terminus by Allen et al. (1989) is identical with residues 4–30 of the NGAL sequence. We thus suspected that NGAL was the fMLP-binding protein of 25 kDa. To investigate this, anti-NGAL antibodies coupled to Sepharose beads were incubated with NOG-solubilized fMLFK- 125 I-B-ASD-photolabelled β -band. The suspension was centrifuged, and both pellet and supernatant were subjected to SDS/PAGE and autoradiography as shown in Figure 6. After incubation with anti-(NGAL antibody) beads, the 25 kDa label is precipitated into the pellet in a dose-dependent manner, whereas the receptor label remains in the supernatant (lanes 1–4). Without anti-NGAL antibodies present, both the receptor label and the 25 kDa label are retained in the supernatant after pelleting of the beads (lane 5). NGAL is accordingly recognized as an fMLP-binding protein present in the β -fractions of human neutrophils, and identical with the protein described by Allen et al. (1989). The binding of fMLFK- 125 I-B-ASD to NGAL could not be blocked completely by unlabelled fMLP (Figure 2, lane 3). This is in accordance with the results of Allen et al. (1989), and reflects the great binding capacity of NGAL and the observation that the binding may in part be caused by the SASD group in the probe (Allen et al., 1989).

DISCUSSION

This is to our knowledge the first demonstration of the orientation of the fMLP receptor in the membranes (receptor latency) in subcellular fractions of human neutrophils. The labelling of fMLP receptors in the β -band is significantly increased after solubilization of the granule membrane. However, to maintain membrane integrity in the fMLP receptor assay, we experienced that it was crucial that the granules were assayed immediately. One freeze-thaw cycle or even storage overnight in the refrigerator enhanced the labelling in the absence of detergent considerably. This granule fragility in the fMLP-receptor assay might explain that previous studies have demonstrated presence of fMLP receptors in fractions containing specific/gelatinase granules, without any solubilization or permeabilization of the granule membrane (Graves et al., 1992; Fletcher and Gallin 1983; Jesaitis et al., 1982).

The latency of the fMLP receptors in the γ -band disappeared after stimulation with PAF. This indicates the presence in the γ -band of a mobilizable compartment housing these receptors. Secretory vesicles containing latent alkaline phosphatase can be separated from plasma-membrane vesicles by free-flow electrophoresis (Sengeløv et al., 1992). Performing this procedure on γ -band from unstimulated neutrophils enabled us to show that fMLP- 125 I-B-ASD labelling was in fact co-localizing with latent alkaline phosphatase, which means that about half of the receptors located to light membranes on density gradients of unstimulated neutrophils are in fact located intracellularly in secretory vesicles, analogous with the localization of alkaline phosphatase. Several reported characteristics of fMLP-receptor up-regulation can be explained by receptor residency in secretory vesicles. (i) The spontaneous up-regulation of fMLP receptors while incubating neutrophils at 37 °C (Andersson et al., 1987; Tennenberg et al., 1988) is a typical feature of secretory vesicles (Borreagaard et al., 1992). (ii) Stimulating Ca^{2+} -depleted neutro-

Figure 5 Receptor translocation from the β -band

Marker distribution in Percoll gradients from control (unstimulated) and PAF-stimulated (10 nM) neutrophils (a and b), and from control and PMA-stimulated (2 μ g/ml) neutrophils (c and d). The fMLP-receptor autoradiograph of labelling from the NOG-solubilized fractions containing activity is depicted below each fractionation. The densitometry scanning values are plotted too. Symbols: ●, myeloperoxidase (MPO); ▲, lactoferrin; ★, gelatinase; +, fMLP receptor (FMLPR); ■, latent alkaline phosphatase (Lat. Alk. Phos.); ◆, HLA class I.

phils with secretagogue still results in fMLP-receptor up-regulation (Sengeløv et al., 1993a), and, in line with this, secretory vesicles constitute the only compartment which is still exocytosed under these conditions (Sengeløv et al., 1993a). Finally, (iii) the rapid receptor up-regulation (Norgauer et al., 1991; Van Epps et al., 1990) matches the mobilization kinetics of secretory vesicles (Sengeløv et al., 1993a).

Inflammatory stimuli known to mobilize secretory vesicles without major release of other granules are fMLP, PAF, zymosan-opsonized serum, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF), interleukin 8 and leukotriene B₄ (Borregaard et al., 1990, 1992), and all of these will consequently up-regulate fMLP receptors. In accordance, fMLP-receptor up-regulation has been demonstrated for fMLP (Norgauer et al., 1991), TNF and PAF (O'Flaherty et al., 1991), and GM-CSF (English et al., 1988;

Graves et al., 1992). Interestingly, Graves et al. (1992) described parallel up-regulation characteristics of fMLP receptors and Mac-1 (CD11b/CD18) and concluded that the gelatinase granules were the common housing compartment for these receptors. However, the existence of a mobilizable light-membrane compartment in subcellular-fractionation experiments was not considered in that study, although the surface up-regulation of fMLP receptors and Mac-1 was far greater than translocation observed in subcellular fractions of the density gradient. In accordance with the observed parallel up-regulation of fMLP receptors and Mac-1, we have found secretory vesicles to contain Mac-1 as well (Sengeløv et al., 1993b; Calafat et al., 1993). However, stimulation with inflammatory mediators results in 15–30% gelatinase release (Kjeldsen et al., 1992a), and indeed there is some translocation of the receptor labelling from the β - to the γ -band in the gradient (Figures 5a and 5b). Massive

Figure 6 Immunoprecipitation of NGAL

A β -band from neutrophils fractionated as described in the Materials and methods section was solubilized with NOG and photolabelled with fMLFK- 125 I-B-ASD as described in the Materials and methods section. Lanes 1 and 2 are pellet (P) and supernatant (S) from labelled membranes incubated with 25 μ l of anti-(NGAL) antibody-coupled Sepharose beads as described in the Materials and methods section, and lanes 3 and 4 are supernatant and pellet from membranes incubated with 50 μ l of the beads. Lanes 5 and 6 are supernatant and pellet from labelled membranes incubated with Sepharose beads coupled with pre-immune serum.

translocation of the fMLP receptor from the β -band to the γ -band, however, is achieved only with potent stimuli. Our interpretation is that fMLP receptors are present in the plasma membrane, in secretory vesicles, in gelatinase granules, and perhaps in specific granules. The specific granules remaining after PMA stimulation obviously do not contain fMLP receptors, and the translocation pattern indicates that the receptors in the β -band are mainly located in the membrane of gelatinase granules. In earlier studies, the specific granules were identified as the intracellular reservoir of fMLP receptors (Jesaitis et al., 1982; Fletcher and Gallin, 1983). However, the existence of gelatinase granules was not evaluated in those studies. A final determination of the content of fMLP receptors in gelatinase granules awaits development of fractionation techniques that allow separate isolation of secretory vesicles, gelatinase granules and specific granules.

The surface recruitment of fMLP receptors after weak inflammatory stimuli is mainly acquired from secretory vesicles. Most of the neutrophil GTP-binding proteins (G-proteins) are found in fractions containing plasma-membrane markers, and the rest in the cytosol and with specific granules (Volpp et al., 1989; Rotrosen et al., 1988). An intriguing question is whether the fMLP receptors stored in secretory vesicles are already G-protein-coupled, or if the G-proteins found in the light membranes are solely located in the plasma membrane.

The 25 kDa fMLP-binding protein was identified as NGAL (Kjeldsen et al., 1993b). This demonstrates the ability of a novel lipocalin in neutrophils to bind fMLP. However, the functional significance of this binding remains to be investigated. The

labelling of NGAL illustrates the usefulness of a receptor assay in which the molecular mass of the labelled species can be evaluated. Furthermore, it casts some doubt on the accuracy of the quantification of fMLP receptors done on subcellular fractions by [3 H]fMLP binding in previous studies (Fletcher and Gallin, 1983; Graves et al., 1992).

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