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Adenovirus mediated expression "in vivo" of the chemokine receptor CXCR1

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Abstract

A major hurdle in the structural analysis of membrane proteins is the expression of a functional and homogeneous form of the protein. Except for rhodopsin, most G protein-coupled receptors (GPCRs) are endogenously expressed at very low levels. Heterologous expression of GPCRs in bacteria, yeast, insect cells or mammalian cell lines often yields proteins with large amounts of misfolded proteins and heterogeneous posttranslational modifications. Here, we report a novel mammalian "in vivo" system for the expression of the chemokine receptor CXCR1. This receptor was expressed in liver of mice infected with adenovirus encoding CXCR1. Liver plasma membranes from infected mice displayed high-levels of ^{125}I-labeled human interleukin-8 (IL-8) binding. The pharmacological profile of the recombinant CXCR1 expressed "in vivo" was similar to those expressed in neutrophils. We found that the incorporation of the detergent solubilized CXCR1 into phospholipid vesicles in the presence of Gi/Go proteins is required for the reconstitution of ^{125}I-IL-8 binding. On the basis of the presence of the several endogenous His residues and glycosylation moieties in CXCR1 we fractionated the detergent-solubilized plasma membranes by employing Ni- and Concanavalin A-

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based chromatography. Fractions enriched with CXCR1 were monitored by ^{125}I-IL-8-bound to the receptor and Western blots with anti-CXCR1 antibodies. This robust expression system could be readily applied for the expression of GPCRs and other eukaryotic membrane proteins.

Keywords

Chemokines; GPCR; Adenovirus; Receptors; Reconstitution; Protein expression

Introduction

The genes encoding G-protein-coupled receptors (GPCRs) comprise 1-5% of vertebrate genomes. These receptors translate a multitude of extracellular signals to mediate most biological functions including pain, development, cell death, neurotransmission and immune responses. The clinical importance of GPCRs is underscored by the fact that they represent the targets for more than 50% of drugs currently used in Medicine. GPCRs have a similar architecture, composed of seven transmembrane segments connected by three extracellular and three cytoplasmic loops. GPCRs contain an extracellular N-terminal domain and an intracellular C-terminal domain. Since the high resolution structures of GPCRs are a prerequisite for drug design, the structural analysis of these proteins is limited to the naturally abundant rhodopsins [10,12], although two recombinant mutant GPCR structures have been recently solved [1,14]. The major bottleneck for the structural analysis of GPCRs is the overproduction of sufficient amounts of functional protein. Heterologous expression of GPCRs in bacteria, yeast, insect cells or mammalian cell lines often yield misfolded proteins and heterogenous posttranslational modifications, thus precluding the structural analysis of GPCRs [2,4,8,9,15,16]. A good alternative is to express GPCRs in transgenic animals, in which the receptors are expressed within a physiological context to properly fold the protein. Indeed, mGluR was expressed in Drosophila eyes [3]. Similarly, 5HT $_{1A}$ and EDG1 GPCRs were expressed in the eyes of *Xenopus laevis* [29]. Although the expression of mammalian GPCRs in those non-mammalian animals is a good approach, the yield of recombinant GPCRs may be restricted due to the competition with the high expression of the endogenous visual photoreceptors.

Chemokine receptors belong to the rhodopsin-like GPCRs subfamily and mediate a plethora of cellular functions, including the trafficking of leukocytes from circulation to the sites of inflammation and injury, as coreceptors in the entry of HIV-1, and development of the nervous system [25]. To date approximately 20 receptors have been identified and classified on the basis of the position of the first two cysteine residues of the chemokine ligands. There are two major families of chemokines, the CC chemokines (CCL) in which the cysteine residues are adjacent to each other and their cognate receptors are named CCR, whereas CXC chemokines (CXCL) exhibit a single residue between the first two cysteine residues and their respective receptors are named CXCR. The best characterized chemokine system is the CXCL8 (interleukin-8, IL-8) and their cognate receptors CXCR1 and CXCR2 (CXCR1/2), which are highly expressed in neutrophils and selective neurons. These receptors mediate the migration of neutrophils from circulation to the sites of infection or injury, and promote angiogenesis and tissue repair. The abnormal expression of CXCL8 and its receptors is implicated in human diseases, inflammatory disorders, neurodegenerative diseases and tumorogenesis. On this basis, CXCR1 and CXCR2 are regarded as major drug target for the modulation of inflammation and tissue repair. The resolution of CXCR1/2 structures is required for the structure-based design of drugs targeting these receptors. However, currently the expression of CXCR1/2 represents a major challenge for their structural analysis. Here, we have devised a novel adenovirus system to express “in vivo” the chemokine receptor CXCR1 in mouse liver, a large organ with the capacity to process large amounts of membrane and secreted proteins

[17]. This approach was based on the transgene expression in intact animals via the systemic infection of animals with adenovirus or hydrodynamic-based transfection [5,28].

Material and methods

Animals

The C57BL/6 J mice 6-8 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a 12-h light/dark cycle and standard rodent chow.

DNA expression vector

The rabbit CXCR1 cDNA was inserted downstream of the CMV promoter of the pADTrack-CMV shuttle plasmid, which also drives the expression of GFP under a separate CMV promoter (pAD-CXCR1/GFP). *E.coli* BJ5183 were transformed with both pAD-CXCR1/GFP and the pAd-Easy-1 helper vector to generate the recombinant adenovirus plasmid, which is used to transfect packaging cells (911 or HEK293 cells). Viruses are harvested after 7-10 days post-transfection, as described [6].

Expression of CXCR1 in the liver

Mice were injected via the tail vein with recombinant adenovirus in PBS (2×10^9 pfu/animal). Infected animals were sacrificed 5 days post injection, and the liver was homogenized in buffer (20 mM TrisHCl pH 7.4, 5 mM EGTA, 5 mM EDTA and 250 mM sucrose) using a Teflon-glass homogenizer. The homogenate was centrifuged at 250g for 4 min; the pellet was re-homogenized and centrifuged again at 250g for 4 min. This pellet is referred as the nuclear fraction (N). The supernatants of both centrifugations were combined and centrifuged at 100,000g for 60 min; the pellet is referred as the plasma membrane fraction and the supernatant as the cytosol fraction.

^{125}I-IL-8 binding

Iodination of monomeric IL-8 and [^{125}I] IL-8 binding was carried out as described previously [23]. The specific activity of the [^{125}I] IL-8 stock was 0.5-1 Ci/mmol. Briefly, crude liver plasma membranes were incubated with several concentrations of [^{125}I] IL-8 in binding buffer [1× PBS, 20 mM HEPES (pH 7.4)] in the absence or presence of various concentrations of unlabeled chemokines for 2 h at 22°C. The binding reaction was terminated by filtration on glass membranes (GF/C), which are then washed with 10 ml of ice-cold PBS containing 0.1% bovine serum albumin. The amount of [^{125}I] IL-8 bound to the membranes was determined in a γ counter.

Purification of Gi/Go

Bovine brains obtained from a local slaughterhouse were used to isolate Gi/Go proteins according to the procedure described [20].

Reconstitution of the ^{125}I-IL-8 binding of detergent-soluble CXCR1

Liver plasma membranes were solubilized with 1.25% octylglucoside (Anatrace) in the presence of 5 mg/ml of Soy bean lipids. The detergent soluble extract was obtained by centrifugation at 150,000g for 1 h. The reconstitution of binding of the detergent-soluble CXCR1was carried out by a 30-fold dilution of the detergent soluble extract with binding buffer in the presence of purified bovine brain Gi/Go and ^{125}I-IL-8.

Fractionation of detergent-solubilized CXCR1

Liver plasma membranes (14 mg/ml) from infected mice were incubated for 1 h at 4°C in binding buffer composed of 20 mM phosphate buffer (pH 8.0), 100 mM NaCl, a cocktail of protease inhibitors (Roche), and in the presence of 1nM of ^{125}I-IL-8 to trace the receptor along the fractionation steps. The liver membranes bound to ^{125}I-IL-8 were solubilized with 1% dodecylmaltoside (Anatrace, DDM; detergent/protein ratio (w/w) of 2.5) for 1 h at 4°C. The detergent soluble extract was obtained by centrifugation at 150,000g for 1 h. The solubilized receptor was adsorbed to a 3 ml Probond resin column (Invitrogen) pre equilibrated with the binding buffer supplemented with 0.02% DDM. The receptor was eluted with a linear gradient of Imidazol (0-300 mM), as determined by the radioactivity in the fractions and Western blots with anti-CXCR1 antibodies (C20, Santa Cruz Biotechnology, Inc.). The fractions containing the highest radioactivity were combined and adsorbed to a 3 ml Concanavalin A column (GE Healthcare) pre equilibrated with10 mM phosphate buffer ph 6.5 and 0.02% DDM, washed with 1 M NaCl, and the receptor eluted with a linear gradient of α-D-methyl glucopyranoside (Sigma, 0-400 mM).

Results and discussion

Expression of recombinant rabbit CXCR1

We found that in contrast to the human or mouse CXCR1s, the rabbit CXCR1 is thermally stable, which is an important requirement for the structural analysis of macromolecules, including membrane proteins. In fact, a GPCR variant of the rat neurotensin receptor with high thermal stability expresses high levels of active receptor in heterologous hosts. Also, this receptor demonstrates stability in detergent solutions and conserves its ligand selectivity [18]. Moreover, well-diffracting crystals are only obtained from β_1 and β_2 adrenergic receptor variants with enhanced thermal stability in detergent solutions [1,14,26]. In agreement with these observations we previously showed that the expression of the wild-type and variants of the rabbit CXCR1 are readily expressed in several cell lines [13]. On this basis we selected to express CXCR1 “in vivo” by infecting mice with adenovirus encoding the rabbit CXCR1 fused at its C-terminus to an 8-amino acid epitope for the 1D4 monoclonal antibody raised against bovine rhodopsin [11]. In Fig. 1 we demonstrated that the subcellular fractions of liver homogenates from infected animals displayed high levels of ^{125}I-IL-8 binding, whereas membranes of uninfected animals showed negligible binding to ^{125}I-IL-8, which does not bind with high affinity to the endogenous murine CXCR1/CXCR2 [21]. The subcellular fractions enriched in plasma membranes and nuclei demonstrated the highest specific binding to ^{125}I-IL-8. Similarly, we showed that several cell lines, including COS-1, Rat1 fibroblasts and HL-60 cells were efficiently infected with adenovirus particles and expressed high levels of rabbit CXCR1 as demonstrated by ^{125}I-IL-8 binding and Western blot analysis with anti-CXCR1 antibodies (data not shown). This finding is in agreement with the transgene expression of green fluorescence protein (GFP) by the infection of mouse liver with adenovirus vector encoding GFP [28]; however this is the first demonstration of adenovirus-mediated expression of a GPCR in the mouse liver.

To further characterize the binding properties of the recombinant CXCR1 we performed ^{125}I-IL-8 binding analysis with plasma membranes from liver of mice infected with adenovirus. First, we showed that the binding of ^{125}I-IL-8 was proportional to protein concentration in plasma membranes and that the non-specific binding was less than 20% of the total binding (Fig. 2a). Second, we demonstrated that the ^{125}I-IL-8 bound to liver membranes is readily displaced by unlabeled human IL-8, but not by the related neutrophil activating chemokines MGSA, NAP-2 and GCP-2 (Fig. 2b), indicating that the recombinant CXCR1 expressed in the liver is similar to the native CXCR1, which preferentially binds IL-8 with high affinity but with low affinity to related neutrophil activating chemokines. This pharmacological profile is

distinct to the CXCR1 homologous receptor CXCR2, which binds with high affinity to IL-8 and related chemokines [7]. We estimated that the Kd of binding of ^{125}I-IL-8 to the liver plasma membranes is 9.3 ± 2.3 nM, which is similar to those reported in rabbit neutrophils, but slightly higher to mammalian cell lines expressing the rabbit CXCR1 [7,23,24].

Reconstitution of ^{125}I-IL-8 binding of detergent solubilized CXCR1

Agonists often fails to bind with high affinity to the detergent-solubilized rhodopsin related GPCRs [27], most likely due to the disruption of the receptor/G protein coupling by the detergents, thus precluding assessing the functionality of the receptor along the purification process. Indeed we found that the detergent solubilized liver membranes from infected mice did not show specific binding to IL-8. However, the binding to IL-8 was reconstituted upon incorporation of the detergent solubilized CXCR1 into lipid vesicles by using the octylglucoside dilution method in the presence of purified Gi/Go from bovine brain (Fig. 3). We employed Soy bean lipids for the reconstitution of CXCR1 because these lipids mimic the lipid composition of eukaryotic membranes and have been successfully employed for the reconstitution of membrane processes [22]. Octylglucoside was chosen for the reconstitution of membrane processes by dilution because this detergent has a high CMC (10 mM). On this basis, dilution of the octylglucoside-solubilized membrane proteins in the presence of exogenous lipids reduces the concentration of this detergent below its CMC thus favoring the formation of vesicles. This simple reconstitution method could be suitable to assess the functional integrity of GPCRs during their purification.

Fractionation of the detergent-solubilized plasma membranes containing CXCR1

Analysis of the amino acid sequence of rabbit CXCR1 revealed the presence of several exposed His residues (Fig. 4), which suggested that the native CXCR1 could bind Ni resin columns. In fact, we found that the DDM-solubilized plasma membranes of infected mice bound to a Probond Ni-resin column, as revealed by the elution of the [^{125}I] IL-8 pre-bound to the receptor with a linear gradient of Imidazol (Fig. 5a). Those fractions enriched in ^{125}I-IL-8 were adsorbed to a Concanavalin A-bound agarose column for further purification of CXCR1, as this receptor like most GPCRs are glycosylated. Elution of this column with a linear α-D-methyl glucoside gradient showed ^{125}I-IL-8 enriched fractions (Fig. 5b). SDS-gel electrophoresis of these fractions revealed an enriched protein band of an apparent molecular weight of ~31 kDa (Fig. 6a), which is stained with anti-CXCR1 antibodies (Fig. 6b). We reconstituted the IL-8 binding of the peak fraction eluted from the Concanavalin A agarose column (Fig. 6c). We attempted to further purify the receptor using the 1D4 immunoaffinity column, as we attached to CXCR1 the peptide epitope for the 1D4 Mabs, however the receptor did not bind this column because the 1D4 antibody bound with high affinity other liver proteins. Nevertheless, our results are promising for achieving the homogenous purification of recombinant GPCRs expressed “in vivo” by including suitable tag to facilitate their purification using affinity chromatography.

Conclusion

We have demonstrated the advantages of the “in vivo” expression system for the functional expression of recombinant CXCR1, which could be extended to other GPCRs. In comparison to other expression system of GPCRs, our “in vivo” system is as efficient as those reported in yeast and insect/mammalian cell lines [19]. We estimate that 20 mice livers could produce at least 1 mg of homogenous rabbit CXCR1, whereas it was estimated that 5 L of cell culture media may yield 1 mg of heterogenous GPCRs [19]. It is important to emphasize that the thermo stability of the GPCR is a key factor for obtaining high protein expression, as recently described [18]. This is perhaps the most promising system for the production of functional GPCRs, as it can be readily adapted to large-scale production by using several mice or rats, which have large livers. We have also devised a functional assay to assess the detergent-

solubilized receptor, and provided a simple system for the purification of the recombinant CXCR1.

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Abbreviations

DDM, n-Dodecyl-β-D-maltoside; GFP, Green fluorescence protein; CMV, Cytomegalovirus; IL-8, Interleukin-8; CMC, Critical micellar concentration; GCP-2, Human granulocyte chemotactic protein 2; NAP-2, Neutrophil-activating peptide-2; MGSA, Melanoma growth-stimulating activity.

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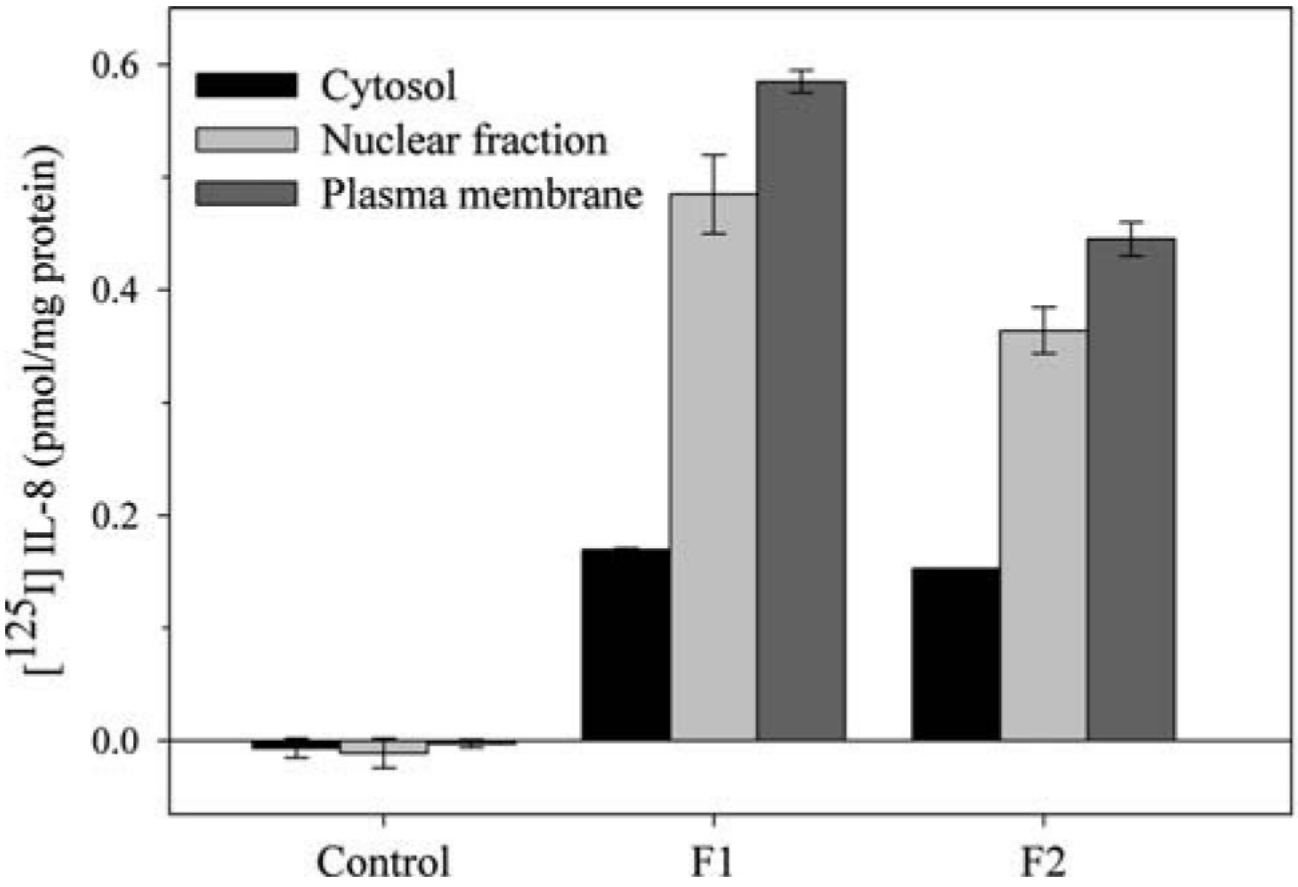

Fig. 1.

Binding of human [^{125}I]IL-8 to subcellular fractions of liver from adenovirus infected mice. Control mice were untreated, F1 and F2 were mice injected with 2×10^6 virus particles/animal

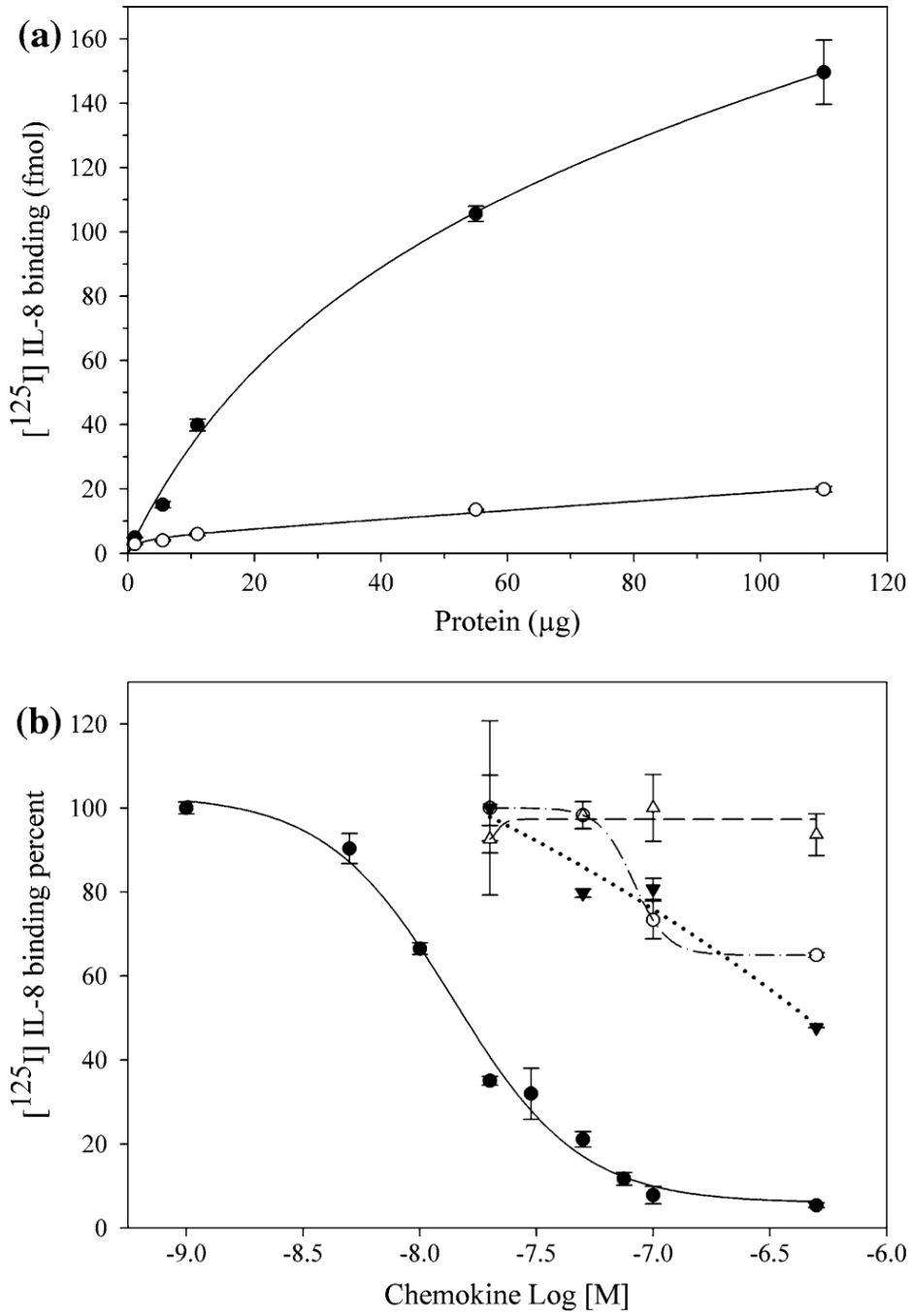

Fig. 2.

^{125}I-IL-8 binding as a function of protein concentration of plasma membranes from infected mice (**a**), and displacement of [^{125}I]IL-8 bound to plasma membranes by unlabeled human IL-8 (●), MGSA (○), NAP-2 (▾) and GCP-2 (Δ) (**b**)

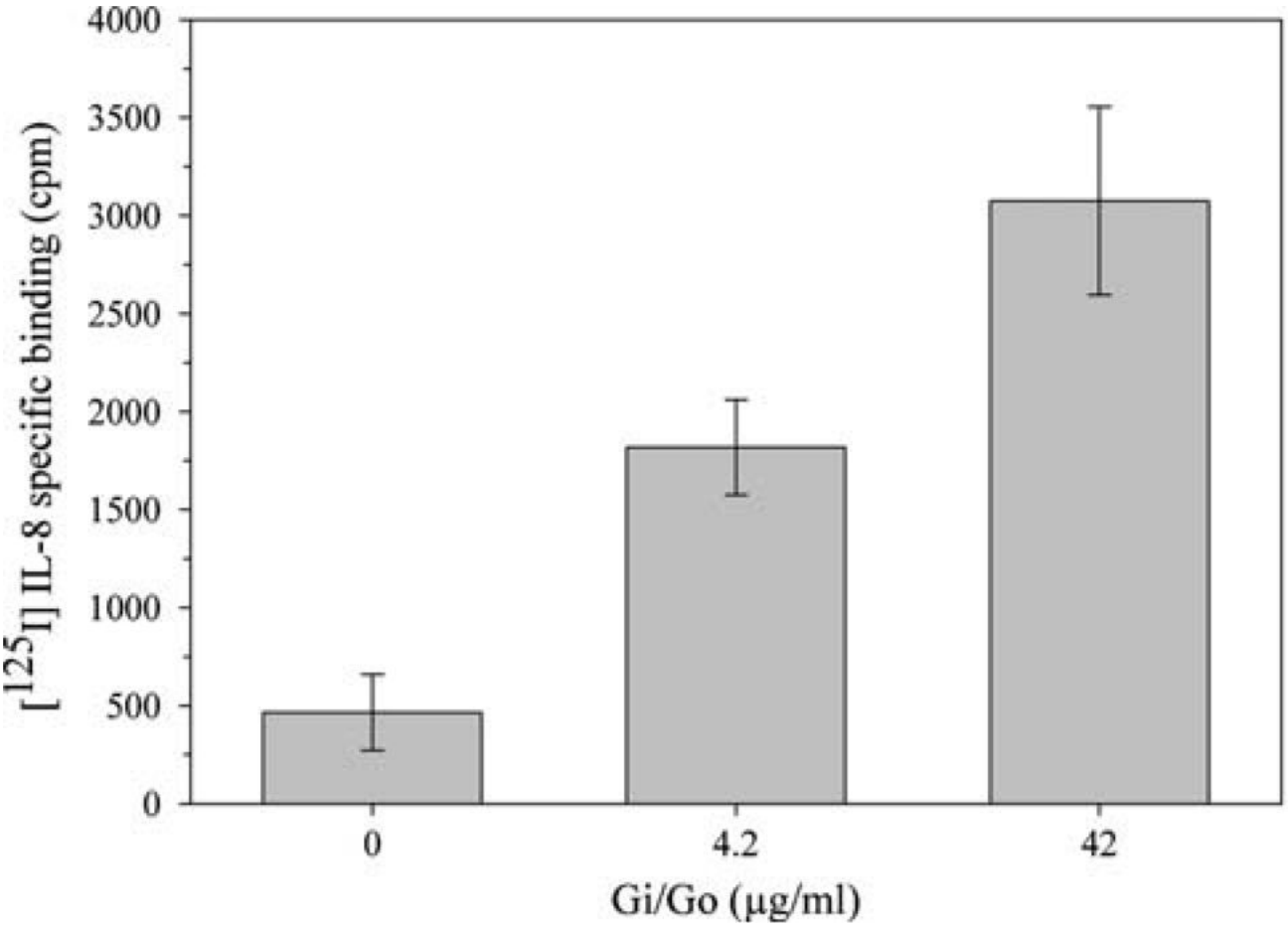

Fig. 3.

Reconstitution of [^{125}I] IL-8 binding of the detergent-soluble liver plasma membranes from adenovirus infected mice. Binding was carried out with an octylglucoside soluble extract from plasma membranes that was incorporated into phospholipid vesicles by the dilution method [22] in the absence or presence of 4.2 and 42 μg/ml of purified Gi/Go protein

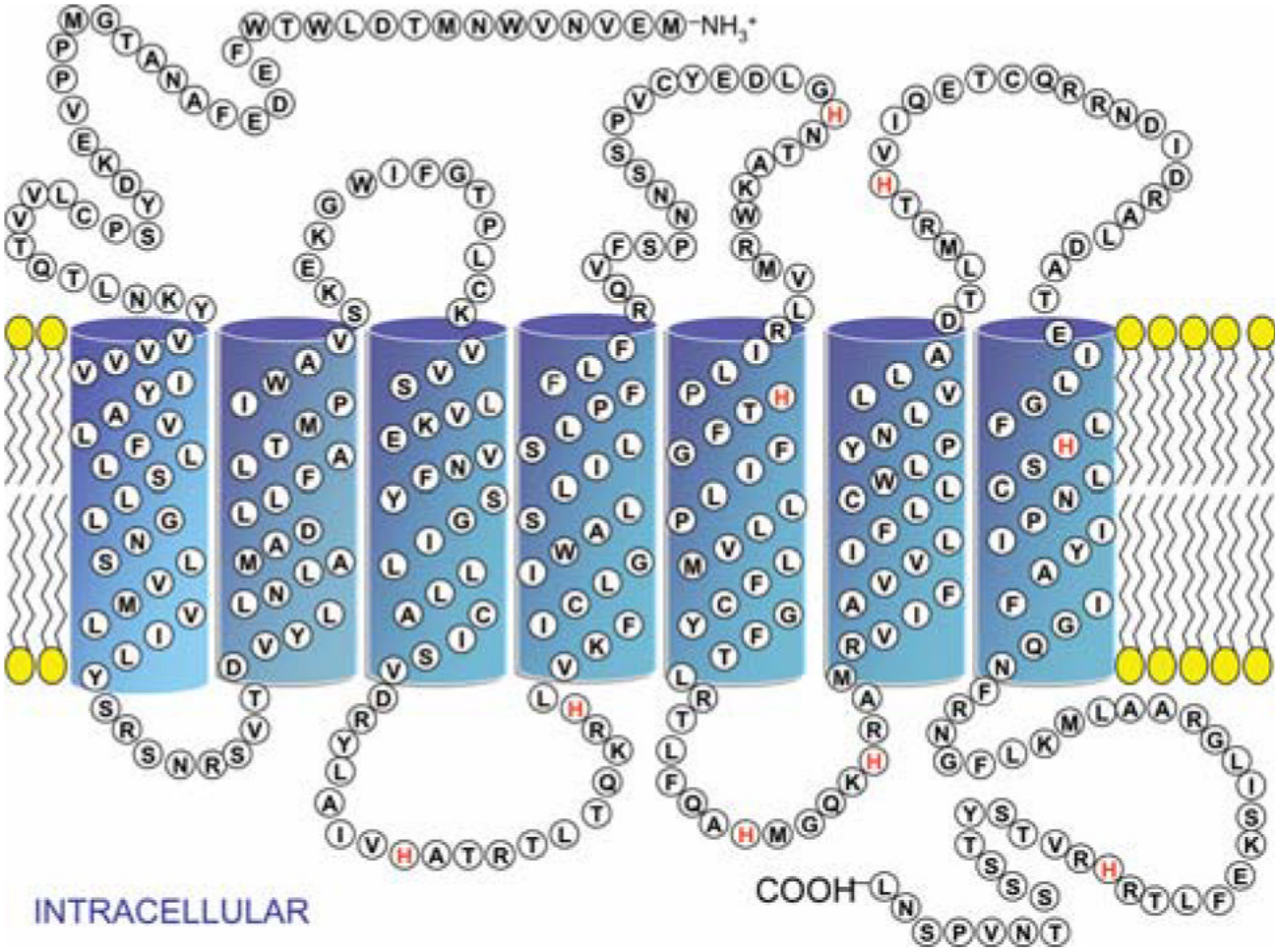

Fig. 4.

Two-dimensional representation of the rabbit CXCR1showing the endogenous His residues (red). The accession number of the rabbit CXCR1 sequence is: P21109 and the two dimensional representation was based on the hydropathy plot (http://www.uniprot.org)

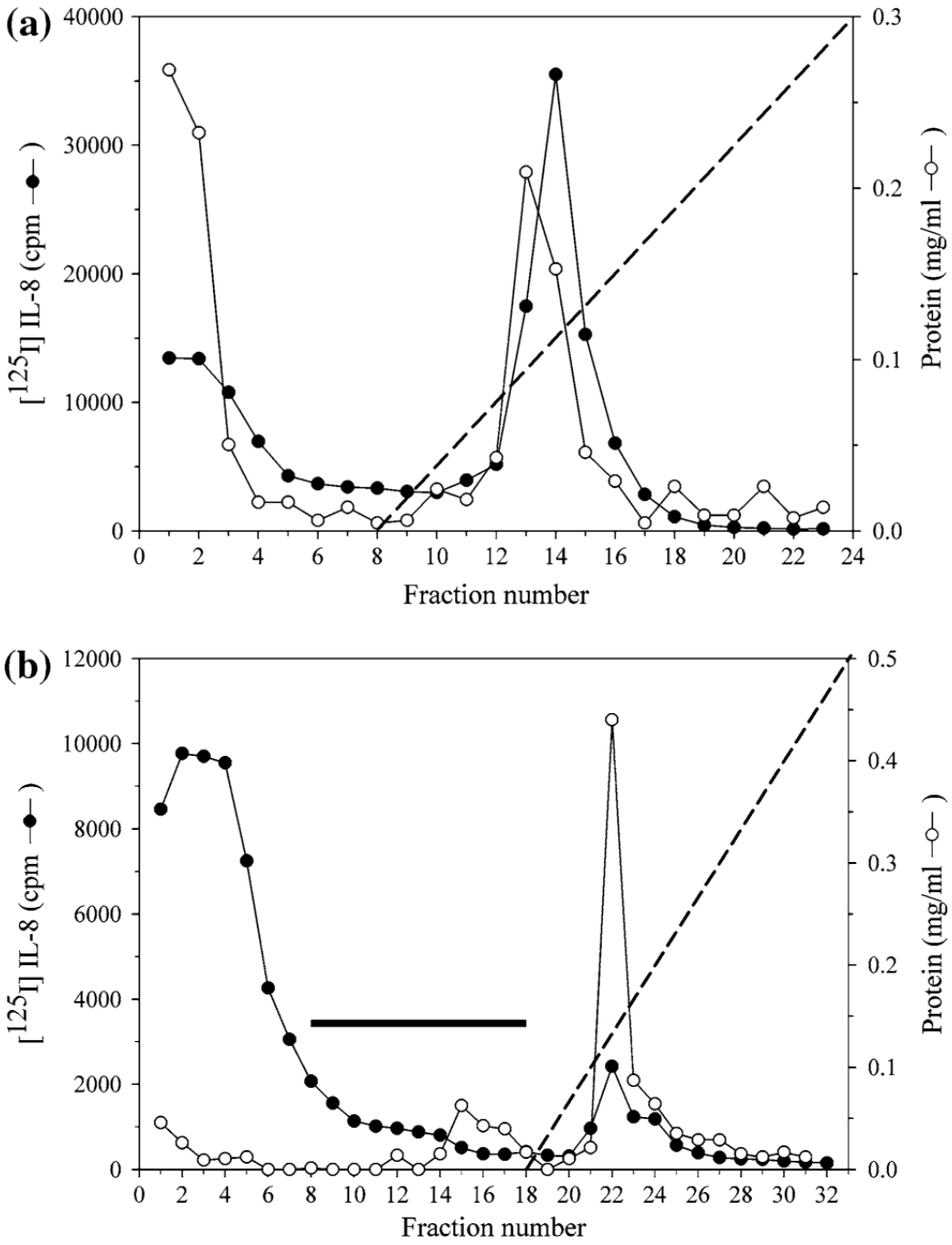

Fig. 5.

Chromatography Fractionation of the Detergent-soluble extract of the liver plasma membrane from adenovirus infected mice. Plasma membranes bound to 1nM of [^{125}I] IL-8 were solubilized with 1% dodecylmaltoside, and the detergent soluble extract was adsorbed to Probond (Ni-resin) column and eluted with a linear gradient of Imidazole (0-300 mM) (**a**); fractions eluted with imidazol and enriched with [^{125}I] IL-8 were adsorbed to a Concanavalin A-agarose column, which was then washed with 1 M NaCl and eluted with a linear gradient of α-methyl glucoside (0-400 mM) (**b**). Fractions were analyzed for radioactivity and protein concentrations. The high levels of radioactivity in the initial fractions of the columns

correspond to free [^{125}I] IL-8, dissociated from the [^{125}I] IL-8-bound to the soluble extract during the chromatography fractionation

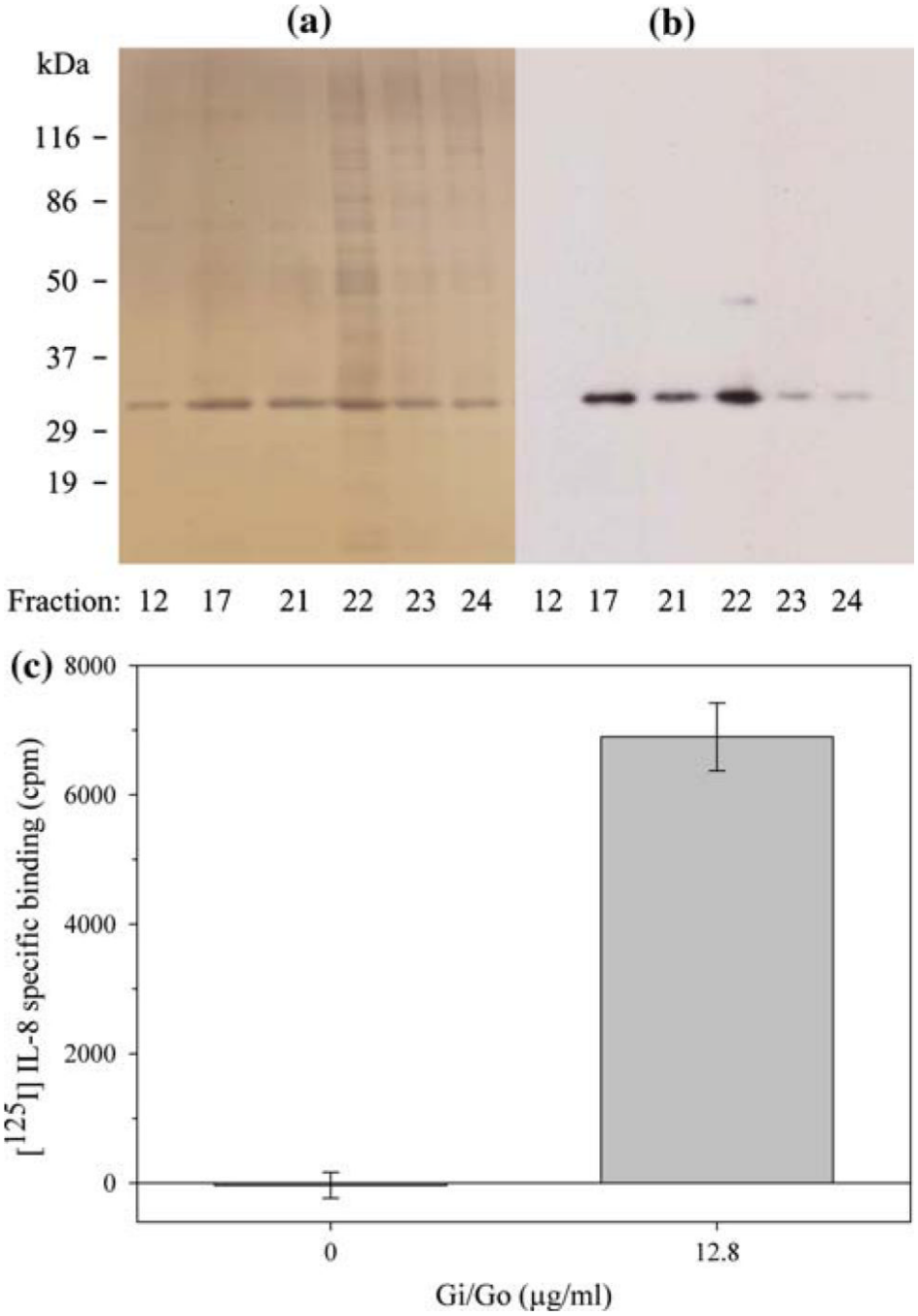

Fig. 6.

SDS-PAGE of the fractions eluted from the Concanavalin A-agarose column, silver stained gel (**a**); Western blots of those fractions using the anti-human CXCR1 antibodies (C20), which cross react with the rabbit CXCR1 (**b**); reconstitution of [^{125}I] IL-8 binding of fraction 22 of Concanavalin A-agarose column by incorporation 13.2 μg of protein into phospholipid vesicles in the absence and presence of 12.8 μg of purified Gi/Go, in a final volume of 1.0 ml (**c**)