Platelet Factor 4 and Interleukin-8 CXC Chemokine Heterodimer Formation Modulates Function at the Quaternary Structural Level*

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The apparent complexity of biology increases as more biomolecular interactions that mediate function become known. We have used NMR spectroscopy and molecular modeling to provide direct evidence that tetrameric platelet factor-4 (PF4) and dimeric interleukin-8 (IL8), two members of the CXC chemokine family, readily interact by exchanging subunits and forming heterodimers via extension of their antiparallel β-sheet domains. We further demonstrate using functional assays that PF4/IL8 heterodimerization has a direct and significant consequence on the biological activity of both chemokines. Formation of heterodimers enhances the anti-proliferative effect of PF4 on endothelial cells in culture, as well as the IL8-induced migration of CXCR2 vector-transfected Baf3 cells. These results suggest that CXC chemokine biology, and perhaps cytokine biology in general, may be functionally modulated at the molecular level by formation of heterodimers. This concept, in turn, has implications for designing chemokine/cytokine variants with modified biological properties.

Platelet factor-4 (PF4) and interleukin-8 (IL8) are members of the CXC chemokine family of small (8–10 kDa) proteins, a subfamily of chemokines within the cytokine superfamily (1). CXC chemokines are generally chemotactic for migratory immune cells, and thus are involved in the regulation of inflammatory processes and wound healing (1–4). In addition, they demonstrate biological activities in hematopoiesis, cell proliferation, angiogenesis, and glycosaminoglycan binding (1–4). CXC chemokines, which are known to self-associate as dimers and tetramers, exhibit high sequence and three-dimensional structural homology at both the tertiary (monomer) and quaternary (dimer and tetramer) levels. Each folded CXC chemokine monomer has an aperiodic, two-disulfide bond-stabilized N-terminal segment, followed by a three-stranded antiparallel β-sheet domain, and a C-terminal α-helix that is folded onto the generally amphipathic β-sheet (5). Dimers are formed by intermolecularly extending the monomer β-sheet into a six-stranded antiparallel β-sheet (originally termed AB-type dimer (6)). Tetramers are formed by β-sheet sandwiching of two AB-type dimers (6). Native PF4 dimers associate asymmetrically into tetramers (6), whereas in an N-terminal PF4 chimera (PF4M2), dimers associate symmetrically into tetramers with little change in biological activity (7). IL8 forms AB-type dimers and is not known to tetramerize (8–10).

Despite sequence and structure similarities, PF4 and IL8 mediate different biological activities. Although first recognized to bind heparin and act as an anticoagulant, PF4 is also known as an anti-angiogenic agent (11). On the other hand, IL8 binds heparin more weakly and can promote angiogenesis (11, 12). The angiogenic functional difference may be due, at least in part, to N-terminal sequence differences. PF4 lacks the IL8 N-terminal tripeptide Glu-Leu-Arg (ELR) motif, which is known to mediate IL8 receptor binding and subsequent signal transduction (13). In addition, PF4 tetramer formation may also contribute to these functional variations. Given biophysically determined dissociation constants (Kd) (14–16) and taking into account normal physiological concentrations (nano- to picomolar range), these CXC chemokines would exist in solution mostly dissociated as monomers for IL8 and as a distribution of monomers, dimers, and tetramers for PF4. However, because Kd values were determined by using chemically pure proteins and nonphysiological conditions, the actual aggregate state distribution in vivo remains unknown because of the milieu of other biomolecules with which these proteins could interact. In vitro, IL8 has been shown to function both as a monomer and dimer (17). In its well known capacity to bind heparin, PF4 functions optimally as a tetramer in vitro (18, 19) and presumably as a tetramer in vivo due both to the presence of heparan sulfate normally found on the surface of cells and because it is released from α-granules of platelets in relatively large quantities upon tissue injury.

Previous studies (20, 21) have shown that mixing various chemokines in solution can significantly modify their biological activities, but no thorough explanation as to why this occurs has been given. Here we use NMR spectroscopy and computational modeling to demonstrate that when present together in solution, CXC chemokines PF4 and IL8 interact with each other and exchange subunits to form AB-type heterodimers. Although structurally highly similar in their monomer folds, native PF4 and the PF4M2 N-terminal chimera were both used in these NMR studies with IL8, because they do show key

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¶ The abbreviations used are: PF4, platelet factor-4; PF4M2, N-terminal chimera of platelet factor 4; IL8, interleukin 8; HSQC, heteronuclear single quantum coherence; PGSE, pulsed field gradient spin-echo; EC, endothelial cell; Baf3, bone marrow-derived pro-B-cells; HUVECs, human umbilical vein-derived endothelial cells; r.m.s.d., root mean square deviation; RFU, relative fluorescence units; ppm, parts per million.
PF4 and IL8 Heterodimers Modulate CXC Chemokine Function

Differences in their quaternary structures at dimer interfaces, as well as in their dimer and tetramer dissociation constants. Moreover, PF4FM2 yields better defined NMR spectra, primarily because of its forming symmetric tetramers and exhibiting less chemical exchange broadening. In biological terms, we demonstrate that formation of PF4/IL8 heterodimers is bio-functionally correlated, by examining the concentration-dependent influence of PF4 on IL8-induced migration of vector-transfected BaF3 cells, as well as the influence of IL8 on anti-proliferative effects of PF4 against endothelial cells (EC).

These findings further suggest that heteroassociation between/ among other structurally homologous members of the chemokine family, and perhaps among members of various cytokine subfamilies, may be a general occurrence and may be responsible for modulating chemokine/cytokine biology on the molecular level in vivo.

**Experimental Procedures**

**Proteins**—Recombinant PF4, PF4FM2, and IL8 were expressed in *Escherichia coli* and purified as described previously (22).

**NMR Spectroscopy**—Uniformly ^15^N-labeled freeze-dried PF4 or PF4FM2 were dissolved in H3O3O3O (9:1) mixture containing 20 mM NaCl. pH was adjusted to 5.0 by adding 1-μl increments of 0.1 M HCl or 0.1 M NaOH to the samples. These solutions of PF4 or PF4FM2 were used alone and with the addition of increasing concentrations of unlabeled IL8. After each addition, the pH of the solution was adjusted to maintain the same value.

Two-dimensional ^1H-^15N HSQC spectra of PF4 and PF4FM2 were recorded to monitor chemical shift changes upon addition of increasing concentrations of IL8. The chemical shifts are quoted in parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentane sulfonate. HSQC spectra were acquired at 40 °C on a Varian Unity Inova 600-MHz spectrometer equipped with an H/C/N triple-resonance probe and x/2/2/2/2 pulse field gradient unit. The solvent deuterium signal was used as a field-frequency lock. Carrier frequencies for ^15^N and ^1H were positioned at 116.5 and 2.5 ppm, respectively. A gradient sensitivity-enhanced version of two-dimensional ^1H-15^N HSQC was applied with 400 (t1) × 2048 (t2) complex data points and spectral widths of 2500 Hz in r1 (^15^N) and 9000 Hz in r2 (^1H) dimensions. Raw data were converted and processed by using NMRPipe (23) and were analyzed by using NMRView (24).

**NMR Diffusion Measurements**—Diffusion measurements were performed on the same NMR spectrometer. The maximum magnitude of the gradient, g, was calibrated using Varian deuterated water standard. Measurements were performed using WIF-PGSE pulse sequence (25), an improved pulsed gradient spin-echo (PGSE) pulse sequence that employs water flip-back and WATERGATE (26) combined with a heteronuclear filter and allows determination of the diffusion coefficient of labeled protein in a mixture.

The diffusion coefficient, D, of the molecule of interest, was estimated from the diffusion attenuation of spin-echo by using Equation 1,

\[
A(g^2) = A(0) \exp(-\gamma^2 g^2 D \Delta - \delta/3) \tag{1}
\]

where γ is the gyromagnetic ratio for protons; δ is the duration of the pulsed field gradient, and Δ is the spacing between the front edges of pulsed field gradients.

**Determination of Equilibrium Constants Using Diffusion Data**—Using diffusion data and known dissociation equilibrium constants for pure native PF4, PF4FM2, and IL8 (7, 14–16, 33), the equilibrium dissociation constant, KD, was estimated for PF4/IL8 and PF4FM2/IL8 heterodimers as described below.

The distribution of PF4 and IL8 homoggregates in the solution is readily obtained by using the following Equations 2 and 3,

\[
c = cM + 2cD + 4cT \tag{2}
\]

\[
K_D = \frac{c_M c_D}{c_D} \quad K_T = \frac{c_M c_T}{c_T} \tag{3}
\]

where c is the total molar concentration of protein; cM, cD, and cT are the concentrations of monomers, dimers, and tetramers, respectively, and KD and KT are the equilibrium dissociation constants for dimer and tetramer, respectively. When PF4 and IL8 are mixed together, aggregate redistribution occurs according to Equation 4,

\[
T_{PF4} \rightleftharpoons D_{PF4} + D_{PF4} \tag{4}
\]

\[
D_{PF4} \rightleftharpoons M_{PF4} + M_{PF4} \tag{5}
\]

\[
K_D = \frac{c_D}{c_T} \tag{6}
\]

where M, D, T, and HD denote monomer, dimer, tetramer, and heterodimer, respectively. Equation 2, which gives the total concentration of protein, can therefore be rewritten to include the concentration of heterodimers, cHD, as shown in Equation 5,

\[
c = c_M + 2c_D + 4c_T + c_{HD} \tag{5}
\]

Here there are four unknowns. Thus, three additional equations are required to calculate the concentration of monomers, homodimers, homotetramers, and heterodimers. By assuming that the equilibrium dissociation constants for homoaggregation remain the same upon mixing IL8 and PF4 (or PF4FM2), two of these equations are known (see Equation 3), and the third (Equation 6) describes the diffusion coefficient for PF4,

\[
P_M = \frac{c_M}{c} \quad P_D = \frac{2c_D}{c} \quad P_T = \frac{4c_T}{c} \quad P_{HD} = \frac{c_{HD}}{c} \tag{6}
\]

\[
D_D = \frac{1}{2} D_M \quad D_T = \frac{1}{3} D_M \tag{7}
\]

Solving Equations 3, 5, and 6 yields the concentration of homodimers, monomers, homotetramers, and heterodimers. The PF4/IL8 heterodimer dissociation constant, KD, was estimated by using Equation 8,

\[
K_D = \frac{c_{HD} c_{M} c_{D} / c_{M} c_{D}}{c_{HD}} \tag{8}
\]

Molecular Dynamics Simulations—The x-ray or NMR structures of PF4 (6), PF4FM2 (7), and IL8 (8) were taken from the Protein Data Bank (27). The Protein Data Bank entries were 1RHP, 1PFN, and 1IL8. Crystallographic water molecules were deleted. Hydrogen atoms were added to the crystal structure by using the HBUILD module of CHARMM (28). The ionization state of the system was set at pH 5.0, the pH value used in experimental studies. At this pH value, the total charge on monomers was +1 (PF4), +11e (PF4FM2), and +6e (IL8).

Molecular dynamics simulations were performed for homodimers of IL8, PF4, and PF4FM2 and heterodimers of PF4/IL8 and PF4FM2/IL8. In all instances, two types of dimers were constructed, AB-type and β-sheet sandwiched type. PF4/IL8 and PF4FM2/IL8 AB-type heterodimers were formed by manually replacing one of the monomer subunits from an AB-type dimer of native PF4 or PF4FM2 with a monomer subunit from IL8, following superposition of PF4 or PF4FM2 and IL8 homodimers. To construct PF4/IL8 and PF4FM2/IL8 β-sheet sandwiched heterodimers, two adjacent and overlapping monomers from each AB-type dimer in the PF4 tetramer were first removed, leaving the β-sheet sandwiched homodimer. Then an IL8 monomer was superimposed onto one of the sandwiched monomer subunits and that PF4 subunit was deleted. All initial structures were built using the Insight-II program (Biosym Technologies Inc., San Diego, CA).

Molecular dynamics simulations were performed using the c29h2 version of CHARMM (28). A one nanosecond trajectory was simulated.
for each hetero- and homodimer. The time step in these simulations was 1 fs, and coordinates were saved at 1-ps time intervals. All dimers were simulated in a 74 × 63 × 63 Å³ explicitly solvated periodic box. After solvation, water molecules closer than 2.8 Å from the protein, as well as from other water molecules, were removed. To make the total charge of the box 0, Cl⁻ ions were added to the water box. Simulations were carried out using the version 22 all-hydrogen force field (29) with a dielectric constant of 1.0. Van der Waals interactions were truncated at 13 Å by using a shifted smoothing function, and electrostatic interactions were calculated with the particle mesh Ewald method (30). Each simulation was initialized with 2000 steps of steepest descent minimization, followed by gradual heating to 300 K and 5000 steps of system equilibration. The temperature during simulations was maintained at 300 K. Bond lengths involving hydrogen atoms were constrained using the SHAKE method (31).

Endothelial Cell Proliferation—Human umbilical vein-derived endothelial cells (HUVECs) were harvested from normal human umbilical cords by perfusion with 0.125% trypsin/EDTA. Harvested HUVECs were cultured in gelatin-coated tissue culture flasks and subcultured 1:3 once a week in culture medium (RPMI 1640 with 20% human serum, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin). Proliferation of basic fibroblast growth factor-stimulated (10 ng/ml) HUVEC cultures was measured by quantification of [3H]thymidine incorporation and expressed as mean counts/min of triplicate cultures in three independent experiments (± S.E.). EC were seeded at 5000 cells/well in flat-bottomed fibronectin-coated tissue culture plates and grown for 3 days in the absence or presence of regulators, in culture medium. During the last 6 h of the assay, the culture was pulsed with 0.3 μCi of [methyl-3H]thymidine/well.

Chemotaxis of Vector-transfected Baf3 Cells—Migration of Baf3 cells, bone marrow-derived pro-B-cell line transfected with the human CXCR1 or CXCR2 receptors, in response to IL8 in the absence and presence of PF4 was tested using Boyden chambers following standard procedure (32). Briefly, 2 × 10⁵ Baf3/hCXCR2 or Baf3/hCXCR1 cells were plated in 100 μl of Cell’s balanced salt solution supplemented with 1 mg/ml of bovine serum albumin into the upper chamber of a 96-well chemotaxis plate (NeuroProbe, Cabin John, MD) equipped with a 5-μm pore size polycarbonate filter. The average number of receptors per cell was estimated to be 1 × 10⁶ as determined by using fluorescein-labeled receptor antibodies and fluorescence-activated cell sorter analysis. Varying concentrations of chemoattractants (IL8 alone or together with PF4) were added to the lower chamber in aliquots of 75 μl. After incubation for 3 h at 37 °C in a 5% CO₂ incubator, cells that migrated to the lower chamber were transferred to a new 96-well plate and stained overnight at 37 °C with Reasurin (R & D Systems). The relative fluorescence from each well was determined using a spectrophotometer equipped with filters for excitation at 544 nm and emission at 580 nm. The number of migrated cells was expressed in relative fluorescence units (RFU).

Chemotaxis dose-response curves were analyzed using the following hyperbolic function shown in Equation 9,

\[
RFU = RFU_{\text{max}} \frac{[D-R]}{[D-R] + K_D} \tag{9}
\]

where [D-R] is the molar concentration of IL8 dimer (D)-receptor (R) complexes, and \( K_D \) is the value of [D-R] that elicits half-maximal RFU. Assuming that IL8 dimers bind to receptor as expressed in Equation 10,

\[
D + R \leftrightarrow DR \tag{10}
\]

the concentration [D-R] is found using the law of mass action and is expressed in terms of the total number of receptors, \( R_0 \), as given in Equation 11,

\[
[D-R] = \frac{[R_0][D]}{K_{DR} + [D]} \tag{11}
\]

where [D] is the concentration of IL8 dimers, and \( K_{DR} \) is the equilibrium dissociation constant for the DR complex.

Combining Equations 9 and 11 yields the following dependence of RFU on the IL8 dimer concentration as shown in Equation 12,

\[
RFU = RFU_{\text{max}} \frac{[R_0] + [D]}{[D] + \frac{1}{K_{DR} + [D]}} \tag{12}
\]

The same equation for PF4/IL8 heterodimer receptor binding was obtained by assuming that binding of the heterodimer to the receptor is described by the following equilibrium shown in Equation 13.

\[
K_{HDR} \quad HD + R \leftrightarrow HDR \tag{13}
\]

RESULTS

Interactions between PF4 (PF4M2) and IL8 Defined Using NMR—To investigate molecular interactions between PF4 and IL8, nonisotopically enriched IL8 was titrated into a solution of uniformly ¹⁵N-enriched PF4, and chemical shift changes in ¹⁵N-PF4 were monitored by recording ¹H-¹⁵N HSQC spectra upon addition of IL8. Fig. 1A shows two overlayed HSQC spectra, one with ¹⁵N-PF4 alone (blue cross-peaks) and the other with ¹⁵N-PF4 and IL8 at a molar ratio of 1:1 (red cross-peaks). Notice in Fig. 1A that PF4 cross-peaks are not all symmetric.
and, in fact, appear as multiple resonances for most spin systems. This arises for two reasons. 1) PF4 exists in solution in a distribution of monomer-dimer-tetramer states that undergo slow exchange on the chemical shift time scale (15, 16). 2) PF4 monomer/dimer subunits associate into tetramers asymmetrically (6). Under conditions of the present experiments, the latter reason is primary because PF4 tetramers predominate (33). In this regard, PF4 resonance assignments cannot be definitively made but can be tentatively made for some residues by analogy with $^1$H-$^{15}$N HSQC spectra of homologous PF4M2 as presented in Fig. 1B (blue cross-peaks). PF4M2 is a PF4 chimera in which the N terminus of PF4 is replaced with the N terminus of IL8. Because PF4M2 forms symmetric tetramers resulting in a well defined set of cross-peaks, complete $^1$H-$^{15}$N resonance assignments and structure analysis were possible (7). By comparing $^1$H-$^{15}$N HSQC spectra for PF4M2 and native PF4, some tentatively assigned cross-peaks for native PF4 have been labeled in the Fig. 1A.

Addition of IL8 to $^{15}$N-PF4 induces chemical shift changes in many resonances. Some sets of multiple resonances for a given residue appear to coalesce or to vary their intensity ratios as exemplified in Fig. 2 for residues His-23, His-35, Ala-43, and Gly-48. In other instances, apparent single cross-peaks merely shift. This is illustrated for residues Ile-42 and Ile-63 at the bottom of Fig. 2. Overall, observed chemical shift changes for $^1$H and $^{15}$N range up to 0.06 and 0.5 ppm, respectively. Some resonances are more affected than others, whereas some apparently are not affected, supporting the idea that interactions between PF4 and IL8 are specific. The change in chemical shift for tentatively assigned residues is plotted in bar graph format in Fig. 3A. The largest chemical shift changes (for cross-peaks that could be assigned) are observed for residues located at the interface of monomer subunits, which from analysis of the x-ray structure of native PF4 (6) are as follows: $\beta$-strands 1 and 2 of the antiparallel $\beta$-sheet along with the loop that connects them (residues 25–45), the hairpin which connects $\beta$-strands 2 and 3 (residues 46–49), a part of the C-terminal $\alpha$-helix (residues 64–70), and various residues within the N terminus like His-23. Tyr-60 changes significantly, most likely because it is a bulky aromatic residue that is sandwiched between the affected C-terminal helix and the $\beta$-sheet.

PF4M2 is a chimera of native PF4 in which the first 11 N-terminal residues of native PF4 are substituted by the first 8 N-terminal residues of IL8. PF4M2 functions biologically like native PF4 (7). However, PF4M2 forms symmetric tetramers, greatly simplifying its NMR spectra and allowing nearly complete $^1$H-$^{15}$N resonance assignments to be made and the structure to be determined (7). Performing the same IL8 titration study using $^{15}$N-labeled PF4M2 has allowed more detailed insight into how PF4 and IL8 interact. Two $^1$H/$^{15}$N HSQC spectra are shown in Fig. 1B for free PF4M2 (blue cross-peaks) and PF4M2 with IL8 at a molar ratio of 1:1 (red cross-peaks). Resonance assignments for PF4M2 are indicated. Addition of IL8 induces numerous PF4M2 resonances to shift. Chemical shift changes however are observed to be less dramatic than with native PF4. As with native PF4, resonances that experi-
ence the largest chemical shift changes are associated with residues located at the interfaces between monomer subunits (Fig. 3B).

Overall, these NMR data indicate that PF4 and PF4M2 subunits are exchangeable with IL8 subunits and can associate to form heteroaggregates. It is important also to realize that although IL8-induced chemical shift changes in PF4 and PF4M2 are significant, they are not major changes to the spectra and indicate that the general fold of the sequence is maintained in heteroaggregates. The reason why some chemical shift differences are greater in PF4 than in PF4M2, is structurally evident. The main quaternary structural difference between PF4 and PF4M2 arises from a difference in electrostatic repulsion between N-terminal segments and other parts of the molecule, essentially resulting in formation of asymmetric and symmetric tetramers for PF4 and PF4M2, respectively. Comparatively, the C-terminal helix in PF4M2 (residues 58–70) is shifted slightly relative to the β-sheet domain, leading to an increased intersubunit distance between helices in two adjacent monomers. Because N-terminal residues in PF4M2 and IL8 are the same, mutual orientation of PF4M2 and IL8 monomers in heteroaggregates differs more from that in the native PF4/PF4 homoaggregate. Therefore, chemical shifts of C-terminal residues are observed to be more perturbed in the native PF4/IL8 heteroaggregate. The same is true for residues in the region around His-23. Because most N-terminal residues in native PF4 could not be assigned, little information about chemical shift changes at the N terminus of native PF4 could be derived. However, based on PF4M2 chemical shift data, N-terminal residues are indeed affected by addition of IL8.

The stoichiometry of subunit association in the heteroaggregate is another crucial point. Because the normalized average change in chemical shift plotted versus the PF4/IL8 ratio (Fig. 4A) plateaus at about a PF4/IL8 molar ratio of 1:1, one can conclude that the stoichiometry of association for PF4 and IL8 is 1:1. The same can be said for formation of PF4M2/IL8 heteroaggregates, as illustrated in Fig. 4B.

Heteroaggregates Are Dimers and Their Formation Disrupts Normal Tetramerization—Although at this point one can conclude that PF4 and IL8 interact to form heteroaggregates with a 1:1 stoichiometry of association, the size of the heteroaggregate has yet to be established. At millimolar concentration, IL8 forms dimers (8), whereas PF4 and PF4M2 both primarily form tetramers (6, 14, 15, 25, 33); therefore, formation of either or both heterodimers and heterotetramers could be possible. To address this question, diffusion coefficients for 15N-labeled PF4 and 15N-labeled PF4M2, before and after addition of IL8, were determined. The diffusion coefficient is inversely proportional to the radius of a diffusing unit, i.e. aggregation state (34–37). Fig. 5 illustrates diffusion coefficients, D, for PF4 and PF4M2, pure and mixed with IL8, versus total protein concentration. For these measurements, the ratio of IL8 to PF4 or PF4M2 was held constant at 1:2 for all concentrations. Data were collected at 40 °C, and solution conditions were kept the same at all concentrations: 90% H2O, 10% D2O, 20 mM NaCl, pH 5.0.
some controversy as to what was the actual highest aggregation state for PF4M2, dimer or tetramer (18), but these data resolve that question, i.e. tetramer is the highest aggregation state for PF4M2, just as it is for native PF4. At some intermediate concentrations, PF4M2 appears to be more dissociated than native PF4, which is consistent with the equilibrium association constants for dimer and tetramer formation being somewhat smaller for PF4M2 than for native PF4 (7).

At any given total protein concentration, the diffusion coefficient of PF4 or PF4M2 to which IL8 was added (Fig. 5, solid and open circles) is greater than the diffusion coefficient of PF4 or PF4M2 alone (Fig. 5, solid and open squares). Because solution conditions are the same in both cases, this increase in the diffusion coefficient can be attributed to formation of heterodimers. This result correlates with the simplification of cross-peak patterns in HSQC spectra of native PF4 observed upon addition of IL8 (see Fig. 1A). Upon addition of IL8 to PF4M2, the diffusion coefficient for 15N-PF4M2 at all concentrations has increased to a value expected for the dimer state as estimated by using Equation 7. By using Equation 7, one is always concerned about changes in shape and how that might affect interpretations of the diffusion coefficient. However, based on structural homology of CXC chemokines and their nearly spherical shape, the effective Stokes radius would be essentially the same for homo- and heterodimers or tetramers. Moreover, AB-type and β-sandwich dimers also have nearly the same overall shape. Thus, one can conclude that addition of IL8 to PF4M2 at a molar ratio of 2:1 leads to apparently complete dissociation of PF4M2 tetramers and formation of PF4M2/IL8 heterodimers.

By using these diffusion data and known dissociation equilibrium constants for pure native PF4 (90 μM (dimers) and 3 μM (tetramers)) (15, 16, 33), for PF4M2 (500 μM (dimers) and 10 μM (tetramers)) (7), and for IL8 (18 μM (forms dimers only) (14), the magnitude of heterodimer equilibrium dissociation constants, $K_{HD}$, was determined by using the approach outlined under “Experimental Procedures.” There are several values reported for the IL8 dimer dissociation constant as determined using sedimentation, fluorescence, and calorimetry, respectively: 10 (17), 18 (14), 21 μM (38). All these values were determined under slightly different solution conditions from those used in the present study. We chose that value that was determined under conditions closest to our own.

Initially, it was apparent that PF4/IL8 or PF4M2/IL8 heterodimers would not form unless the $K_d$ value for heterodimers was smaller than that for PF4 or PF4M2 homotetramers and about the same as that for IL8 homodimers. This restricts the range for the $K_d$ value of heterodimers. Furthermore, from titration studies with IL8 and native PF4, PF4 tetramer dissociation was only partial, whereas PF4M2 dissociation was nearly complete. If one assumes that IL8 forms only heterodimers with native PF4 as it does with PF4M2, then native PF4/IL8 heterodimers must be less stable than PF4M2/IL8 heterodimers, and the heterodimer $K_D$ value for PF4/IL8 heterodimers must have a value larger than that for PF4M2/IL8 heterodimers.

Values for the concentrations of monomers, dimers, and tetramers were calculated by using Equations 2 and 3 for pure PF4, PF4M2, and IL8 and are shown in Table I (columns 2–4) as fractions of total protein concentration. Comparison of monomer fractions of pure PF4 and pure IL8 clarifies what occurs when these two proteins are combined in solution. Initially, there is an excess of PF4 monomers. IL8 monomers dimerize with structurally homologous PF4 monomers, resulting in redistribution of PF4 and IL8 homoaggregates as defined by Equation 4. Solving Equations 3, 5, and 6 yields the concentration of homo- and heteroaggregate species given in Table I (columns 5–7) for the 2:1 ratio of IL8/PF4. Addition of IL8 causes the fraction of PF4 tetramers to decrease and that of heterodimers to increase. A similar process occurs in a solution of PF4M2 and IL8. Dissociation constants, estimated using Equation 7, are found to be about $1 \times 10^{-6}$ M for PF4/IL8 heterodimers and $7 \times 10^{-7}$ M for PF4M2/IL8 heterodimers. These $K_{HD}$ values indicate somewhat stronger subunit associations in the heterodimer compared with the IL8 homodimer and considerably stronger subunit associations compared with those of PF4 and PF4M2 homodimers.

**Heterodimers are AB-type Dimers**—This conclusion arises primarily from two pieces of evidence: correlation of chemical shift changes with structure, and dimer stability derived from molecular dynamics simulations. As noted above for native PF4 and PF4M2 in the presence of IL8, most residues that give rise to the largest chemical shift changes lie at the interface between monomer subunits (Fig. 3). These IL8-induced chemical shift changes with PF4 and PF4M2 nevertheless would be expected for perturbations in both AB-type and β-sandwich-type interactions. However, the IL8 monomer that participates in the other half of the heterodimer is not known to form dimers other than the AB-type (8–10), and PF4 and PF4M2 AB-type dimers are thermodynamically more stable than their β-sandwich-type dimers (6, 7). Overall, this supports the idea that IL8, in the presence of PF4 and PF4M2, induces formation of AB-type heterodimers.

Further support for formation of AB-type heterodimers comes from exhaustive molecular dynamics simulations of PF4-MF and PF4-M2IL8 complexes. Heterodimer structures were first built using the Insight-II program (Biosym Technologies Inc., San Diego) and crystallographic or NMR-derived coordinates for these CXC chemokines. Heterodimers were formed by manually replacing one of the monomer subunits from an AB-type dimer of native PF4 or PF4M2 and a monomer subunit from a β-sheet sandwich dimer of PF4 with a monomer subunit from the IL8 dimer, and aligning the two subunits to maintain the same general inter-subunit orientation as that found in the respective homodimer. These initial heterodimeric structures were then energy-minimized at 300 K and subjected to a 1-ns molecular dynamics simulation in explicit solvent using CHARMM (27). Molecular dynamics trajectories were

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**TABLE I**

Fractional populations of homo- and heteroaggregates in pure solutions of PF4, PF4M2, and IL8, and in solutions of PF4 and PF4M2 to which IL8 is added at a molar ratio of 1:2, respectively.

| Oligomeric state | Pure in solution | Combination of PF4 and IL8 | Combination of PF4M2 and IL8 |
|------------------|------------------|----------------------------|-----------------------------|
|                   | PF4              | PF4M2                      | IL8                         | PF4M2 | IL8 |
| Monomer           | 0.09             | 0.25                       | 0.09                        | 0.08  | 0.01 |
| Dimer             | 0.08             | 0.01                       | 0.91                        | 0.06  | 0.50 |
| Tetramer          | 0.83             | 0.74                       | 0.47                        | 0.39  | 0.19 |

* Fractional populations are calculated for protein molar concentrations of 0.43 mM (PF4 or PF4M2) and 0.86 mM (IL8), when present pure in solution (left side of table) or in combination with each other (right side of table).
analyzed, and backbone heavy atom root mean square deviation (r.m.s.d.) values were calculated as a function of time as plotted in Fig. 6, A and B.

During the entire 1-ns simulation, two monomer subunits remained together in all hetero- and homodimer combinations. Root mean square deviations fluctuated greatly at the beginning of the simulation because initial structures were modeled and taken as being minimized but were not heated and equilibrated. However, r.m.s.d. values did level out by about 200 ps and fluctuated within less than 1 Å over the course of the simulation, indicating that heterodimers form stable complexes. Time-averaged fluctuations, calculated for each residue, are plotted in Fig. 6, C and D. Reasonably good correlations are observed between the amplitude of fluctuation and the location of a given residue within elements of secondary structure. As expected, residues experiencing the largest fluctuations were terminal residues or residues within loops. Root mean square deviations for residues within β-strands or the α-helix were less than 1 Å. Moreover, the amplitude of fluctuations was essentially the same in AB-type PF4/IL8 and PF4M2/IL8 heterodimers as in homodimers, further indicating formation of stable structures. Note that residues within the β-strands or α-helix in β-sheet-sandwiched dimers experienced larger fluctuations than in AB-type dimers, indicating that they are less stable than AB-type heterodimers. This supports NMR data for the formation of AB-type heterodimers. The resulting AB type PF4/IL8 heterodimer is inset into Fig. 6A.

PF4 and IL8 Heterodimerization Modulates Biological Function—Aside from its ability to bind heparin, PF4 is a known anti-angiogenic agent, acting via inhibition of endothelial cell proliferation (11). At a concentration of 2.5 μM, PF4 normally inhibits proliferation of cultured EC by about 40–50% (39). Alternatively, IL8, depending on its concentration, can be a stimulator of angiogenesis (12). To demonstrate functional significance of PF4/IL8 heterodimer formation, we tested the endothelial cell proliferation in the presence of PF4 or IL8 alone and in combination. These data are presented in Fig. 7A. PF4 alone (2.5 μM) inhibits EC proliferation, as expected. At similar concentrations, IL8 has no significant effect on EC proliferation. Most interestingly, the combination of PF4 and IL8 exhibits a dramatic effect by increasing the antiproliferative effect of PF4. This effect was found to be dependent on the molar ratio of PF4 to IL8. Thus, if a small amount of IL8 is added to the PF4 solution (a PF4/IL8 ratio of up to 1:0.5), only a weak effect on EC proliferation, if any at all, is observed.

By using dissociation constants for homoaggregates and heterodimers of PF4 and IL8 discussed above, the distribution of aggregate states was calculated for the concentrations of PF4 and IL8 used in this study (Fig. 7B). As the concentration of IL8 is increased, the amount of PF4 monomers, dimers, and tetramers is decreased because of the formation of heterodimers. The population of heterodimers increases up to a PF4/IL8 molar ratio of 1:1 and then levels out as more IL8 is added. Because IL8 alone does not show any significant effect on EC proliferation, the enhanced effect observed at these PF4/IL8 ratios can be explained simply by the presence of heterodimers.

The modulation in biological activity of IL8 associated with the presence of PF4 was assessed through the measurement of the IL8-induced migration of vector-transfected BaF3/hCXCR2 and BaF3/hCXCR1 cells. As expected, BaF3/hCXCR2 cells migrated toward pure IL8 in a dose-dependent manner (Fig. 8A, solid circles), whereas they do not migrate toward pure PF4 at any PF4 concentration studied (solid squares). If we perform the same migration assay varying the concentration of PF4 at a fixed concentration of IL8 (1 ng/ml open triangles) and 0.1 ng/ml (open diamonds)), we find that the presence of PF4 has a
significant effect on Baf3/hCXCR2 cell migration. Although a constant number of these cells migrate toward IL8 (1 ng/ml) over the PF4 concentration range of 0.01 to near 1 ng/ml, the number of migrating cells increases dramatically as the concentration of PF4 is increased from 1 to 100 ng/ml (Fig. 8A, open triangles). Essentially the same observation is made for chemotaxis data acquired at the fixed IL8 concentration of 0.1 ng/ml as the PF4 concentration is varied over the same range (Fig. 8A, open diamonds).

The enhancement in chemotaxis of Baf3/hCXCR2 cells upon addition of PF4 can be readily explained by considering populations of IL8 and PF4 monomers, dimers, tetramers, and PF4/IL8 heterodimers derived using the $K_{DH}$ values as discussed above. Some of these values are shown in Fig. 8B. PF4/IL8 heterodimers form when PF4 is added to the IL8 solution. From the results presented in Fig. 8A, there are populations for two fixed IL8 concentrations to be considered, 1 and 0.1 ng/ml. First, the higher fixed IL8 concentration of 1 ng/ml will be analyzed. In this case, the population of heterodimers (Fig. 8B, solid squares) is negligibly small at low PF4 concentrations from 0.01 to 1 ng/ml. Therefore, the concentration of IL8 monomers (Fig. 8B, open circles) and dimers (crossed circles) remains essentially constant, and the chemotactic response of Baf3/hCXCR2 cells remains unchanged (see Fig. 8A). The concentrations of IL8 monomers and dimers are shown expanded in Fig. 8B, insets. However, as the concentration of PF4 is increased further to 100 ng/ml, the population of heterodimers becomes substantial. The same results are found when varying the PF4 concentration at the fixed IL8 concentration of 0.1 ng/ml (Fig. 8B, half-open squares). If heterodimers had no functional effect, chemotactic activity should have remained the same or have decreased from 1 ng/ml of IL8 or from 0.1 ng/ml of IL8 because the number of IL8 monomers and dimers decreased (Fig. 8B, insets); however, it increased. Therefore, these data allow us to conclude that PF4/IL8 heterodimers are responsible for modulating IL8-mediated chemotaxis of Baf3/hCXCR2 cells.

We then tried the same chemotaxis experiments using Baf3/hCXCR1 cells to assess effects from the other IL8 receptor. The chemotaxis curve from IL8 alone using Baf3/hCXCR1 cells was essentially the same as that observed using Baf3/hCXCR2 vector-transfected cells. However, there was no difference on migration of Baf3/hCXCR1 cells when PF4 was added at any concentration investigated (Fig. 8A, half-opened triangles). This observation reflects the difference in IL8 binding to CXCR1 and CXCR2 receptors. Recently, it was shown that IL8 dimer dissociation is essential for IL8 binding to the CXCR1 receptor. With this IL8 receptor, only IL8 monomers apparently bind CXCR1 (40). Because the concentration of IL8 monomers dominates at any concentration of PF4 investigated, the number of migrating Baf3 cells transfected with CXCR1 receptors remains constant. On the other hand, it was clear above that the number of Baf3 cells transfected with CXCR2 receptors increases upon addition of PF4 at a fixed concentration of IL8. A plausible explanation for this is that IL8 dimers, rather than monomers, bind the CXCR2 receptor (41). The concentration of IL8 dimers calculated when IL8 alone is present in solution is indicated by crosses in Fig. 8B. When IL8 and PF4 are present together in solution, the population of heterodimers at PF4 concentrations above 1 ng/ml becomes larger than that of IL8 dimers and comparable with that of IL8 dimers when IL8 is alone in solution. Therefore, heterodimers probably interact with the CXCR2 receptor the same as IL8 dimers.

If we assume that only IL8 dimers or PF4/IL8 heterodimers bind the CXCR2 receptor, we can estimate the dissociation constant for the heterodimer-CXCR2 receptor complex by using these chemotaxis data. Fig. 8C shows the number of migrating Baf3/hCXCR2 cells expressed in terms of relative fluorescence units replotted here from Fig. 8A as a function of the concentration of IL8 dimers in the case of pure IL8 (circles) and of PF4/IL8 heterodimers in the case of a mixture of IL8 and PF4 (triangles for 1 ng/ml IL8 and diamonds for 0.1 ng/ml IL8). These two dependences were simultaneously fit to Equation 12 by using the parameters $RF_{max}$, $KE$, $K_{DR}$, and $K_{HDR}$, as defined in Experimental Procedures. $KE$ was shared for all curves during fitting, and $K_{DR}$ was constrained to a range of $1 \times 10^{-6}$ to $1 \times 10^{-5} \text{M}$ as given in the literature (42). The CXCR2 receptor number was $1 \times 10^{5}$/cell, and the number of cells was $2 \times 10^{5}$. The lines shown in Fig. 8C are the results of fits to Equation 12. The value for the heterodimer/CXCR2 receptor binding constant, $K_{HDR}$, is larger than the IL8/CXCR2 receptor binding constant, $K_{DR}$, and is estimated to be $1 \times 10^{-5} \text{M}$. In other words, IL8 binds CXCR2 receptor more strongly than does the heterodimer.

**DISCUSSION**

From the present study, we can make two biologically relevant conclusions concerning these CXC chemokines. 1) PF4 tetramers/dimers and IL8 dimers can readily exchange subunits to form...
FIG. 8. Effect of heterodimerization on IL8-induced chemotaxis of Baf3 cells, vector-transfected with the human CXCR1 or CXCR2 receptors. A, chemotactic response of Baf3/hCXCR1 cells to pure IL8 (solid circles). Chemotactic response of Baf3/hCXCR1 cells to IL8 at 1 ng/ml upon addition of PF4 (half-opened triangles), and from Baf3/hCXCR2 cells to IL8 at 1 ng/ml (open triangles) or at 0.1 ng/ml (open diamonds) upon addition of PF4. Chemotactic response of Baf3/hCXCR2 cells to pure PF4 (solid squares). Solid lines represent the best fit of experimental dose-response curves. B, with the concentrations used in this assay and the known $K_d$ values for homo- and heteroaggregate formation, concentrations of all possible monomer, dimer, and tetramer species were calculated. Concentrations of IL8 monomers (open circles), dimers (crossed circles), and PF4/IL8 heterodimers for IL8 fixed at 1 ng/ml (solid squares); PF4/IL8 heterodimers for IL8 fixed at 0.1 ng/ml (half-opened squares); and IL8 dimers for pure IL8 (+) are shown. The concentrations of IL8 monomers and dimers are shown expanded in the insets and to B, C, the number of migrating Baf3/hCXCR2 cells replotted from A as a function of the concentration of IL8 dimers in the case of pure IL8 (circles) and of PF4/IL8 heterodimers in the case of a mixture of IL8 and PF4 (triangles for 1 ng/ml IL8, and diamonds for 0.1 ng/ml IL8). Solid lines represent results of fitting the data using Equation 12.
effects resulting from mixed CXC and CC chemokines on proliferation of human myeloid progenitor cells. In essence, they found that whereas certain chemokines (CXC chemokines GRO-β, PF4, IL8, as well as CC chemokines MIP-1α and MCAF) could suppress colony formation of immature subsets of myeloid progenitor cells stimulated by granulocyte-macrophage colony-stimulating factor and Steel Factor, various combinations of these chemokines demonstrated synergistic suppression of colony formation. Furthermore, whereas other chemokines (CXC chemokines GRO-α, NAP-2, and MIP-2β or GRO-γ, as well as CC chemokines MIP-1β and RANTES) had no suppressive effects alone or in combination, a molar excess of MIP-1β blocked the suppressive effects from MIP-1α, and a molar excess of MIP-2β or GRO-α could block the suppressive effects from IL8 or PF4. These authors offered no mechanistic explanation for these observations, expect to propose that “the mechanisms involved in these chemokine suppressive and synergistic activities are presently unknown . . . but probably involve intracellular signaling events such as protein phosphorylation and/or immediate early or later gene responses.” Our present studies provide a plausible explanation of heterodimer formation to their very interesting observations.

The extension of these findings to what occurs in vivo is less clear for two main reasons. First of all, relative concentrations of PF4 and IL8 are different in vivo from those used in this study. In blood serum, PF4 is present at micromolar to nanomolar concentrations, whereas IL8 has been reported to be present at nanomolar to picomolar levels (14, 17). Second, relative association binding constants, which dictate the extent of heterodimerization, can be different in vivo from those values determined here, because solution conditions vary considerably, i.e., temperature, pH, salt, and the presence of numerous other biomolecules in the serum milieu. In addition, chemokines are co-localized in vivo, and for example, under pathologic conditions following tissue damage, they are immediately available locally at micromolar concentrations (46, 47). Therefore, it is highly probable that CXC chemokine heteroaggregates do form in vivo. It is merely difficult to quantify that formation. PF4, for example, is found associated in vivo with heparan sulfate on the surface of endothelial cells. However, the presence of IL8, and probably other CXC chemokines, could shift that cell surface-bound state equilibrium by formation of PF4/IL8 heteroaggregates. Considering IL8 alone, the influx of this CXC chemokine would have to be on the micromolar scale to have a significant effect on PF4-mediated biology. As mentioned above, IL8 is normally present in serum at nanomolar to picomolar levels, and other CXC chemokines would have to be present in order to effect formation of significant amounts of PF4 heteroaggregates in serum.

On the other hand, it would appear that IL8, which is mostly monomeric at its reported physiologic concentration, would be mostly sequestered as PF4/IL8 heterodimers in serum, also depending on the serum concentration of PF4. IL8 monomers interact with CXC CR1 receptors (40), whereas IL8 dimers and PF4/IL8 heterodimers interact with CXC CR2 receptors. Changing IL8 aggregate states and heterodimer formation could be the switch for IL8 between its two receptors. At low IL8 concentration in the serum where mostly IL8 monomers exist, IL8 would act primarily through CR1, but when PF4 is added to the mix and heterodimers form, CR2 would be brought more into play. At higher IL8 concentrations, when CR2 receptors are in play, addition of PF4 might modulate that response as well. This biological control mechanism certainly depends on the relative concentrations of IL8 and PF4, as well as possibly on other CXC chemokines. Another finding here is that the IL8 dimers bind CR2 receptors more strongly than do the PF4/IL8 heterodimers. Presumably, the reason for this lies in structural differences between the IL8 dimer and the PF4/IL8 heterodimer. However, we also showed above that the overall folds of IL8 dimers and heterodimers are conserved; therefore, it must be that both subunits from either dimer interact with the CR2 receptor and that those contacts from the PF4 subunit in the heterodimer somehow weaken interaction with the CR2 receptor by about 10-fold. This differential binding to CR2 presents an additional level of biological control.

Heterodimer formation also raises another question. IL8 in serum is usually quantified by using anti-IL8 antibodies in an enzyme-linked immunosorbent assay. If the antibody epitope on IL8 is somehow modified in the chemokine heterodimer, the amount of IL8 in serum may be underestimated. If this were true, the actual concentration of IL8 in serum could be much higher, even falling into the micromolar regime.

Formation of PF4/IL8 heterodimers also suggests a possible effect of PF4 on hematopoiesis by PF4 interacting with IL8 and/or other CXC chemokines, and thus attenuating (or enhancing) the capacity to induce signaling. Formation of PF4/IL8 heterodimers clearly enhances the ability PF4 to act as an anti-proliferative agent against endothelial cells. This observation alone has therapeutic implications for the use of PF4 in the clinic as an anti-angiogenic and/or anti-tumor agent. PF4 by itself exhibits limited effectivity as an anti-cancer agent in the clinic; however, if PF4 and IL8, for example, were administered jointly and/or as a conjugate or fusion product, the effects should be synergistic as observed here in vitro.

In conclusion, we propose that heteroaggregate formation between other structurally homologous chemokines, as well as possible in other structurally homologous cytokine subfamilies in general, modulates biologic function on the molecular level. Further study into and validation of this seminal hypothesis are required.

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Note Added in Proof—Soon after this paper was prepublished online, Paulettd et al. (48) reported that several other mixed chemokines synergistically enhance chemotaxis, and using immunoprecipitation experiments that the CC chemokine SLC (secondary lymphoid tissue chemokine) and the CXC chemokine BCA-1 (B cell-attracting chemokine-1) form heteromeric complexes. Their findings further support our hypothesis that heteromerization and concomitant functional modulation is a general phenomenon among chemokines and is not limited to IL8 and PF4.

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