A comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2 Chemokine mediated activation of p42/p44 MAP-kinase (ERK-2)

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Abstract The CXC chemokine, IL-8, is a potent chemoattractant of neutrophils and binds to two distinct receptors, termed IL-8R1 and IL-8R2. These receptors share high affinity for IL-8, however, only IL-8R1 is specific for IL-8 whereas IL-8R2 binds other related chemokines, including GROα with high affinity. Stable Jurkat transfectants were generated expressing either functional IL-8R1 or IL-8R2 (J-IL8R1 and J-IL8R2). Both J-IL8R1 and J-IL8R2 exhibited high affinity IL-8 binding (K_d 3–5 nM) with respective receptor densities of 23,000 ± 3,000 and 18,500 ± 1,500. Pre-treatment of both transfectants with 1.0 μg/ml *B. pertussis* toxin (PTx) resulted in inhibition of IL-8 mediated intracellular Ca^{2+} mobilisation and chemotaxis, without altering the receptor's affinity for its ligand. This indicates that both receptors couple to a PTx-sensitive G-protein. Further studies showed that IL-8R1 and IL-8R2 could mediate time-dependent phosphorylation of p42/p44 MAP-kinase. In both transfectants, phosphorylation was maximal at 1–2 min after IL-8 stimulation and could be inhibited by PTx. Stimulation of J-IL8R1 and J-IL8R2 with GROα revealed that this chemokine was a more potent activator of MAP-kinase in J-IL8R2, an observation reflected in the high affinity binding of GROα to IL-8R2. These studies indicate that chemokines are capable of activating protein kinases and with regards to PTx-sensitivity and MAP-kinase stimulation, no significant differences between IL- 8R1 and IL-8R2 post-receptor signalling occur during cell activation by IL-8.

Key words: Receptor; Interleukin-8; MAP-kinase; Pertussis toxin

1. Introduction

A characteristic feature of the inflammatory response is the recruitment of leukocytes by chemotactic cytokines (chemokines) to areas of tissue injury. Chemokines are low molecular weight (8–10 kDa) proteins and consist of two distinct families, which differ from one another with respect to their target cell specificity [1,2]. These families are classified according to the position of the first two cysteine residues, which are either separated by a single amino acid (CXC-chemokines) or adjacent to each other (CC-chemokines). Interleukin-8 (IL-8), the most widely studied CXC chemokine, is a potent chemoattractant of neutrophils and induces a variety of cellular responses, including chemotaxis, mobilisation of intracellular Ca^{2+}, exocytosis and activation of NADPH oxidase [2–4]. Characterisation of IL-8 binding has revealed that neutrophils express two IL-8 receptors; IL-8R1 and IL-8R2 [5,6]. Both receptors share high affinity for IL-8 (K_d 0.2–2.5 nM), however only IL-8R2 is capable of binding other CXC chemokines with similar high affinity [7–9]. IL-8 receptors consist of seven putative trans-membrane domains (7-TMD) and belong to a family of G-protein coupled receptors [2,10].

A general model for chemokine mediated signal transduction events in phagocytes proposes that an appropriate ligand-receptor interaction causes activation of a *B. pertussis* toxin (PTx)-sensitive G-protein and induction of phospholipase C activity [3]. This leads to an accumulation of diacylglycerol and cytosolic inositol trisphosphate [11], which in turn initiates mobilisation of intracellular Ca^{2+} and protein kinase C activation [12,13]. It has recently been shown that 7-TMD receptors, such as the f-MLP receptor [14,15], the M2-muscarinic receptor [16] and the α2-adrenergic receptor [17] can mediate activation of the mitogen-activated protein (MAP)-kinase signalling cascade [18]. However, chemokine activation of this protein kinase pathway has not been demonstrated. MAP-kinases are known to be responsible for the regulation of proto-oncogenic transcription factors (c-Jun, c-Myc, c-Fos), cytoskeletal elements, phospholipase A2 and other protein kinases [19].

IL-8R1 and IL-8R2 share a high degree of sequence homology, particularly within the 7-TMD, however a significant degree of amino acid variation occurs at both their extracellular amino-terminus and intracellular carboxyl-terminus. This suggests that both receptors not only possess different ligand-binding properties but may also initiate different post-receptor signal transduction events. Leotscher et al. [20] previously demonstrated the chemokine-induced migration of Jurkat cells transfected with cDNA encoding for either IL-8R1 or IL-8R2. In the present communication we have compared IL-8 mediated post-receptor signalling events in this human T-cell line expressing IL-8 receptors. Both functional receptors mediated PTx-sensitive cellular responses and chemokine stimulation resulted in the activation of p42/p44-MAP kinase. Our results indicate no significant difference between the immediate post receptor signalling of IL-8R1 and IL-8R2, at least with respect to their susceptibility to PTx and activation of MAP-kinase.

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Abbreviations: 7-TMD, seven putative trans-membrane domains; IL-8R1/2, human interleukin-8 receptors type 1/2; J-IL8R1/2, Jurkat transfectants expressing IL-8R1/2; GROα, IL-8 related chemokine; PTx, *B. pertussis* toxin; MAP-kinase, mitogen-activated protein kinase.

2. Materials and methods

2.1. Materials

All reagents were purchased from Merck, Fluka and Sigma chemical companies. RPMI 1640 media and additional cell culture supplements, including G418 (Geneticin) were obtained from Gibco-BRL. The

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expression vectors used for the transfection of IL-8 receptors were from the Invitrogen Corporation, *B. pertussis* toxin from List Biochemicals and ^{125}I-radiolabelled NaI was obtained from Amersham. For Western blot analysis, Immobilon-P (PVDF) transfer membrane was from Millipore and the p42/p44 MAP-kinase (ERK-2) polyclonal antibody (c14) was purchased from Santa Cruz Biotechnology. Polyclonal antibodies specific for p47-*phox* (JW-1) and p67-*phox* (JW-2), cytosolic components of the NADPH oxidase, were kindly provided by Professor O.T.G. Jones, Department of Biochemistry, University of Bristol.

2.2. Jurkat cell culture

Jurkat cells were maintained at 37°C, 5% CO_2 in culture medium consisting of RPMI-1640, 10% (v/v) feotal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Stable Jurkat transfectants were cultured in the same growth medium supplemented with 0.8 mg/ml G418.

2.3. Preparation of Jurkat transfectants expressing either IL-8R1 or IL-8R2

Jurkat transfectants expressing IL-8R1 were generated using the pcDNA-3 expression vector, which contains an inherant neomycin (G418) resistance gene. A 1,112 bp cDNA fragment encoding for IL-8R1 was generated as outlined by Leotscher et al. [20] and sub-cloned into pcDNA-3. Briefly, 20 μg of linearised pcDNA-3/IL-8R1 was transfected into Jurkat cells (5–8 × 10^6 cells/800 μl PBS) by electroporation. Two weeks later G418-resistant (0.8 mg/ml) Jurkat clones were selected by limited dilution and characterized for expression of IL-8R1 by ^{125}I-IL-8 binding. Of four independent clones, the clone with the highest binding capacity was selected and termed J-IL8R1. Jurkat cells transfected with cDNA encoding for human IL-8R2 were generated as previously described by Leotscher et al. [20] and is refered to here as J-IL8R2.

2.4. B. pertussis *toxin (PTx) treatment of Jurkat cells*

Jurkat cells were washed and resuspended at a concentration of 10^7 cells/ml in 130 mM NaCl, 4.6 mM KCl, 5.0 mM $NaHCO_3$, 0.05 mM $CaCl_2.2H_20$, 20 mM HEPES (pH 7.4) and 1 g/l D-glucose. The cells were incubated for 90 min at 37°C in the presence or absence of 1 μg/ml PTx, pelletted and then resuspended in an appropriate buffer for binding and functional assays.

2.5. ^{125}I-IL-8 binding and functional assays

Chemically synthesised IL-8 [21] was iodinated as previously outlined [7]. Saturation titration binding studies were performed on 1.25–1.5 × 10^6 PTx-treated/non-treated J-IL8R1 and J-IL8R2 cells. Transfectants were incubated in a range of ^{125}I-IL-8 concentrations (0.2–20 nM) and specific receptor binding compared to cells incubated with the same range of ^{125}I-IL-8 concentrations in the presence of 500 nM non-radiolabelled IL-8. The binding constant (K_d) and receptor numbers were subsequently determined using the method described by Schumacher and von Tscharner [22].

Calcium mobilisation and in vitro chemotaxis assays were performed as previously described by Loetscher et al. [20].

2.6. Western blot analysis of p42/p44 MAP kinase (ERK-2)

Cells were washed twice with sterile PBS and finally resuspended at a concentration of 10^7 cells/ml. Aliquots (200 μl) of the cell suspension were incubated for 10–15 min at 37°C before stimulation with IL-8 or GROα and the reactions were stopped by the addition of TCA (at a final concentration of 10%). Precipitated proteins were recovered by centrifugation, washed twice in cold acetone and air dried. Protein pellets were resuspended in SDS-PAGE sample buffer, boiled, separated by electrophoresis in a 10% acrylamide gel and transferred to an Immobilon-P (PVDF) membrane by electroblotting. Transfer membranes were blocked and probed with an anti-p42/p44 MAP-kinase antibody followed by an alkaline phosphatase conjugated secondary antibody.

3. Results and discussion

3.1. Characterisation of Jurkat transfectants

Saturation binding studies, using ^{125}I radiolabelled IL-8 revealed that the J-IL8R1 and J-IL8R2 stable transfectants expressed 23,000 ± 3,000 and 18,500 ± 1,500 receptors/cell respectively. Both cell lines were found to bind IL-8 with similar high affinity (Table 1), comparable with IL-8 binding to human neutrophils [7,9]. Although, Loetscher et al. [20] previously demonstrated the migratory characteristics of Jurkat cells transfected with IL-8 receptors, other chemokine-induced neutrophil functions were not addressed. Here we were unable to detect IL-8 stimulation of superoxide production in our transfectants and Western blot analysis of Jurkat cell lysates confirmed the absence of p47-*phox* and p67-*phox*, cytosolic factors of the superoxide-generating NADPH oxidase, (data not shown). Similarly, by contrast to neutrophils chemokine stimulation of J-IL8R1 and J-IL8R2 did not result in release of the proteases *N*-acetyl-glucosaminidase or esterase (data not shown).

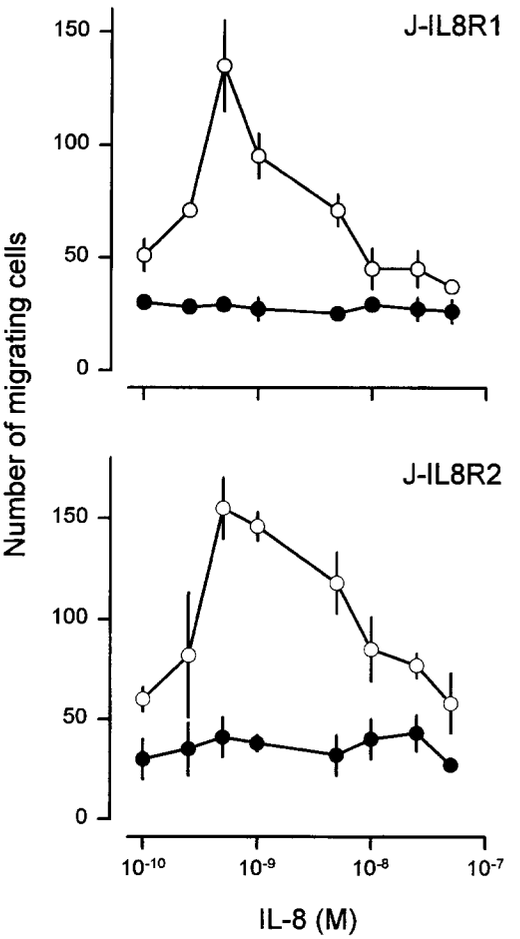

Fig. 1. PTx Inhibition of IL-8 mediated chemotaxis of J-IL8R1 and J-IL8R2. The IL-8 induced migration of PTx-treated (●) and non-treated (○) Jurkat transfectants was assessed in 48-well micro-chemotaxis chambers. Each assay point was measured in triplicate and the values represent the mean number (± S.D.) of migrating cells per five high-power fields of view. The average number of cells incubated in the absence of IL-8 was 25 ± 4.

3.2. Assessing the sensitivity of Jurkat transfectants to PTx

Signalling by chemoattractant and chemokine receptors is inhibited by ADP-ribosylation with PTx [3,10] indicating that the receptors are coupled to G_i subclass of G-proteins [23].

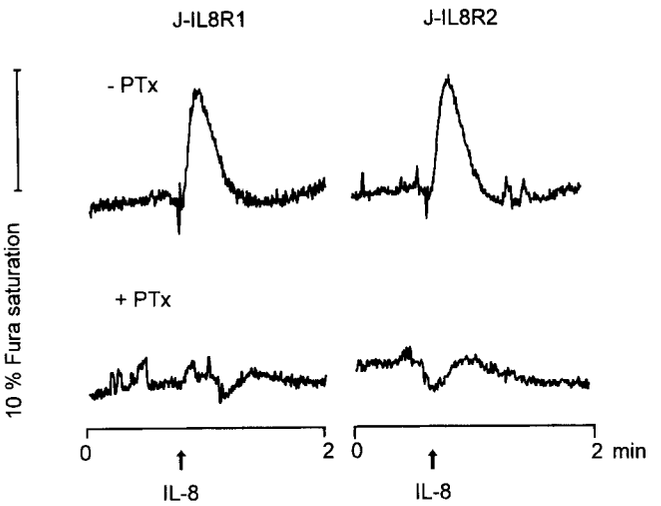

Fig. 2. PTx inhibition of IL-8 induced intracellular Ca^{2+} mobilisation. PTx-treated and non-treated Jurkat transfectants (5×10^6 cells/ml) were loaded with fura-2 and stimulated with 100 nM IL-8. Fluorescence changes are represented as a percentage of the total fura-2 saturation and were standardised as previously described [39].

Granulocytes predominantly express the $G_{\alpha 2i}$ protein [24–26] and the closely related $G_{\alpha 3i}$ [27]. The $G_{\alpha 2i}$ protein has previously been shown to directly couple to the C5a and f-MLP receptors [24,28]. Co-transfection of COS-7 cells with IL-8 receptors and an accompanying PTx-resistant G_α-protein revealed however that IL-8R1 and IL-8R2 may also couple to the Gq subclass of G-proteins, $G_{\alpha 14}$ and $G_{\alpha 16}$ [29]. Although T-lymphocytes and cells of myelomonocytic lineage express Gα16 [30], the emigration of lymphocytes from the thymus has been shown to be PTx-sensitive, indicating a requirement for the G_i subclass of G-proteins [31].

Pretreatment of J-IL8R1 and J-IL8R2 with 1.0 μg/ml PTx resulted in the inhibition of IL-8 stimulated cell migration (Fig. 1) and similarly, inhibited the mobilisation of intracellular Ca^{2+} (Fig. 2). The IL-8 receptors expressed in these transfectants therefore directly couple to the G_i subclass of G-proteins, closely resembling their coupling in neutrophils. Saturation binding studies were subsequently performed in the presence and absence of PTx in order to test whether ADP-ribosylation of the G-protein interfered with receptor-ligand interactions. Table 1 shows that binding of ^{125}I-IL-8 to both receptors is unaltered by PTx treatment, suggesting that although PTx ribosylation leads to uncoupling of the G_α-subunit [32,33], the interaction of IL-8 with its receptor binding site remains unaffected.

3.3. *Chemokine induced activation of p42/p44 MAP-kinase (ERK-2)*

MAP-kinases, also known as extracellular-signal-regulated kinases (ERK), are activated by phosphorylation of tyrosine/threonine residues [34,35] and are responsible for the regulation of several different proteins, including oncogenic transcription factors and other protein kinases. In particular, MAP-kinase activation of phospholipase A_2 [36] and cytoskeletal elements [37] suggests a role for this protein kinase signalling cascade in chemokine-induced cellular responses. Stimulation of human neutrophils with f-MLP activates the Ras/Raf pathway [38], leading to phosphorylation of MAP-kinase [14,15]. Phosphorylated MAP-kinase is easily detected by its characteristic retarded electrophoretic mobility and can be distinguished from the unphosphorylated form by immuno-blotting. Using the J-IL8R1 and J-IL8R2 transfectants, we investigated whether IL-8 could induce phosphorylation of p42/p44 MAP-kinase (ERK-2). Western blot analysis of J-IL8R1 and J-IL8R2 showed that both chemokine receptors can activate p42/p44 MAP-kinase in a PTx-sensitive manner (Fig. 3), indicating the involvement of a G_i-protein. Both Jurkat transfectants mediated an apparent transient activation of MAP-kinase, with maximum IL-8-dependent phosphorylation occurring 1–2 min after stimulation (Fig. 3). A similar time-dependent activation of this protein kinase has been shown in f-MLP [14] and IL-8 stimulated human neutrophils (Wirthmueller and Thelen, unpublished observation). Stimulation of the transfectants with GROα resulted in a more potent activation of MAP-kinase in J-IL8R2 (data not shown). A finding which correlates with the binding characteristics of the IL-8 receptors, since IL-8R2 possesses a higher binding affinity, than IL-8R1, for GROα.

In conclusion, stable transfection of IL-8 receptors into Jurkat cells resulted in their coupling to PTx-sensitive G-proteins resembling the immediate signalling system found in neutrophils. Our data however, suggest no significant difference between the immediate post-receptor signalling of IL-8R1 and IL-8R2. On the other hand, the stable transfection of chemo-

Table 1
Characterisation of IL-8 binding to Jurkat transfectants in the presence and absence of PTx

Transfectant	Receptors/cell	Binding constant (nM)	
		− PTX	+ PTX
J-IL8R1	23,000 ± 3,000	2.5	3.4
J-IL8R2	18,500 ± 1,500	2.1	2.0

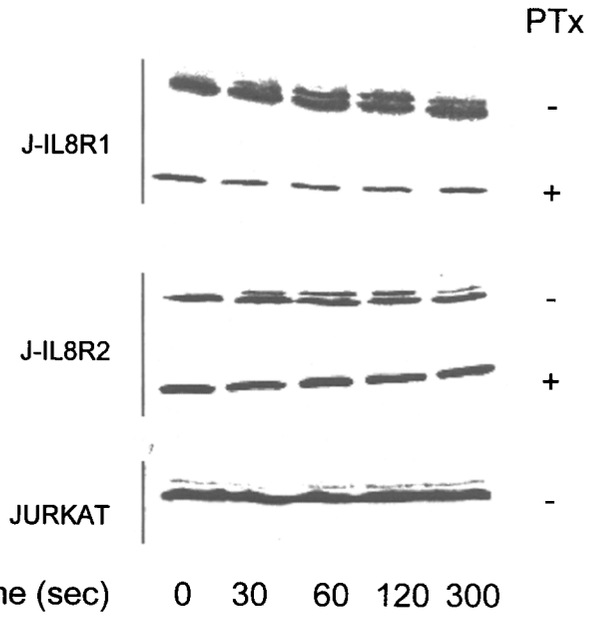

Fig. 3. The time-dependent phosphorylation of p42/p44 MAP-kinase by IL-8. Jurkat cells (2×10^6 cells/assay condition) were stimulated at 37°C with 100 nM IL-8. At the indicated time intervals total cellular proteins were precipitated by the addition of 10% TCA and separated by SDS-PAGE. The proteins were electroblotted and probed with a polyclonal anti-p42/p44 MAP-kinase antibody (c14).

kine receptors into cells of haemopoeitic lineage, in this case Jurkat cells, provides a useful system to evaluate receptor signalling events, particularly since the degree of receptor expression, in this heterologous system, remains at a physiological level. Thus the modes of signalling can be examined in an environment comparable with that found in the short lived neutrophils.

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