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Association of Nucleophosmin Negatively Regulates CXCR4-Mediated G Protein Activation and Chemotaxis

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ABSTRACT

CXCR4, the primary receptor for CXCL12, plays a critical role in the development of hematopoietic, vascular, central nervous, and immune systems by mediating directional migration of precursor cells. This mechanism promotes homing of tumor cells to metastatic sites that secrete CXCL12, and CXCR4 expression is a negative prognostic factor in acute myelogenous leukemia (AML). To elucidate mechanisms that regulate CXCR4 signaling, we used a proteomic approach to identify proteins physically associated with CXCR4. Analysis of CXCR4 immune complexes identified nucleophosmin (NPM), which was confirmed by reciprocal coimmunoprecipitation for NPM. Constitutively active CXCR4 variants bound higher levels of NPM than the wild-type receptor, which was reversed by T140, an inverse agonist. NPM binding to CXCR4 localized interactions to the C terminus and cytoplasmic loop (CL)-3, but not CL-1 or CL-2. Alanine scanning mutagenesis demonstrated that positively charged amino acids in CL-3 were critical for NPM binding. Recombinant NPM decreased GTP binding in membrane fractions after activation of CXCR4 by CXCL12. Suppression of NPM expression enhanced chemotactic responses to CXCL12, and, conversely, overexpression of a cytosolic NPM mutant reduced chemotaxis induced by CXCL12. This study provides evidence for a novel role for NPM as a negative regulator of CXCR4 signaling induced by CXCL12 that may be relevant to the biology of AML.

CXCR4 is a G protein-coupled receptor (GPCR) that is the primary receptor for CXCL12, a CXC chemokine previously known as stromal cell-derived factor-1 (Loetscher et al., 1994; Bleul et al., 1996). During embryogenesis, this ligand provides a chemotactic gradient that programs the directed migration of cells expressing CXCR4 to form portions of the central nervous system and the cardiovascular system, and it promotes localization of hematopoietic precursors to the bone marrow and B-lymphopoiesis, which are critical for viability (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). In adults, this receptor-ligand pair functions to promote the recruitment of leukocytes to sites of pathology and to maintain stem cells in a microenvironmental niche (Loetscher et al., 1994; Sugiyama et al., 2006). Expression of CXCR4 by tumor cells induced by hypoxia, loss of von Hippel-Lindau factor, or activation of receptor tyrosine kinases confers a mechanism for metastatic homing to target organs that secrete CXCL12 (Staller et al., 2003; Phillips et al., 2005). In addition to its role in normal and pathological physiology, CXCR4 has been shown to function as a coreceptor for T-tropic strains of human immunodeficiency virus (HIV) type 1, which occur late in the evolution of HIV-1 infection in many patients (Bleul et al., 1996).

Because CXCR4 plays key roles in the pathogenesis of inflammatory disorders, metastatic spread of tumors, mobilization of stem cells, and HIV-1 infection, it is important to gain insight into critical mechanisms for CXCR4 function. Binding of CXCL12 to CXCR4 results in the activation of heterotrimeric G proteins, typically $G\alpha_i$ subunits that are inhibited by exposure to pertussis toxin (Zhang et al., 2002; Papayannopoulou et al., 2003). There is increasing evidence

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ABBREVIATIONS: GPCR, G protein-coupled receptor; HIV, human immunodeficiency virus; C-ter, C terminus; NPM, nucleophosmin; CL, cytoplasmic loop; ΔT, C terminus-truncated; GST, glutathione transferase; siRNA, small interfering RNA; CHO, Chinese hamster ovary; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDF, stromal cell-derived factor; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; siNPM, small interfering RNA targeting nucleophosmin; WT, wild type; EGFP, enhanced green fluorescent protein.

that proteins that regulate receptor signaling and trafficking associate with cytosolic domains of GPCRs (Bockaert et al., 2004). β-Arrestin functions as a scaffold for cytoplasmic complexes, including the association of Akt and PP2A with the dopamine receptor (Beaulieu et al., 2005), and formation of β-arrestin, c-src, and dopamine receptor complex is a potential mechanism that links D2 dopamine receptor to nuclear factor κB activation (Yang et al., 2003). After CXCL12 binding, CXCR4 undergoes down-modulation and ubiquitination of the C terminus (C-ter) by E3 ubiquitin ligase, thereby promoting targeting of the receptor for degradation rather than recycling via the endosomal pathway (Marchese and Benovic, 2001; Marchese et al., 2003).

To gain insight into mechanisms that regulate CXCR4 signaling, we undertook a proteomic approach to identify proteins that associate with cytosolic domains of CXCR4. Nucleophosmin (NPM/B23) was identified in immune complexes with constitutively active CXCR4 signaling mutants. The binding, which mapped to C-ter and CL-3 of CXCR4, was decreased when the active conformation was reversed by exposure to an inverse agonist. Recombinant NPM decreased CXCR4 signaling, and suppression of NPM expression increased the chemotactic response to CXCL12. On the contrary, overexpression of a cytosolic NPM mutant decreased chemotaxis induced by CXCL12. These findings provide evidence that physical association of NPM with CXCR4 negatively regulates the induction of signaling by CXCL12. This mechanism may provide an approach to block the function of CXCR4 in the pathophysiology of tumor metastasis and the biology of leukemic myeloblasts, as well as in HIV-1 infection.

Materials and Methods

Plasmids. The construction of pcDNA3-Myc-CXCR4-WT, 119S, and 119A was described previously (Zhang et al., 2002). C-ter truncation mutants WTΔT, 119SΔT, and 119AΔT were generated by mutating Thr318 to Ala followed by an artificial stop codon. Segments encoding the CL-1 (G64-K75) and the CL-3 (Ser224-Lys239) of CXCR4 were generated by annealing two complementary oligonucleotides that encode the corresponding amino acid residues. Segments encoding the CL-2 (Asp133-K154) and CXCR4 C-ter (Ala303-Ser352) were achieved from pcDNA3-Myc-CXCR4-WT using polymerase chain reaction. Segments were cloned into pGEX-4T2 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) at BamHI/XhoI sites to make GST fusion protein constructs. The pGEX-4T2-CL-3 mutants 1, 2, 3, and 4 were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by replacing codons for Lys225, Lys230, KRK(234–236), and Lys239 with codons for alanine. pcDNA3-Myc-CXCR4-AAA mutant was constructed using the QuikChange site-directed mutagenesis kit by replacing codons for KRK(234–236) with sequences programming AAA. pQE30-His-NPM was a kind gift from Dr. M. O. J. Olson (University of Mississippi Medical Center, Jackson, MS) (Hingorani et al., 2000). The pcDNA3-FLAG-NPM-cyto mutant was constructed using polymerase chain reaction by replacing the C-ter WQWRKSL sequence with CLAVEEVSLRK in a pFLAG-NPM plasmid kindly provided by Dr. Grover C. Bagby (Oregon Health Sciences University, Portland, OR). The sequences of constructs were confirmed by nucleotide sequencing. The siRNA for NPM was constructed using the sequence described by Dhar. et al. (2004). The oligonucleotide AAGATAACTGGTGCTCATT, which corresponds to nucleotides 112 to 130 of the human NPM, was cloned into the pENTR/U6 vector using the BLOCK-iT U6 RNAi Entry Vector kit (Invitrogen, Carlsbad, CA) using standard techniques. A self-ligated pENTR/U6 vector was used as negative control.

Tissue Culture and Transfection. CHO-K1 cells stably expressing wild-type CXCR4 (CHO-CXCR4-WT) and the constitutively active mutant CXCR4 (CHO-CXCR4–119S) were constructed and grown as described previously (Zhang et al., 2002). pcDNA3-Myc-CXCR4-WT and the pcDNA3-Myc-CXCR4-AAA mutant were transiently expressed in CHO-K1 cells using Lipofectamine (Invitrogen). Cells were replated 24 h after transfection, and they were used for experiments at 48 h. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum. Transfection was performed with Lipofectamine 2000. Transfectants with stable expression of Myc-CXCR4 were selected by resistance to G-418 (Geneticin; Invitrogen) followed by magnetic sorting (Miltenyi Biotec Inc., Auburn, CA). For siRNA knockdown experiments, HEK-293 cells were replated 48 h after transfection, and experiments were carried out at 72 h. For overexpression of FLAG-NPM-cyto plasmid, HEK-293 cells were replated 24 h after transfection, and experiments were carried out at 48 h.

Coimmunoprecipitation and Western Blot. HEK-293 stable transfectants were detached with 1 mM EDTA-PBS, resuspended in DMEM containing 0.5% BSA, and incubated in the presence or absence of T140 for 15 min. The reaction was stopped by adding ice-cold PBS. Cells were centrifuged at 300*g* and extracted into lysis buffer containing 20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM NaCl, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Membranes were collected by centrifugation at 21,000*g* at 4°C for 30 min, and they were solubilized in solubilization buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% *n*-dodecyl β-D-maltoside, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail by rocking at 4°C for 30 min. The insoluble cell fraction was removed at 21,000*g* for 20 min. The cell lysate was incubated with Sepharose 4B conjugated to 9E10 (anti-c-Myc antibody; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 3 h or with anti-NPM antibody (Invitrogen) for 2 h followed by addition of protein G-Sepharose beads for a further 2 h. After washing with solubilization buffer, proteins bound to the beads were eluted in SDS sample buffer, and then they were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie Blue, and specific bands were identified by LC-MS/MS (The W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT). For Western blot experiments, the proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare), which were incubated with primary antibody overnight and secondary antibody for 1 h at room temperature. Finally, proteins were detected using the ECL Plus system (GE Healthcare) and quantitated by digital imaging (LAS-3000; Fuji Medical Systems, Stamford, CT).

Cytoplasmic Extracts Preparation. Cytoplasmic extracts were prepared according to the method described by Dignam et al. (1983) with minor modifications. HEK-293 cells (5×10^6) were resuspended in 125 μl of hypotonic buffer. After incubation for 10 min on ice, cells were spun at 7000 rpm for 5 s at 4°C, and the cell pellet was resuspended in 50 μl of hypotonic buffer. Ten microliters of sample was taken as total cell lysate. The remaining cells were homogenized for 10 strokes in a cell homogenizer (mortar and pestle), centrifuged at low speed (500*g*) for 10 min, and separated between supernatant and pellet. Ten microliters of the supernatant was taken as cytoplasmic fraction 1. The rest of the supernatant was further centrifuged at high speed (21,000*g*) for 10 min to remove the membranes from cytoplasmic fraction. This final supernatant was designated cytoplasm extract 2. The pellet from low-speed centrifuge was washed three times with hypotonic buffer, resuspended in 40 μl of hypotonic buffer, and designated as the nuclear fraction.

Immunofluorescence Microscopy. Cells were grown on cover slides coated with poly(lysine). After fixation with 4% paraformaldehyde, cells were permeabilized with 0.1% Triton and stained with 9E10 for Myc-tagged CXCR4 and the M2 monoclonal mouse anti-FLAG antibody (Sigma-Aldrich) for flag-NPM-cyto, followed by in-

cubation with Alexa Fluor 488-labeled secondary antibody (Invitrogen). Immunofluorescent images were taken by confocal microscopy (LSM510 Meta; Carl Zeiss, Thornwood, NY) or with a fluorescent microscope (TE2000; Nikon, Melville, NY).

Fusion Protein Purification. GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells grown in Luria broth. His-tagged NPM protein was expressed in *E. coli* M15 grown in Terrific Broth. The culture was induced with 100 μM isopropyl-β-D-thiogalactoside and grown at 37°C for 3 h. Cells were lysed by sonication in lysis buffer containing 50 mM sodium phosphate, pH 7.0, 0.5% Triton X-100, 5 mM NaCl, protease inhibitor cocktail, 1 mM PMSF, and 1 mM β-mercaptoethanol. GST fusion proteins were affinity-purified using glutathione-Sepharose 4B beads (GE Healthcare) and His-tagged NPM was affinity-purified using Talon beads (Clontech, Mountain View, CA) using standard techniques. Purified GST fusion proteins were dialyzed in buffer containing 20 mM Tris,

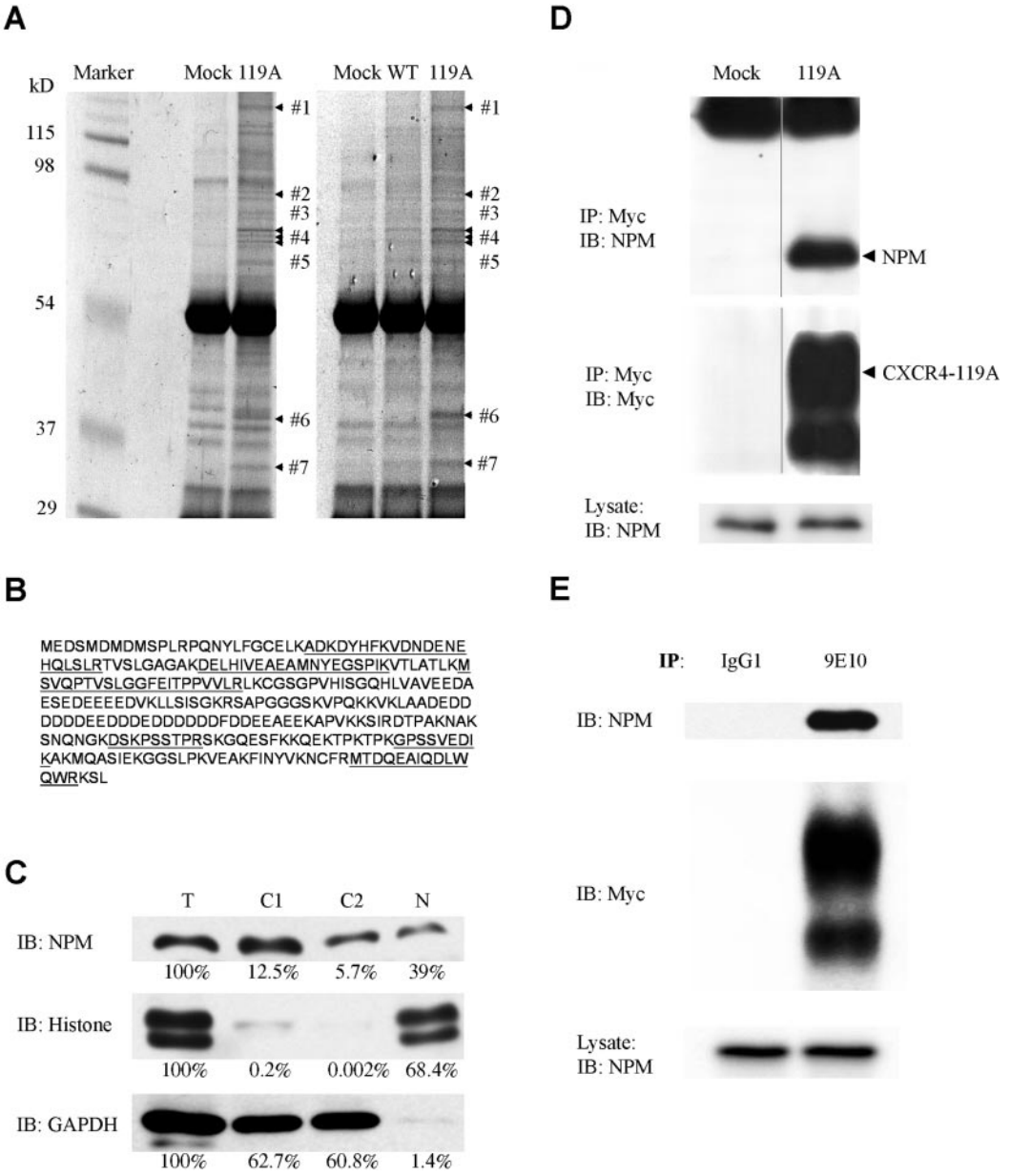

Fig. 1. Identification of CXCR4 as a nucleophosmin interacting protein. A, lysates of HEK-293 stably expressing EGFP (Mock) or Myc-CXCR4-N119A (119A) or Myc-CXCR4-WT (WT) were incubated with 9E10, a monoclonal antibody to the Myc epitope tag, covalently linked to Sepharose 4B, as described under *Materials and Methods.* Proteins eluted from the CXCR4 immune complexes were separated by SDS-PAGE in gels containing 10% acrylamide, which were stained with Coomassie Blue. Gels from two independent experiments are shown. Seven distinct bands were cut from the gel and identified by LC-MS/MS. B, human NPM protein sequence. Underlined sequences are those revealed by LC-MS/MS. C, HEK-293 stably expressing Myc-CXCR4-WT cells were separated into cytoplasmic and nuclear fractions, as described under *Materials and Methods.* The quantitative data take into account the percentage of the totality of each fraction loaded on the gel. Because NPM and histone are highly abundant in the total cell (T) and nuclear (N) fractions relative to the cytoplasmic fractions (C1 and C2), the volume of T and N loaded was 10% of the volume loaded for C1 and C2 for the NPM and histone immunoblots. D, immunoprecipitation was performed as described in A. Immune complexes were resolved by SDS-PAGE and immunoblotted with anti-NPM and anti-Myc tag antibodies. NPM in the solubilized cell lysates is shown. The lane between Mock and 119A contained WT-CXCR4. It was removed from this figure because these data are included in an extended analysis of NPM interacting with various CXCR4 conformations in Fig. 2. E, lysates of HEK-293 stably expressing Myc-CXCR4-N119A were incubated with control antibody (IgG1) or 9E10. The immune complexes were adsorbed on Sepharose 4B-protein G, resolved by SDS-PAGE, and immunoblotted with anti-NPM and anti-Myc tag antibodies. NPM from solubilized cell lysates is shown.

pH 7.5, 1 mM EDTA, and 0.25 mM dithiothreitol. Purified His-NPM protein was dialyzed in the buffer containing 20 mM Tris, pH 7.5, 300 mM NaCl, 1 mM EDTA, and 0.25 mM dithiothreitol. His-NPM was further concentrated using Microcon (Millipore Corporation, Billerica, MA). The flow-through buffer was used together with BSA as the control for [^{35}S]GTPγS binding experiment. Protein quantification was performed by measuring the intensity of protein bands in SDS-PAGE stained with Coomassie Blue.

GST Pull Down. HEK-293 cells were solubilized in lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05 mM EDTA, 1 mM PMSF, protein inhibitors, phosphatase inhibitors, and 1% CHAPS by rocking at 4°C for 1 h. Cells debris was removed by centrifugation at 21,000*g* for 20 min. Cell lysates containing 200 μg of protein or 1 μg of purified recombinant His-NPM in 500 μl were incubated with 10 μg of GST fusion protein prebound to glutathione-Sepharose 4B beads at 4°C for 2 h. The beads were subsequently washed, and bound proteins were eluted in SDS sample buffer. The GST fusion protein complex was detected by Western blotting or Coomassie Blue staining.

[^{35}S]GTPγS Binding Assay. The [^{35}S]GTPγS binding experiments were performed as described previously (Zhang et al., 2003) with the following minor modifications: the cell membrane fraction was preincubated with His-NPM (or a BSA control) and CXCL12 in a final volume of 90 μl at 37°C for 10 min before initiating the reaction by adding 10 μl of 2 nM [^{35}S]GTPγS (1000 Ci/mmol) (GE Healthcare). Specific binding was calculated by subtracting nonspecific binding from total binding. Data were presented as femtomoles of [^{35}S]GTPγS-specific binding per milligram of membrane protein.

Chemotaxis Assay. Chemotaxis assays were performed using a transwell apparatus (8.0-μm pore size; Corning, New York, NY) coated with collagen type IV (Sigma-Aldrich), as described previously (Navenot et al., 2005). The lower compartment was filled with 600 μl of DMEM containing 0.5% BSA in the presence or absence of CXCL12 (Leinco Technologies, St. Louis, MO). The upper compartment was filled with 1×10^5 HEK-293-CXCR4-WT cells in 100 μl of DMEM containing 0.5% BSA, which had been transfected with siNPM or vector control for 72 h, or transfected with Flag-NPM-cyto or pcDNA3 for 48 h. After incubation at 37°C for 4 h, cells were fixed with 0.5% crystal violet in 20% ethanol. The cells on the upper side of the membrane were wiped off, and those that had migrated to the lower side were counted in 10 random microscope fields (20× objective). The net migration was calculated by subtracting random migration in the absence of CXCL12 from the total migration in the presence of CXCL12.

Quantification and Statistical Analyses. All films and enhanced chemiluminescence images were processed by using a Fuji LAS-3000 digital imaging system, and they were quantified with the Muti-Gauge software (Fuji Medical Systems). Data are presented as mean ± S.D. of at least three independent experiments, unless specified. Statistical analyses were carried out by Student's *t* test. Statistical significance was accepted at $p < 0.05$.

Results

Physical Association of NPM with Constitutively Active CXCR4 Mutants. A proteomics approach was taken to identify proteins that interact with cytoplasmic domains of the activated CXCR4 receptor using CXCR4(N119A), a constitutively active mutant that is chronically desensitized in transfectants (Zhang et al., 2002). This signaling variant mimics many aspects of CXCR4 after CXCL12 binding, including G protein activation, constitutive phosphorylation, and constitutive internalization. Using CXCR4(N119A) facilitates the study of proteins interacting with CXCR4 when this association either requires chronic desensitization of receptor or requires a stably active CXCR4 conformation, because the interaction between SDF-1 and CXCR4 may not be durable during solubilization and immunoprecipitation. Proteins physically associated with CXCR4 were identified in immune complexes containing this GPCR.

Transfectants expressing CXCR4(N119A) or wild-type CXCR4 containing an N-terminal c-Myc epitope tag were solubilized under conditions to preserve protein-protein interactions using buffer containing 1% dodecyl maltoside. Immune complexes obtained with a monoclonal antibody to the c-Myc tag were isolated from HEK-293 cells stably expressing CXCR4(N119A), wild-type CXCR4 (WT), and control cells programmed to express EGFP. As shown in Fig. 1A, resolution of immune complexes containing CXCR4(N119A) in polyacrylamide gels containing SDS stained with Coomassie Blue revealed seven bands in lysates from CXCR4(N119A) transfectants that were absent in control cells. It is noteworthy that bands 1 and 6 showed a more specific association to CXCR4(N119A) compared with wild-type CXCR4. The identity of multiple bands was determined by LC-MS/MS analysis (Table 1).

Among the candidates that were identified, repressor of estrogen receptor activity was confirmed by Western blot analysis using specific antibody (data not shown), and NPM, a multiple-function nuclear-cytosol shuttle protein (Grisendi et al., 2006), was selected for further study. The peptide sequences determined by LC-MS/MS are shown in the amino acid sequence of NPM (Fig. 1B). The presence of cytoplasmic NPM was demonstrated by cell fractionation and Western blot (Fig. 1C). Nucleophosmin activity was detected in cytoplasmic fractions that were positive for glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic marker) activity and negative for histone (nuclear marker) activity. Western blot analysis of the CXCR4 immune complexes was performed to confirm the proteomic data. As shown in Fig. 1D, immuno-

TABLE 1
Identification of proteins associating with CXCR4(N119A) by LC-MS/MS

Band	Protein	Peptide Match		Molecular Mass
		No.	Coverage	
			%	*kDa*
1	Kinectin variant 1	2	1.8	156.1
2	Mannosyl-oligosaccharide glucosidase	3	3.6	91.8
3	CoA dehydrogenase α-subunit of trifunctional protein	7	14.7	82.9
4	Heat shock 70-kDa protein cognate	1	1.9	70.8
5	Heat shock 70-kDa protein 1B	9	21.5	70.0
6	Nucleophosmin (B23)	7	33.2	30.9
7	Repressor of estrogen receptor activity	11	43.1	33.4

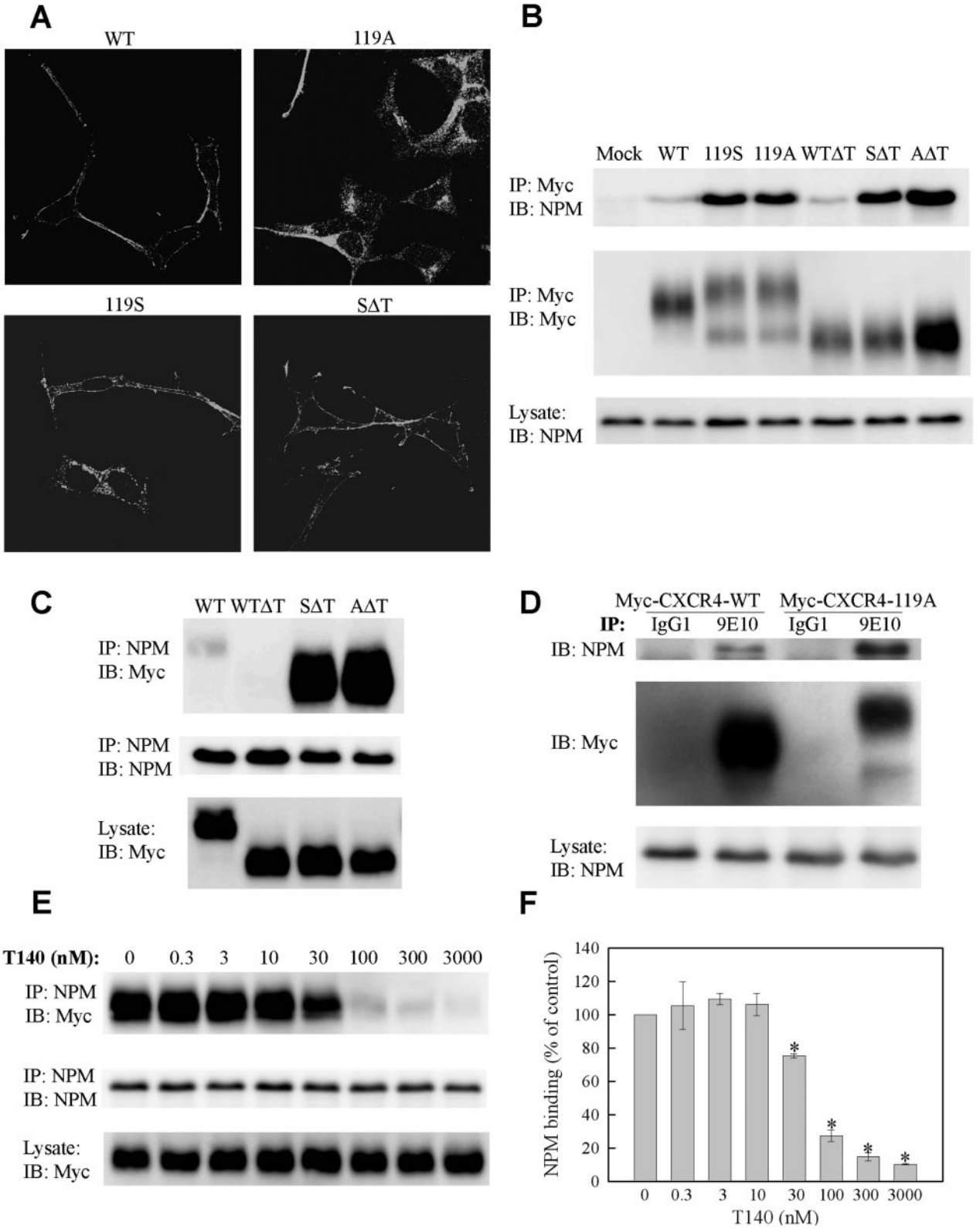

Fig. 2. NPM interaction with active CXCR4 conformation is greater than with the wild-type receptor. A, CXCR4 variants were stained with the anti-Myc tag antibody (9E10) followed by Alexa Fluor 488-conjugated anti-mouse antibody staining. Images were taken by confocal immunofluorescent microscopy, and representative fields are shown. B, CXCR4 variants were immunoprecipitated from HEK-293 stable transfectants or control cells expressing EGFP as described under *Materials and Methods*. The immune complexes were resolved by SDS-PAGE and immunoblotted with anti-NPM and anti-Myc tag antibodies. NPM content of solubilized cell lysates is also shown. The blots are representative of four independent experiments. C, lysates from HEK-293 cells stably expressing the indicated CXCR4 mutants were immunoprecipitated using anti-NPM antibody. The immune complexes were resolved by SDS-PAGE and immunoblotted with anti-Myc tag and anti-NPM antibodies. Myc-CXCR4 from solubilized cell lysates is shown. The bands shown correspond to the predicted migration of CXCR4 and the respective truncation mutants. Bands corresponding to the predicted electrophoretic mobility of c-Myc were inconspicuous in these experiments. The blot is representative of two independent experiments. D,

blotting of CXCR4 immune complexes with a monoclonal antibody to NPM revealed a specific band at the predicted molecular weight of NPM. This band was not detected in control reactions. The specific association with CXCR4(N119A) was further confirmed by immunoprecipitation using control IgG and 9E10, which showed a specific NPM band in the 9E10 immune complexes, but not with control IgG.

The CXCR4 Active Conformation Enhanced Association with NPM. Because NPM was discovered in immune complexes with constitutively active variants of CXCR4, experiments were performed to compare the ability of NPM to associate with CXCR4 in active and inactive conformations. The locations of these epitope-tagged signaling variants of CXCR4 were assessed by confocal microscopy after staining of the Myc tag with 9E10. Consistent with our previous observation (Zhang et al., 2002), CXCR4 was present primarily on the cell surface, but constitutively active mutants N119A and N119S were present both on the cell surface and in intracellular compartments as a result of constitutive internalization (Fig. 2A). It is noteworthy that truncation of the C-ter of N119A (data not shown) and N119S abolished the internalization (Fig. 2A), although the constitutive activity was maintained in regard to G protein activation (data not shown). The association of NPM with epitope-tagged signaling variants of CXCR4 was determined in immunoprecipitation experiments using lysates from stable HEK-293 cell transfectants. As shown in Fig. 2B, immune complexes containing wild-type CXCR4 or a C-ter truncation of this receptor had low levels of NPM. In contrast, two constitutively active CXCR4 mutants, CXCR4(N119S) and CXCR4(N119A), were associated with high levels of NPM, which was not altered by C-ter truncation. Reciprocal immunoprecipitation experiments revealed that immune complexes obtained with anti-NPM antibodies also contained the C-ter-truncated forms of these constitutively active mutants but only minimal levels of wild-type CXCR4 and the C-ter truncation of the wild-type receptor (Fig. 2C). Moreover, immunoprecipitation experiments using HEK-293 expressing lower levels of CXCR4(N119A) also revealed that wild-type CXCR4 associated with less NPM than CXCR4(N119A), despite higher levels of expression (Fig. 2D). In addition, no NPM was immunoprecipitated by the control antibody IgG1, indicating the specificity of the association of CXCR4 with NPM.

T140 is a polypeptide inverse agonist that binds to CXCR4 and CXCR4 constitutively active mutants and favors the inactive conformation (Zhang et al., 2002). CXCR4(N119SΔT), the noninternalized variant of N119S, was used to study the effect of T140 because the N119S mutant responds to CXCL12 and the constitutively active conformation is reversible, whereas the response of the N119A mutant is minimal. The reversal of CXCR4 active conformations by T140 decreased the association with NPM in a dose-dependent manner, as shown in Fig. 2E. Densitometric analysis of the Western blot revealed that 50% inhibition of NPM coimmunoprecipitation with CXCR4(N119SΔT) occurred at concentrations between 30 and 100 nM (Fig. 2F). Exposure of HEK-293 transfectants stably expressing wild-type CXCR4 to CXCL12 did not significantly increase levels of NPM in CXCR4 immune complexes (data not shown).

Basic Amino Acids in the Third Cytoplasmic Domain (CL-3) of CXCR4 Were Required for Association with NPM. Sequences in CXCR4 involved in NPM binding were mapped in pull down experiments using recombinant GST fusion proteins containing amino acid residues predicted to be in the respective cytoplasmic domains of the receptor (Fig. 3A) with HEK-293 cells lysates. As shown in Fig. 3B, the GST fusion protein containing sequences corresponding to CL-3 showed the highest level of interaction with NPM, and the recombinant GST-C-ter fusion protein had weak binding to NPM. GST fusion proteins containing CL-1 and CL-2 sequences lacked significant interactions with NPM, as was observed for wild-type GST alone.

Because HEK-293 lysates contain multiple proteins, it is possible that the association between CXCR4 domains and NPM is direct or indirect. To determine whether NPM interacts directly with CXCR4, pull down experiments were performed with the GST fusion proteins containing CXCR4 cytoplasmic domains and recombinant NPM. Purified recombinant His-tagged NPM migrated as a single band of the predicted molecular mass in SDS-PAGE followed by staining with Coomassie Blue (Fig. 3C). Pull down experiments revealed that GST-CL3 and CST-C-ter, but not the GST fusion proteins containing CL-1 or CL-2, bound directly to NPM (Fig. 3D).

Sequences in the CXCR4 CL-3 necessary for association with NPM were determined in mutagenesis experiments. Alanine scanning mutations were introduced into GST-CL3 fusion proteins (Fig. 3E), and binding to His-NPM was determined in pull down experiments. As shown in Fig. 3F, substitution of Ala for any individual basic amino acid in CL-3 decreased binding to recombinant NPM, which was greatest in CXCR4 (K230A). Replacement of the Lys-Arg-Lys sequence with Ala-Ala-Ala resulted in complete loss of His-NPM binding.

NPM Negatively Regulated Induction of GTP Binding by $G\alpha_i$ Subunits after CXCR4 Activation. The effect of recombinant NPM on the binding of [^{35}S]GTPγS induced by 10 nM CXCL12 in CHO-K1 transfectants expressing CXCR4 was determined. CHO-K1 transfectants expressing CXCR4 were used for these experiments because HEK-293 transfectants expressing CXCR4 showed poor signal-to-noise ratio in this assay (data not shown). Overexpression of CXCR4 did not change the basal binding of [^{35}S]GTPγS compared with CHO-K1 that lacked CXCR4 expression (Fig. 4A). Whereas incubation of membrane fractions with recombinant NPM did not alter the basal binding of [^{35}S]GTPγS induced by wild-type CXCR4, a statistically significant de-

lysates of HEK-293 cells stably expressing Myc-CXCR4-WT (WT) or Myc-CXCR4-N119A (119A) were incubated with control antibody (IgG1) or 9E10. The immune complexes were absorbed with Sepharose 4B-protein G, resolved by SDS-PAGE, and immunoblotted with anti-NPM and anti-Myc tag antibodies. NPM from solubilized cell lysates is shown. The blot is representative of two independent experiments. E, HEK-293 cells stably expressing Myc-CXCR4–119SΔT were exposed to T140 at the indicated concentrations for 15 min at 37°C. The cells were lysed and immunoprecipitated with anti-NPM antibody as described above. Immune complexes were resolved by SDS-PAGE and immunoblotted with anti-Myc tag (9E10) and anti-NPM antibodies. The Myc-CXCR4 levels from solubilized cell lysates are also shown. The blot is representative of three independent experiments. F, quantitative and statistical analysis of data presented in E. Coimmunoprecipitated NPM from cells in the absence of T140 was used as control for normalization. Data are mean ± S.D. of three independent experiments. *, $p < 0.05$ compared with control.

crease in the binding of [^{35}S]GTPγS to Gα_i proteins was observed in membrane fractions from transfectants expressing CXCR4(N119S) in the presence of NPM, as shown in Fig. 4A. Increasing doses of recombinant NPM decreased [^{35}S]GTPγS binding after stimulation of wild-type CXCR4 by 10 nM CXCL12 in a dose-dependent manner, reaching statistical significance at recombinant NPM concentrations of 3, 10, and 30 μg/ml (Fig. 4B).

Exposure of membrane fractions that expressed wild-type CXCR4 to recombinant NPM (20 μg/ml) shifted the CXCL12 dose-response curve for [^{35}S]GTPγS binding to the right, with differences that achieved statistical significance (Fig. 4C). The presence of 20 μg/ml recombinant NPM increased the EC_{50} values from 11 to 27 nM in this experiment. The functional importance of the basic residues of CL-3 was demonstrated by measuring the binding of [^{35}S]GTPγS to G proteins in membrane fractions of CHO-K1 cells transiently expressing CXCR4 or a CXCR4 mutant containing the conversion of the Lys-Arg-Lys sequence to Ala-Ala-Ala (CXCR4-AAA) after exposure to 100 nM CXCL12. The AAA mutation itself reduced the activity of CXCR4 induced by CXCL12 (Fig. 4D). However, [^{35}S]GTPγS binding was not altered by the addition of NPM to the CXCR4-AAA mutant (Fig. 4D), which was demonstrated to lack binding to NPM. In contrast, NPM significantly reduced wild-type CXCR4 induced binding of [^{35}S]GTPγS to G proteins at both 1 and 100 nM CXCL12.

NPM Negatively Regulated CXCR4-Mediated Chemotactic Responses: Enhancement with NPM Suppression and Decrease with Augmented Cytosolic NPM Expression. To establish whether the negative regulation of CXCR4 signaling by NPM has biological relevance, the chemotactic response to CXCL12 was determined in the presence and absence of siRNA that suppresses NPM expression. As shown in Fig. 5A, transient expression of a hairpin siRNA for NPM in HEK-293 transfectants stably expressing CXCR4 resulted in a consistent decrease of NPM detected by Western blotting in two independent experiments, 50 and 41%, respectively. The knockdown of NPM did not affect cell viability as assessed by propidium iodide exclusion or the level of CXCR4 expression as measured by flow cytometry (data not shown). Transient expression of the siRNA in HEK-293 CXCR4 transfectants resulted in an increase in the chemotactic response to incremental concentrations of CXCL12 (Fig. 5B). Quantitative analysis of the chemotaxis experi-

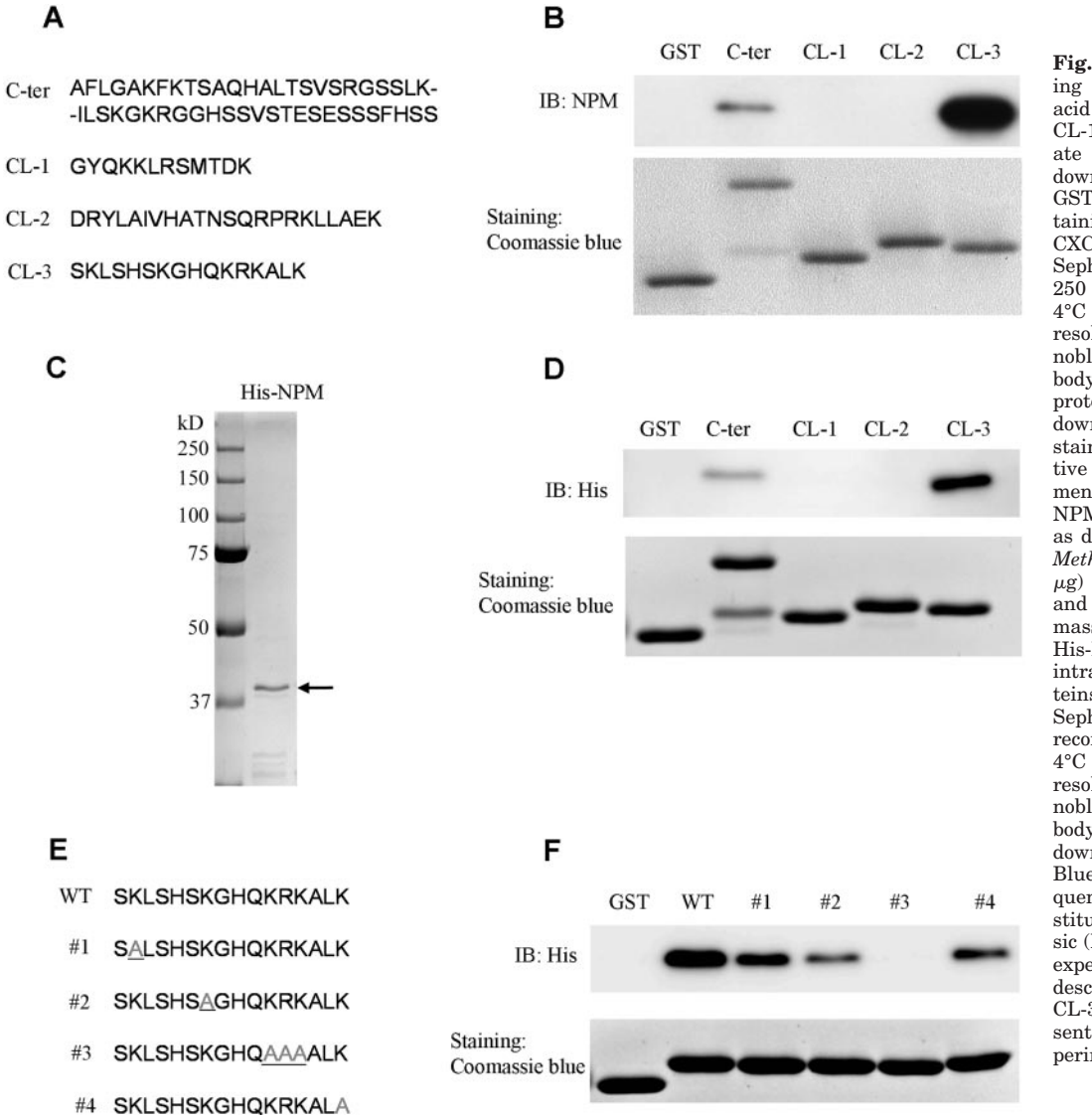

Fig. 3. Mapping of the NPM binding region in CXCR4. A, amino acid sequence of CXCR4 C-ter and CL-1, CL-2, and CL-3 used to create GST fusion proteins for pull down experiments. B, 10 μg of GST or GST fusion proteins containing cytoplasmic domains of CXCR4 bound to glutathione-Sepharose 4B for incubation with 250 μg of HEK-293 cell lysates at 4°C for 2 h. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-NPM antibody. Equal amount of GST fusion proteins were present in each pull down, as shown by Coomassie Blue staining. The data are representative of three independent experiments. C, recombinant His-tagged NPM was expressed and purified as described under *Materials and Methods*. Purified His-NPM (0.3 μg) was subjected to SDS-PAGE and revealed by staining with Coomassie Blue. The arrow indicates His-NPM. D, GST or GST-CXCR4 intracellular domain fusion proteins (10 μg) bound to glutathione-Sepharose 4B were incubated with recombinant His-NPM (1 μg) at 4°C for 2 h. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-His tag antibody. Fusion proteins in each pull down are shown by Coomassie Blue staining. E, CXCR4 CL-3 sequence and the mutants with substitution of Ala (underlined) for basic (K or R) residues. F, pull down experiments were performed as described above using different CL-3 mutants. The data are representative of four independent experiments.

ments resulted in an increase in the number of cells that migrated to the bottom of the transwell membrane in four independent experiments, but the bell-shaped dose-response relationship was maintained. In the representative experiment shown in Fig. 5C, knockdown of NPM resulted in a statistically significant increase in the chemotactic response to CXCL12 at concentrations of 0.1, 1, and 10 nM, which confirms that the negative regulation of CXCR4 signaling by NPM may have relevant physiological consequences. Further experiments demonstrated that overexpression of a Flag-tagged NPM mutant that is primarily localized to the cytosolic compartment (Fig. 5D), significantly reduced the chemotactic response to CXCL12 compared with pcDNA3 control transfectants (Fig. 5, E and F). This suggests that the amount of NPM present in the cytosol can modulate the functional activity of CXCR4.

Discussion

CXCR4 plays a key role in directing the trafficking of precursor cells during embryogenesis and adulthood and in the recruitment of mature inflammatory cells to sites of pathological insult in response to a chemotactic gradient of the exclusive ligand CXCL12, as well as in the entry of CD4-positive target cells by T-tropic strains of HIV-1 (Loetscher et al., 1994; Bleul et al., 1996; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). The range of pathological processes that involve CXCR4 has generated a significant interest in mechanisms that regulate CXCR4 signaling, although the insight is limited. The physical interaction of cytoplasmic domains of CXCR4 with a spectrum of proteins, including heterotrimeric G proteins and β-arrestin, regulates signaling and desensitization, respectively (Brelot

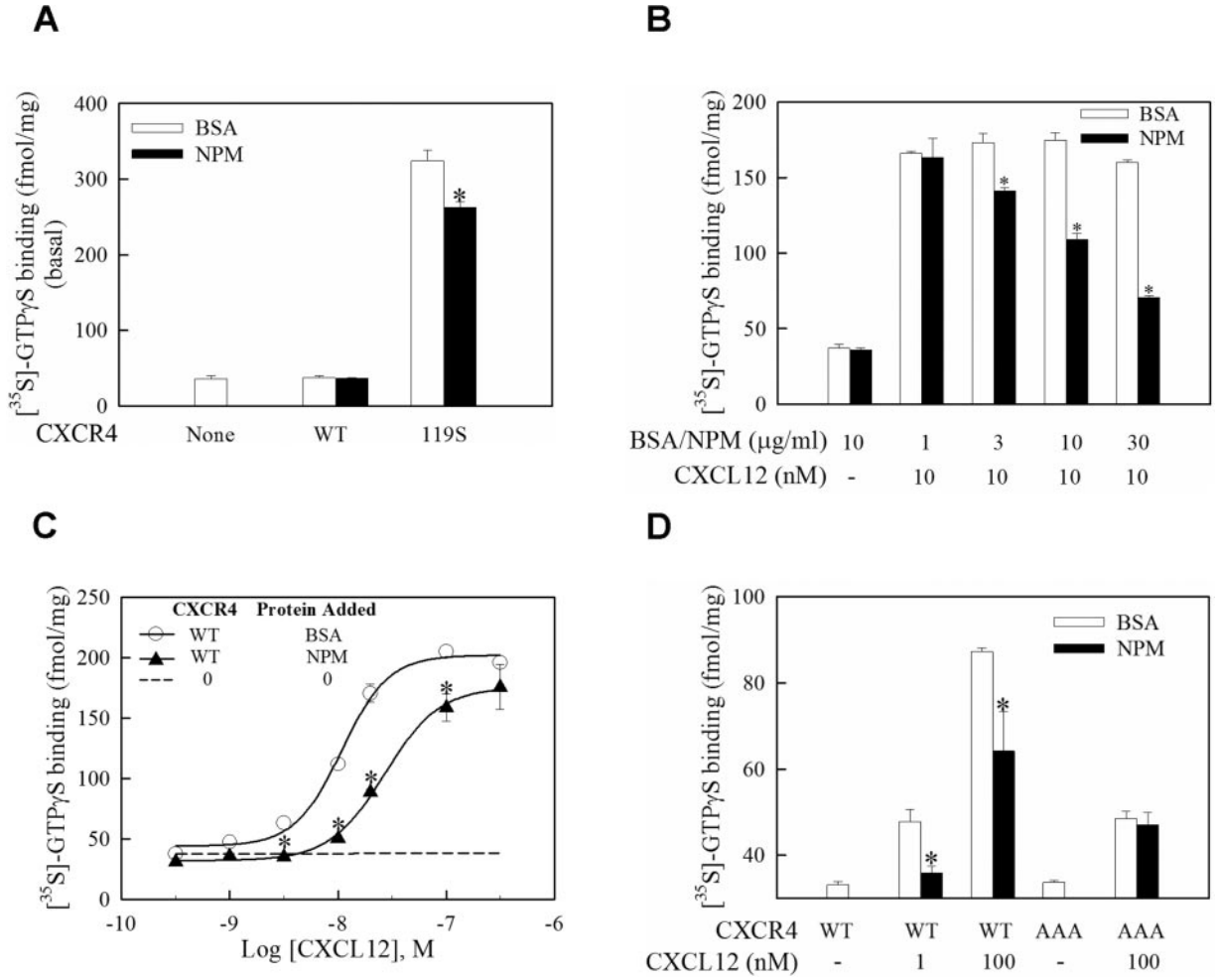

Fig. 4. Inhibition of CXCR4-mediated G protein activation by recombinant His-NPM. CXCR4-mediated G protein activation was measured by [^{35}S]GTPγS binding assays as described under *Materials and Methods*. A, basal [^{35}S]GTPγS binding of membrane fractions from CHO-K1 cells transfected with pcDNA3 vector control (None) or CHO-K1 cells stably expressing CXCR4-WT or CXCR4–119S in the presence of 20 μg/ml BSA or His-NPM protein. The values are the means ± S.D. of duplicate samples. The results are representative of two independent experiments. *, $p < 0.05$ compared with BSA control. B, membranes from CHO-K1 cells stably expressing CXCR4-WT were incubated with BSA or His-NPM at the indicated concentrations in the absence or presence of 10 nM CXCL12. The values are the means ± S.D. of duplicate samples. The results are representative of three independent experiments. *, $p < 0.05$ compared with the BSA at the corresponding protein concentration. C, membranes from CHO-K1 cells stably expressing CXCR4-WT (solid line) were treated with the indicated concentrations of CXCL12 in the presence of 20 μg/ml BSA (○) or His-NPM (▲). The basal [^{35}S]GTPγS binding of cells that did not express CXCR4 is shown (dashed line). The values are the means ± S.D. of duplicate samples. The results are representative of three independent experiments. *, $p < 0.05$ compared with the control at the corresponding CXCL12 concentration. D, membranes from CHO-K1 cells transiently expressing CXCR4-WT or the CXCR4-AAA mutant were incubated with 20 μg/ml BSA (control) or His-NPM protein in the presence of CXCL12 as indicated. The values are the means ± S.D. of duplicate samples. The results are representative of two independent experiments. *, $p < 0.05$ compared with the control.

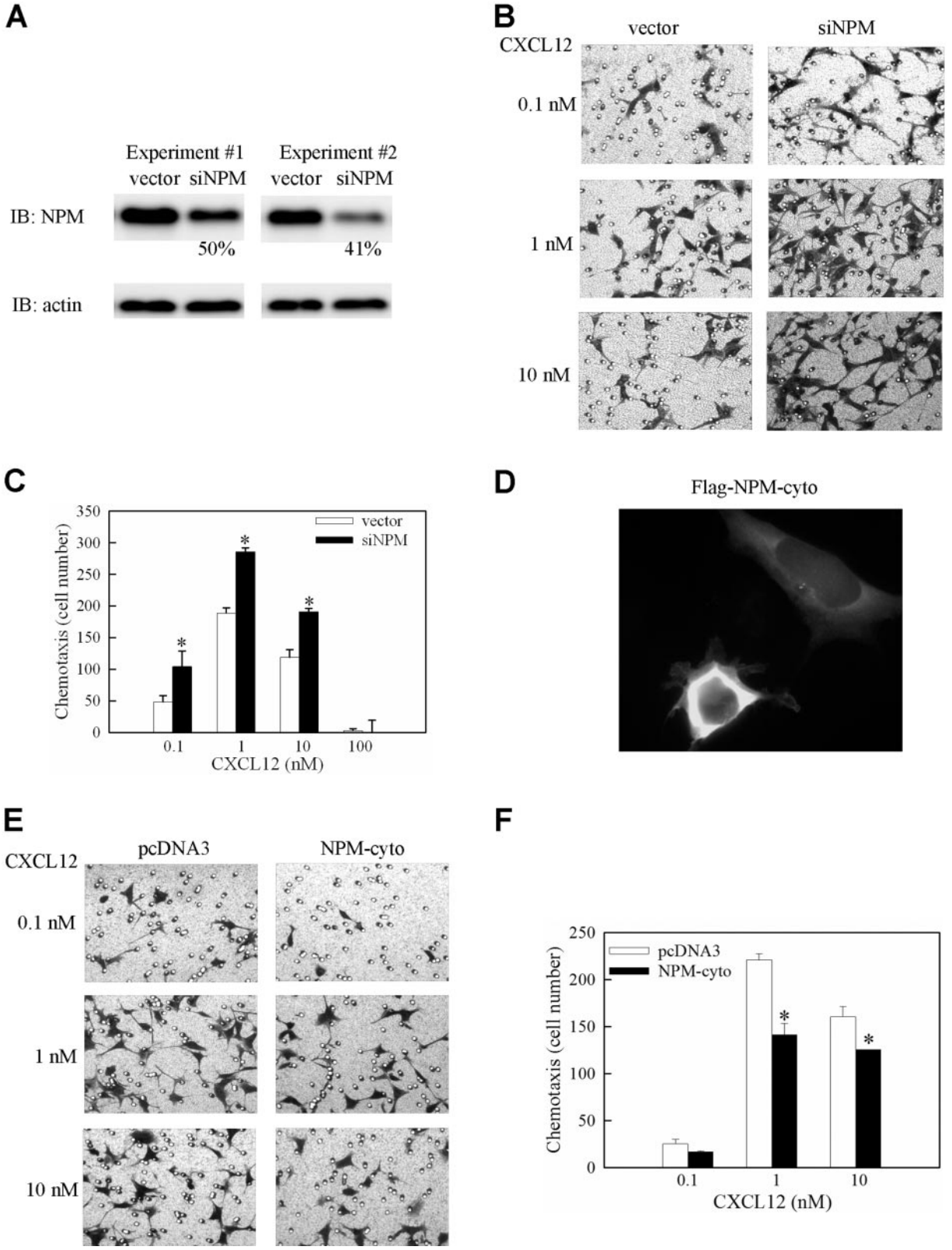

Fig. 5. NPM negatively regulates CXCR4-mediated chemotaxis. A, HEK-293-CXCR4 cells were transfected with a pENTRTM/U6 vector-based siRNA for human NPM (siNPM) or control pENTRTM/U6 vector for 72 h. Matched amounts of cell lysates (2.5 μg) from cells exposed to siRNA, and controls were resolved by SDS-PAGE and immunoblotted with anti-NPM antibody or anti-actin antibody. B, HEK-293-CXCR4 cells were transfected with pENTRTM/U6 vector or siNPM. Chemotaxis assays with different concentrations of CXCL12 were performed 72 h after transfection, as described under *Materials and Methods*. Representative chemotactic migrations are shown. C, quantitative data from 10 random fields from B. Basal cell migration in the absence of CXCL12 was subtracted from each data point. The values are the means ± S.D. of triplicate samples. The results are representative of four independent experiments. $*$, $p < 0.05$ compared with the vector transfectants. D, HEK-293-CXCR4 cells transiently expressing Flag-NPM cytosolic mutant (Flag-NPM-cyto) were fixed, permeabilized, and incubated with a mouse monoclonal antibody for Flag tag antibody (M2),

et al., 2000; Cheng et al., 2000; Roland et al., 2003; Akekawatchai et al., 2005). Based on the importance of the cytoplasmic interhelical loops and carboxyl tail in signaling and desensitization, we used a proteomic approach to identify cytosolic proteins that physically associate with these domains of CXCR4. Seven proteins were identified to interact with CXCR4, including heat shock 70-kDa protein cognate, which has been reported to regulate CXCR4 trafficking (Ding et al., 2006). Here, we report that NPM and repressor of estrogen receptor activity are physically associated with cytoplasmic domains of CXCR4 and that NPM regulates signal transduction through this receptor and chemotaxis, the principal biological response thereof.

NPM is a phosphoprotein that is located primarily in the nucleolus and that has been implicated in a variety of functions, including ribosome biogenesis, maintenance of genomic integrity, and prevention of protein aggregation through a molecular chaperone function (Hingorani et al., 2000; Grisendi et al., 2006). Although the majority of NPM protein resides in the nucleus, it functions as a molecular shuttle to the cytoplasm and variant forms that result from chromosomal translocations or mutations may be redirected to the cytosol (Grisendi et al., 2006; Falini et al., 2007). There is little insight available regarding the roles of NPM outside of the nucleus, aside from regulation of cell cycle progression by interaction with the centrosome (Okuda et al., 2000), regulation of apoptosis via binding to Bax (Kerr et al., 2006), and functioning as a component of the fructose lysin-specific binding proteins in the plasma membrane (Brandt et al., 2004). In the current study, we identified the physical association of NPM with CXCR4 mutants that assume a conformation that confers autonomous and constitutive G protein activation, suggesting that it may regulate signal transduction. The finding that knockdown of NPM expression enhanced CXCR4-mediated chemotaxis supports this potential physiological role. This is, to our knowledge, the first report that NPM forms a complex with a GPCR and functions to regulate receptor signaling. An analogous role has been demonstrated recently for β-arrestin, a scaffold protein that modulates GPCR signaling and trafficking, which was shown to be translocated to the nucleus after GPCR activation and to modulate transcription by interacting with nuclear regulatory factors (Kang et al., 2005).

CXCR4 mutants that are constitutively coupled to G proteins and chronically phosphorylated (Zhang et al., 2002) showed greater association with NPM than was observed for the wild-type receptor. Reversal of this conformation with T140, a potent inverse agonist that switches receptor architectural equilibrium to the "off" state, resulted in a decrease in NPM interaction with CXCR4 in immunoprecipitation experiments. In contrast, activation of wild-type CXCR4 by CXCL12 binding did not have a dramatic effect on the association of NPM with cytoplasmic loops of the receptor, raising the possibility that the interaction may be an indirect interaction that involves additional subunits present in the milieu of chronic desensitization. This possibility was excluded by the finding that recombinant NPM actively associated with recombinant GST fusion proteins containing CL-3 or the C-ter domains, as would be observed in a direct interaction. The direct nature of the interaction is also supported by the finding that NPM binding to CL-3 inhibits the activation of Gα subunits, which also require CL-3 for pertussis toxin-sensitive functions (Brelot et al., 2000; Roland et al., 2003). An alternative possibility is the SDF-1-induced active receptor conformation is not maintained well under the conditions used for immunoprecipitation. Transition to an active conformation of CXCR4 requires the high-affinity engagement of the N-loop of SDF-1 with the extracellular loops of CXCR4 followed by the interaction of N terminus of SDF-1 with transmembrane helices of CXCR4, a critical step but with lower affinity (Huang et al., 2003). This transition may be transient, depending on the stability/durability of the ligand-receptor interaction. In contrast, the active conformation of constitutively active mutants does not require this interaction, and it is not transient in nature.

Here, we provide a novel mechanism for suppression of CXCR4-mediated G protein activation via directly interacting with NPM in the CL-3 of CXCR4. In the presence of NPM, the EC_{50} value of CXCL12-induced G protein activation was increased, and NPM inhibited CXCL12-induced G protein activation in a dose-dependent manner. This effect is unlikely to have been due to alterations in CXCL12 binding to the receptor because NPM also reduced CXCR4-constitutively active mutant activation of G proteins, which occurs in the absence of CXCL12. Moreover, deletion of NPM binding sites in the CL-3 significantly reduced the inhibition of G protein activation by NPM. Positively charged amino acid residues in CL-3 of GPCRs have been thought to be the important sites for G protein binding and activation, and partial truncation of this domain in CXCR4 abolishes the calcium mobilization response to CXCL12, although it has no effect on receptor expression (Brelot et al., 2000). Our results suggest that NPM may compete with G protein subunits for binding to CL-3 with a mechanism analogous to the negative regulation of GPCR activation by calmodulin (Wang et al., 1999) and cytoskeletal protein 4.1G (Lu et al., 2004). However, based on the reports that other proteins, such as filamin, bind to the CL-3 of the dopamine D2 receptor to modulate receptor trafficking (Lin et al., 2001), we cannot exclude the possibility that NPM binds to other proteins to form a receptor complex that modulates GPCR trafficking and also plays a positive role in the other signaling cascade induced by CXCR4.

We demonstrated that NPM was involved in CXCR4-mediated cell chemotaxis by showing enhanced CXCL12-elicited cell migration after knockdown of NPM expression with specific siRNA for NPM. This is the first report that CXCR4-mediated cell migration is negatively regulated by a protein that interacts with cytoplasmic domains of the receptor. Mechanisms underlying this regulation may be the result of the competition of G protein binding by NPM. Interruption of G protein activation by pertussis toxin has been shown to

followed by a Alexa Fluor 488-conjugated anti-mouse secondary antibody. After immunostaining, cell images were taken by fluorescence microscopy. Representative image is shown. The transfection efficiency is approximately 40% by counting the ratio of fluorescent cells to the total cells. E, HEK-293-CXCR4 cells were transfected with pcDNA3 vector or Flag-NPM-cyto. Chemotaxis assays with different concentrations of CXCL12 were performed 48 h after transfection, as described under *Materials and Methods*. Representative chemotactic migrations are shown. F, quantitative data from 10 random fields from E. Basal cell migration in the absence of CXCL12 was subtracted from each data point. The values are the means ± S.D. of duplicate samples. The results are representative of two independent experiments. *, $p < 0.05$ compared with the pcDNA3 transfectants.

suppress CXCR4-mediated chemotaxis in peripheral blood cells and bone marrow cells, which indicates activation of G protein is an indispensable step for this function (Papayannopoulou et al., 2003). However, our observation also did not rule out the possibility that NPM regulates CXCR4-mediated chemotaxis via modulation of receptor trafficking or competition for recruitment of Akt and activation by binding to phosphatidylinositol-(3,4,5)-trisphosphate (Ahn et al., 2005).

Directed migration mediated by CXCR4 has been implicated in the metastatic spread of tumor cells, and CXCL12 secretion by stromal cells in the microenvironment may provide a niche for malignant cells (Müller et al., 2001; Burger and Kipps, 2006). Expression of CXCR4 has been associated with a poor prognosis in acute myelogenous leukemia (Spoo et al., 2007). Inhibition of CXCR4 was found to enhance chemotherapy-induced apoptosis in a subset of leukemic myeloblasts that carry Flt3 mutations and to overcome chemoresistance associated with stromal activity (Zeng et al., 2006). NPM variants with cytoplasmic localization represent the most common mutations detected in myeloid malignancies and are associated with a favorable clinical outcome (Döhner et al., 2005; Falini et al., 2007). We showed here that expression of this mutant results in a decrease in CXCR4-mediated chemotaxis, which suggests that the interruption of myeloid cell trafficking from decreased CXCR4 function due to inhibition by NPM could decrease the ability of CXCL12 to program a microenvironment that supports the homeostasis of leukemic cells (Burger and Kipps, 2006). This provides a rationale for investigating the role of CXCR4 blockade in the therapy of acute myelogenous leukemia.

Here, we present evidence for a new association between a GPCR for a chemoattractant, CXCR4 (Loetscher et al., 1994; Bleul et al., 1996), and NPM, a multifunctional phosphoprotein with known functions, in the nucleus in normal physiology and unscheduled localization to the cytoplasm in the pathological physiology of acute myelogenous leukemia (Grisendi et al., 2006; Falini et al., 2007). The physical association of CXCR4 and NPM was demonstrated using multiple independent approaches, and it was shown to have a significant negative impact on the chemotactic response to the cognate ligand CXCL12. Insight into mechanisms to disrupt CXCR4 function by extending physiological or pathological mechanisms and/or using pharmacological approaches may open important avenues for developing therapeutic approaches for myeloid malignancies, as well as HIV-1 infection.

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