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Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg

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ABSTRACT **Amino acid deletion and mutagenesis experiments have indicated that the sequence Glu-Leu-Arg (ELR) preceding the first cysteine at the N terminus of interleukin 8 (IL-8) is required for receptor binding and neutrophil activation. Platelet factor 4 (PF4) is structurally related to IL-8 (35% sequence identity) but lacks the N-terminal ELR sequence and comparable effects on neutrophils. We introduced the ELR sequence at the N terminus of PF4 and found that the modified protein was a potent neutrophil activator and attractant. On the other hand, when the ELR sequence was introduced into the corresponding positions of two other proteins related to IL-8, γ-interferon-inducible protein IP10 and monocyte chemoattractant protein 1, neither of them acquired neutrophil-activating properties, indicating that besides ELR additional structural determinants of IL-8 and PF4 are important for binding to IL-8 receptors. The conservation of these binding determinants suggests that PF4 may have evolved from a neutrophil activating protein.**

Interleukin 8 (IL-8) is a proinflammatory cytokine that is produced by a wide variety of cell types and promotes the recruitment and functional activation of neutrophil leukocytes (1, 2). Several human proteins with sequence similarity to IL-8 have been described and divided into two subfamilies depending on whether the first two cysteines are adjacent (CC) or separated by one amino acid (CXC). IL-8, neutrophil-activating peptide 2 (NAP-2) (3), the growth-related proteins GROα, -β, and -γ (4, 5), ENA-78 (6), platelet factor 4 (PF4) (7, 8), and γ-interferon inducible protein IP10 (9) belong to the CXC subfamily. Monocyte chemoattractant protein 1 (MCP-1) is the best known representative of the CC subfamily (10).

IL-8, NAP-2, GROα, and ENA-78 bind to common receptors on neutrophils and induce chemotaxis, exocytosis, and the respiratory burst (3, 6, 11, 12). Comparable neutrophil responses were not observed with PF4 (3, 13), although chemotaxis and exocytosis have been reported with concentrations that were 1000- to 10,000-fold higher than those required for IL-8 (14–16). PF4 has been studied extensively in the context of thrombosis and circulatory disorders (17). Its affinity for heparin and other sulfated glycans suggests that it may function as a prothrombotic agent. Effects of PF4 on cellular functions, such as inhibition of megakaryocytopoiesis (18) and angiogenesis (19), as well as modulation of cellular immune responses (17), have been described, but as yet no cell surface receptor has been identified. The finding that PF4 modulates growth factor receptor interactions (20, 21) suggests that at least some of its effects are indirect. No neutrophil stimulating (22) or other biological activities have been observed with IP10, whereas MCP-1 has been characterized as a chemotactic protein that stimulates monocytes (23) and basophils (24), but not neutrophils.

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The three-dimensional structure of IL-8 reveals a noncovalently linked homodimer (25, 26). The monomer has a conformationally flexible N-terminal region that is anchored by two disulfide bridges to the protein core, which consists of three antiparallel β-strands and a C-terminal α-helix (25). Although bovine PF4 crystallizes as a tetramer, the monomer structure is similar to that of IL-8 (27). In solution, human PF4 is in equilibrium among monomers, dimers, and tetramers (28). PF4 and IL-8 share 35% sequence identity, including the four cysteines. Molecular modeling studies (I.C.-L., unpublished work) suggest that a similar folding pattern will be found for all members of the CXC subfamily. Even for MCP-1, which belongs to the CC branch, the same tertiary structure has been proposed (29).

MATERIALS AND METHODS

Chemical Synthesis. IL-8, GROα, PF4, IP10, MCP-1, and their analogs were synthesized by solid-phase methods using the *tert*-butyloxycarbonyl and benzyl protection strategy (30). After deprotection with hydrogen fluoride, the material was folded by air oxidation and purified by reverse-phase HPLC. Purity was determined by reverse-phase HPLC and isoelectric focusing. Amino acid incorporation was monitored during synthesis, and the final composition was determined by amino acid analysis. That the proteins had the correct covalent structure was inferred from the results of ion-spray mass spectrometry (SCIEX APIII). A detailed description of the procedure has been reported (30).

Biological Assays. Human neutrophils were isolated from buffy coats of donor blood (31). The final suspension, containing 10^8 cells per ml in 0.15 M NaCl supplemented with 0.05 mM $CaCl_2$, was kept at 10°C until use. Elastase release from cytochalasin B-pretreated neutrophils (31), cytosolic changes in free calcium concentration (31), and neutrophil chemotaxis (32) were determined according to established methods.

Receptor Binding Assays. IL-8 iodination, binding assays, and the calculation of the K_d values were performed as described (12).

RESULTS AND DISCUSSION

ELR Motif. Previous studies have shown that the N-terminal region of IL-8 is critical for receptor binding and neutrophil activation (33). Truncation of the N terminus yielded IL-8 derivatives with higher potency, and a maximum was reached with an analog having the N-terminal sequence Glu-Leu-Arg-Cys (Fig. 1). Further truncation resulted in drastic loss of activity: The analog without Glu was much less potent, and those without Glu-Leu or Glu-Leu-Arg were

Abbreviations: ELR, Glu-Leu-Arg; IL-8, interleukin 8; MCP-1, monocyte chemoattractant protein 1; NAP-2, neutrophil-activating peptide 2; PF4, platelet factor 4.

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IL-8	**SAKELRCQC.....**
ELR-IL-8	**ELRCQC.....**
PF4	**EAEEDGDLQCLC.....**
DLQ-PF4	**DLQCLC.....**
ELR-PF4	**ELRCLC.....**
[E^7,R^9]PF4	**EAEEDGELRCLC.....**
IP10	**VPLSRTVRCTC.....**
ELR-IP10	**ELRCTC.....**
MCP-1	**QPAINAPVTC-C.....**
ELR-MCP-1	**ELRC-C.....**

FIG. 1. N-terminal sequences of the IL-8-related proteins and their analogs. Shown are the N-terminal sequences through the second cysteine of IL-8 (34, 35), PF4 (7, 8), IP10 (9), MCP-1 (36), and chemically synthesized analogs.

inactive (33). Similar results were obtained in mutagenesis experiments showing that replacement of either Glu, Leu, or Arg by Ala greatly reduced the receptor binding affinity (37). These studies demonstrate the importance of the N-terminal sequence Glu-Leu-Arg, which we term the ELR motif.

The ELR motif is conserved in all IL-8-related proteins that exhibit neutrophil-activating properties, but not in PF4, IP10, or the members of the CC subfamily such as MCP-1 (Fig. 1). To assess the importance of this region, we have synthesized and tested IL-8 analogs with single replacements of the ELR residues, as well as PF4, IP10, and MCP-1 with ELR at the N terminus. Throughout this manuscript, the variants are referred to by the first three residues in one-letter code followed by the symbol of the parent molecule (see Fig. 1).

Substitutions in the ELR Motif. The relative contribution of the individual ELR residues for IL-8 activity was determined in a comparison of 18 ELR-IL-8 analogs with single amino acid replacements. Although the activity was sensitive to modification of each of the three residues, a clear hierarchy was evident (Table 1). The arginine was the most sensitive,

Table 1. Relative effects of single substitutions of Glu, Leu, or Arg of ELR-IL-8 on neutrophil-stimulating activity

Substitution	Fold decrease	Substitution	Fold decrease	Substitution	Fold decrease
Glu	1	Leu	1	Arg	1
Asp	22 ± 4	Ile	9 ± 1	His	150 ± 60
Gln	24 ± 10	Val	41 ± 11	Gln	—
Ser	4,000	Gln	45 ± 10	Leu	—
Ala	>10,000	Phe	250 ± 105	Nle	—
		Pro	480 ± 309	Lys	—
		Ala	2000	Orn	—
		Trp	3000		
		Gly	3000		

Chemically synthesized analogs with the indicated substitution were tested at concentrations ranging between 0.1 and 1000 nM for stimulation of elastase release from cytochalasin B-pretreated neutrophils. The concentration required for 30% (EC_{30}) of the maximal elastase release that was obtained with ELR-IL-8 was determined. EC_{30} rather than EC_{50} values are given because of the severe decrease in activity caused by most substitutions. Data are expressed as the ratio of the EC_{30} of the analog to the EC_{30} of ELR-IL-8. Values shown are the means ± SD of at least four experiments performed with different neutrophil preparations. —, No activity was detected at concentrations up to 1000 nM. Nle, norleucine; Orn, ornithine.

since all substitutions tried (except for histidine, which led to a 150-fold loss in potency) yielded derivatives without detectable activity. At all three positions, substitution with small-side-chain amino acids resulted in a loss of activity of at least 2000-fold, a finding that is in agreement with the observations with alanine mutants (37). Taken together, these results suggest that the order of importance of the three residues for receptor binding and neutrophil activation is Arg > > Glu > Leu.

ELR-PF4 Is a Neutrophil-Activating Protein. To further examine the role of the ELR motif for receptor binding and activity, we synthesized an analog of PF4 with ELR at the N terminus, designated ELR-PF4 (Fig. 1). When assayed for neutrophil-stimulating activity, ELR-PF4 induced the release of elastase over the same concentration range as ELR-IL-8 (Fig. 2*A*). Its potency was about 1/10th that of ELR-IL-8, but about 5-fold greater than that of GROα. The average ratio of the EC_{30} values (defined in Table 1) for ELR-PF4 and ELR-IL-8 obtained with neutrophils from 10 different donors was 9.7 (range, 6.0–17.2). In contrast, with full-length PF4 or its truncated form (DLQ-PF4), no release response was observed up to a concentration of 1 μM. ELR-PF4 also induced neutrophil chemotaxis (Fig. 2*B*). In four experiments with cells from different donors, a characteristic biphasic response was obtained. Maximum migration occurred at approximately 10 nM ELR-IL-8 and 100 nM ELR-PF4, consistent with the somewhat lower potency of the PF4 analog, whereas full-length PF4 and DLQ-PF4 were inactive. In addition, ELR-PF4 competed with labeled IL-8 for binding to IL-8 receptors (Fig. 3), and analysis of the data revealed

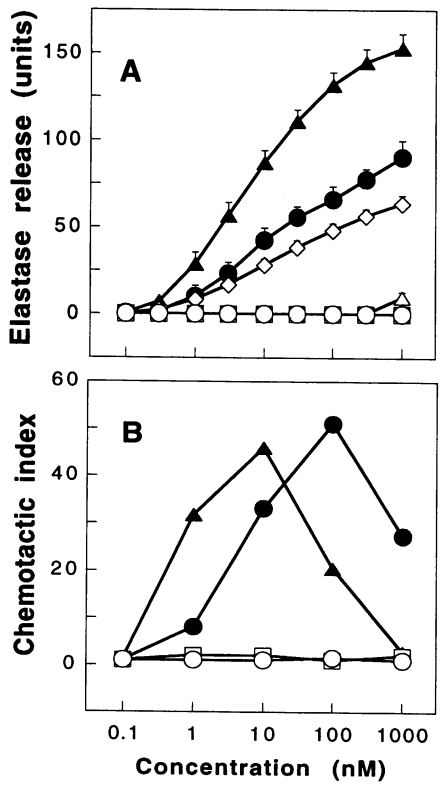

FIG. 2. ELR-PF4 activates human neutrophils. (*A*) Release of elastase from cytochalasin B-treated neutrophils stimulated with ELR-PF4 (●), PF4 (□), DLQ-PF4 (○), [E^7,R^9]PF4 (△), ELR-IL-8 (▲), and GROα (◇). Values are means ± SD from five experiments performed with neutrophils from different donors. (*B*) Neutrophil chemotaxis induced by ELR-PF4 (●), PF4 (□), DLQ-PF4 (○), and ELR-IL-8 (▲) as determined in multiwell chambers. One of four similar experiments is shown. Mean values ± SD for the chemotactic index at the optimum concentration were 53.1 ± 5.2 for ELR-PF4 (100 nM) and 48.8 ± 5.8 for ELR-IL-8 (10 nM).

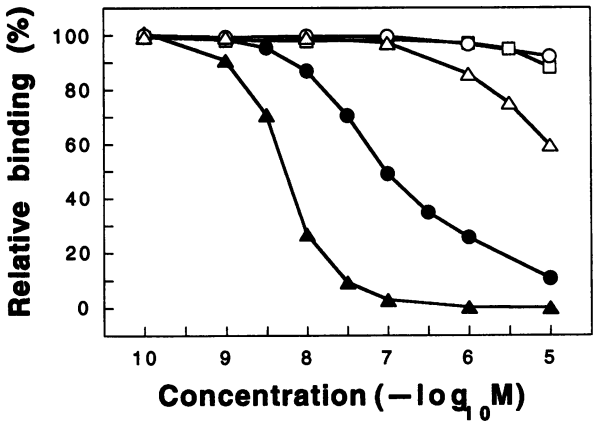

FIG. 3. ELR-PF4 binds to IL-8 receptors. Competition for ^{125}I-IL-8 binding to neutrophils by the indicated concentrations of ELR-PF4 (●), PF4 (□), DLQ-PF4 (○), [E^7,R^9]PF4 (△), and ELR-IL-8 (▲) was assessed. One of two experiments performed with intact neutrophils is shown. Similar results were obtained in two additional experiments performed with isolated neutrophil membranes.

that the affinity was 1/15th that of ELR-IL-8. Cross-desensitization between ELR-PF4 and ELR-IL-8, as assessed by the stimulus-dependent rise of intracellular calcium, also indicated that the two proteins acted through the same receptor (data not shown).

When the ELR motif was introduced into full-length PF4, the resulting analog, [E^7,R^9]PF4, had marginal activity and competed only slightly for IL-8 receptor binding (Figs. 2 and 3). This finding is in agreement with former studies showing that extension of the N terminus of NAP-2 (3) and IL-8 (33) results in a marked reduction in activity.

ELR-Containing Peptides. The neutrophil-activating properties of ELR-PF4 highlights the role of the ELR motif as a determinant of IL-8 receptor activation. As shown by NMR spectroscopy, the N-terminal domain of IL-8 has a high degree of flexibility in solution (25), and the ELR domain may thus have some characteristics of a free peptide. Therefore, several linear (ELR, ELRA, AVCPRSAKELRA) and cyclic (CPGELRC, CPGGKELRC, CGPKELRC, CPGELRAQC, CLPRSAKELRC; terminal cysteines in disulfide linkage) synthetic peptides were tested, but none competed for binding to the IL-8 receptor or had functional activity when tested at up to millimolar concentrations.

ELR Modification of IL-8-Related Cytokines. To determine whether the ELR motif can confer neutrophil-activating properties on other IL-8-related proteins, we synthesized IP10 and MCP-1 (which both share 24% identity with IL-8) and their analogs with ELR at the N terminus (Fig. 1). Neither the natural nor the ELR-modified proteins, ELR-IP10 and ELR-MCP-1, were active on neutrophils, as determined by the lack of detectable elastase release or chemotaxis (data not shown).

Conclusions. Collectively our results show that the ELR motif is necessary, but not sufficient, for IL-8 receptor binding and neutrophil activation and imply that other regions of the IL-8 protein are also important. When ELR is deleted, no binding is observed, showing that these three residues are essential for the recognition of the receptor. In addition, the ELR sequence is highly sensitive to single amino acid substitutions, which decrease or abrogate the biological activity of IL-8. The strongest argument for the importance of this motif for receptor interaction is provided by the fact that the introduction of the ELR sequence converts PF4 to a neutrophil-activating protein that has potency comparable to that of IL-8. Due to the flexibility of the N-terminal domain, ELR may bind to the receptor like a small ligand (e.g., fMet-Leu-Phe). It is clear from this study, however, that IL-8 and the other neutrophil-stimulating proteins must interact with the receptor through additional binding sites to ensure signaling.

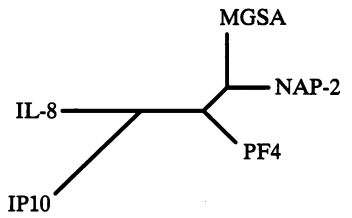

FIG. 4. Relatedness of human PF4 and IL-8 homologs. The distances of the evolutionary distance tree were derived from an amino acid substitution probability matrix (38). MGSA, melanoma growth stimulatory activity, equivalent to GROα.

It was surprising to find that PF4, a molecule with an activity profile distinct from that of IL-8, has all the structural features, except for the ELR motif, required to trigger the IL-8 receptor. This observation suggests that PF4 may have evolved from a neutrophil-activating protein and that, therefore, its IL-8 receptor-binding properties have been largely conserved. Indirect support for this hypothesis is provided by evolutionary distance comparisons of the sequences of IL-8-related proteins, which show that PF4, NAP-2, and GROα are equally close to each other but that all three are more distant from IL-8 (Fig. 4). A key step in the evolution of PF4 could have been the substitution of glutamine for the arginine preceding the first cysteine, resulting in the abrogation of its neutrophil-stimulating properties (Table 1). In this regard it is noteworthy that in porcine PF4 the corresponding residue is an arginine (39).

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