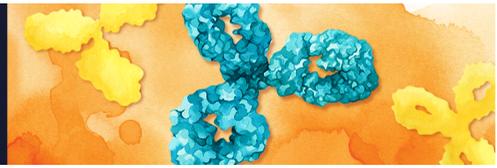


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A Chondroitin Sulfate Proteoglycan on Human Neutrophils Specifically Binds Platelet Factor 4 and Is Involved in Cell Activation¹

Frank Petersen,² Lothar Bock, Hans-Dieter Flad, and Ernst Brandt³

Platelet factor 4 (PF-4), a member of the α -chemokine subfamily of cytokines, activates human neutrophils independently of intracellular free calcium mobilization or binding to IL-8R. In the present study, we have identified and partially characterized a receptor for PF-4 on human neutrophils, which displays weak cross-reactivity with the IFN- γ -inducible protein 10, but not with other α -chemokines such as IL-8, neutrophil-activating peptide 2, or melanoma growth-stimulatory activity (GRO α). Binding studies revealed that human neutrophils express a high number of receptors ($B_{\max} \sim 7.6 \times 10^6$ sites/cell) of moderate affinity ($K_d \approx 650$ nM). The kinetics of PF-4-binding correlates with the proportion of PF-4 tetramers in solution and with the activation of neutrophils for exocytosis. Reduction of PF-4 binding and PF-4-induced exocytosis in the presence of various glycosaminoglycans or following treatment of cells with chondroitinase ABC (but not other glycosaminoglycan-degrading enzymes) altogether demonstrates that the PF-4 receptor is a proteoglycan of the chondroitin sulfate class. Cross-linking experiments with radiolabeled PF-4 revealed a receptor-ligand complex of ~ 250 kDa. Taken together, our data show that a distinct chondroitin sulfate proteoglycan represents specific receptors for tetrameric PF-4 on human neutrophils. *The Journal of Immunology*, 1998, 161: 4347–4355.

Among the various mediators that control functional activation of polymorphonuclear neutrophil granulocytes (neutrophils, PMN⁴), several members of the α -chemokine subfamily of chemotactic and proinflammatory cytokines have been recognized to play important roles. The prototype of these polypeptides, the IL-8 as well as the structurally related polypeptides melanoma growth-stimulatory activity (GRO α), neutrophil-activating peptide 2 (NAP-2), and others were shown to activate neutrophils, but not monocytes, to undergo directed chemotactic migration, adherence to endothelial cells, and degranulation of lysosomal enzymes (reviewed in Refs. 1 and 2). Moreover, it was found that these mediators elicited PMN functions through interaction with two common receptors on the cell surface (3, 4), the two IL-8R, A and B (5, 6), which are now termed CXCR-1 and CXCR-2 and belong to the family of seven-transmembrane-domain (7-TMD) receptors.

Since it was shown that the presence of the so-called ELR (Glu-Leu-Arg-) sequence motif within the N terminus of α -chemokines is critical for binding to and signaling through these receptors (7–9), the question arose as to by which structures and mechanisms those α -chemokines that do not contain the ELR motif might elicit cellular functions. Two representatives of the latter group that are presently under intense investigation are the IFN- γ -inducible protein (IP-10) (10) and platelet factor-4 (PF-4) (11, 12). While the former polypeptide is an inducible product of many different cells (13, 14), PF-4 is preformed and stored within granules of blood platelets and mast cells, and becomes released in high amounts upon appropriate activation (12, 15). To date, no direct evidence has been reported that neutrophils express specific receptors for either of these mediators. While this is in principle conceivable with IP-10, because to date no neutrophil-directed biologic functions have been demonstrated for this polypeptide, the proven activation of certain PMN functions such as secondary granule exocytosis and adhesion (16) by PF-4 demands for structures that can function as cellular binding sites. These structures cannot be identical to the IL-8R mentioned above, as has become clear from our previous findings that PF-4 even at extremely high molar excess (up to 200,000-fold) is unable to compete for receptor binding with IL-8 (16), as well as from another report by Clark-Lewis et al. (7), demonstrating that PF-4 can only bind to these receptors after artificial introduction of an ELR motif into its N terminus.

Moreover, several further observations indicate that the prospective PF-4 receptor may not even belong to the family of 7-TMD proteins. Most strikingly, we did not find PF-4-dependent elevation of intracellular free calcium concentration in PMN (16), a typical signal that is induced by all chemokines and other mediators interacting with 7-TMD receptors (17–21). In addition, the PF-4 functional spectrum in neutrophils differs considerably from that elicited by ELR-containing chemokines. While in earlier studies chemotactic and degranulation activity as well as enhancement of PMN adhesion to various surfaces has been claimed for PF-4

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⁴ Abbreviations used in this paper: PMN, polymorphonuclear; BS³, Bis(sulfosuccinimidyl) suberate; bFGF, basic fibroblast growth factor; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; D-PBS, Dulbecco's PBS; GAG, glycosaminoglycan; GRO α , melanoma growth-stimulatory activity α ; HSPG, heparan sulfate proteoglycan; ¹²⁵I-PF-4, ¹²⁵I-labeled platelet factor 4; IP-10, IFN- γ -inducible protein 10; NAP-2, neutrophil-activating peptide 2; PF-4, platelet factor 4; 7-TMD, seven-transmembrane domain.

(22–25), more recent studies failed to demonstrate any PMN-stimulating capacity for this chemokine (7, 26–28). In an approach to clarify this situation, we could recently demonstrate that highly purified PF-4 *in vitro* is in fact not chemotactic for PMN and does not induce relevant degranulation or adhesion by itself (16). However, a specialized role for PF-4 different from that of IL-8 was indicated by its cooperation with physiologically relevant amounts of TNF- α . In the presence of the latter cytokine, PMN responded to PF-4 by the selective exocytosis of secondary granule contents (but not of primary granule contents), and underwent enhanced adhesion to gelatin or plasma proteins. The latter function is probably a direct consequence of secondary granule exocytosis and might be important to localize PMN at inflammatory sites. The fact that neither chemotaxis nor an intracellular Ca²⁺ signal was generated even in cooperation with TNF- α provided a further hint that the potential PF-4 receptor(s) was different from 7-TMD proteins.

To date, there exist only few reports dealing with cellular binding structures for PF-4. The information available is almost exclusively limited to endothelial and connective tissue cells. As a common feature, there is evidence that heparan sulfate proteoglycans (HSPGs) are involved, as inferred from the sensitivity of PF-4 binding to treatment of cells with heparinase (29, 30). In a more defined approach, Watson and coworkers described interference of PF-4 with basic fibroblast growth factor (bFGF) for binding to the low affinity bFGF receptor (31), which was also identified as a HSPG (31). While it is not clear from this work whether there also exist HSPGs with strict specificity for certain ligands, others could recently provide evidence for the occurrence of HSPGs exhibiting at least a certain degree of selectivity. This was demonstrated by Luster et al. by describing a receptor on endothelial cell lines with cross-reactivity for PF-4 and IP-10, but not with chemokines of the β -subfamily (32). However, until now, there are no reports dealing with the problem of whether these receptors are simply heterogeneous populations of proteoglycans or whether there may exist structurally defined molecules with individual functions.

In the present study, we have addressed these questions by investigating the binding of PF-4 to neutrophils and by attempting to biochemically characterize specific receptors. Our results indicate that PF-4 interacts with a chondroitin sulfate proteoglycan (CSPG) of defined size. These receptors were found to exhibit specificity for PF-4, which appears to be determined by the oligomeric state of the chemokine. Furthermore, our data suggest that the integrity of this receptor is required for the induction of PF-4-mediated biologic responses in neutrophils.

Materials and Methods

Cytokines

Human rTNF- α was a gift from Dr. H. Gallati (Hoffmann-LaRoche, Basel, Switzerland). Human monocytic rIL-8 (i.e., the 72-residue isoform), rIP-10, and rGRO α were obtained from Pepro Tech (Rocky Hill, NJ). Human natural PF-4 was purified in our laboratory from release supernatants of thrombin-stimulated platelets in a three-step procedure, as previously described (16). Briefly, the major contaminant β -thromboglobulin Ag was removed by immunoaffinity chromatography. PF-4 in the flow-through was further enriched using a heparin-Sepharose affinity column (Pharmacia/LKB, Freiburg, Germany) and was finally purified to homogeneity by HPLC on an analytical cyanopropyl column (4.6 \times 250 mm, 5 μ m, wide pore; Baker Research Products, Phillipsburg, NJ). Eluates and fractions were screened for the presence of PF-4 and potential contamination by β -thromboglobulin Ag by ELISA, as described elsewhere (16). The final PF-4 preparation exceeded 99% purity, containing no detectable protein contaminants according to analyses in silver-stained SDS-polyacrylamide gels and by automated N-terminal amino acid sequencing (kindly performed by Dr. A. Petersen, Department of Clinical Medicine, Forschungszentrum Borstel, Borstel, Germany). Human natural NAP-2 was prepared as described elsewhere (33).

Preparation of human neutrophils

PMN were routinely isolated from citrated blood of healthy single donors by gradient centrifugation on Ficoll-Hypaque to a purity greater than 95% in all events, as previously described (34). Viability was examined by trypan blue exclusion and exceeded 98% in all experiments.

Measurement of exocytosis

A total of 1×10^7 /ml PMN was suspended in Dulbecco's PBS (D-PBS)/0.1% BSA (low endotoxin BSA (Serva, Heidelberg, Germany)). A total of 100 μ l volumes of cells was then distributed to 96-well microassay plates, containing 100 μ l of rTNF- α (9 ng/ml) and various concentrations of secondary stimulus PF-4, in buffer supplemented with CaCl₂ and MgCl₂ to yield final concentrations of 0.9 and 0.5 mM, respectively. In some experiments, PF-4/rTNF- α stimuli were supplemented with various concentrations of glycosaminoglycans (GAG) (CSA, CSB, or CSC; all from Sigma, Deisenhofen, Germany) appropriately diluted in D-PBS/0.1% BSA. Controls were performed with rTNF- α and either of the CS alone. After an incubation period of 30 min at 37°C, the cells were sedimented, and harvested supernatants were assayed for contents of lactoferrin using a quantitative sandwich ELISA, as described elsewhere (3). Release rates for lactoferrin were expressed as the percentages of total contents determined in detergent-treated PMN lysates prepared in 0.1% hexadecyl-trimethylammonium bromide.

Iodination of chemokines and binding experiments

PF-4 and IL-8 were iodinated using the chloramine T method, as reported previously (3). Routinely, PF-4 was labeled to a sp. act. of 1.5 to 2 Ci/mmol. This tracer served to perform binding experiments designed to establish binding isotherms for PF-4 and was used in competition experiments performed with a fixed dosage of 1 μ M ¹²⁵I-PF-4. In experiments in which very low concentrations of tracer were used, ¹²⁵I-PF-4 of high sp. act. (450–550 Ci/mmol) was employed. This material served to perform competition experiments with a fixed dosage of 5 nM ¹²⁵I-PF-4, to trace the incorporation of 0.5 nM ¹²⁵I-PF-4 into oligomers.

Binding experiments with PMN were performed as described in detail elsewhere (3). Briefly, cells were suspended at 2×10^7 cells/ml in D-PBS supplemented with 20 mg/ml BSA (binding buffer), and duplicate samples of 2×10^6 cells were incubated on ice for 2 h with ¹²⁵I-PF-4 at various concentrations in the presence or absence of unlabeled competitors (PF-4, rGRO α , NAP-2, rIL-8, rIP-10). Nonspecific binding was determined by incubating parallel samples in the presence of a 100-fold molar excess of unlabeled ligand. For practical reasons, the molar excess in samples containing more than 500 nM labeled PF-4 was limited to 50-fold (at 1–2 μ M ¹²⁵I-PF-4), to 20-fold (at 2.5–5 μ M ¹²⁵I-PF-4), or to 10-fold (at 10 μ M ¹²⁵I-PF-4). After removal of the unspecifically bound material by washing, radioactivity was determined in a gamma counter. The binding data were curve fit with the computer program EBDA/LIGAND (35) to determine affinity constants (K_d) and numbers of binding sites (B_{max}). Binding of IL-8 was determined as described previously (3).

Cross-linking experiments

For the analysis of PF-4 oligomer formation, 0.5 nM ¹²⁵I-PF-4 was incubated in PBS with increasing concentrations of the unlabeled peptide for 60 min at room temperature to achieve equilibrium in oligomer formation. Samples were cross-linked by addition of Bis(sulfosuccinimidyl) suberate (BS³) (Pierce, Oud-Beijerland, The Netherlands) from a stock solution of 40 mM in PBS to yield a final concentration of 2 mM. After 30 min, the reaction was quenched by addition of Laemmli buffer, and 50 μ l vol were separated electrophoretically on a 15% SDS-polyacrylamide gel under reducing conditions. Rainbow marker proteins (low m.w. marker; Amersham, Braunschweig, Germany) served as m.w. standard. Gels were fixed, dried, and subsequently exposed on x-ray film (Cronex 4; DuPont, Bad Homburg, Germany) for 24 to 48 h. Furthermore, all gels were scanned and quantitatively analyzed for radioactivity using the PhosphorImager System (Molecular Dynamics, Krefeld, Germany) in combination with the program ImageQuaNT Version 4.2 (Molecular Dynamics). The relative amount of monomers and oligomers was then calculated from the amount of radioactivity detected in the individual bands in relation to the total activity present on the respective lane.

For cross-linking of PF-4 to PMN membranes, 4×10^6 cells/tube were incubated with 10 nM ¹²⁵I-PF-4 in binding buffer alone or in the presence of increasing concentrations of unlabeled PF-4 for 90 min on ice in a total volume of 200 μ l. After repeated washing with PBS, cell-bound material was cross-linked with 1.5 mM BS³ in PBS for 1 h on ice. Cells were sedimented by centrifugation and pellets were lysed by adding 250 μ l Laemmli buffer containing 1 mM PMSF, 5 μ g/ml E-64 (both from Sigma),

and 4 mM EDTA, and subsequently homogenized by sonification (10–15 pulses for 1 s at 1.5 W) (Branson, Carouge-Genève, Switzerland). After extraction of proteins at 37°C for 30 min, the supernatants were cleared by centrifugation (230,000 × g, 10 min at 10°C) and directly loaded on a 7.5% SDS-polyacrylamide gel, and electrophoresis was run under nonreducing conditions. Gels were analyzed for radioactivity, as described above.

Enzymatic digestion of PF-4 binding sites

All enzymes were purchased from Sigma. PMN (2×10^7 cells/ml in PBS) were incubated for 30 min at 37°C under agitation with 2000-benzoyl-L-arginine ethyl ester (BAEE) U/ml tosyl-L-lysine-chloromethyl ketone (TLCK)-treated trypsin, 6 U/ml tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated chymotrypsin, or different concentrations of heparinase I (from *Flavobacterium heparinum*, EC 4.2.2.7), heparinase III (heparitinase I, from *F. heparinum*, EC 4.2.2.8), chondroitinase ABC (protease free, from *Proteus vulgaris*, EC 4.2.2.4), or hyaluronate lyase (from *Streptomyces hyalurolyticus*, EC 4.2.2.1), as indicated in the text. After washing, cells were directly used in binding assays or cross-linking experiments.

Statistics

Statistical significance was analyzed by the two-tailed *t* test. The mean values given in figures and table represent results obtained from three experiments. Each experiment was conducted with neutrophils from a different individual.

Results

Specific binding of PF-4 to neutrophils is characterized by positive cooperation

Experiments designed to explore the time course of PF-4 binding to neutrophils were performed by using a constant concentration of 1 μM of the iodinated chemokine. This dosage was chosen with respect to our former observation that PF-4 in the 0.3- to 10-μM range was effective in inducing the exocytosis of secondary granule markers from PMN (16). Binding of 1 μM 125 I-PF-4 to PMN at 4°C increased over time with 40 to 50% of maximal binding achieved at 10 min, and 95% of maximal binding achieved after 90 min of incubation (data not shown). Thus, binding of PF-4 to PMN occurred considerably slower than that of other α-chemokines, e.g., of NAP-2 and IL-8, either of which displayed 80% of maximal binding at 10 min and achieved equilibrium after 60 min, as seen in previous work (3). Therefore, in subsequent equilibrium-binding experiments with PF-4, the incubation time was extended to 2 h.

As shown in Figure 1, specific PF-4 binding to PMN using increasing concentrations of 125 I-PF-4 was measurable from 0.15 μM on and was near saturation with 10 μM of the ligand. Nonspecific binding (data not shown) continued to increase in a linear manner even at higher concentrations of labeled PF-4. Scatchard analysis of the data (Fig. 1, inset) revealed an unusual binding pattern composed of essentially two phases. A typical linear relationship between the transformed data was only obtained when data derived from PF-4 binding at 300 nM (corresponding to 8.1 pmol of bound 125 I-PF-4/2 × 10⁶ cells) and at higher concentrations were considered. Based on this set of data, PMN expressed a single class of binding sites with an apparent K_d of 648 ± 14 nM and a B_{max} of $7.6 \times 10^6 \pm 1 \times 10^6$ sites/cell. By contrast, transformed data derived from PF-4 binding below 300 nM of the ligand did not fit into this relationship, but revealed a decrease of binding affinity with decreasing concentration of the chemokine. Examination of the data by Hill transformation yielded a Hill coefficient (nH) of 3.701, indicating significant positive cooperation in the binding of PF-4 to its receptor(s).

Part of PF-4 binding sites on neutrophils is shared by IP-10

In additional experiments, we sought to obtain more direct evidence for positive cooperation in PF-4 binding to its receptor(s), as well as to examine whether specific PF-4 binding sites were shared

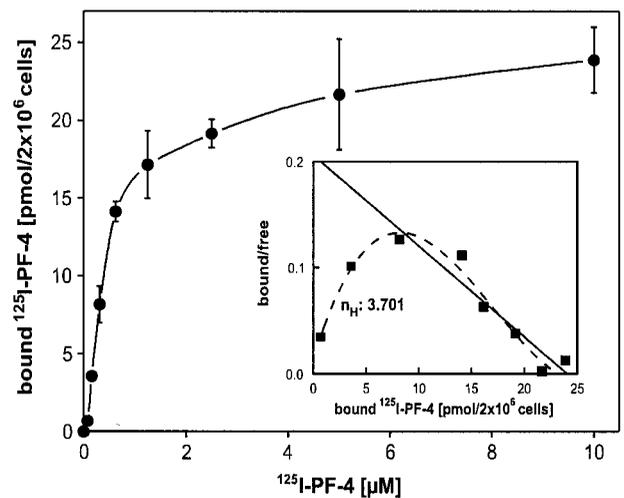


FIGURE 1. Specific binding of 125 I-PF-4 to receptors on human neutrophils. PMN were incubated with increasing concentrations of 125 I-PF-4. Nonspecific binding was determined in the presence of an excess of the unlabeled ligand. Specific binding was calculated by subtraction of nonspecific binding from total binding. *Inset*, Data were transformed according to Scatchard for specifically bound 125 I-PF-4 (dashed line). Only values representing >8 pmol bound 125 I-PF-4/2 × 10⁶ cells were used for the determination of K_d and B_{max} (solid line). The data represent mean ± SD of three independent experiments, each performed in duplicate.

by other α-chemokines. To approach the former issue, a very low (nonbinding) concentration of 125 I-PF-4 at 5 nM was incubated with PMN in the presence of increasing concentrations of unlabeled PF-4, and its binding kinetics was analyzed. As shown in Figure 2A, at dosages of cold PF-4 higher than 50 nM, 125 I-PF-4 binding dramatically increased over background levels, reaching a maximum with 1.25 μM of cold PF-4. Further enhancement of cold ligand concentration led to a dose-dependent decrease in the amount of bound 125 I-PF-4 down to background levels. The initial increase in 125 I-PF-4 binding in the presence of cold PF-4 confirmed that positive cooperation existed between PF-4 molecules, leading to enhanced interaction with binding sites, while competition of this binding by more elevated dosages of cold PF-4 indicated that the binding was specific. The selectivity of cooperation between PF-4 molecules was confirmed in experiments in which 5 nM 125 I-PF-4 was coincubated with increasing concentrations of other unlabeled α-chemokines (IL-8, NAP-2, GROα, IP-10). Under these conditions, none of the chemokines enhanced or competed the binding of labeled PF-4 (Fig. 2A).

The selectivity of binding sites for PF-4 was further investigated by performing competition experiments using a high concentration of labeled PF-4 (1 μM, reproducibly resulting in about 65% of maximal binding). As expected, upon coincubation with increasing concentrations of unlabeled PF-4, classic competition kinetics was obtained, with 50% of competition achieved at a dosage of 1 to 1.5 μM of competitor, and maximal competition at about a 30-fold molar excess (Fig. 2B). By contrast, neither unlabeled rIL-8, nor rGROα or NAP-2 used as competitors at dosages up to 30 μM changed the binding of 125 I-PF-4 to PMN. Unexpectedly, the non-ELR-α-chemokine IP-10 at concentrations from 1.1 to 30 μM competed for binding with labeled PF-4, although with a potency about threefold lower than PF-4 competed for itself. Moreover, competition by IP-10 was only partial, achieving a maximum of 71% (at 10 μM) as compared with total competition by PF-4 itself (at 30 μM). A further increase in cold IP-10 concentration did not result in further reduction of bound 125 I-PF-4, suggesting that

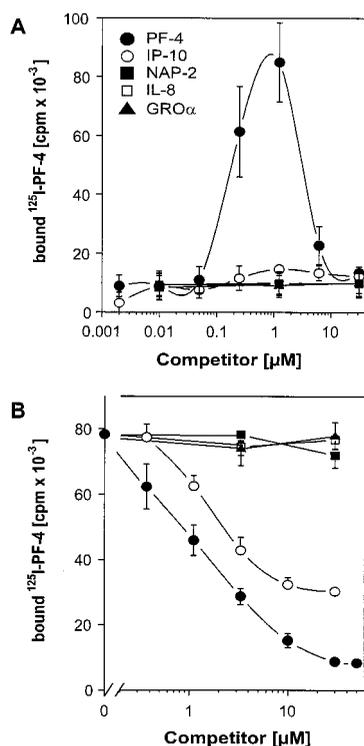


FIGURE 2. Competition of unlabeled PF-4 and other α -chemokines (NAP-2, IL-8, GRO α , IP-10) for binding to receptors with a low and high dosage of ^{125}I -PF-4. PMN were incubated with 5 nM ^{125}I -PF-4 (sp. act. 450–550 Ci/mmol) (A) or 1 μM ^{125}I -PF-4 (sp. act. 1.5–2 Ci/mmol) (B) in the presence of increasing concentrations of PF-4 (●), rIP-10 (○), rIL-8 (□), NAP-2 (■), or GRO α (▲). Data represent total bound radioactivity and are given as mean \pm SD of three independent experiments.

IP-10 interacted with only part of the PF-4 receptors. Thus, within the range of different α -chemokines tested, binding sites were selective for non-ELR- α chemokines PF-4 and IP-10, while ELR chemokines exhibited no detectable binding to these receptors.

PF-4 binding and biologic activity for neutrophils correlate with the formation of PF-4 tetramers in solution

With respect to former reports that PF-4 tends to undergo noncovalent oligomerization with increasing concentration (36, 37), we wondered whether the phenomenon of positive cooperation in PF-4 binding to PMN could be caused by the formation of oligomers, exhibiting enhanced affinity for specific receptors. To examine whether oligomerization took place within the concentration range of PF-4 used for receptor-binding experiments, a constant concentration of radiolabeled PF-4 (0.5 nM) was incubated alone or in the presence of increasing concentrations of the unlabeled chemokine. Potentially formed oligomers were then stabilized by adding cross-linker BS³, and identical volumes of samples were subsequently separated by SDS-PAGE. In Figure 3C, the appearance of a representative polyacrylamide gel following visualization of radioactive protein bands by autoradiography is given. At concentrations of up to 50 nM, PF-4 existed in a monomer-dimer equilibrium, as indicated by the presence of two bands with a molecular mass of 7.9 and 16.2 kDa, respectively (lanes 2 and 3). From a concentration of 250 nM on, two further bands with apparent molecular masses of 26.3 and 36.2 kDa became detectable (lane 4), indicating the formation of PF-4 trimers and tetramers, respectively. With further increasing concentrations of cold PF-4 added, tetramer formation was clearly at the expense of monomers,

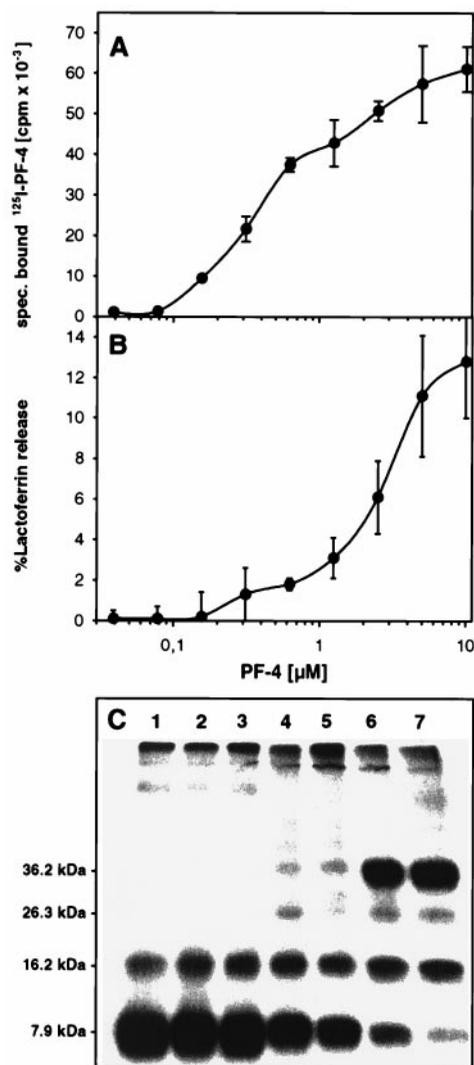


FIGURE 3. Comparison of concentration-dependent PF-4 tetramer formation with PF-4 receptor binding and PF-4/rTNF- α -mediated exocytosis. A, Specific binding of ^{125}I -PF-4 to receptors on human neutrophils. The data are derived from the binding isotherm given in Figure 1B, PF-4-induced marker exocytosis in TNF- α -costimulated neutrophils. Cells were incubated with a constant concentration of 9 ng/ml TNF- α in the presence of increasing concentrations of PF-4, and the percentage of lactoferrin released into the supernatant was determined. Assay backgrounds ($4.6 \pm 1.3\%$) were subtracted. The data represent mean \pm SD of three independent experiments. C, Incorporation of ^{125}I -PF-4 into oligomers of unlabeled PF-4. A total of 0.5 nM ^{125}I -PF-4 was incubated alone (lane 1) or with unlabeled PF-4 at concentrations of 0.01 μM (lane 2), 0.05 μM (lane 3), 0.25 μM (lane 4), 1.25 μM (lane 5), 6.25 μM (lane 6), or 31.25 μM (lane 7), respectively, and subsequently cross-linked with 2 mM BS³. Identical volumes (60 μl) of each sample were then separated electrophoretically on a 15% SDS-polyacrylamide gel under reducing conditions, and radioactive bands were visualized by autoradiography. The data from one representative experiment of three are given.

while the relative proportions of dimers and trimers remained largely unchanged (lanes 5–7). The proportion of a minor amount of high m.w. material appearing at the top of the gel in the presence of cross-linker (compare to untreated control) remained constant over the entire concentration range of PF-4. Scanning analysis of radioactive bands and determination of their individual contribution to total radioactivity detected on the respective lanes revealed negative correlation of the amounts of tetramers and monomers present. Thus, within the range of 0.25 to 31.25 μM of

Table I. Effect of proteases, high ionic strength and low pH on the binding of PF-4 and rIL-8 to receptors on neutrophils

	% Sp. Bound ^{125}I -PF-4 ^a	% Sp. Bound ^{125}I -IL-8 ^a
Untreated control	100	100
Chymotrypsin digestion ^b	109.2 ± 16.1	42.4 ± 9.2
pH 3.0 ^c	98.4 ± 6.8	0.8 ± 0.8
0.5 M NaCl ^c	1.8 ± 0.9	96.8 ± 5.6

^a Cells were analyzed for binding of 1 μM ^{125}I -PF-4 or 1 nM ^{125}I -rIL-8 at 4°C. Data were corrected for unspecific binding, determined in the presence of a 50-fold (PF-4) or 200-fold (rIL-8) molar excess of the unlabeled ligand, respectively. Values were expressed as the percentage of specific binding obtained with the respective ligand in untreated control cells. Unspecific binding was 15.8 ± 4.9% (PF-4) and 3.6 ± 1.4% (IL-8) of total binding by control cells. Data represent mean ± SD of three independent experiments, each performed in duplicate.

^b PMN were treated with 6 U/mL TPCK-chymotrypsin for 30 min at 37°C or left untreated prior to the binding assay with labeled chemokines.

^c Neutrophils loaded with the labeled chemokines for 2 h at 4°C were subsequently exposed to PBS buffer containing 0.5 M NaCl or glycine buffer (50 mM glycine 0.1 M NaCl, pH 3.0) for 1 min at 4°C and residual binding was determined. Cells exposed to PBS only served as controls.

cold PF-4 added, the relative amount of tetramers increased from about 7 to 63%, while at the same time that of monomers decreased from about 67 to 10%. Comparison of these data with those obtained for concentration-dependent binding of PF-4 to neutrophils (Fig. 3A) as well as with the dose-response kinetics of PF-4-induced exocytosis of lactoferrin from the same cells (Fig. 3B) revealed that both binding and biologic activity were not detectable in the absence of PF-4 tetramers. In fact, the threshold concentration of 0.25 μM PF-4 for tetramer formation was similar (within the limits of the assay) to that obtained for binding (0.15 μM) and for the induction of exocytosis (0.31 μM), and either of the latter parameters continued to increase with increasing tetramer concentration.

PF-4 binding to PMN is resistant to proteolysis and low pH, but sensitive to high ionic strength

To obtain initial information whether PF-4 binding sites were biochemically similar to other α -chemokine receptors on neutrophils, the effects of proteolytic enzymes, high ionic strength, and low pH on the specific binding of PF-4 and IL-8 to PMN were compared. As shown in Table I, pretreatment of PMN with 6 U/ml chymotrypsin for 30 min at 37°C had no effect on the subsequent binding of ^{125}I -PF-4 (at 4°C), while the binding of ^{125}I -labeled IL-8 was reduced by more than 50%. Likewise, PF-4 binding was not affected following pretreatment of cells with 2000 BAEE U/ml of TLCK-treated trypsin (data not shown). A drastic effect on PF-4 binding was, however, observed when cells preloaded with ^{125}I -PF-4 were subsequently washed with buffer of high ionic strength (PBS containing 0.5 M NaCl). Under these conditions, PF-4 binding was reduced by more than 95%, while IL-8 binding remained totally unaffected (Table I). Surprisingly, washing a parallel sample of the same chemokine-loaded cells with an acidic buffer of pH 3 (a procedure commonly used to detach polypeptide ligands from membrane receptors) did not reduce the binding of ^{125}I -PF-4, while no detectable ^{125}I -labeled IL-8 remained associated with the cells (Table I). Altogether these results demonstrate that interaction of PF-4 and IL-8 with their respective receptors is governed by quite different principles. Resistance of PF-4 binding to the action of proteases and to acidic conditions, as well as its susceptibility to high ionic strength, all suggest biochemical characteristics of the PF-4 receptor(s) diverging from those of the common seven-transmembrane-segment receptors known for chemokines.

PF-4 binds to CS GAG neutrophils

The data presented above provided further evidence that PF-4 binding sites on PMN were quite different from those for other α -chemokines. In particular, our observation that high salt buffer abrogated the binding of PF-4 pointed to the involvement of proteoglycans, inasmuch as others have previously demonstrated that binding of chemokines to, for example, heparan sulfate proteoglycans (HSPGs) exhibited significant lability under corresponding

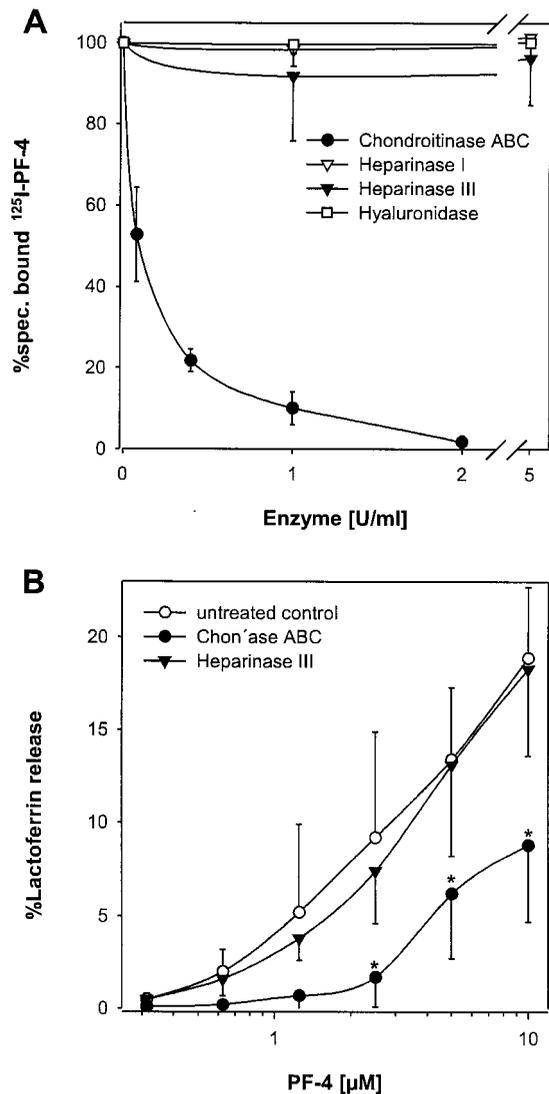


FIGURE 4. Effect of GAG-degrading enzymes on the binding of ^{125}I -PF-4 to receptors on neutrophils and PF-4/rTNF- α -mediated exocytosis. **A**, Specific binding of ^{125}I -PF-4 to receptors on human neutrophils. PMN were preincubated with increasing concentrations of chondroitinase ABC (●), heparinase I (▽), heparinase III (▼), or hyaluronidase (□), for 30 min at 37°C. After removal of the enzymes by washing with PBS, binding assay was performed with a fixed concentration of 1 μM ^{125}I -PF-4 at 4°C. Values were expressed as the percentage of specific binding of untreated controls. Specific binding was determined as described in Table I. **B**, PF-4-induced marker exocytosis in TNF- α -costimulated neutrophils. Cells were incubated with a constant concentration of 1 U/ml chondroitinase ABC (●), heparinase III (▼), or left untreated (○), as described above. After removal of the enzyme, exocytosis assays were performed as described in Figure 3B. *, Indicates significant difference ($p < 0.02$) to the untreated control. Data represent mean ± SD of three (A) or four (B) independent experiments, each performed in duplicate.

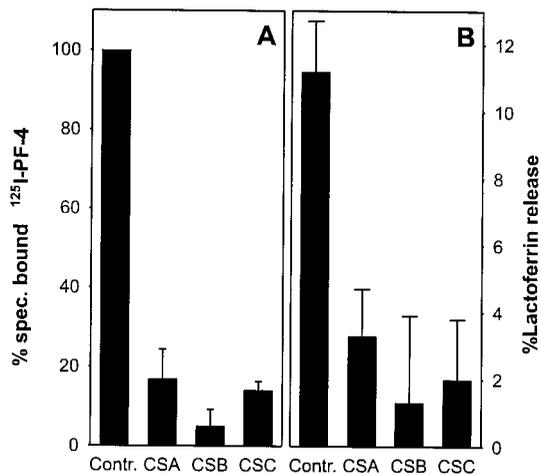


FIGURE 5. Effect of soluble GAG on the binding of ^{125}I -PF-4 to receptors on neutrophils and on PF-4/rTNF- α -mediated PMN activation. Cells were incubated with 1 μM ^{125}I -PF-4 (A) at 4°C alone or in the presence of 10 $\mu\text{g}/\text{ml}$ CSA, CSB, or CSC, and analyzed for specific binding. Values were expressed as the percentage of specific binding to control cells receiving no GAG. PMN activation (B) was induced with 2 μM PF-4 and 9 ng/ml rTNF- α alone or in the presence of 10 $\mu\text{g}/\text{ml}$ of the respective GAG. Assay backgrounds ($3.8 \pm 1.1\%$) were subtracted. The data represent mean \pm SD of three independent experiments.

experimental conditions (32). To investigate whether cell surface-associated proteoglycans could be responsible for PF-4 binding, neutrophils were incubated with different GAG-degrading enzymes for 30 min and subsequently examined for their capacity to specifically bind radiolabeled PF-4 as well as to respond to PF-4 by exocytosis of secondary granule contents. As shown in Figure 4A, pretreatment of cells with increasing concentrations (up to 5 U/ml) of hyaluronidase, heparitinase I, or heparinase I did not affect ^{125}I -PF-4 binding as compared with untreated control cells. However, a drastic decrease in binding of the chemokine was obtained following incubation with chondroitinase ABC. Reduction by about 50% was achieved with only 0.08 U/ml of the enzyme, and binding was reduced to background levels after treatment with 2 U/ml. Furthermore, treatment of PMN with 1 U/ml chondroitinase ABC, but not heparinase III, reduced significantly PF-4-induced exocytosis in TNF- α -costimulated cells (Fig. 4B). Upon stimulation with up to 2.5 μM PF-4, the biologic response in chondroitinase-digested PMN was reduced to background levels, while at more elevated dosages of PF-4 residual responses amounting to 29% (at 5 μM PF-4) and 47% (at 10 μM PF-4) of that of untreated control cells were obtained. These data clearly demonstrate that CSPG are responsible for the binding of PF-4 to PMN, and binding to CSPGs is involved in PF-4-induced neutrophil activation.

The efficacy of CSPG binding to PF-4 was further investigated by examining the ability of soluble CSA, CSB, and CSC to interfere with PF-4 binding to PMN as well as with PF-4-induced PMN biologic function. As shown in Figure 5A, specific binding of 1 μM ^{125}I -PF-4 was inhibited in the presence of all three types of CS, i.e., by 83% with CSA, by 95% with CSB, and by 86% with CSC as compared with the untreated control. Because preincubation of PMN with CS, followed by washing, was without effect on subsequent PF-4 binding to the cells (data not shown), direct complex formation of these GAG with PF-4 is likely to be responsible for their inhibitory effects. Corresponding results were obtained when the effect of CS on PF-4-induced lactoferrin release was examined (Fig. 5B). In this study, as compared with untreated control cells, inhibition by 71%, by 90%, and by 82% was observed

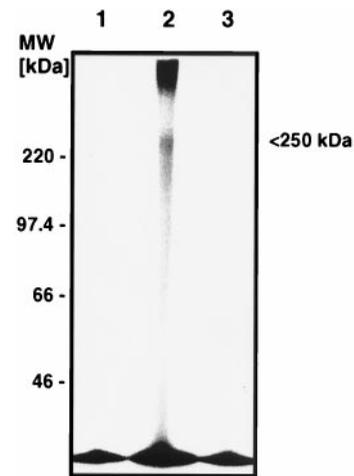


FIGURE 6. Cross-linking of ^{125}I -PF-4 to PMN membranes. PMN were incubated with 10 nM ^{125}I -PF-4 in assay buffer alone (lane 1), or in the presence of unlabeled PF-4 at 1 μM (lane 2), or at 10 μM (lane 3) for 90 min at 4°C. Bound material was cross-linked with 1.5 mM BS³ for 1 h on ice, and extracts prepared in Laemmli buffer were directly loaded on a 7.5% SDS-polyacrylamide gel and run under nonreducing conditions. Radioactive bands were visualized by autoradiography.

with CSA, CSB, and CSC, respectively. Altogether these results demonstrate that CSPGs are efficient competitors of PF-4 binding and PF-4 biologic activity, providing further evidence that CSPG-like structures may function as cellular receptors for the chemokine.

PF-4 specifically binds to a 250-kDa membrane CSPG

To better define the biochemical characteristics, i.e., the molecular size and number of species of PF-4 binding sites, radiolabeled PF-4 was covalently cross-linked to intact PMN and the solubilized membrane fraction subsequently separated by SDS-PAGE under nonreducing conditions. To examine the specificity of ^{125}I -PF-4 incorporation into radioactive bands detected on the gel, parallel samples of cells were cross-linked in the presence of 1) 10 nM ^{125}I -PF-4 (a dosage exhibiting no specific binding to PMN; see Fig. 1), 2) a mixture of 10 nM ^{125}I -PF-4 and 1 μM cold PF-4 (in which, due to oligomerization, high specific binding was to be expected; see Fig. 2A), and 3) a mixture of 10 nM ^{125}I -PF-4 and 10 μM cold PF-4 (in which, due to the excess of unlabeled PF-4 oligomers, competition of specific binding should occur; see Fig. 2A). As expected, cross-linking with 10 nM labeled PF-4 alone did not lead to detectable radioactivity on the gel, except for that caused by free ligand itself at the bottom of the gel (Fig. 6, lane 1). By contrast, a broad band typical for proteoglycans and corresponding to an approximate molecular mass of 250 kDa became visible with 10 nM ^{125}I -PF-4/1 μM PF-4 (lane 2). This band disappeared upon increasing the dosage of cold PF-4 to 10 μM (lane 3). In a parallel set of experiments, PMN were pretreated with GAG-degrading enzymes (each at 2 U/ml for 30 min at 37°C) before cross-linking with 10 nM ^{125}I -PF-4/1 μM PF-4. Neither hyaluronidase nor heparinase treatment abolished the 250-kDa band, while treatment with chondroitinase ABC led to its disappearance (data not shown). However, a lot of poorly separated material remaining at the top of lane 2 (Fig. 6) and forming a broad smear indicated that at least part of the receptor-ligand complexes had become precipitated during the cross-linking procedure. Thus, it cannot be excluded that further bands representing specific binding sites may be hidden within this unseparated material. Exactly the same results were obtained when cross-linked cellular proteins were separated under reducing conditions (data not shown).

Discussion

In the present study, we report on the discovery of a specific receptor for PF-4 on human neutrophils and its unusual biologic and biochemical properties as compared with all other chemokine receptors described to date. Human neutrophils express about 55,000 α -chemokine receptors/cell for IL-8 (3), which comprise two related molecular species, termed CXCR-1 and CXCR-2. Both molecules are G protein-coupled 7-TMD receptors displaying similarly high affinities for IL-8 and diverging affinities for other α -chemokines such as NAP-2 and GRO α (3, 4). However, as we could recently show by cross-competition analyses, these receptors are not able to bind PF-4 (16). In our present approach using labeled PF-4 for direct binding analyses, we nevertheless found a surprisingly high number of PF-4 binding sites (7.6×10^6 /cell) of relatively moderate affinity ($K_d = 650$ nM). Even with respect to our findings that PF-4 apparently binds as a tetramer, resulting in a calculated density of 1.9×10^6 sites/cell, this amount outnumbers that of IL-8R by more than 30-fold. An explanation for this enormous number of binding sites could be derived from the biochemical composition of the receptor. First, indirect evidence for its proteoglycan nature arose from experiments in which the sensitivity of PF-4 binding to different dissociating agents was examined. At variance with IL-8, cell-associated PF-4 could be dissociated by brief exposure to high salt buffer (0.5 M NaCl), indicating the predominant involvement of ionic bonds in receptor binding. Moreover, the stability of PF-4 binding to treatment with moderately acidic buffer (pH 3) suggested a crucial role for moieties of considerably low pI. More direct evidence that the PF-4 binding sites are indeed located within the (usually negatively charged) carbohydrate portion of a CSPG was provided by our observation that pretreatment of intact neutrophils with chondroitinase ABC (but not with heparinase I, heparitinase I, nor hyaluronidase) strongly reduced PF-4 binding. Because several carbohydrate side chains may be expressed on a single PG core protein, and one side chain will possibly bind more than one PF-4 tetramer, the actual number of receptor molecules is probably much lower than that inferable from the calculated number of binding sites.

Due to the heterogeneity in length of their GAG chains, proteoglycans do not migrate as sharp bands in SDS-PAGE, and therefore, determination of the molecular size of the PF-4 receptor was difficult. Our approach to covalently cross-link iodinated PF-4 to intact neutrophils revealed a specifically labeled receptor-ligand complex migrating at approximately 250 kDa in SDS-PAGE, a size that has also been found for a variety of different PGs such as syndecans or glypican (38, 39). Current studies are underway for the identification and characterization of the receptor core protein. Nevertheless, the strict requirement for detergent to achieve solubilization indicates that PF-4 receptors are integral membrane molecules.

To our knowledge, the present report is the first to show specific binding of a chemokine to proteoglycans on neutrophils. Although others have shown previously that PF-4 binds to membrane-associated HSPGs on bovine aortic endothelial cells (30) as well as to human umbilical cord vein-derived cells (29), none of these reports provided information on the number of species or the molecular size of the proteoglycans involved. Interestingly, Rot and coworkers recently could demonstrate interaction of IL-8 with proteoglycans on endothelium *in vivo* (40–42), and Luster et al. found that PF-4 and IP-10 share the same HSPGs for binding to human endothelial cell lines (32). Our data demonstrating that human neutrophils likewise express proteoglycans cross-reactive to PF-4 and IP-10 are nevertheless surprising, since we found CSPG rather than HSPG to be responsible for binding of the chemokines. In addition, we found IP-10 to induce an exocytosis response in TNF-

α -costimulated PMN within a similar concentration range as characteristic for PF-4, although with considerably lower efficacy (unpublished data). This indicates that IP-10 is able to induce functional activation of neutrophils through a pathway similar or identical to that activated by PF-4. On the other hand, it is rather unlikely that IP-10 constitutes a physiologically relevant activator of PMN functions, since its binding and biologic activity occur at dosages much higher than those that are observed to become released by its producer cells (43). Considering this, PF-4 receptors will be physiologically monospecific for PF-4, a chemokine found at micromolar concentrations. In general, our results strengthen the view that cell-associated proteoglycans may exhibit selectivity for certain chemokines, since no competition of PF-4 binding was found with the closely related α -chemokines IL-8, NAP-2, and GRO α .

That chemokines in fact may exhibit specificity for individual GAG also was shown recently by Witt and coworkers (44), who demonstrated divergent binding affinities of IL-8, NAP-2, GRO α , and PF-4 for subpopulations of fractionated heparin and heparan sulfates. With respect to these observations, we were not surprised to find that exogenous CS (A, B, and C) in solution prevented the binding of PF-4 to neutrophils, and inhibited PF-4/TNF- α -mediated neutrophil exocytosis. Obviously, the soluble GAG acted as scavengers (by binding free PF-4) and did not directly impair the capacity of cell-associated receptors to interact with the chemokine, since PF-4 binding and PF-4/TNF- α -mediated biologic activity remained unchanged in cells preexposed to and subsequently washed free of soluble GAG. Interestingly, Webb et al. (45) reported quite different phenomena when investigating the effect of exogenous GAG in solution on IL-8-mediated neutrophil activation. These authors described enhancement of IL-8-mediated chemotactic migration as well as of intracellular calcium fluxes, suggesting that GAG-bound IL-8 was still able to interact with its receptor, and that matrix PGs could serve to immobilize and present IL-8 to migrating PMN. It is thus conceivable that the distinct physical natures of IL-8 receptors (7-TMD proteins) and PF-4 receptors (CSPGs) will have a different impact on the ability of the bound ligand to interact with accessory molecules such as exogenous GAG.

A further parameter determining the selectivity of chemokine receptors for their ligands may be the oligomeric state of the chemokines themselves. Noncovalent oligomer formation has been found to represent a typical feature of all chemokines analyzed to date. While with increasing concentration IL-8, NAP-2, GRO α , and MIP-1 β form dimers in solution (46–49), PF-4 forms tetramers (36, 37) and IP-10 associates in oligomers of even higher order (32). The question whether dimerization of IL-8 is essential for its biologic activity on neutrophils has been investigated exhaustively, until Rajarathnam et al. (50) could show that synthetic IL-8 analogues modified in a way to prevent oligomer formation retained full biologic activity and receptor-binding capacity. According to our results, conditions appear to be quite different with PF-4. Scatchard analyses of PF-4 binding to its receptor revealed significant positive cooperation, indicating that PF-4 oligomer formation might be essential for binding. This assumption was further supported by the observation that a marked shift in monomer-dimer-tetramer equilibrium took place over the concentration range of PF-4 used for the binding experiments. As visible after covalent cross-linking and SDS-PAGE analysis, the proportion of tetramers in solution dramatically increased with increasing PF-4 concentration at the expense of monomers, while that of dimers remained unchanged. As has been reported by other authors before (36), PF-4 trimers (that became detectable in parallel to tetramers) are most likely due to incomplete cross-linking of tetramers, and thus

are not normally present in solution. More direct evidence for a role of oligomers in PF-4 binding to its receptor could be derived from the analysis of competition kinetics, that resulted in an unusual bell-shaped binding curve: while ^{125}I -PF-4 alone did not bind at low concentrations (<150 nM), addition of increasing dosages of cold PF-4 led to a successive increase in cell-bound radioactivity, suggesting that mixed oligomers of iodinated and cold PF-4 had formed and interacted with the receptor. Displacement of the ligand at further increased dosages of cold PF-4 was most likely due to the formation of unlabeled oligomers that competed for binding. Similar bell-shaped competition kinetics was reported recently by Hoogewerf et al. for the binding of oligomeric IL-8 and other chemokines to isolated heparin as well to GAG on the surface of endothelial cells (51). Furthermore, these authors suggested that the oligomeric state of chemokines may play a crucial role for their sequestration by GAG-coated surfaces. It should be noted that in the presence of increasing concentrations of IP-10, the binding of labeled PF-4 was not enhanced. This indicates that IP-10 does not associate with PF-4 to generate functional heterooligomers. Finally, since PF-4 did not significantly bind to or stimulate biologic activity in neutrophils at dosages (up to 50 nM) in which only monomers and dimers were present in solution, but only from concentrations on which tetramers became detectable (250 nM), all evidence obtained to date is in favor of the tetramer representing the functional PF-4 oligomer.

The circumstance that PF-4 receptors appear to selectively bind tetramers imposes several problems on the correct determination of receptor affinity. Calculation of dissociation constants (K_d) based on Scatchard transformation of binding data is limited to bimolecular interactions defined by the equilibrium according to the mass action law $[\text{Rec.}] + [\text{Ligand}] \leftrightarrow [\text{RecLigand}]$. However, PF-4 has to oligomerize before binding, and the proportion of tetramers formed is a result of various monomer-dimer-tetramer equilibria. Because the association of PF-4 into tetramers is far from complete at concentrations in which binding to receptors occurs (e.g., 16% at 1.25 μM), the calculated affinity of $K_d = 650$ nM can only be addressed as an apparent K_d , but does not give the true affinity of receptors for tetramers. Nevertheless, it may provide a measure for the relevance of PF-4 binding under physiologic conditions. Affinities of PF-4 for other GAG were reported to be significantly higher. Witt et al. (44) demonstrated that PF-4 binds to heparin with a K_d of 30 nM, and Gallagher and coworkers reported divergent affinities (K_d of 15.9 and 200 nM, respectively) for its binding to heparan sulfate (52). Although in comparison with these findings the affinity of PF-4 for CSPG on PMN appears rather low, local concentrations of PF-4 after platelet activation (normal serum concentration: 1.3–2.5 μM) (53) are likely to be more than sufficient to effect more than half-maximal receptor occupation and biologic responses in neutrophils. Furthermore, restriction of the receptor to tetramer binding could serve as a mechanism to protect neutrophils from improper activation, e.g., by low amounts of PF-4 that constantly arise from decaying platelets. The sharp decrease in the relative proportion of tetramers with decreasing PF-4 concentration will probably also help to focus neutrophil activation to distinct inflammatory sites, i.e., to areas in which massive platelet aggregation, followed by substantial release of the chemokine, takes place. Even a slight decrease in PF-4 levels in the close vicinity of such sites would greatly reduce the neutrophil inflammatory response and protect intact tissue from becoming destroyed.

It is still an open question whether the PF-4 receptor described has a signaling function on its own. One could imagine that the CSPGs that we have identified are not signaling by themselves, but that they function as coreceptors, presenting the bound PF-4 to a

second receptor with signaling capacity. Such a relationship has been described for bFGF, which binds to a HSPG on fibroblast and becomes then presented to its signaling receptor (54). Interestingly, it was found that PF-4 can also bind to this coreceptor and thereby modulate bFGF biologic activity (31). The coreceptor hypothesis might be supported by our observation that PF-4-induced exocytosis in chondroitinase ABC-treated cells was completely abrogated only at low concentrations of the stimulus, while there was still residual activity with high dosages of PF-4. On the other hand, to avoid cytotoxic side effects, we limited the concentration of the enzyme to 1 U/ml, and this dosage might not have been sufficient to effect total degradation of all GAG chains. Thus, a direct signaling function of the receptor cannot be excluded. Nevertheless, our data demonstrating that removal of cell-associated CS by chondroitinase treatment as well as the presence of excess soluble CS both inhibit PF-4 binding and PF-4-induced exocytosis strongly suggest that interaction of PF-4 with membrane-bound CS proteoglycans has a crucial impact on PF-4-mediated neutrophil activation.

Our present investigations are directed to the identification of the receptor(s) and the mechanism that are involved in PF-4 receptor signaling.

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