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Neutrophil-activating Peptide-2 and Melanoma Growth-stimulatory Activity Are Functional as Monomers for Neutrophil Activation*

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Neutrophil-activating peptide-2 (NAP-2) and melanoma growth-stimulatory activity (MGSA) are members of the chemokine family of inflammatory proteins. The structures of NAP-2, determined by x-ray crystallography, and MGSA, elucidated by NMR spectroscopy, revealed a tetramer and dimer, respectively. In order to address the relevance of multimeric species to their activities on neutrophils, analogs of NAP-2 and MGSA were synthesized in which the backbone amide proton of Leu-22 in NAP-2, and Val-26 in MGSA, was substituted with the bulky methyl group ($NH \rightarrow NCH_3$). These analogs were shown to be monomeric by sedimentation equilibrium ultracentrifugation studies and were similar to the corresponding native protein in assays for neutrophil elastase release and Ca^{2+} mobilization from IL-8R1 and IL-8R2 transformed cells. Sedimentation equilibrium studies of the native NAP-2 and MGSA were also carried out to address the association behavior. For NAP-2, there was no evidence for the tetramer, but an equilibrium between monomers and dimers and the dissociation constant was calculated to be 50–100 μM. Similarly, MGSA showed a monomer-dimer equilibrium with a K_d of ~5 μM. The data from the monomeric analogs and also the calculation of dissociation constants indicate that NAP-2 and MGSA have a tendency to associate above the concentrations required for maximal activity or for receptor activation, but at functional concentrations they are predominantly monomers.

Recruitment and accumulation of circulating leukocytes at the site of inflammation are mediated in part by a family of small molecular weight proteins called chemokines (1–3). They have four conserved cysteines and are called either C*X*C or CC chemokines, depending on whether the first two cysteines are separated by one amino acid (C*X*C) or are adjacent (CC). Functionally, the C*X*C chemokines predominantly attract neutrophils, whereas CC chemokines attract monocytes, lymphocytes, and eosinophils.

Three well studied proteins of the C*X*C chemokine family are interleukin-8 (IL-8)[1], melanoma growth-stimulatory activity (MGSA), and neutrophil-activating peptide-2 (NAP-2). The structure of IL-8 has been solved by both NMR and x-ray methods, and both approaches indicated it to be a dimer (4, 5). NMR studies of MGSA (6, 7) and x-ray studies of a variant of NAP-2 (M6L) (8) indicated them to be a dimer and tetramer, respectively. The tertiary and quaternary structures of IL-8 and MGSA are similar, and NAP-2 adopts a similar structure while showing an additional level of complexity by forming a tetramer. The tertiary structure consists of a series of turns and loops in the N-terminal region followed by three β-strands and a C-terminal α-helix. In the dimeric structure, the three β-strands from each monomer form a six-stranded β-sheet with the helices transversing the β-sheet. On the basis of the dimeric structure of IL-8, it was previously thought that dimerization is essential for function. However, we subsequently demonstrated that an analog in which Leu-25 was substituted with *N*-methylleucine (L25NMe) was monomeric and had similar functional properties as the native IL-8 (19). The structure of this synthetic IL-8 monomer was solved by ^{1}H NMR spectroscopy and was shown to be largely similar to that of the monomeric unit in the NMR and x-ray structures of the native dimer (16). Taken together, these observations suggested that dimerization of IL-8 is essential neither for structural integrity nor for functional activation.

Two receptors (IL-8R1 and IL-8R2) that bind C*X*C chemokines have been identified and characterized in neutrophils, and they belong to the superfamily of seven transmembrane domain-containing proteins that bind to G-proteins. IL-8R2 binds IL-8, MGSA, and NAP-2 with equal affinity. In contrast, IL-8R1 binds only IL-8 with high affinity, but it does bind MGSA and NAP-2 with low affinity (9–11). The N-terminal residues Glu-4, Leu-5, and Arg-6[2] (the "ELR" motif) (Fig. 1) are conserved and are absolutely essential for receptor binding in MGSA, NAP-2, and IL-8 (12–15). Differential binding to the two receptors has been attributed to a region of about 10 residues immediately preceding the first β-strand, and these residues are similarly located away from the interface (17, 18). Mutation of charged residues at the dimer interface in IL-8 had no affect on activity (18). Thus, functional data indicate that the residues at the dimer interface are not directly involved in receptor binding.

In order to address the relevance of higher order states

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[1] The abbreviations used are: IL-8, interleukin-8; MGSA, melanoma growth-stimulatory activity; NAP-2, neutrophil-activating peptide-2; RANTES, regulated upon activation, normal T-cell expressed, and presumably secreted.

[2] The numbering corresponds to the sequence in IL-8. The corresponding positions are 2, 3 and 4 in NAP-2 and 6, 7 and 8 in MGSA.

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                5   10   15   20   25   30   35   40   45   50   55   60   65   70
IL-8       SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS
MGSA      ASVATELRCQCLQTLQ-GIHPKNIQSVNVKSPGPHCAQTEVIATLKNGRKACLNPASPIVKKIIEKMLNSDKSN
NAP-2       AELRCMCIKTTS-GIHPKNIQSLEVIGKGTHCNQVEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
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FIG. 1. **The alignment of the amino acid sequences of IL-8, MGSA, and NAP-2.** The residues *highlighted* in the different proteins constitute the dimer interface.

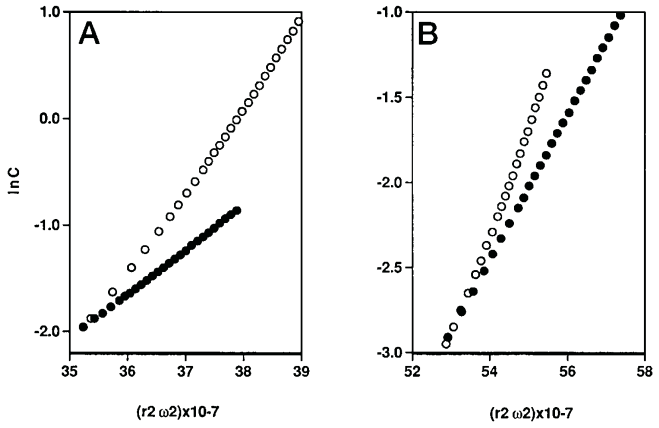

FIG. 2. **The average molecular weight determination by sedimentation equilibrium of MGSA (○) and the V25NMe MGSA analog (●) (*A*) and native NAP-2 (○) and the L22NMe NAP-2 analog (●) (*B*).** Plots of ln *C versus* $r^2\omega^2$ are shown, where *C* is the concentration in fringe displacement units, *r* is the distance from the axis of rotation, and ω is the angular velocity. All of the samples were in 50 mM sodium phosphate, 100 mM sodium chloride at pH 7.0. Molecular weights were calculated from the slopes of the plot (Equation 1). The native MGSA was run at an initial concentration of 1.0 mg/ml at 26,000 rpm; the V25NMe MGSA at an initial concentration of 2.1 mg/ml at 26,000 rpm; native NAP-2 at an initial concentration of 1.0 mg/ml at 26,000 rpm; and the L22NMe NAP-2 at an initial concentration of 1.4 mg/ml at 32,000 rpm. For clarity of presentation, a constant value of 1 and 2 were added to ln *C* and $r^2\omega^2$, respectively, for MGSA, and a value of 17.5 was added to $r^2\omega^2$ for NAP-2.

observed in structures of NAP-2 and MGSA, monomeric analogs of NAP-2 and MGSA were synthesized using a similar strategy employed in the synthesis of the monomeric IL-8 (19). For MGSA, Val-26 was substituted with *N*-methylvaline, and for NAP-2, Leu-22 was substituted with *N*-methylleucine. In addition to disrupting hydrogen bonding across the dimer interface, the modification also introduces a bulky methyl group, which prevents monomers from coming close to each other. We show here that the monomeric analogs of NAP-2 and MGSA have an activity similar to that of the corresponding native proteins for neutrophil elastase release and induce a similar rise in free Ca^{2+} in Jurkat cells transfected with either IL8-R1 or IL8-R2, indicating that dimerization is not essential for receptor binding and functional activation. Furthermore, calculations of K_d by sedimentation equilibrium for both NAP-2 and MGSA indicate that they are predominantly monomeric at functional nanomolar concentrations.

EXPERIMENTAL PROCEDURES

Protein Synthesis and Biological Assay—All proteins were chemically synthesized, purified, and characterized as discussed in detail previously (20). The monomeric MGSA and NAP-2 were synthesized using the same strategy as that used for IL-8 (19). Val-26 in MGSA and Leu-22 in NAP-2, the residues corresponding to Leu-25 in IL-8, were substituted with *N*-methylleucine and *N*-methylvaline, respectively. The synthesis was performed using *t*-butoxycarbonyl chemistry, and longer reaction times were required for the addition of the protected amino acid following the *N*-methyl-amino acid (NH_2-terminal amino acid), apparently due to slow reaction kinetics. The analogs were purified by HPLC and characterized by ion spray mass spectroscopy (SCIEX AP III) as described previously. MGSA was synthesized without the last Asn residue, and this analog was shown to have the same activity as the full-length protein. Elastase release from freshly purified neutrophils

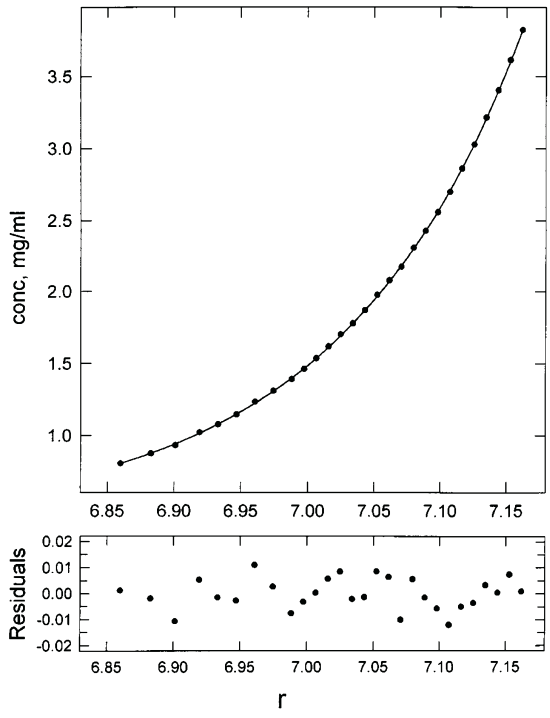

FIG. 3. **Calculation of dissociation constant by sedimentation equilibrium of native NAP-2 at 100 mM NaCl, 50 mM phosphate, pH 7.0.** The concentrations calculated in fringe displacement units (in mg/ml) are plotted *versus* r^2. The dissociation constants were calculated from curve fitting the data using Equation 2. Residuals of the corresponding least square fit are random, indicating the goodness of the fit.

(21) and Ca^{2+} induction from Jurkat cells transfected with IL-8R1 and IL-8R2 receptors were performed as described previously (22).

Ultracentrifugation Studies—Sedimentation equilibrium studies were performed on a Beckman Spinco model E analytical ultracentrifuge using Raleigh interference optics. The samples were extensively dialyzed for a period of 48 h against the appropriate buffer. 100 μl of the samples was loaded into 12-mm double-sector, charcoal-filled Epson cells equipped with sapphire windows. Sedimentation equilibrium runs of all of the proteins were carried out at a concentration of ~1 mg/ml in 100 mM NaCl, 50 mM sodium phosphate, pH 7.0. Runs were carried out at rotor speeds between 24,000 and 32,000 rpm, depending on the sample, for a period of 48 h, and photographs were taken when fringes could be resolved across the boundary region between the protein solution and the solvent. For both native NAP-2 and MGSA, data were collected using at least two different rotor speeds, two different pH values, and/or two different starting concentrations.

The average molecular weights (M_{av}) from sedimentation equilibrium runs were calculated using the equation,

$$M_{av} = \frac{2RT\ d(\ln C)}{(1-\bar{v}\rho)\omega^2\, d(r^2)} \qquad \text{(Eq. 1)}$$

where *R* is the universal gas constant and is equal to 8.314×10^7 erg/mol/K; *T* is the temperature in K; ρ is the solvent density, which was calculated to be 1.005; ω is the angular velocity (rpm $\times$ $2\pi/60$) in radians/s; $\bar{v}$, the partial specific volume of the protein, was calculated from the amino acid composition (23); *C* is concentration in units of mg/ml, which is calculated from vertical fringe displacement. An average refractive increment of 4.1 fringes/mg/ml was used in the calculation of the concentrations (24), and $d(\ln C)/dr^2$ is the slope from the plots in Fig. 2.

The dissociation constant for the monomer-dimer equilibrium was calculated from the equation (25),

$$C_r = C_o\exp[HM\delta] + C_o^2K_a\exp[2HM\delta] + E \qquad \text{(Eq. 2)}$$

where δ is $(r^2 - r_o^2)$ and C_r and C_o are the concentrations as measured by the scattering method at positions *r* and r_o, respectively. $H = (1-\bar{v}\rho) \times \omega^2/2RT$, *M* is the molecular weight of the monomer, K_a is the association constant for the monomer-dimer equilibrium, and *E* is the offset. The calculated equilibrium constant (K_a) is in units of mg/ml and is converted into molar units (K_A) by multiplying with the molecular

TABLE I
Dissociation constants of NAP-2, MGSA, and IL-8
Dissociation constants were measured by sedimentation equilibrium ultracentrifugation unless otherwise indicated.

Chemokine	Dissociation constant	Experimental conditions	Reference
	M		
NAP-2	$5.3 (\pm 2.0) \times 10^{-5}$	50 mM sodium phosphate, 100 mM NaCl, pH 7.0, 20 °C	This study
	$10.2 (\pm 3.6) \times 10^{-5}$	50 mM sodium phosphate, 100 mM NaCl, pH 5.0, 20 °C	This study
	$K_{d1} = \sim 3 \times 10^{-4}$ $K_{d2} = \sim 9 \times 10^{-4}$	250 mM NaCl, pH 7.0, 30 °C	26[b]
	$3.2 (\pm 0.7) \times 10^{-7}$	Phosphate-buffered saline, pH 7.4, 20 °C	27[c]
MGSA	$4 (\pm 3) \times 10^{-6}$	50 mM sodium phosphate, 100 mM NaCl, pH 7.0, 20 °C	This study
	$4.3 (\pm 1.4) \times 10^{-5}$	50 mM sodium phosphate, 100 mM NaCl, pH 5.0, 20 °C	This study
IL-8	$21 (\pm 10) \times 10^{-6}$	Phosphate-buffered saline, pH 7.4, 8 °C	34
	$14 (\pm 4) \times 10^{-6}$	20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 25 °C	33
	$18 (\pm 6) \times 10^{-6}$	20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 37 °C	33[d]

[b] Dissociation constants were measured by NMR. K_{d1} stands for monomer-dimer equilibrium, and K_{d2} stands for dimer-tetramer equilibrium.
[c] Dissociation constant was measured by chemical cross-linking.
[d] Dissociation constant was measured by microcalorimetry.

weight of the monomer ($K_A = K_a \times M/2$).

The data were fitted using the curve-fitting routine in Table Curve (Jandel Scientific). The estimated K_d is given at a 95% confidence interval. For the monomeric proteins, the data could be fitted to a single species, and for the native proteins, the data could be fitted to a simple monomer-dimer equilibrium. There was no need to fit the data to a more complex monomer-dimer-tetramer equilibrium, and when this was tried there was no significant improvement of the residuals, indicating that the monomer-dimer equilibrium adequately fits the data.

RESULTS AND DISCUSSION

Characterization of Monomeric and Native MGSA—Sedimentation equilibrium ultracentrifugation data of V26NMe MGSA (Fig. 2*A*) indicated that this analog is a monomer, since the calculated molecular weight (7,880) (Equation 1) was very similar to the experimental molecular weight (7,640). The data could be fitted to a single species with no evidence of a dimer form. The M_{av} of native MGSA at pH 7.0 was calculated from Equation 1 to be 13.7 kDa (monomer molecular weight, 7,865) with a mass distribution between 11 and 15 kDa, indicating that it was in equilibrium between monomers and dimers (Fig. 2*A*). The data could be fitted to a monomer-dimer equilibrium with a dissociation constant (K_d) of $4 \pm 3 \times 10^{-6}$ M. The K_d at pH 5.0 was calculated to be $4.3 \pm 1.4 \times 10^{-5}$ M and indicated a weaker dimer interface at pH 5.0 than at pH 7.0. Consistent with these results, NMR at pH 5.5 (7) and at pH 5.1 (6) indicated that the last four residues are unstructured. The dependence of dimer association as a function of pH indicates that electrostatic interactions play a role in stabilizing the dimer structure.

Characterization of Monomeric and Native NAP-2—Ultracentrifugation data of L22NMe NAP-2 (Fig. 2*B*) indicated that this analog is a monomer (calculated molecular weight, 7,646; experimental molecular weight, 7,775) with no evidence for dimeric species. The average molecular mass of native NAP-2 at pH 7.0 (Fig. 2*B*) was calculated to be 11.6 ± 0.4 kDa (monomer molecular weight, 7,632), with a mass distribution from 10 to 14 kDa, indicating that it exists in an equilibrium between monomers and dimers. The data could be fitted adequately to a monomer-dimer equilibrium with a K_d value of $5.3 \pm 2.0 \times 10^{-5}$ M (Fig. 3). Unlike the case for MGSA, there was no appreciable difference in the K_d values between pH 5.0 and 7.0 (~100 and ~50 μM, respectively).

These values indicate a relatively weak dimer interface compared with MGSA and IL-8. Lack of a pH effect is consistent with the NMR studies of NAP-2, which showed no differences

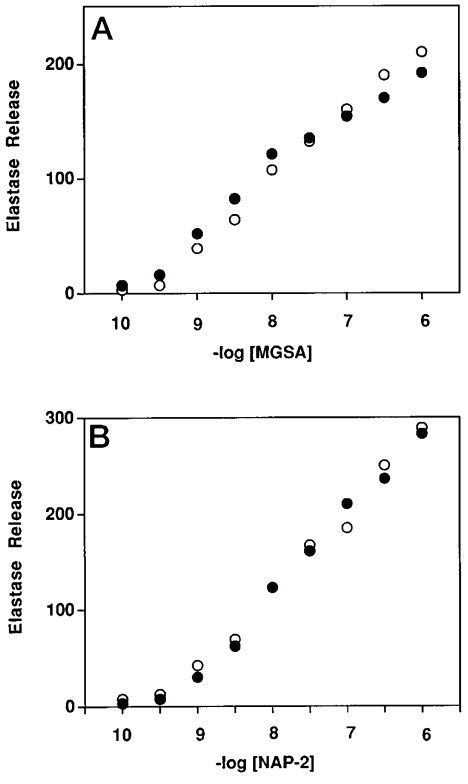

FIG. 4. **Neutrophil elastase release activity of the MGSA (●) and the monomeric V26NMe MGSA analog (○) (*A*) and NAP-2 (●) and the monomeric L22NMe NAP-2 analog (○) (*B*).** Data are representative of three assays using different neutrophil preparations

in the K_d value for pH values between 5 and 7 (26). At 250 mM NaCl and pH 7.0, the authors calculated a dissociation constant for the monomer-dimer equilibrium as ~300 μM and for a dimer-tetramer equilibrium as ~900 μM (Table I). However, from chemical cross-linking studies, a dissociation constant of ~0.3 μM was reported for NAP-2 (27). Ultracentrifugation data are largely consistent with the NMR studies, and the ~6-fold difference is within the experimental error of the two techniques. It is likely that tetramers were not detected at the concentrations used in the sedimentation equilibrium experiment due to the large K_d value for the dimer-tetramer equilibrium. Overall, data from this study and those reported by NMR

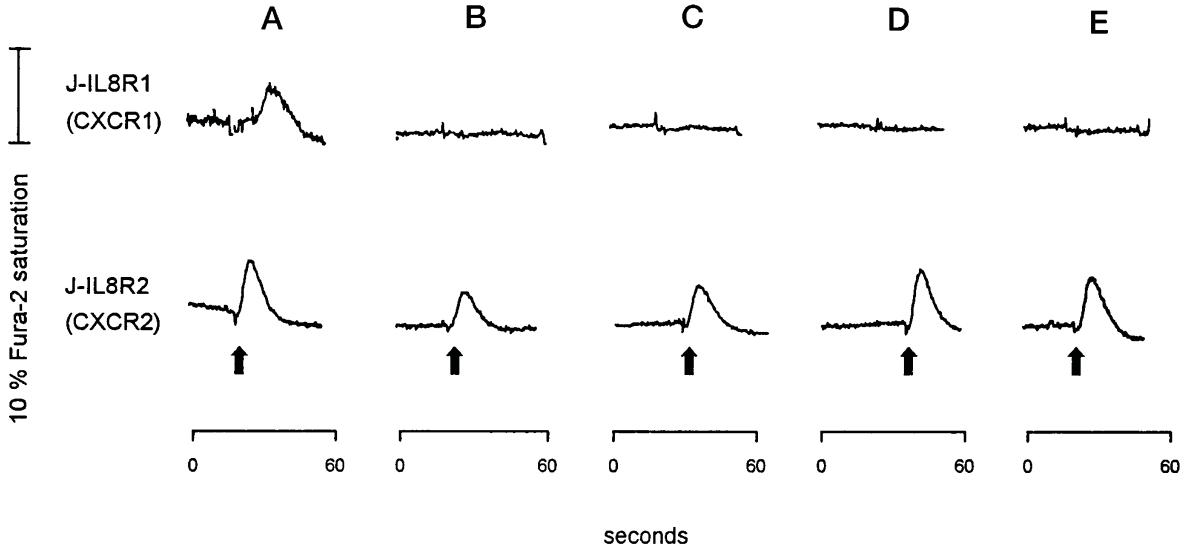

FIG. 5. **The monomeric analogs of MGSA and NAP2 activate IL-8R2 but not IL-8R1.** Changes in intracellular Ca^{2+} were monitored in Jurkat cells that express either IL-8R1 (JIL8R1) or IL-8R2 (JIL8R2). The cells were preloaded with Fura-2, a Ca^{2+}-sensitive fluorophor, and at the point indicated by the *arrows*, a 100 nM concentration of IL-8 (*A*), NAP-2 (*B*), monomeric L22NMe NAP-2 analog (*C*), MGSA (*D*), or monomeric V26NMe MGSA analog (*E*) was added. Data are representative of one of two similar experiments.

studies (26) are inconsistent with the low K_d values measured by cross-linking studies.

X-ray studies of a NAP-2 variant, having the substitution Met-6 to Leu (M6L), showed the structure to be a tetramer (8). In addition to having a dimer interface like that for IL-8 (designated as A/B interface), the second interface involves residues in the N-terminal region (designated as A/C interface). In solution structures of macrophage inflammatory protein-1β (28) and RANTES (29, 30), which are CC chemokines, N-terminal residues constitute the dimer interface. The crystal structure of platelet factor-4, a C*X*C chemokine, showed it to be a tetramer, and it has two dimer interfaces similar to NAP-2, an A/B interface like IL-8 and an A/C interface like RANTES (31, 32). By necessity, the crystals of NAP-2 were grown at high concentrations of the protein (17 mg/ml in 100 mM sodium acetate, 200 mM ammonium acetate, pH 4.6), and this could be the reason for the formation of the tetramer. It has been suggested by the authors that the mutation M6L may have favored interaction at the A/C interface compared with the native NAP-2, since platelet factor-4 has a Leu at the corresponding position. Since L22NMe NAP-2 is a monomer, the sedimentation equilibrium data suggest that the A/B interface is stronger than the A/C interface for NAP-2.

Structure-Function—Neutrophil elastase release activity of native MGSA and NAP-2 and the monomeric L22NMe MGSA and V26NMe NAP-2 analogs are shown in Fig. 4. The data indicate that the activities of the monomeric analogs are indistinguishable from those of the native proteins. Neutrophils have two receptors that bind ELR motif-containing chemokines, and both receptors elicit chemotaxis exocytosis and a rise in Ca^{2+} level response. It has been shown that IL-8, NAP-2, and MGSA elicit the same transient induction of Ca^{2+} from Jurkat cells transfected with IL-8R2, whereas up to 100 nM, only IL-8 elicits Ca^{2+} release response from cells transfected with IL-8R1 (22). Similar experiments were performed with the monomeric analogs (Fig. 5). The data clearly indicate that both the monomeric analogs elicit similar functional response as the native protein. The relative distribution of monomers and dimers for different total concentrations of NAP-2 calculated from the K_d at pH 7 is shown in Fig. 6. It is observed that at functional concentrations (0.1–10 nM), NAP-2 exists predominantly as a monomer, and at concentrations used for structural

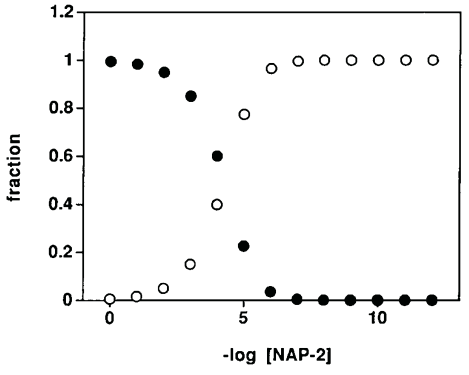

FIG. 6. **Mole fraction of the monomer (○) and dimer species (●) as a function of NAP-2 concentration.** The relative amounts of the monomer and dimer were calculated using a K_d of 102 μM calculated from sedimentation equilibrium studies at pH 7.

determination (~1 mM) it exists predominantly as a dimer. The distribution profile for MGSA, calculated for a K_d of 4 μM, shows a similar trend and indicates that it is predominantly monomeric at functional concentrations. The K_d for IL-8 has been determined by sedimentation equilibrium and microcalorimetry studies as 10–30 μM (33, 34) (Table I), which indicates that IL-8 also exists as a monomer at physiological concentrations (35, 36). The data from this study for MGSA and NAP-2 and previous studies for IL-8 provide experimental evidence that these chemokines have a strong propensity to associate at high concentrations but bind as monomers to the neutrophil receptors.

Implications for Receptor Binding—Various models for interaction between chemokines and their receptors have been discussed (19, 37). The data presented here are consistent with a model in which a monomeric chemokine ligand binds to a monomeric receptor. Several lines of evidence indicate that the active species is also the monomer for some of the CC chemokines. At high concentrations, I-309 and monocyte chemoattractant protein-3 exist as monomers with no evidence of dimerization (34, 37). Equilibrium constants for monocyte chemoattractant protein-1 and -2 are in the μM region (34, 37), and a variety of studies have shown that the functionally active species for macrophage inflammatory protein-1α is a monomer

(38).

Our data do not rule out the possibility that dimerization of the native chemokine may occur during or after initial binding of a monomer to the receptor. This is not possible for the monomeric chemokines discussed here, since the same mechanisms that prevent dimerization in solution will prevent dimerization on the receptor. However, there is no reason to believe that dimerization of the ligand is necessary for G-protein-coupled seven-transmembrane receptors. Most of the ligands for this family of receptors tend to be small molecular weight compounds such as formylmethionylleucylphenylalanine and epinephrine. For the growth factor type receptors, which have a distinctly different topology, dimerization of the receptor, and not the ligand, has been shown to be essential (39). All of the data presented here are from *in vitro* studies, and it is possible that these proteins may dimerize *in vivo* due to local high concentrations in the microenvironment at the receptor binding site. The dimerization may regulate the active concentration available for receptor binding or may stabilize the protein from proteolytic cleavage. A number of *in vivo* and *in vitro* studies seem to indicate that heparin binding on the cell surface is critical to generate a concentration gradient for leukocyte attraction and, hence, receptor binding (40–43). Preliminary studies from our laboratory suggest that the interaction of monomeric and native dimeric IL-8 with heparin is highly differential.[3] Presently, all of the available data from our study and other studies indicate that chemokines have a tendency to associate at high concentrations but that the monomeric unit is sufficient to bind the receptor for functional activation.

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[3] K. Rajarathnam, C. M. Kay, I. Clark-Lewis, and B. D. Sykes, unpublished results.