Chemokine Receptor−Ligand Interactions Measured Using Time-Resolved Fluorescence

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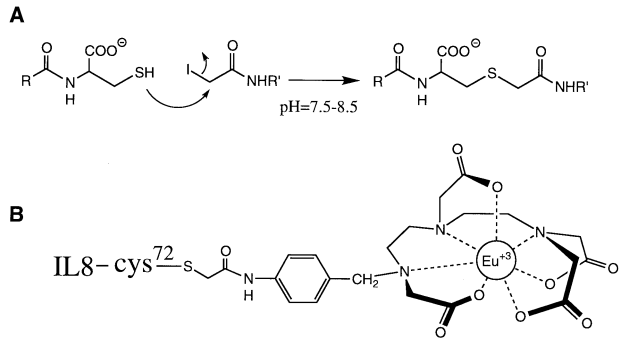
ABSTRACT: Two G protein-coupled receptor subtypes (CXCR1 and CXCR2) mediate Interleukin-8 (IL8) action in cells. A nonradioactive lanthanide−chelate derivatized IL8 ligand was developed to measure the binding activity of the chemokine receptors, CXCR1 and CXCR2. Site-specific mutagenesis of the carboxyl-terminal serine of IL8 to cysteine resulted in a mutant IL8 (IL8-S72C) having a single free sulfhydryl. Using an iodoacetamide derivative of the Eu^{3+}-chelate of *N*-(*p*-benzoic acid)diethylenetriamine-*N*,*N*′,*N*″-tetraacetic acid (DTTA), incorporation of one Eu^{3+} per IL8 molecule ([Eu^{3+}]IL8-S72C) was achieved. The dissociation constant for this conjugate was similar to that measured for [^{125}I]IL8 (∼2 nM) when measured by time-resolved fluorometry using CHO cell lines stably expressing CXCR1 or CXCR2 receptors. The sensitivity, stability, and high specific activity of europium-labeled IL8 demonstrate the usefulness of lanthanide-labeled proteins in the measurement of receptor−ligand interactions and may be extended to other peptide ligands.

Interleukin-8 (IL8),[1] the best-characterized chemokine, is produced by a variety of cell types involved in inflammatory settings. It is known to act mostly on neutrophilic polymorphonuclear leukocytes (neutrophils), promoting their migration to the site of inflammation and causing their subsequent activation (*1*, *2*). IL8 (together with other chemokines) is elevated in a variety of pathophysiological conditions, including lung diseases such as cystic fibrosis and adult respiratory distress syndrome, atherosclerosis, psoriasis, rheumatoid arthritis, and reperfusion injury (*3*). The causal role of IL8 in the onset of inflammation was also demonstrated in animal models (*4*, *5*). This vast body of evidence has led toward efforts to discover antagonists of IL8 binding to its specific receptors on the cell surface as therapeutic agents in IL8-dependent disorders. To devise an alternative to radiolabeled-chemokines in high-throughput drug screening, we developed a rationally designed, fully active fluorescent IL8 derivative with a detection sensitivity similar to [^{125}I]IL8.

Two receptor subtypes (CXCR1 and CXCR2) mediate IL8 action in cells (*6*, *7*). Together with the other chemokine receptors, these subtypes belong to the large family of seven-transmembrane G protein-coupled receptors. While CXCR1 binds only IL8 with high (nM) affinity, CXCR2 binds a broader spectrum of chemokines equally including IL8, GROα, GRO*β*, ENA-78, and GCP-2 (*8*). The structural and functional features of the chemokines, including specifically IL8, are well-characterized through a combination of NMR, X-ray crystallography, and site-directed mutagenesis (*9*, *10*). Multiple regions of the IL8 molecule are essential for receptor binding and activation (*11*). However, many residues can be substituted in the C-terminal region with no loss of activity, suggesting that modifications of the C-terminus of IL8 should not impair its interaction with its receptors.

Scheme 1



We modified the carboxyl terminus of IL8 by replacing serine72 with a reactive cysteine residue. The reactions of sulfhydryl groups with α-haloketones, amides, and acids in the physiological pH range (pH 6.5−8.0) are well-known (reviewed in ref 12; Scheme 1A) and allow for the specific modification of cysteines in proteins (*13*). Since the four naturally occurring cysteines of IL8 form two intramolecular disulfide bonds, a fifth cysteine should exist as a reduced sulfhydryl. Furthermore, based on the ^{1}H NMR solution structure of the IL8 carboxyl terminus (*14*), this residue is surface localized and should be accessible to modification by an appropriate α-haloacetamide.

Lanthanide-ion-chelates (Scheme 1B) are ideal reporter groups because of their unique fluorescent properties, which

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[1] Abbreviations: ACN, acetonitrile; BODIPY, (N^1-B)-$N^{1'}$-(difluoroboryl)-3,5′-dimethyl-2,2′-pyrromethene-5-propionic acid, *N*-succinimidyl ester; BSA, bovine serum albumin; DTTA, *N*-(*p*-benzoic acid)diethylenetriamine-*N*,*N*′,*N*″-tetraacetic acid; IL8, Interleukin-8; IL8-S72C, ser72cys Interleukin-8 mutant; IL8-S72C^{ox}, intermolecular cysteine72 disulfide of Interleukin-8; TBS, Tris-buffered saline.

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can be measured using time-resolved fluorometric microplate readers. The long-lived and sharp emission spectrum of certain lanthanide ions, e.g., europium, has allowed antibody labeling with detection sensitivities similar to those obtainable using radioisotopes, thus making lanthanides useful in immunodetection assays (*15*). These highly sensitive nonradioactive probes should have broad applicability in the study of receptor−ligand interactions.

EXPERIMENTAL PROCEDURES

Materials. Eu^{3+}-chelate of *N*-(*p*-iodoacetamidobenzyl)-diethylenetriamine-*N*,*N*′, *N*′′-tetraacetic acid (DTTA), Enhancement Solution for measuring Eu^{3+}, and Optiphase scintillation fluid were from Wallac. Vector pET30b was from Novagen. pcDNA3 was from Invitrogen. Microtiter plates were from Costar. [^{125}I]IL8 was obtained from Dupont/NEN. Chinese hamster ovary cells (CHO-K1) cells were from American Type Culture Collection. The Jupiter reverse phase high-performance liquid chromatography (HPLC) column was from Phenomenex. Europium−streptavidin (Wallac) was conjugated to a stoichiometry of 7 Eu^{3+}-chelates per streptavidin; fluorescein-deglyco-avidin (Biomeda) was conjugated to a stoichiometry of 5 fluoresceins per avidin; and rhodamine- and BODIPY-NeutaLite avidin (Molecular Probes) were both conjugated to a stoichiometry of 3.3 fluorophores per avidin.

Methods. Receptor cDNAs and Cell Lines. CXCR1 cDNA was amplified from dibutyryl cAMP-differentiated HL-60 cells and cloned into pcDNA3; CXCR2 cDNA was obtained from P. Murphy (NIH) and recloned into pcDNA3. cDNAs encoding CXCR1 (IL8RA) or CXCR2 (IL8RB) were transfected into CHO-K1 cells. Cell clones expressing >100 000 binding sites/cell were isolated using G418 selection (1 mg/mL) and subsequently maintained in Ham's F12 medium supplemented with 10% fetal bovine serum and 0.25 mg/mL G418.

Construction of an Expression Plasmid for Human IL8 and IL8-S72C. The cDNA for the coding sequence of human IL8 (amino acids 1−72 of the mature IL8) was synthesized by PCR from human thymus cDNA (Clontech). PCR primers [5′-IL8 primer 5′ ggg cac gca tat gag tgc taa aga act tag atg tca g 3′ and 3′-IL8 primer 5′ cgg gat cct tat gaa ttc tca gcc ctc ttc aa 3′] containing Nde1 and BamH1 sites were used to construct the IL8 coding sequence and clone this product into the Nde1-BamH1 sites of the *E. coli* expression plasmid pET30b to give pET30b-IL8 and the pET30b-IL8-S72C. Construction of the IL8-S72C employed a mutagenic 3′-primer containing the following sequence: 5′ cgg gat cct tag caa ttc tca gcc ctc ttc aaa aac 3′. The 5′-primer contained an initiation Met directly preceding serine1 and the 3′-primer contained a stop codon directly following S72 or C72. PCR was performed with Pfu polymerase. The constructs were introduced in the *E. coli* strain BL21(DE3).

Expression and Purification of Recombinant Human IL8 and IL8-S72C. The pET30b-IL8 and pET30b-IL8-S72C plasmids were used to transform competent *E. coli* strain BL21 (DE3) employing standard protocols (Novagen's protocol). Wild-type and mutant IL8 were produced in *E. coli* as inclusion bodies. The following purification method was identical for both proteins. Cells pelleted from a 1 L culture induced with 0.5 mM IPTG for 4 h were resuspended in lysis buffer A (Tris−HCl, 5 mM pH 8.0; EDTA, 2 mM; Tween-20, 0.5%) to approximately 5−10 mL/g of cells and subjected to three to four freeze/thaw cycles. Chromosomal DNA was sheared by repeated passage through syringe needles of increasing gauge. Insoluble material was collected by centrifugation and pellets were resuspended in urea wash buffer (Tris−HCl, 20 mM, pH 8.0; urea, 0.75 M; Tween-20, 0.5%) and centrifuged (85000*g* for 20 min). Resuspension in urea and centrifugation were repeated twice or until the clarified supernatant was colorless. The urea-washed pellet was taken up in 40 mL of 6 M guanidine HCl, 20 mM PBS, pH 6.0, and resuspended until no further solubilization occurred, and centrifuged at 85000*g* for 1 h at 4 °C. The supernatant was dialyzed (Spectra/Por Membrane MWCO 2,000) against 20 mM sodium phosphate buffer, pH 6.5 at 4 °C, with buffer changes every 4 h until the guanidine concentration was calculated to be below 1 μM. The resultant flocculent solution was centrifuged (23000*g* for 20 min at 4 °C) and the supernatant carefully removed and stored at 4 °C until further purified by cation exchange chromatography.

Perfusion Cation-Exchange Chromatography. Perfusion chromatography was performed at room temperature using a BioCAD Perfusion Chromatography Workstation (PerSeptive Biosystems) connected to an SF-2120 Super Fraction Collector (Advantec). The clarified dialyzate (total protein ∼45 mg) was loaded onto a POROS 20CM column (4.6 mm diameter × 100 mm length). The sample was eluted with a NaCl gradient (0−800 mM NaCl over 20 column volumes) buffered with 20 mM sodium phosphate, pH 6.5. The IL8 was eluted at ∼400 mM NaCl. The column flow rate was 10 mL/min (3600 cm/h) and the UV monitor set at 280 nm. The fraction sizes were 1 mL of which 2 μL samples were analyzed by SDS−PAGE on a 16% Tris-Gly gel (Novex) to ascertain purity. Protein concentration was determined for each fraction by Bradford assay (*16*) using BSA as a standard.

HPLC Conditions. Purification of IL8 and IL8-S72C and analysis of the labeling reaction were carried out using a Dynamax HPLC System (Rainin) with a reverse phase column (particle size, 5 μm; stationary phase, C5; pore size, 300 Å; 250 × 4.6 mm). The mobile phase was water, 0.1% trifluoroacetic acid/acetonitrile, 0.1% trifluoroacetic acid. The gradient was linear from 20 to 60% acetonitrile at 1%/min. For analysis of labeling reactions, 200−500 pmol of sample was injected. For purification of IL8 or IL8-S72C, samples ranging from 1 to 30 nmol (8−240 μg) were injected. Separations were monitored at both 215 and 280 nm. For purification, protein peaks were collected by hand, flash-frozen, and dried on a Speed Vac (Savant Instruments).

Preparation of [Eu^{3+}]IL8-S72C. To 300 nmol (227 μg) of Eu^{3+}−*N*-iodoacetamide (Wallac; 756.3 g/mol; $C_{21}H_{25}N_4O_9$-EuI) was added 160 μg (19 nmol in 40 μL of PBS) of IL8-S72C. The reaction was studied at pH 7.5 and 8.5. The tube was vortexed and placed at ambient temperature. Aliquots (1 μL, ∼0.5 nmol) were removed at indicated times to monitor labeling progress by HPLC. When the reaction was judged complete, the sample was diluted 10-fold into Tris-buffered saline (TBS), pH 7.5, dialyzed (Spectra/Por, MWCO = 2000) against TBS (4 × 4 L) at 4 °C and filtered through P-2 Bio-Gel (Bio-Rad) in Ultrafree centrifuge tubes

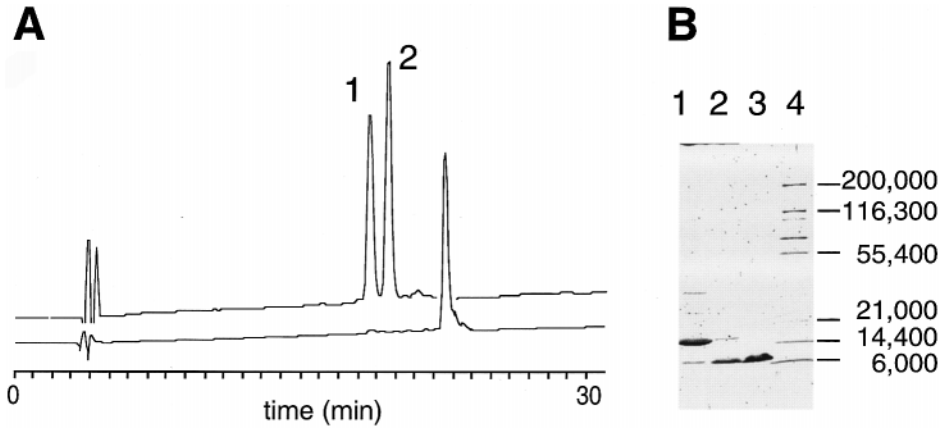

FIGURE 1: Analysis of IL8, IL8-S72C, and oxidized IL8-S72C (IL8-S72C^{ox}). (a) (upper trace) HPLC chromatogram of a co-injection of IL8 (peak 1) and IL8-S72C (peak 2) obtained after perfusion cation-exchange chromatography purification; (lower trace) sample of oxidized IL8-S72C^{ox}. (b) Nonreducing SDS−PAGE analysis of IL8-S72C^{ox}, IL8-S72C, and IL8: (lane 1) 10 μg of IL8-S72C^{ox}; (lane 2) 10 μg of IL8-S72C; (lane 3) 10 μg of IL8; (lane 4) molecular weight makers.

(Millipore) to remove excess Eu^{3+}−DTTA label. The protein concentration was measured and the specific activity was determined using a europium standard solution (Wallac).

Fluorescence Plate Reader. Standard fluorophores were counted on a FluoStar fluorescence microplate reader (SLT instruments) using the appropriate excitation/emission wavelength settings for each fluorophore (fluorescein 485/538 nm; rhodamine 544/590 nm; BODIPY 485/538 nm). The gain was set automatically against the well containing the highest concentration of fluorophore. A stock solution of each labeled protein was made in TBS/0.1% BSA based on the fluorophore molarity.

Ligand Binding Assay. CHO cells expressing either CXCR1 or CXCR2 were seeded in 96-well tissue culture plates. When [^{125}I]IL8 was used as a ligand, microtiter plates with opaque walls/black mask were used (Wallac). Confluent cells (∼40000/well) were washed with binding buffer (25 mM Hepes, 75 μM EDTA; 11.5 mM KCl, 115 mM NaCl, 6 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 0.25% BSA) and incubated with the ligands for 1 h at room temperature. Plates were then washed three times with binding buffer. Optiphase scintillation fluid (Wallac) was added (80 μL/well), and the plates were shaken for 5 min. Plates were counted in a Microbeta liquid scintillation counter, Model 1450 (Wallac). When [Eu^{3+}]IL8-S72C was used as a ligand, cells were seeded in transparent polystyrene culture plates (Costar), and enhancement solution (80 μL/well) was added prior to counting in a DELFIA fluorometer Model 1234 (Wallac). The europium counting protocol was used, with a 320 nm excitation pulse at a frequency of 1000 s^{-1} and detection at 615 nm (emission wavelength). Specific fluorescence was measured after a 400 μs time delay (for decay of background fluorescence) for 400 μs between each excitation pulse. Nonspecific binding was defined using 3 μM IL8. Each determination was in three to five replicates.

Mass Spectroscopy. Mass spectra of IL8, IL8-S72C, and IL8-S72C^{ox} were obtained on a Finnigan MAT LCQ spectrophotometer by injecting 5 μL of a 12.5 μM solution (60 pmol) of the chemokine in 50% ACN/50% H_2O containing 0.5% HOAc.

Data Analysis. Ligand binding data were analyzed by nonlinear least-squares regression using Prizm (GraphPad Software). Saturation data were fitted to a rectangular hyperbola and competition data were fitted to a sigmoidal curve with a slope of 1. Inhibition constants (K_i) were determined from IC_{50} values using the Cheng−Prusoff equation (*17*).

RESULTS

Protein Expression, Purification, and Characterization. The *trp* promoter-driven bacterial expression and purification of IL8 have been reported previously (*18*). The preparation of IL8 and IL8-S72C reported here was accomplished using a bacterial T7 expression system (*19*). The proteins were overexpressed as inclusion bodies, solubilized with guanidine, refolded, and purified by fast-flow cation-exchange perfusion column chromatography with yields comparable to Furuta and co-workers (*18*). For example, 1 L of cells resulted in ∼30 mg of >90% pure IL8 or IL8-S72C as assessed by HPLC and SDS−PAGE analysis (Figure 1). Purification to homogeneity was achieved on a small scale using HPLC. The single substitution of the carboxyl terminal serine (S72) by cysteine resulted in a shift in retention time (+0.9 min) on reverse phase HPLC (Figure 1A).

Intermolecular disulfide oxidation of cysteine72 (C72) in the mutant IL8 (IL8-S72C^{ox}) occurred slowly over time at pH 6.5 or rapidly at pH above 7.0. Formation of this species was easily detected by an increase in retention time of 2.8 min on HPLC (Figure 1A). Storage of 1 mM IL8-S72C at pH 6.5 in phosphate buffer containing 200 mM NaCl for ∼1 year at 4 °C resulted in 34% conversion to the oxidized form. IL8-S72C^{ox} could be converted to the reduced form (IL8-S72C) by incubation with 1 mM β-mercaptoethanol. The β-mercaptoethanol could then be removed by rechromatographing on HPLC. The dimeric nature of this species was confirmed by migration of IL8-S72C^{ox} on a nonreducing SDS gel at a mass approximately twice that of IL8 or IL8-S72C (Figure 1B). The C72 mutant had a binding affinity similar to that of IL8 prepared using the same expression and purification procedure (Table 1).

IL8, IL8-S72C, and IL8-S72C^{ox} were characterized by electrospray mass spectroscopy. IL8 is a basic protein containing 10 acidic and 16 basic side chains. The resulting net charge of +6 falls within the range of charged species seen in the protein mass ion envelope (data not shown), the predominant mass ion being a +7 species. The spectroscopic

Table 1: Binding Parameters for [Eu^{3+}]IL8-S72C and [^{125}I]IL8[a]

	[Eu3+]-IL8 S72C	[^{125}I]-IL8
CXC R1 K_D	1.4 ± 0.5 (3)	1.9 (1)
B_{max} (×10^3)	180 ± 100 (3)	260 (1)
K_i (IL8)	5 (1)	3.3 ± 2.3 (4)
K_i (GROα)	>300 (1)[b]	>300 (1)[b]
CXC R2 K_D	2.4 ± 1.1 (4)	2.8 (1)
B_{max} (×10^3)	500 ± 100 (4)	600 (1)
K_i (IL8)	3.7 ± 2.4 (4)	2.3 ± 0.8 (4)
K_i (GROα)	3.3 ± 0.8 (3)	1.5 ± 0.6 (2)
K_i (IL8S72C)	nd	2.2 ± 1.1 (2)

[a] K_D and K_i values are reported in nM and B_{max} in thousands of ligand binding sites per cell. Shown are means ±SD of *n* experiments done in triplicate. [b] GROα failed to displace [Eu^{3+}]IL8-S72C in the concentration range tested. See Figure 5.

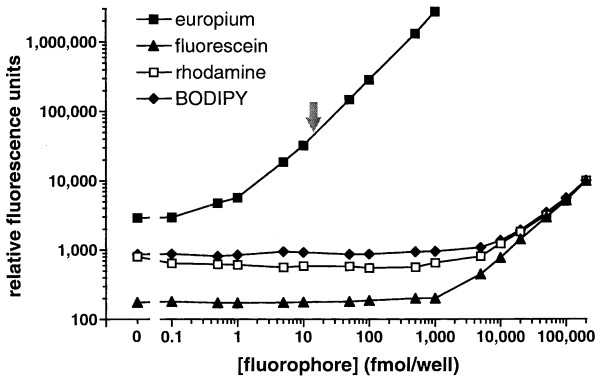

FIGURE 2: Comparison of the detection sensitivity of europium, fluorescein, rhodamine, and BODIPY. The arrow indicates the receptor density expected when the receptor expression level is ~250 000 binding sites per cell.

masses obtained were: IL8, 8382.74 ± 2.09 (calcd 8382); IL8-S72C, 8399 ± 1.7 (calcd 8398); IL8-S72C^{ox}, 16 796 ± 3 (calcd 16 794).

Comparison of the Detection Sensitivity of Europium, Fluorescein, Rhodamine, and BODIPY. Several fluorescent molecules have been used to label G protein-coupled receptor ligands for the study of receptor−ligand interactions (*20*−*22*). To identify a fluorophore suitable for a binding assay performed in a 96-well microtiter plate format, we compared the detection sensitivities of europium, fluorescein, rhodamine, and BODIPY (Figure 2). Various concentrations of commercially available streptavidin or avidin conjugates of europium, fluorescein, rhodamine, and BODIPY were added to a standard tissue culture 96-well microtiter plate containing a monolayer of CHO cells. Fluorescence emission intensities were measured using the appropriate excitation and emission filters for each fluorophore on a Fluostar fluorescence microplate reader. Europium fluorescence was measured on a time-resolved fluorescence plate reader.

A ~2-fold increase in signal above background was used to determine the detection threshold for each fluorophore. The amount of fluorophore required to reach this threshold, as shown in Figure 2, is ~10 000 fmol for rhodamine and BODIPY and ~5 000 fmol for fluorescein, but only ~1 fmol for europium. The G protein-coupled receptor concentration range for a CHO cell monolayer expressing ~250 000 receptors per cell (~40 000 cells per well) is on the order of ~16 fmol/well. Thus, of the three common fluorophores tested, none provides sufficient sensitivity for this assay format. In contrast, a receptor density of 10−20 fmol/well is well within the dynamic range of europium detection (see arrow in Figure 2). Europium is thus the fluorophore of choice.

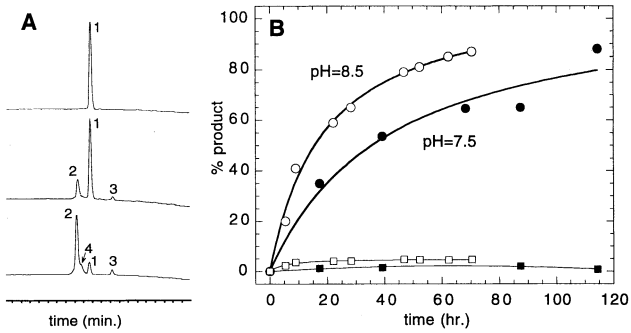

FIGURE 3: IL8-S72C labeling reaction. (a) representative HPLC chromatograms for three time points from the labeling reaction: (top) time zero; (middle) 5 h; and (bottom) 47 h. Peak 1, IL8-S72C; peak 2 is [Eu^{3+}]IL8-S72C; peak 3 is IL8-S72C^{ox}, and peak 4 is possibly a multiply labeled product. (b) Labeling time course of IL8-S72C by Eu^{3+}−DTTA iodoacetamide. Solid symbols represent % product formation at pH = 7.5 for IL8-S72C Eu^{3+}−DTTA adducts (circles) and IL8-S72C^{ox} (squares). The open symbols represents measurements made at pH = 8.5. Reaction products were measured at given times by analysis on HPLC (see Methods) from ~1 nmol of total protein per injection.

Labeling with Eu−DTTA Iodoacetamide. IL8-S72C was labeled with Eu^{3+}-DTTA iodoacetamide under two pH conditions (pH 7.5 and 8.5) at a molar ratio of protein:(Eu^{3+}-DTTA iodoacetamide) of 1:15. Analysis of the reaction products by HPLC (Figure 3A) indicated that the conjugate of IL8-S72C (peak 1) with Eu^{3+}−DTTA ([Eu^{3+}]IL8-S72C; peak 2) reacted completely at both pH values, although a 2-fold increase in reaction rate occurred at the higher pH (Figure 3B). The major product, [Eu^{3+}]IL8-S72C, eluted on HPLC 1.4 min earlier than the unmodified IL8-S72C. Byproducts included oxidized IL8-S72C (i.e., IL8-S72C^{ox}, peak 3; ~3% at pH 7.5 and ~5% at pH 8.5) and approximately 10−16% of a possibly more highly conjugated product (Figure 3A) appearing as a shoulder on the product peak. Increased reactivity of lysine and histidine can be expected in the pH range of 7 and higher, but accompanying increases in thiolate concentration hasten cysteine reactivity as well (*12*) and may explain the similar product and byproduct ratios seen at the two pH values studied.

Characterization of Eu-Labeled IL8. The specific activity of [Eu^{3+}]IL8-S72C was determined to be 4.5×10^6 counts s^{-1} $pmol^{-1}$. This value indicates that IL8-S72C was labeled with ~1 Eu^{3+} ion per molecule (spec. act. of Eu^{3+} = (3.6 ± 0.8) × 10^6 counts s^{-1} $pmol^{-1}$ = 11). Absolute counts s^{-1} are dependent on instrument settings and enhancement buffer composition (*15*), both of which were optimized by the manufacturer.

Europium-labeled and radiolabeled IL8 were compared under identical binding conditions, using CHO cell clones expressing CXCR1 or CXCR2. The binding reached saturation in 60 min at room temperature (Figure 4A). Comparison of [Eu^{3+}]IL8-S72C and [^{125}I]IL8 binding to CXCR2 indicates that the signal-to-noise ratios are comparable (7−10-fold; Figure 3B). [Eu^{3+}]IL8-S72C showed saturable binding to both receptors, and the dissociation constants of both ligands were in agreement (Figure 5 and Table 1). Likewise, the B_{max} determined using both ligands were similar. Dissociation constants for [Eu^{3+}]IL8-S72C also agreed with published

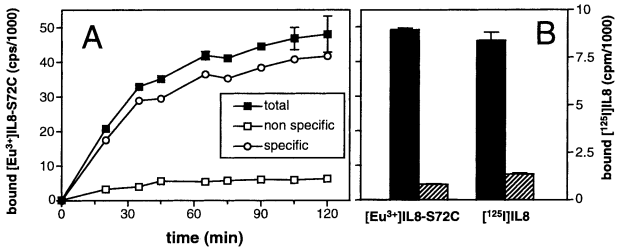

FIGURE 4: Binding of [Eu^{3+}]IL8-S72C to CXCR2. Binding of [Eu^{3+}]IL8-S72C to CHO cells expressing CXCR2 was as described in Methods. (a) Time-course of 1 nM [Eu^{3+}]IL8-S72C binding. (b) Comparison of the binding of 1 nM [Eu^{3+}]IL8-S72C and 1 nM [^{125}I]IL8: filled bars, total binding; hatched bars, nonspecific binding. Data shown are representative experiments.

K_D values for [^{125}I]IL8 which ranged from 1.4 to 7 nM (*6*, *23*−*25*). In addition to showing specific, saturable binding, [Eu^{3+}]IL8-S72C could be displaced by unlabeled chemokines with the expected potencies: while IL8 and GROα were equipotent at displacing [Eu^{3+}]IL8-S72C from CXCR2, GROα failed to displace [Eu^{3+}]IL8-S72C at CXCR1 in the concentration range tested (Figure 5 and Table 1). Thus, [Eu^{3+}]IL8-S72C and [^{125}I]IL8 identify the same binding sites and present the same pharmacological properties.

DISCUSSION

Assays of G protein-coupled receptors typically measure the displacement of radiolabeled ligands, both in receptor pharmacological/biochemical studies and in high throughput drug screening. Although there are a number of well-established, nonradioactive detection strategies for measuring intermolecular interactions, both separation-based (e.g., using standard fluorophores such as fluorescein) and homogeneous (e.g., using environmentally sensitive fluorophores such as dansyl), these methods generally suffer from lack of general applicability or low sensitivity. Thus, there has been no convenient alternative to radioactivity in G protein-coupled receptor binding assays.

As reviewed by Soini and Lövgren (*26*), specific trivalent lanthanide ion (Eu^{3+}, Tb^{3+}, and Sm^{3+}) chelates have fluorescent properties that make them useful as probes in biological assays. The extended fluorescence lifetime (millisecond range) of Eu^{3+} allows measurement of signal to be made after the decay of the shorter-lived biological and plastic fluorescence that is often a source of background in many fluorescence measurements (*15*). First used in immunofluorometric detection assays (*27*) the scope of assays for which lanthanide ions are being used is expanding. Recently, Eu^{3+}-labeled anti-phosphotyrosine has provided the basis for a sensitive protein tyrosine kinase assay (*28*), and Takeuchi et al. (*29*) have described a lanthanide-labeled benzodiazepine for use in measuring ion channel receptor−ligand binding in membranes prepared from rat and cow brain homogenates.

In this report, we describe the site-specific conjugation of IL8 with Eu^{3+}−DTTA and show it has a detection sensitivity equivalent to [^{125}I]IL8 in measuring CXCR1 and CXCR2

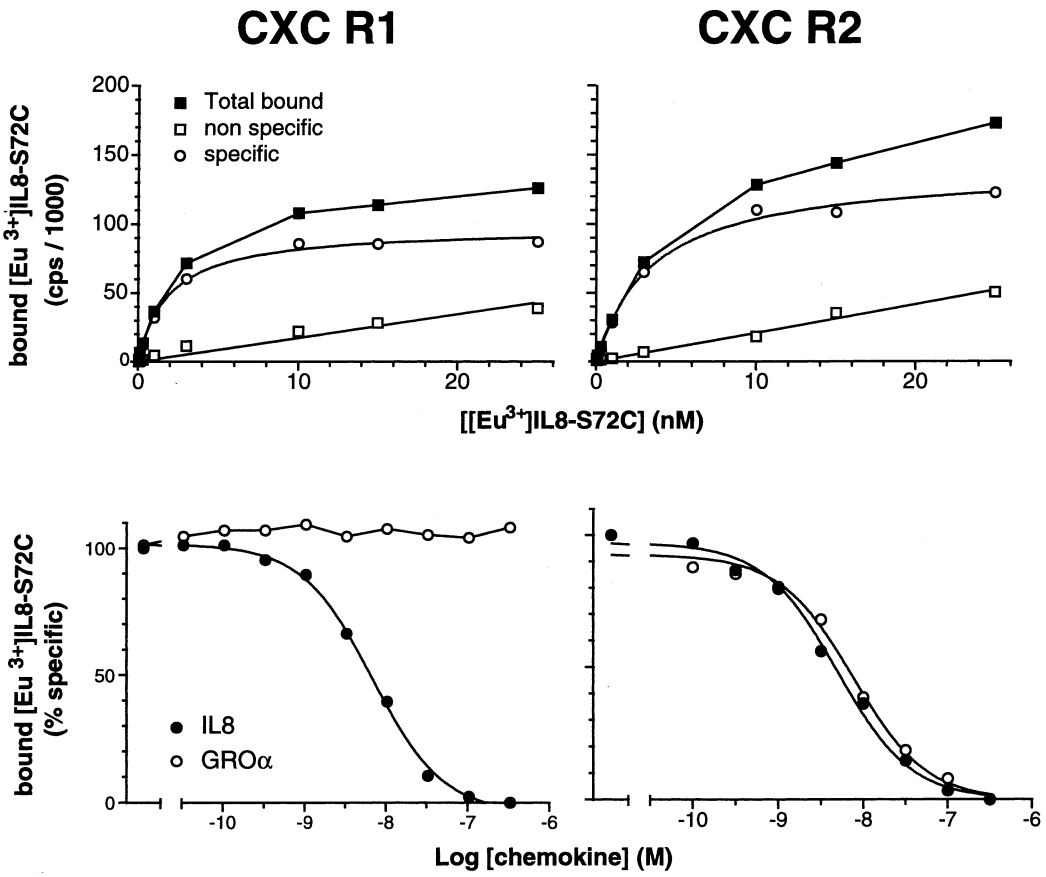

FIGURE 5: Binding of [Eu^{3+}]IL8-S72C to CXCR1 and CXCR2. (top) Saturation binding for CXCR1, K_D = 1.9 nM, B_{max} = 260 000 receptors/cell; for CXCR2, K_D = 4 nM, B_{max} = 360 000 receptors/cell: closed squares, total binding; open squares, nonspecific binding; circles, specific binding. (bottom) Competition of [Eu^{3+}]IL8-S72C by IL8 or GROα. [[Eu^{3+}]IL8-S72C] = 1 nM; for CXCR1 K_i (IL8) = 5 nM; for CXCR2 K_i (IL8) = 2 nM; K_i (GROα) = 4 nM. Data shown are representative experiments.

receptors on whole cells. Replacing the carboxyl terminal serine of IL8 with cysteine allowed us to generate a ligand with a single free cysteine which reacted specifically with an iodoacetamide derivative of Eu^{3+}−DTTA. Modification of IL8-S72C by other iodoacetamide reagents (e.g., iodoacetamides of biotin and the sulfoindocyanine dye, Cy5) resulted in IL8 bioconjugates with affinities similar to IL8-S72C and Eu^{3+}-labeled IL8 (data not shown), thus suggesting that the carboxyl terminus of IL8 can tolerate a wide range of moieties with minor effect on receptor binding.

Since IL8 contains two intramolecular disulfide bonds the potential for disulfide bond scrambling with the additional engineered cysteine existed. Nevertheless, this apparently did not prevent the formation of milligram amounts of properly folded product. While the carboxyl-terminal cysteine will oxidize with time, the resultant disulfide can be reduced with β-mercaptoethanol without reducing the structural intramolecular disulfide bonds of IL8 (data not shown).

The use of published structure−function studies was critical to designing a bioconjugate which retained the high affinity of the wild-type ligand. Indeed, modifications near the amino terminus can alter the binding properties of IL8. For example, Alouani and co-workers (*30*) specifically labeled the amino terminus of IL8 by first oxidizing serine1 under mild periodate oxidation conditions and reacting the resultant glyoxylyl with an amino-oxy derivative of fluorescein. This fluorescein-labeled IL8 had a ∼10-fold lower affinity than [^{125}I]IL8 for IL8 receptors on neutrophils or expressed recombinantly on HEK293 cells, suggesting that ligand−receptor contacts at the amino terminus of IL8 are sensitive to chemical modification. Another study (*31*) has demonstrated that Leu25 can be replaced by a cysteine with no effect on the binding properties of IL8, but modification of this cysteine with a fluorescent nitrobenzoxadiazole iodoacetamide lowers the affinity of the IL8 conjugate for the IL8 receptors by 2 orders of magnitude. Interestingly, this modification also alters the receptor specificity such that the conjugate can bind to the CCR1 receptor, a related chemokine receptor which recognizes a different class of chemokines.

In conclusion, we have demonstrated the site-specific labeling of a chemokine with a europium−DTTA chelate using a site-specific mutant of IL8. This bioconjugate retains the binding properties of the unmodified ligand and can be used in place of [^{125}I]-labeled IL8. Whereas radiolabeled IL8 has a limited shelf life, the europium-labeled ligand does not lose specific activity with time and has been stored for over 1 year with no loss in activity. On the basis of the similar structure of other chemokines, we anticipate that this modification method should be generally applicable to this family of ligands, now numbering more than 30 members. In our laboratory, we are extending the usefulness of lanthanide detection methods to additional G protein-coupled receptors and other ligand receptor classes.

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