Receptor-mediated Endocytosis of CC-chemokines*

(Received for publication, January 15, 1997, and in revised form, February 11, 1997)

Roberto Solari‡‡, Robin E. Offord‡, Sandrine Remy§, Jean-Pierre Aubry§§, Timothy N. C. Wells, Erik Whitehorn*, Thim Oung**, and Amanda E. I. Proudfoot‡‡‡

From the Cell Biology Unit, Glaxo Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, United Kingdom, the Département de Biochimie Medicale, Centre Medical Universitaire, 1224 Champel, Geneva 1224, Switzerland, Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, 14 Chemin des Aulx, 1228 Plan les Quarates, Geneva 1228, Switzerland, and **Affymax Research Institute, Palo Alto, California 94304

Chemokines are chemotactic proteins which play a central role in immune and inflammatory responses. Chemokine receptors are members of the seven transmembrane G-protein coupled family and have recently been shown to be involved in the entry of human immunodeficiency virus (HIV) into target cells. To study chemokine endocytosis in detail we have used novel site-specific chemistry to make a fluorescently labeled CC-chemokine agonist (rhodamine-MIP-1α) and antagonist (NBD-RANTES). We have also generated a CHO cell line stably expressing a hemagglutinin-tagged version of the CC-chemokine receptor 1 (CCR1), and using these reagents we have examined the receptor-mediated endocytosis of CC-chemokines by confocal microscopy. Our studies reveal that the agonist was internalized and accumulated in transferrin receptor-positive endosomes whereas the antagonist failed to internalize. However, receptor-bound antagonist could be induced to internalize by co-administration of agonist. Analysis of receptor redistribution following chemokine addition confirmed that sequestration was induced by agonists but not by antagonists.

Chemokinase are a large family of chemotactic proteins which play a central role in immune and inflammatory responses. They can be divided into two main classes, the chemokine agonist (rhodamine-MIP-1α) and antagonist (NBD-RANTES). We have also generated a CHO cell line stably expressing a hemagglutinin-tagged version of the CC-chemokine receptor 1 (CCR1), and using these reagents we have examined the receptor-mediated endocytosis of CC-chemokines by confocal microscopy. Our studies reveal that the agonist was internalized and accumulated in transferrin receptor-positive endosomes whereas the antagonist failed to internalize. However, receptor-bound antagonist could be induced to internalize by co-administration of agonist. Analysis of receptor redistribution following chemokine addition confirmed that sequestration was induced by agonists but not by antagonists.

Chemokine receptors are members of the seven transmembrane G-protein coupled family and have recently been shown to be involved in the entry of human immunodeficiency virus (HIV) into target cells. To study chemokine endocytosis in detail we have used novel site-specific chemistry to make a fluorescently labeled CC-chemokine agonist (rhodamine-MIP-1α) and antagonist (NBD-RANTES). We have also generated a CHO cell line stably expressing a hemagglutinin-tagged version of the CC-chemokine receptor 1 (CCR1), and using these reagents we have examined the receptor-mediated endocytosis of CC-chemokines by confocal microscopy. Our studies reveal that the agonist was internalized and accumulated in transferrin receptor-positive endosomes whereas the antagonist failed to internalize. However, receptor-bound antagonist could be induced to internalize by co-administration of agonist. Analysis of receptor redistribution following chemokine addition confirmed that sequestration was induced by agonists but not by antagonists.
Endocytosis of CC-chemokines

To study CC-chemokine endocytosis we generated fluorescently labeled RANTES and MIP-1α using site-specific chemistry to attach the fluorophore specifically to the terminal amino group of the polypeptide chain (23, 26). Using this technique we conjugated rhodamine to MIP-1α and 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) to RANTES. These fluorescently labeled CC-chemokines retained their ability to bind specifically to CCR1 although the binding affinity was slightly reduced compared with the unmodified agonist (23, 26). Prior to using these ligands to study endocytosis we assessed their biological activity in chemotaxis assays. Rhodamine-MIP-1α acted as a full agonist and induced maximal chemotaxis at a concentration of 100 nM (Fig. 1A). However, NBD-RANTES had no biological activity in a chemotaxis assay. We have previously shown that modification of the amino terminus of RANTES has a profound influence on its biological properties (25). Failure to cleave the initiating methionine from bacterially expressed RANTES (Met-RANTES) produced a protein which was devoid of agonist activity but was a potent antagonist. Similarly, conjugation of the fluorophore NBD to the amino terminus of RANTES also converted it from an agonist to an antagonist. As shown in Fig. 1B both Met-RANTES and NBD-RANTES can fully antagonize the chemotactic activity of RANTES on THP-1 cells.

For endocytosis studies the human CCR1 cDNA was cloned by reverse transcriptase-PCR, and an HA epitope tag was placed at the extreme amino terminus. The tagged receptor was transfected into CHO cells, and lines stably expressing the receptor were cloned. Radioligand binding assays confirmed that the tagged CCR1 expressed in CHO cells (CHO-CCR1) retained high affinity ligand binding and ligand specificity (data not shown). In the initial experiments the fluorescent rhodamine-RANTES-1α and NBD-RANTES were bound to CHO-CCR1 cells for 4 h at 4 °C at a final concentration of 500 nM. Unbound ligand was washed off with ice-cold buffer prior to warming the cells to 37 °C for timed periods up to 30 min (Fig. 2). The agonist, rhodamine-MIP-1α, was effectively internalized and accumulated in perinuclear vesicles (panels A–C) whereas the antagonist, NBD-RANTES, remained almost entirely on the cell surface (panels D–F). This is consistent with other GPCRs for which it has been shown that receptor internalization is dependent upon agonist stimulation and is inhibited by antagonists (4, 6, 9, 10). However, since we have made NBD-RANTES we were able to perform the experiment to study the effect of agonist stimulation upon the receptor-mediated endocytosis of a fluorescent antagonist. Binding of NBD-RANTES in the presence of an equimolar concentration of RANTES showed that the antagonist could be induced to cluster and internalize in the presence of agonist (panels G–I). Although the internalized antagonist appeared in more peripheral endosomes rather than in the perinuclear structures in which the agonist accumulated, this observation nonetheless demonstrated that agonist-bound receptor could induce the sequestration of antagonist-occupied receptor. These findings suggest that activation of the GPCR kinase by agonist will induce phosphorylation, and consequently sequestration, of receptors occupied by agonists or antagonists. The significance of this relates to how a virus uses a GPCR to gain entry to a cell. These findings suggest that HIV would not have to act as an agonist to be internalized via CC-chemokine receptors provided there is sufficient chemokine agonist present to induce receptor internalization. This is consistent with recent findings, using chimeric receptors, that the regions of CCR5 required for viral entry and for chemokine signal transduction are distinct (27). It may also explain the observation that in certain macrophage cultures addition of CC-chemokines actually enhances rather than inhibits HIV replication (28).

To define the endocytic compartment into which rhodamine-MIP-1α was being delivered we performed dual-labeling studies with the transferrin receptor. Rhodamine-MIP-1α was bound to CHO-CCR1 cells at 4 °C, and receptor-bound ligand was subsequently allowed to internalize for 30 min at 37 °C. Cells were fixed, permeabilized, and stained using an antibody to the transferrin receptor (Fig. 3). Analysis of the cells by confocal microscopy revealed that at 4 °C, the rhodamine-MIP-1α decorated the plasma membrane (panel B) whereas the transferrin receptor staining was both on the plasma mem-
Endocytosis of CC-chemokines 9619

FIG. 2. Receptor-mediated endocytosis of fluorescent chemokines. Chemokines were bound to the surface of CHO-CCR1 cells at 4 °C. Unbound chemokine was washed off, and cells were either fixed immediately or rapidly warmed to 37 °C for 15 or 30 min prior to fixation. Following fixation the cells were examined by confocal microscopy. Panels A, D, and G show chemokine distribution with no warmup, panels B, E, and H show distribution after a 15-min warmup, and panels C, F, and I show distribution after a 30-min warmup. Rhodamine-MIP-1α (panels A, B, and C), NBD-RANTES (panels D, E, and F), and NBD-RANTES plus RANTES (panels G, H, and I). (Bar = 10 μm).

bran and in numerous endocytic vesicles (panel A). Following a 30-min warm up the rhodamine-MIP-1α was internalized into perinuclear endosomal vesicles (panel D) and showed an exact co-localization with the transferrin receptor (panel C). Since it is well documented that the transferrin receptor is a marker for the clathrin-coated pit endocytic pathway it is reasonable to conclude that the CCR1 is also internalized via this mechanism.

As a final study we decided to examine the receptor redistri-

FIG. 4. CC-chemokine receptor 1 redistribution following addition of chemokine agonists and antagonists. CHO-CCR1 cells were incubated for 30 min at 37 °C with a range of CC-chemokines. Following this period the cells were fixed and permeabilized, and the HA-tagged receptor was detected with the anti-HA antibody 12CA5 followed a biotinylated rabbit anti-mouse antibody and a streptavidin-Texas Red conjugate. Panel A, no chemokine addition; panel B, 50 nM NBD-RANTES; panel C, 1 μM NBD-RANTES; panel D, 50 nM Met-RANTES; panel E, 1 μM Met-RANTES; panel F, 50 nM MIP-1α; panel G, 1 μM MIP-1α; panel H, 50 nM RANTES; panel I, 1 μM RANTES.

bution following addition of agonist or antagonist. For these experiments various chemokines were added to CHO-CCR1 cells at 37 °C for 30 min after which the cells were washed, fixed, and permeabilized, and the CCR1 receptor was visualized by staining with the anti-HA monoclonal antibody (Fig. 4). The various chemokines were added at a concentration of either 50 nM or at 1 μM. Without chemokine addition the receptor was predominantly seen on the plasma membrane (panel A) and addition of the antagonists NBD-RANTES (panels B and C) or Met-RANTES (panels D and E) did not induce any significant receptor redistribution. However the agonists MIP-1α (panels F and G) and RANTES (panels H and I) both induced receptor sequestration, although MIP-1α appeared more effective in this respect than RANTES. Even at 50 nM, MIP-1α induced most of the CCR1 to redistribute from the plasma membrane to perinuclear vesicles, and at 1 μM receptor down-regulation was almost complete.

These observations are the first detailed description of chemokine and chemokine receptor endocytosis, and they show that CCR1 is likely to be internalized via clathrin-coated pits. We demonstrate that both ligand and receptor accumulate in perinuclear endosomes and that receptor internalization is dependent upon agonist stimulation. Provided that all CC chemokine receptors behave like CCR1, does this give us any insight into how we might use chemokines as therapeutic agents to prevent HIV entry into cells? The protective effects of chemokines may be due to two factors. The chemokine may act as a competitive inhibitor preventing binding of the virus to the receptor or it may down-regulate surface receptors so that there are no receptors available for the virus. If the second explanation were true then antagonists, which do not induce receptor sequestration, would not be expected to block HIV entry. Recently published data (29) and our own studies3 confirm that chemokine antagonists are effective at inhibiting HIV

entry into target cells. Consequently our study strongly sug-
uggests that the observed HIV suppressive effects of chemokines
are due to competitive inhibition for receptor binding and not
due to receptor down-regulation. These findings have signifi-
cant implications for the design and discovery of novel thera-
peutic agents targeted at blocking HIV entry into cells.

Acknowledgments—We thank Emily Tate, Frederic Borlat, Rapha-
eille Buser, and Marc-Olivier Montjovent for technical assistance.

REFERENCES
2. Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B.,
3. Raposo, G., Dunia, I., Delavie-Klutchko, C., Kaveri, S., Strusberg, A. D., and
5. Grady, E. F., Slice, L. W., Brant, W. O., Walsh, J. H., Payan, D. G., and
J. Biol. Chem. 271, 18302–18305
306–313
13. Deng, H., Liu, R., Lellmeier, W., Choe, S., Unutmaz, D., Burkhardt, M., Di
Martino, P., Marmon, S., Sutton, B. E., Hill, C. M., Davis, C. B., Peiper, S. C.,
A., Cytanis, N. D., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A.
15. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L.,
Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and
17. Bleul, C. G., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and
18. Oberlin, E., Amara, A., Bacherer, F., Bessia, C., Viréuzier, J-L., Arenzana-
Seisdedos, F., Schwartz, O., Heard, J-M., Clark-Lewis, I., Legler, D. F.,
22. Proudfoot, A. E. I., Power, C. A., Hogewerf, A., Montjovent, M-O., Borlat, F.,
Chem. 268, 12247–12249
29. Arenzana-Seisdedos, F., Viréuzier, J-L., Rousset, D., Clark-Lewis, I.,
Receptor-mediated Endocytosis of CC-chemokines
Roberto Solari, Robin E. Offord, Sandrine Remy, Jean-Pierre Aubry, Timothy N. C. Wells, Erik Whitehorn, Thim Oung and Amanda E. I. Proudfoot

doi: 10.1074/jbc.272.15.9617

Access the most updated version of this article at http://www.jbc.org/content/272/15/9617

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 13 of which can be accessed free at http://www.jbc.org/content/272/15/9617.full.html#ref-list-1