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Specific detection by flow cytometry of histidine-tagged ligands bound to their receptors using a tag-specific monoclonal antibody

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Abstract

Engineering proteins to contain a histidine (His)-tag has proved to be very useful for the purification and analyses of these molecules. In the present study, we demonstrate that the binding of His-tagged ligands to their receptors may be visualised by flow cytometry making use of a selected monoclonal antibody (mAb) against the His-tag. Employing this method, a recombinant C3a (rC3a) anaphylatoxin with a His-tag at its N-terminus could be shown to bind to C3a receptor (C3aR)-expressing RBL-2H3 transfectants with a half-maximal effective concentration (EC_{50}) of about 3 nM which is well within the range of published affinity constants. Binding of a recombinant interleukin-8 (rIL-8) molecule with a C-terminal His-tag to RBL-2H3 cells which stably express the IL-8 receptors CXCR1 or CXCR2 could also be demonstrated using the tag-specific mAb. Furthermore, aminoterminally tagged C5a molecules of rat or human origin could be shown to bind to the human C5a receptor (C5aR). However, the fluorescence signal of the binding of rat rC5a to the human C5aR was distinctly higher over a wide range of ligand concentrations than the signal of human rC5a binding although both ligands were equally potent in the induction of chemotaxis in C5aR-expressing cells. Thus, the tag-specific mAb was able to interfere with the binding of human but not rat rC5a to the human C5aR. This observation is in agreement with the hypothesis of a two binding site model for the interaction of human C5a with its receptor whereas a different binding mode may apply for rat C5a. Our data demonstrate that the selected His-tag specific mAb may be a valuable tool for the visualisation of the binding of recombinant ligands to their receptors and may also provide useful information on the specific binding properties of the ligands. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The purification of recombinant proteins has been greatly facilitated in recent years by affinity interactions with specific protein sequence recognition 'affinity tags' genetically engineered into the protein of interest. This allows the expressed tagged protein to

Abbreviations: mAb: monoclonal antibody; rC3a: recombinant C3a; rC5a: recombinant C5a; rIL-8: recombinant interleukin-8; C3aR: C3a receptor; C5aR: C5a receptor; EC_{50}: half-maximal effective concentration; His: histidine

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be purified by affinity chromatography techniques (Jones et al., 1995). One of the most widely used tagging systems consists of five or six consecutive histidines (Arnold, 1991). The adjacent imidazole side chains can bind to free coordination sites of metal ions such as Ni^{2+}, which are attached to a solid support by a chelator complex. This interaction is highly specific in the presence of high salt which eliminates nonspecific ionic interactions, and many proteins can be purified to near homogeneity in a single step (Lindner et al., 1997). Tag technology has also been adapted for plate assays and other solid phase techniques. Recently, monoclonal antibodies (mAbs) which specifically react with the His-tag have been generated (Pogge v. Strandmann et al., 1995; Zentgraf et al., 1995). Possible applications for the detection of His-tagged proteins using tag-specific mAbs are immunoblotting, ELISA techniques or flow cytometric analysis (Lindner et al., 1997).

The family of anaphylatoxins (C3a, C4a and C5a) comprises low-molecular inflammatory mediators which are cleaved from the inactive precursor proteins C3, C4 and C5 in the course of complement activation (Hugli and Müller-Eberhard, 1978). The proinflammatory effects of the anaphylatoxins are based largely on the activation of granulocytes and monocytes/macrophages. Leukocytes may also be activated by chemokines, an expanding group of small proteins to which IL-8 belongs and which are similar in size to the anaphylatoxins (Murphy, 1994).

The cellular receptors CXCR1 and CXCR2 (Holmes et al., 1991; Murphy and Tiffany, 1991) which bind IL-8 with high affinity as well as the receptors for the human anaphylatoxins C3a (Ames et al., 1996; Crass et al., 1996) and C5a (Gerard and Gerard, 1991) have been cloned. They belong to the large family of G-protein coupled receptors with seven transmembrane segments. Different binding characteristics of small peptide ligands to their receptors have been reported ranging from essentially one receptor binding domain in the case of C3a (Lu et al., 1983) to two or more in the case of C5a (Siciliano et al., 1994) and IL-8 (Schraufstätter et al., 1993).

It was the purpose of the present study to investigate whether a His-tag attached to small protein ligands (i) might be exploited for the visualisation by flow cytometry of ligand–receptor interactions using tag-specific mAbs and (ii) may provide useful information on specific binding characteristics of a ligand to its receptor.

2. Materials and methods

2.1. Recombinant anaphylatoxins

Human recombinant C3a (rC3a) was generated as described and contained an additional N-terminal tag of 11 amino acids (Zwirner et al., 1997). Human and rat recombinant C5a (rC5a) have been described recently and contained an aminoterminal tag of 12 amino acids each (Rothermel et al., 1997a). The addition of the aminoterminal amino acids to human rC3a and rC5a, respectively, has been shown to be without influence on the functional activity of the molecules since recombinant and serum-derived C3a and C5a stimulated the release of *N*-acetyl-β-D-glucosaminidase from dibutyryl-cAMP-treated U937 cells equally well (Rothermel et al., 1997a; Zwirner et al., 1997). These recombinant anaphylatoxins were generated using the bacterial expression vector pQE30 (Qiagen) coding for the tag sequence MRGSHHHHHHGS. Purification of native C5a from human serum was carried out as previously described (Schulze and Götze, 1986).

Recombinant IL-8 was prepared by cDNA synthesis on RNA from monocytes and a subsequent PCR using oligonucleotide primers that were designed according to the human monocyte-derived IL-8 cDNA sequence (Matsushima et al., 1988). The PCR product was cloned into the bacterial expression vector pQE60 (Qiagen) with the additional C-terminal nine amino acid sequence GGSHHHHHH coded for by the vector. Expression conditions for recombinat interleukin-8 (rIL-8) were the same as for the recombinant anaphylatoxins. The recombinant protein was purified from the bacterial lysate by Ni^{2+}-chelate chromatography and refolded in a renaturation buffer containing oxidized and reduced glutathione. All recombinant proteins used in this study were purified to homogeneity by FPLC applying cation-exchange-chromatography (Mono S, Pharmacia, Freiburg).

2.2. Antibodies

The mAb Maximilian (mouse IgG1, kappa) against the His-tag recognizing the amino acid sequence XGSHHH was obtained from Connex (Martinsried). The anti-hexa histidine and anti-penta histidine mAbs were purchased (Qiagen, Hilden). Non-relevant mouse IgG1 (Sigma-Aldrich, Deisenhofen) was used for control purposes.

2.3. Cells

Rat basophilic leukemia RBL-2H3 cells (American Type Culture Collection, Rockville, MD, USA) which stably express the human C3a receptor (C3aR), C5aR receptor (C5aR), IL-8 receptor type A (CXCR1) and IL-8 receptor type B (CXCR2), respectively, have been generated recently (Rothermel et al., 1997b; Zahn et al., 1997; Zwirner et al., 1998).

2.4. Flow cytometric analysis

The binding of rC3a, rC5a and rIL-8 was analyzed by indirect immunofluorescence using a FACstar instrument (Becton Dickinson, Heidelberg). RBL-2H3 transfectants (2×10^5) were washed with PBS containing 1.5% fetal calf serum and 10 mM sodium azide. They were then blocked with 200 μg/ml heat aggregated human IgG and incubated with the recombinant anaphylatoxins in 100 μl for 45 min on ice. After washing three times with PBS/1.5% fetal calf serum/10 mM sodium azide, cells were incubated with mAb Maximilian (1 μg/100 μl) reactive against the N-terminal tag of the recombinant molecules for another 45 min. After washing three times, cells were finally incubated with fluorescein-labeled $F(ab')_2$ fragments of goat-anti-mouse Ig (Dakopatts, Hamburg) for 45 min. Labeled cells were washed as described above, resuspended in PBS containing 1% formaldehyde and analyzed.

2.5. Cell migration assay

Chemotaxis was evaluated in a 48-well chemotaxis microchamber (Costar, Bodenheim) using PVP-free polycarbonate membranes (8 μm-pore size). Cells were washed and resuspended in GBSS buffer containing 2% BSA at 1×10^6 cells/ml. Fifty microliters of cell suspension were added to the upper wells and 27 μl of anaphylatoxin diluted in GBSS/2% BSA were added to the lower wells of the microchamber. The incubation time was 90 min at 37°C in a 5% CO_2 atmosphere. After wiping cells off the upper side of the membrane, cells on the lower side were visualised using the Hemacolor staining kit (Merck, Darmstadt). Each experiment was performed in triplicate and the numbers of migrating cells were counted in three high power fields (magnification ×400).

3. Results

3.1. Binding of rC3a to the C3a receptor

Three different anti-His tag mAbs were tested for their sensitivity to detect, by flow cytometry, the binding of rC3a to RBL-2H3 cells expressing the human C3aR. Fig. 1 demonstrates that mAb Maximilian (Connex) was the superior reagent in this assay offering the highest signal-to-noise ratio at a concentration of 10 μg/ml. This antibody was therefore used in all subsequent experiments. Next, binding of

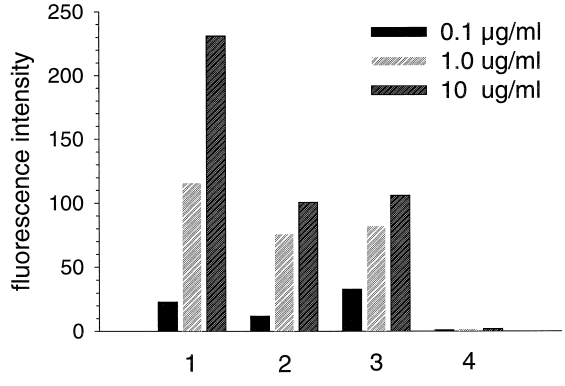

Fig. 1. Binding of different His-tag specific mAbs to rC3a on C3aR-expressing cells. Recombinant C3a (100 ng) was incubated with RBL-2H3 cells expressing the human C3aR. In a second step, cells were incubated with three different monoclonal antibodies against the His-tag of rC3a in three different concentrations. Finally, cells were incubated with a fluorescein-conjugated anti-mouse Ig and analyzed by flow cytometry. (1) mAb Maximilian, (2) mAb anti-tetra histidine, (3) mAb anti-penta histidine, (4) IgG1 control mAb.

increasing concentrations of rC3a to C3aR-expressing RBL-2H3 cells was analyzed using the His-tag specific mAb. Fig. 2 demonstrates that the half maximal effective concentration (EC_{50}) of rC3a binding to the RBL-2H3 transfectants was approximately 3 nM. As a control for the specific nature of the interaction between rC3a and its receptor, we could demonstrate that rC5a which contains the same N-terminal tag as rC3a did not bind to C3aR-expressing RBL-2H3 cells (Fig. 2). Furthermore, no binding of rC3a to human C5aR-expressing RBL-2H3 transfectants was detectable (not shown).

3.2. Binding of rC5a to the C5a receptor

Binding of human and rat rC5a to human C5aR-expressing RBL-2H3 cells was analyzed using the His-tag specific mAb. Fig. 3 demonstrates that the EC_{50} of rat rC5a binding to the RBL-2H3 transfectants was approximately 3 nM. In contrast to rat rC5a, the binding of human rC5a to human C5aR-expressing RBL-2H3 as detected by the His-tag specific mAb did not reach saturation (Fig. 3), thus no EC_{50} values could be calculated. Furthermore, the fluorescence signal of the binding of rat rC5a to the human C5aR was distinctly higher over a wide range of ligand concentrations than the signal of human rC5a binding. A similar pattern of staining intensities for human and rat rC5a bound to the human C5aR was detected using the mAbs anti-tetra or anti-penta histidine (not shown). To exclude the possibility that human rC5a was not as functionally potent as rat rC5a we tested the induction of chemotaxis by human rC5a in RBL-2H3 transfectants expressing the human C5aR (Fig. 4). Human rC5a was chemotactically active with an EC_{50} of about 0.3 nM compared

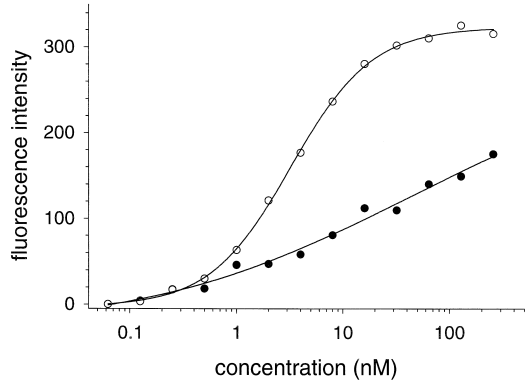

Fig. 3. Binding of human or rat rC5a to RBL-2H3 transfectants expressing the human C5aR. Increasing concentrations of human rC5a (filled circles) or rat rC5a (open circles) were incubated with human C5aR-expressing RBL-2H3 cells. In a second step cells were incubated with the mAb Maximilian which reacts with the aminoterminal tag of the recombinant molecules. Finally, cells were incubated with a fluorescein-conjugated anti-mouse Ig and analyzed by flow cytometry. Mean values of two independent experiments are shown.

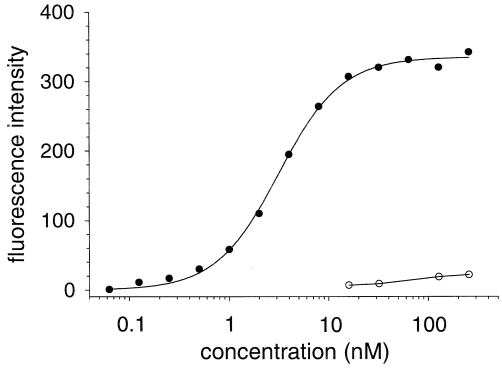

Fig. 2. Binding of rC3a to C3aR-expressing RBL-2H3 cells. Increasing concentrations of rC3a (filled circles) or rC5a (open circles) were incubated with RBL-2H3 cells expressing the human C3aR. Then, cells were incubated with mAb Maximilian which reacts with the aminoterminal tag of the recombinant molecules. Finally, cells were incubated with a fluorescein-conjugated anti-mouse Ig and analyzed by flow cytometry. One representative experiment out of two is shown.

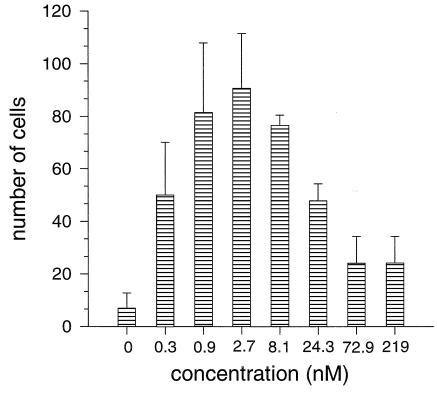

Fig. 4. Migration of RBL-2H3 transfectants expressing the human C5aR towards human rC5a. Cells were placed in the upper chambers of a 48-well chemotaxis device in the presence of increasing concentrations of human rC5a in the lower chambers. Migration is expressed as the number of migrated cells counted in three high power fields of triplicate experiments. Mean values ± SEM are shown ($n = 4$).

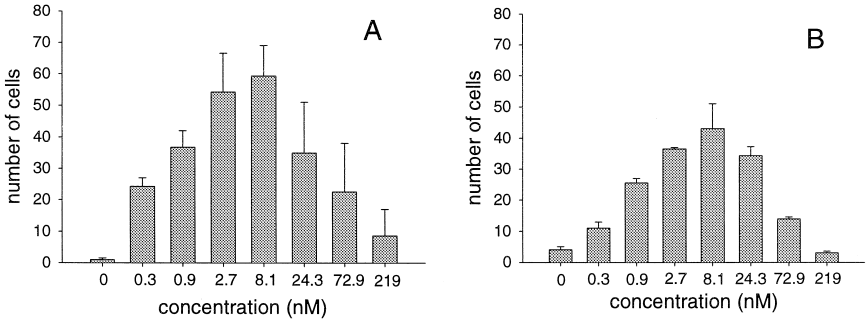

Fig. 5. Migration of RBL-2H3 transfectants expressing the human IL-8 receptors CXCR1 (A) or CXCR2 (B) towards rIL-8. Cells were placed in the upper chambers of a 48-well chemotaxis device in the presence of increasing concentrations of rIL-8 in the lower chambers. Migration is expressed as the number of migrated cells counted in three high power fields of triplicate experiments. Mean values ± SEM are shown ($n = 2$).

to the similar EC_{50} values of 0.14 nM and 0.15 nM reported recently for the induction of chemotaxis in human C5aR-expressing RBL-2H3 cells by rat rC5a and human serum-derived C5a, respectively (Rothermel et al., 1997a,b). Furthermore, the binding of human and rat rC5a to human C5aR-expressing RBL-2H3 cells as detected by the His-tag specific mAb could be blocked by a 100-fold molar excess of human serum-derived C5a (not shown).

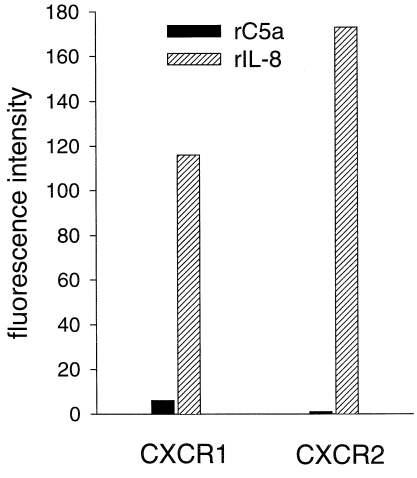

Fig. 6. Binding of rIL-8 to RBL-2H3 transfectants expressing the IL-8 receptors CXCR1 or CXCR2. Recombinant IL-8 (100 ng; grey columns) or rC5a (100 ng; dark columns) were incubated with CXCR1- or CXCR2-expressing RBL-2H3 cells. In a second step cells were incubated with the mAb Maximilian which reacts with the aminoterminal tag of the recombinant molecules. Finally, cells were incubated with a fluorescein-conjugated anti-mouse Ig and analyzed by flow cytometry.

3.3. Binding of rIL-8 to IL-8 receptors CXCR1 and CXCR2

A recombinant IL-8 molecule was constructed with six histidine residues as part of a nine amino acid tag at the C-terminus. Recombinant IL-8 was tested for its functional activity in the induction of chemotaxis in RBL-2H3 transfectants which express either the IL- 8 receptor type A (CXCR1) or type B (CXCR2). Fig. 5 demonstrates that rIL-8 induces chemotaxis in both transfectants equally well with EC_{50} values of 0.5 versus 0.8 nM, respectively. Again, binding of rIL-8 to RBL-cells expressing the IL-8 receptors CXCR1 or CXCR2 could be detected by flow cytometry using the His-tag specific mAb Maximilian (Fig. 6).

4. Discussion

In the present study we demonstrate that the binding of His-tagged small protein ligands to their receptors may be visualised by flow cytometry using a selected mAb against the His-tag. Using this method, rC3a with a His-tag at its N-terminus could be shown to bind to C3aR-expressing RBL-2H3 transfectants with an EC_{50} of about 3 nM which is well within the range of published affinity constants. In the past, affinity constants between 0.3 and 4.5 nM have been calculated for the binding of C3a to RBL-2H3 cells transfected with C3aR cDNA (Ames et al., 1996; Crass et al., 1996). Our data suggest that

binding of the His-tag specific mAb Maximilian to the N-terminus of rC3a did not interfere with the interaction of the ligand with its receptor. The receptor binding site of C3a is suggested to reside primarily in the C-terminal part of the molecule since a synthetic peptide encompassing residues 57–77 of human C3a was found to possess biological activity nearly equivalent to that of the natural molecule (Lu et al., 1983).

It could also be demonstrated that aminoterminally tagged rC5a molecules of rat or human origin bound to the human C5aR as detected by the tag-specific mAb. Using this method, rat rC5a bound to human C5aR-expressing RBL-2H3 transfectants with an EC_{50} of about 3 nM which is similar to the published affinity constant of 7.2 nM (Rothermel et al., 1997b). Interestingly, over a wide range of ligand concentrations, a higher fluorescence signal was obtained for the binding of rat rC5a, as compared to human rC5a, to the human C5aR. In contrast, rat and human rC5a were equally effective in inducing chemotactic mobility of C5aR-expressing RBL-2H3 cells. These results suggest that the tag-specific mAb Maximilian may interfere with the binding of human but not rat C5a to the human C5aR. An alternative explanation may be that after binding of human rC5a to its receptor the His-tag is rendered less accessible to the mAb. For the binding of human C5a to its cellular receptor, a binding model consisting of two different subsites has been proposed (Siciliano et al., 1994). According to this concept the first binding domain comprises the N-terminal and possibly the first external loop of the receptor. This site interacts with the globular core of C5a, provides the energy necessary for high affinity binding, and facilitates the interaction between the C-terminus of C5a and the second binding domain of the C5a receptor (De-Martino et al., 1994, 1995; Siciliano et al., 1994). It has also been suggested that due to the low sequence homology between the N-termini of the human and rat C5aR, rat rC5a may bind to the human C5aR without direct particitpation of the aminoterminal receptor region (Rothermel et al., 1997b). Our results corroborate the hypothesis of different binding modes for the interaction between human and rat rC5a, respectively, and the human C5aR.

Interleukin-8 binds to two different receptors (CXCR1 and CXCR2) with high affinity. It has been demonstrated that different binding sites of IL-8 are responsible for the binding to these receptors except for the amino acid motif ELR close to the N-terminus which is essential for the binding to both receptors (Hebert et al., 1991). To circumvent any interference with the aminoterminal binding site, we generated a recombinant IL-8 molecule with a C-terminal His-tag. This recombinant molecule induced chemotaxis in CXCR1- or CXCR2-expressing RBL-2H3 transfectants very efficiently. Binding of rIL-8 to both its high affinity receptors expressed on RBL-2H3 transfectants could be demonstrated by flow cytometry using the tag-specific mAb Maximilian. Thus, this antibody which bound to the C-terminal tag of rIL-8 did not markedly interfere with its binding to both IL-8 receptors and is, therefore, a suitable reagent for the detection of cellular rIL-8 binding.

It should also be noted that three different mAbs recognizing His-tags of recombinant proteins were tested for their sensitivities in the detection of rC3a binding to C3aR-expressing RBL-2H3 cells. The fluorescence signals obtained with mAb Maximilian were distinctly higher compared to the other mAbs tested. The reason for these differences remains unclear. It may be speculated that the three tag-specific mAbs react differently with the secondary reagent used in the flow cytometric experiments.

In summary, our data demonstrate that the His-tag specific antibody Maximilian may be a valuable tool for the visualisation of the binding of recombinant ligands to their receptors and may also provide useful information on the specific binding properties of the ligands.

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