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Importance of the Carboxy-Terminus of the CXCR2 for Signal Transduction[1]

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The CXCR2 is phosphorylated at the C-terminal intracytoplasmic portion within 15 sec following the addition of IL-8 or MGSA. Cells transfected with a truncated form of the receptor missing the last 12 amino acids (T3) showed normal binding affinity, but were no longer phosphorylated; individual alanine replacement indicated that Ser346 and 348 were the primary sites of phosphorylation. In studies of the importance of phosphorylation in CXCR2 desensitization, cells expressing wild type CXCR2 lost GTPγS binding above basal rate after the first exposure to IL-8, while cells with the T3 mutant retained 60% of their capacity to induce GTPγS exchange upon a second exposure to IL-8. In contrast, receptor internalization was not affected by the loss of phosphorylation of the T3 mutant. Further receptor truncation led to decreasing binding affinities for IL-8 and MGSA and a decreased rate of GTPγS exchange following addition of excess ligand which suggests involvement of this region in G-protein coupling.

Interleukin-8 (IL-8) is a potent neutrophil stimulating peptide that causes neutrophil accumulation in numerous inflammatory diseases (1-4). The neutrophil expresses two IL-8 receptors (5,6) which are 77% identical, with most of the amino acid differences in the NH_2- and C-termini. While the IL-8 receptor 1 (CXCR1) is specific for IL-8, the IL-8 receptor 2 (CXCR2) reacts with similar affinities with MGSA and NAP2 (7,8). Blockade of the CXCR1 or 2 with anti-receptor antibodies in the neutrophil indicated possible functional differences of the two receptors in spite of equal IL-8 affinities (9-12). Furthermore it has been shown that replacement of the last 27 amino acids of the CXCR1 with CXCR2 sequence increases its rate of internalization (13). Thus it seems likely that the C-terminus of each receptor may interact with its respective signalling targets in different ways.

Activation of G-protein coupled receptors is terminated by the phosphorylation of the receptor which initiates uncoupling of the receptor from the heterotrimeric G-protein (14). A phosphorylated receptor becomes a target for the binding of an arrestin-type protein which uncouples the receptor from its G-protein, resulting in homologous desensitization (15) and sequestration of the receptor. The CXCR2 contains a cluster of serines and threonines in its C-terminus, which were mutated in this report.

Another characteristic of the CXCR2 is the short-lived nature of its activation as compared to the formyl peptide or C5a receptor. Functions that require prolonged receptor activation such as the oxidative burst (16), are poorly activated by IL-8 (17,18). Thus understanding the mechanism of phosphorylation and inactivation of CXCR2 may shed light on the determining factors in the unique CXCR2 function.

This study explores the role of the C-terminus of the CXCR2 in signal transduction, and examines regions in it associated with receptor phosphorylation and G-protein interaction.

MATERIALS AND METHODS

Reagents. [^{32}P] Orthophosphate (8500-9120 Ci/mmol) and [^{125}I] iodine (17Ci/mg) were purchased from NEN. Geneticin (G418) and all tissue culture reagents were obtained from Gibco. IL-8 and MGSA were produced in E. coli and purified as reported previously (8).

Cell culture. RBL2H3 rat basophilic leukemia cells were grown in RPMI 1640 containing 15% fetal calf serum and transfected with CXCR2 or receptor mutants in pSFFV.neo as described (8). Stable cell-lines were selected with G418. Receptor surface expression was

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List of abbreviations: FACS: fluorescent activated cell sorter; FITC: fluorescein isothiocyanate; IL-8: interleukin 8; CXCR1: IL-8 receptor 1; CXCR2: IL-8 receptor 2; MGSA: melanoma growth stimulatory activity; MW: molecular weight; NAP2: neutrophil activating protein 2; PBS; phosphate buffered saline; PMSF: phenyl methylsulfonyl fluoride; SDS: sodium dodecyl sulfate.

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confirmed in all transfected cell lines by FACS using rabbit anti-CXCR2 (19).

Receptor mutants. Truncations and point mutations of the CXCR2 were introduced by PCR and cloned into pSFFV.neo. Their correctness was verified by automated DNA sequencing. The specific mutations are outlined below:

-RHGLLKILAIHGLISKD	T1
-RHGLLKILAIHGLISKDSLPKD	T2
-RHGLLKILAIHGLISKDSLPKDSRPSF	T3
-RHGLLKILAIHGLISKDSLPKDSRPSFVGSSSGH**NANA**L	P35
-RHGLLKILAIHGLISKDSLPKDSRPSFVG**A**SSGHTSTTL	P36
-RHGLLKILAIHGLISKDSLPKDSRPSFVGS**A**SGHTSTTL	P37
-RHGLLKILAIHGLISKDSLPKDSRPSFVGSS**A**GHTSTTL	P38
-RHGLLKILAIHGLISKDSLPKDSRPSFVGSSSGHTSTTL	wild type
320 330 340 350	

Phosphorylation assay for CXCR2 and mutants. RBL2H3 cells were washed 2× in PO_4-free RPMI 1640 and resuspended at 10^7 cells/ml in the same media containing 0.3 mCi/ml of ^{32}P-phosphate and incubated for 90 min at 37°. 500 μl samples of cell suspension were stimulated at 37°. The reaction was stopped by placing the samples on ice. All of the following steps were performed at 4°. The cells were microfuged and once washed in PBS. The cell pellet was lysed in 300 μl lysis buffer (1% octylglucoside, 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM NaF, 10 mM Na-pyrophosphate, 1 mM PMSF, 48 mU aprotinin) and rotated for 15 min. Insoluble material was removed by centrifugation at 10,000 × g for 5 min. The supernatant was precleared by incubating with 10 μl normal rabbit serum for 1 hour, followed by addition of 20 μl protein A Sepharose beads (Pierce). After 30 min the beads were removed by centrifugation. 1 μl rabbit CXCR2 antiserum was added, and the samples were rotated for 1 hr, followed by another 1 hr incubation in the presence of 20 μl protein A Sepharose beads. The samples were microfuged, washed 3 × in lysis buffer, resuspended in 40 μl SDS-sample buffer and boiled for 2 min. Proteins were separated on a 10% SDS polyacrylamide gel and analyzed by autoradiography.

Lactoperoxidase labelling. RBL2H3 cells (10^7c./500 μl PBS) were surface iodinated in the presence of 0.1 U lactoperoxidase and 0.5 mCi Na^{125}I with 10 additions of 1 μM H_2O_2. The cells were washed 3 × in PBS containing 0.1 % BSA and immune-precipitated as described above.

[^{35}S]GTPγS binding assay. For the preparation of membranes for the [^{35}S]GTPγS binding assay, RBL2H3 cells (10^7/ml) were suspended in 20 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM NaF, 1 mM PMSF and 0.25 U/ml aprotinin at 4°. The cells were N_2 cavitated for 20 min at 500 psi, centrifuged for 20 min at 30,000 × g, and the resulting membrane pellet was resuspended in the same buffer containing 0.2 M sucrose. In some experiments cells were treated with 2 ×10^{-7} M IL-8 for 15 min at 37°, or for 3 hrs with 300 ng/ml of pertussis toxin prior to the preparation of membranes. [^{35}S]GTPγS binding was carried out as described by Richardson (20).

Receptor internalization. Receptor surface expression was determined in cells incubated for different time intervals with 10^{-7} M IL-8 at 37°C. The cells were put on ice to stop the reaction, incubated with anti-CXCR2 antibody followed by anti rabbit-FITC IgG (Biosource) and FACS analysis as described in (19,21). Baseline cell fluorescence analyzed on cells incubated only with the second antibody was subtracted to determine % internalization.

Additional methods. Ca^{2+} mobilization was determined as previously described (8). The method for ^{125}I-IL-8 binding was as reported in (8). Protein was detected with the BCA reagent (Pierce) using bovine serum albumin as a standard.

RESULTS

Receptor expression and affinity. In order to determine the role of the C-terminus of the CXCR2 in receptor function, several truncations of this receptor were expressed in RBL2H3 cells. FACS analysis with anti-receptor antibody (19) indicated that all receptors were expressed. Scatchard analysis of the mutants showed an affinity similar to wild type for P35 to P38 and T3, an eightfold lower affinity for T2 and an affinity of >20 nM for T1 (Table 1).

Receptor function. All of the mutant receptors elicited Ca^{2+} mobilization in the presence of IL-8 or MGSA, although the response in T1 was significantly diminished (Fig. 1A and B). Dose responses followed the pattern expected from the binding affinities. In wild type and P35 receptor mutant cells, Ca^{2+} mobilization was transient and the Ca^{2+} concentrations quickly fell back to background level. In contrast Ca^{2+} stayed elevated over several minutes in the receptor truncations T3 and T2 (Fig. 1C and D), suggesting that the last twelve amino acids were important for signal termination.

Receptor phosphorylation. Since receptor phosphorylation leads to desensitization of other receptors (22), we hypothesized that the continued stimulation seen in T3 and T2 was due to the loss of phosphorylation sites in these mutants.

When wild type receptor of surface iodinated cells was immune precipitated with anti-CXCR2 antibody, a wide band of approximately 60,000 MW was detected (Fig. 2A). An additional band of MW 40,000 was noted. The same bands were immune precipitated in [^{32}P] phosphate labeled cells following stimulation with IL-8 (Fig. 2B) or MGSA, but not in the absence of ligand. The reaction could be detected as early as 10 sec after the addition of ligand and reached its maximum by about 30 sec for IL-8 and between 3-5 min for MGSA (Fig. 2B). Phosphorylation became measurable with a dose of 1nM IL-8 and was maximal around 10^{-7} M ligand.

Phosphorylation of the P35 mutant was similar to wild type receptor (Fig. 3), but T3 and all of the shorter truncations could not be phosphorylated, indicating that serines 346, 347 and 348 - the only three hydroxyl-

TABLE 1

Binding Affinities for the Wild Type and Mutant CXCR2s

Receptor	K_d	Receptors/cell
CXCR2	0.9×10^{-9} M	40,000
T1	$>2 \times 10^{-8}$ M	~6000
T2	5.9×10^{-9} M	38,000
T3	1.1×10^{-9} M	48,000
P35	0.8×10^{-9} M	35,000
P36	2.0×10^{-9} M	12,000
P37	1.2×10^{-9} M	49,000
P38	1.0×10^{-9} M	34,000

Note. Binding of ^{125}I-IL-8 was determined on 2×10^6 RBL2H3 cells expressing the receptor mutants for 90 min at 4° ± a 1000-fold excess of unlabelled IL-8 (8). The receptor number for T1 is a FACS estimate.

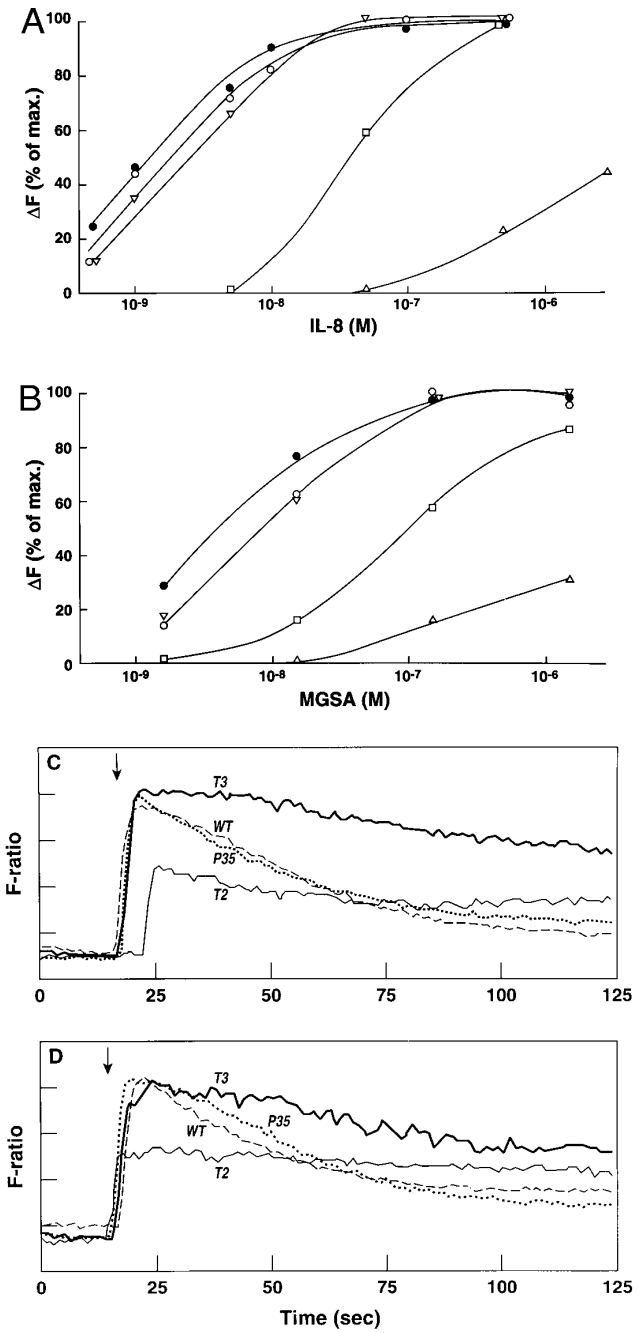

FIG. 1. Ca^{2+} mobilization in RBL2H3 cells expressing the CXCR2 or receptor mutants. Ca^{2+} mobilization was followed kinetically in indo-1-AM labelled cells as described (8). In panels A and B the maximal change in fluorescence ratio seen in IL-8 (panel A) or MGSA (panel B) stimulated cells was compared to that in Triton-X lysed cells. ▽ wild type receptor, △ T1, □ T2, ● T3 and ○ P35. Each point is the mean of 3 determinations. In panels C and D Ca^{2+} fluxes are shown following the injection of 1×10^{-7} M IL-8 (panel C) or 1.5×10^{-7} M MGSA (panel D) at the arrow.

ated amino acid differences between P35 and T3 - are a prerequisite for receptor phosphorylation. To confirm that the lack of phosphorylation in T3 was due to the presence of these specific amino acids, we replaced these 3 serines individually with alanines. Ser348 was absolutely necessary for receptor phosphorylation (Fig. 3), and P36, in which Ser346 is mutated to Ala did not appear phosphorylated, while replacement of Ser347 by Ala showed no effect on receptor phosphorylation.

Lack of phosphorylation in T3 correlated with continuous activation following the addition of ligand (see Fig. 1C and D).

Ligand induced [^{35}S]*GTPγS binding.* In the neutrophil both CXCRs are coupled to Gi2α (23), although they can also associate with Gα14, Gα15 and Gα16 in cells overexpressing these proteins (24). It is therefore important to analyze the function of the receptor mutants

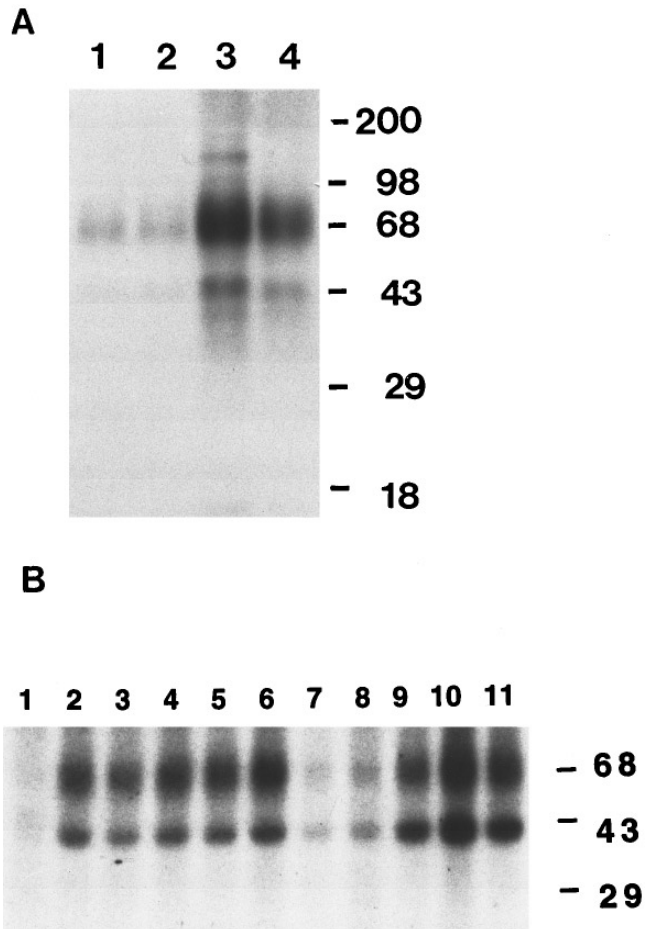

FIG. 2. Immunoprecipitation of wild type CXCR2 in RBL2H3 cells. In panel A cells were surface iodinated. Lane 1: untransfected cells + anti-receptor antibody, lane 2: CXCR2 expressing cells + preimmune serum, lane 3: Cells incubated with 10^{-7} M IL-8 for 2 min prior to immunoprecipitation with anti-receptor antibody, lane 4: unstimulated CXCR2 cells + anti-receptor antibody. Panel B: Time course of phosphorylation of the wild type CXCR2 in cells stimulated with 10^{-7} M IL-8 or MGSA at 37°. Lane 1: 5 min incubation without the addition of ligand, lanes 2–6: IL-8 added for 15 sec, 30 sec, 1 min, 5 min and 10 min, respectively, lanes 7–11: MGSA added for 15 sec, 30 sec, 1 min, 5 min and 10 min, respectively.

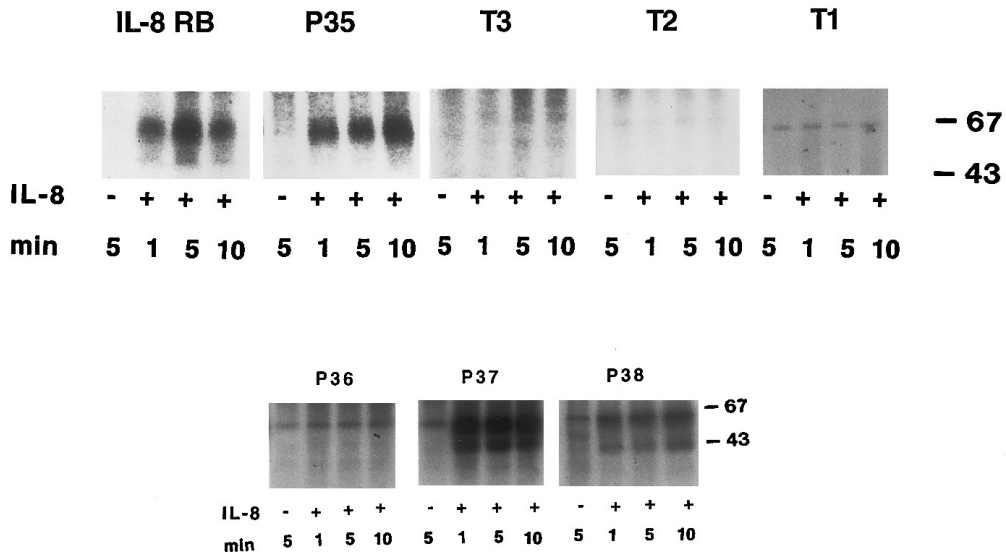

FIG. 3. Phosphorylation of receptor mutants. RBL2H3 cells expressing wild type CXCR2 or receptor mutants were [^{32}P]phosphate labelled, stimulated with 10^{-7}M IL-8 for 1 to 10 min and immuneprecipitated. One experiment representative of 3. Top panel: The autorad exposure was 24 hrs for CXCR2 and P35, and 36 hrs for T1, T2 and T3 to verify the absence of specific label in these cells. The bottom panel shows a 60 hour exposure of single amino acid substitution mutants.

in hematopoietic cells such as RBL2H3 cells that express a signal transduction cascade similar to the neutrophil.

Ligand-induced [^{35}S]GTPγS binding in membranes has been used extensively to study the effect of receptor phosphorylation on desensitization (20). Wild type receptor membranes pretreated with 2×10^{-7} M IL-8 showed a loss of GTPγS exchange following restimulation (Fig. 4A). P35 expressed a small increase in GTPγS exchange rate upon a second exposure to IL-8, and T3 cells retained most of the GTPγS response following a second stimulation (Fig. 4B and 4C). These findings are consistent with the concept that receptor phosphorylation is necessary for homologous receptor desensitization and that the T3 mutant lacks phosphorylation properties and the capacity of desensitization. Further truncation of the receptor (T2, T1) decreased the rate

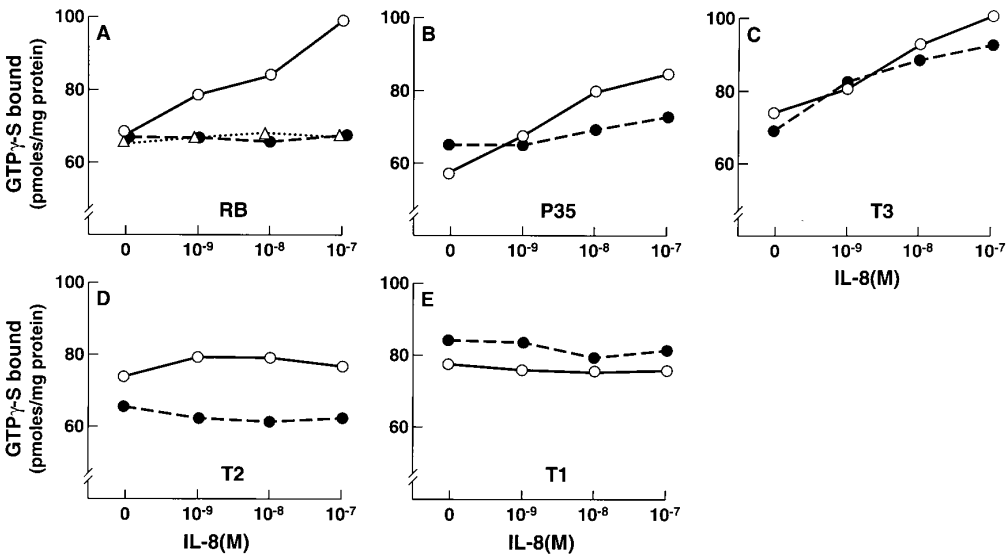

FIG. 4. Effect of IL-8 on GTPγS exchange. Plasma membranes prepared from RBL2H3 cells expressing the CXCR2 (panel A) or receptor mutants (panels B to E) were incubated in GTPγ^{35}S binding buffer with the concentrations of IL-8 indicated on the abscissa, and bound GTPγ^{35}S was quantified as described previously (20) (○). Closed circles represent the same assay performed on plasma membranes from cells that were prestimulated for 15 min with 2×10^{-7} M IL-8 prior to membrane preparation. The triangles show the effect of pertussis toxin treatment of the membranes.

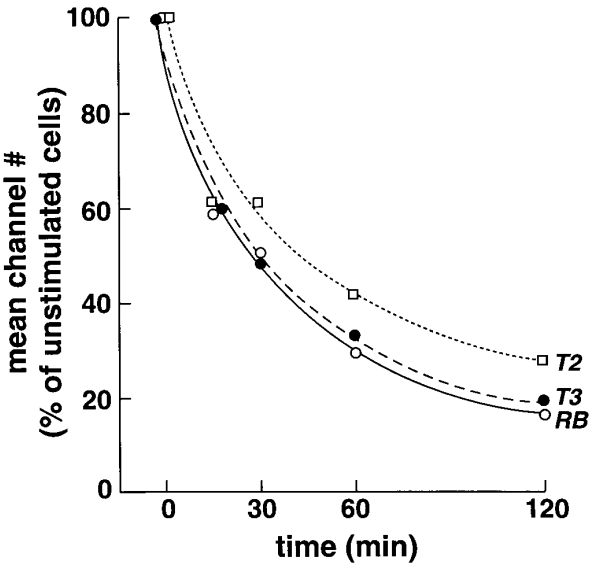

FIG. 5. Internalization of the CXCR2 and receptor mutants. 10^6 RBL2H3 cells carrying the CXCR2 or receptor mutants were incubated with 10^{-7} M IL-8 at 37° for the times indicated on the abscissa. The cells were put on ice and surface receptor expression was determined by FACS analysis as previously described (19). n=3. All standard deviations were less than 5%.

of GTPγS exchange during the first exposure to IL-8, which barely increased above baseline (Fig. 4D and 4E), suggesting that amino acids 334 to 343 are important for G-protein interaction.

Receptor internalization. In the β_2 adrenergic system, phosphorylation of the receptor has been shown to allow β-arrestin binding (25) and internalization of the receptor. In the case of the CXCR2, internalization did not depend on prior phosphorylation of the receptor. T3 receptors, which could not be phosphorylated were internalized at the same rate as wild type receptor (Fig. 5), and T2 cells showed only a marginally slower rate of internalization.

DISCUSSION

Our results indicate that serines 346 to 348 close to the C-terminus of the CXCR2, are necessary for receptor phosphorylation and inactivation (26). Following completion of this study a similar observation was reported by Mueller et al. (27). Interestingly, alanine replacement of the corresponding Thr/Ser/Ser/Ser sequence in the CXCR1 attenuated receptor phosphorylation by about 40%, but did not abolish it (28), while the truncation of the CXCR2 did in the present study.

Phosphorylation of the CXCR2 in RBL2H3 cells following stimulation with IL-8, reached its maximum within 30 sec, which is fast in comparison with other neutrophil stimulating receptors which take 3-5 min to be fully phosphorylated. Interestingly, MGSA induced CXCR2 phosphorylation followed slower kinetics. Rapid phosphorylation and hence desensitization of the CXCR2 may be a reason for the greater efficiency of the anti-CXCR1 antibody compared to anti-CXCR2 antibody (9-11) in blocking IL-8 induced neutrophil chemotaxis in spite of similar ligand affinities and receptor numbers for both receptors.

The phosphorylation site of the CXCR2 is close to an area of the receptor that is necessary for G-protein coupling as assessed by ligand induced enhancement of the exchange rate for GTPγS. Reducing the receptor by 5 (T2) or 10 (T1) amino acids from the phosphorylation site partially or completely abolished G-protein stimulation and led to decreased ligand-receptor affinity, a function of G-protein coupling. While Ben-Baruch et al. concluded that this area is not necessary for G-protein coupling, their results show a nearly complete loss of chemotaxis, between a receptor truncation at amino acid 324 and one at amino acid 335, the area that corresponds to our T1 (29). Since the $\beta\gamma$-subunit of G-proteins target receptor kinases to their ligand bound receptors (14) by binding to the pleckstrin homology domain of the receptor kinase (30), one may hypothesize that all 3 molecules assemble around the C-terminus of the CXCR2.

The unphosphorylated receptor mutant T3 stayed coupled to the G-protein as evidenced by the continuous high GTPγS exchange rate following a 2nd exposure to ligand. This prolonged duration of receptor-G-protein coupling was associated with continued high levels of free Ca^{2+} as would be expected if receptor phosphorylation is the first step in desensitization.

The effect of receptor phosphorylation on internalization varies for different receptors and/or cell types. It was shown previously that receptor phosphorylation is associated with sequestration both for the β_2-adrenergic receptor (25), and the m2 muscarinic receptor expressed in COS7 cells (31). But desensitization and internalization occurred as two independent events for the m2 muscarinic receptor expressed in 293 cells (32), the angiotensin II receptor (33), and the α_{1B}-adrenergic receptor (34). Similarly, in the CXCR2, receptor internalization did not depend on phosphorylation and occurred at the same rate in the T3 mutant as in wild type receptor. The mechanisms involved in endocytosis of the 7-membrane-spanning receptors are only starting to be elucidated, and fall into two different pathways. In the first pathway, β-arrestin binds to the phosphorylated receptor followed by internalization (35). A second pathway, observed for the bradykinin receptor involves uptake via caveolae (36). One may speculate that the CXCR2 falls into the latter category of receptors.

The amino acid sequence of the CXCR2 accounts for a molecular weight of 40,123 (6). The NH_2 terminus and the 2nd extracellular loop contain 3 possible glycosylation sites. Other members of the 7-membrane-

spanning receptor family of similar MW, appear as a wide band of a molecular weight of ~60,000 on SDS gels (28,37,38). In agreement with this, earlier cross-linking experiments of the CXCR2 showed a wide band with a molecular weight around 60,000 (39,40). In contrast, in later phosphorylation studies of the CXCR2, a tight band of 40,000 molecular weight was immune-precipitated with antibody against the receptor (27,41), which probably represents degraded receptor, as we observed a similar coprecipitating band on occasion (see Fig. 2).

In summary, the progress made in the understanding of G-protein-coupled receptor function which was originally defined in the β-adrenergic system has greatly contributed to the understanding of G-protein coupled receptors generally, but the details of the receptor/signaling cascade vary for different receptors, as shown here for the CXCR2, and deserve further elucidation to eventually define the rules that govern the interaction between each receptor and its transduction machine.

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