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CCL3, acting via the chemokine receptor CCR5, leads to independent activation of Janus kinase 2 (JAK2) and G_i proteins

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Abstract The interaction of the chemokine receptor, CCR5, expressed in recombinant cells, with different G proteins was investigated and CCR5 was found to interact with G_i, G_o and G_q species. Interaction with G_i leads to G protein activation, whereas G_q does not seem to be activated. Additionally, CCR5 activation also leads to phosphorylation of Janus kinase 2 (JAK2). Activation of JAK2 is independent of G_i or G_q activation. G_i protein activation was not prevented by inhibition of JAK, showing that heterotrimeric G protein activation and activation of the JAK/signal transducer and activator of transcription (STAT) pathway are independent of each other.

Keywords: CCR5; JAK2; G protein; [^{35}S]GTPγS binding assay; Calcium ion release

1. Introduction

The chemokine receptor, CCR5, belongs to the superfamily of G protein coupled receptors (GPCRs) and activation of CCR5 by chemokines has been reported to lead to changes in a number of cellular processes, e.g., activation of G proteins [1], increases in intracellular calcium ion concentration [2–7], inhibition of adenylyl cyclase [1], activation of MAP kinase [8–10] and Jun-N-terminal Kinase (JNK) [9]. These processes are typical of responses dependent on activation of pertussis toxin (PTX)-sensitive G proteins of the G_i/G_o family, being related to effects of either α or βγ subunits of these G proteins.

Many cytokine receptors and growth factor receptors mediate their effects by activation of a common signal transduction pathway, the STAT pathway [11,12]. Binding of the ligand to its specific transmembrane receptor results in the catalytic activation of receptor-associated cytoplasmic protein–tyrosine kinases, Janus kinases (JAKs), and phosphorylation-activation of latent monomeric STAT proteins [13–15]. There is now emerging evidence that GPCRs are also capable of activating these JAK/STAT signalling pathways. A report by Marrero et al. showed for the first time that the JAK/STAT pathway can be stimulated by angiotensin II via its specific GPCR (AT1) [16]. Recently, it has been shown that GPCRs are able to activate kinases independent of G protein activation [17–19]. Activation of some STAT transcription factor family members has been described in T cells after CCL5 or CCL3 stimulation which may be associated with CCR5 [20]. In PM1 T cells CCL5 leads to phosphorylation of JAK2 and JAK3 [21]. This phosphorylation is PTX insensitive, indicating that CCL5-CCR5 mediated tyrosine phosphorylation events are not mediated via G_i/G_o proteins [21]. It has also been suggested that JAK activation is necessary for receptor and $G_{\alpha i}$ proteins to associate and induce signalling [17].

It seems, therefore, that the activation of some GPCRs, including CCR5, leads to activation of at least two intracellular signalling systems, those dependent on heterotrimeric G proteins and those dependent on JAK/STAT proteins. In this study, therefore, we have examined the activation of these two systems for CCR5.

2. Materials and methods

Cells and materials. Chinese hamster ovary cells (CHO cells) stably expressing CCR5 (CHO.CCR5) or CCR5 and CD4 (CHO.CCR5.CD4) have been described previously [22,23]. HEK.CCR5 cells were a generous gift from British Biotech (Oxford, UK) and were grown in DMEM with 10% foetal calf serum (FCS) and 2 mM glutamine in the presence of hygromycin. THP-1 cells were from the MRC Aids Repository Programme (Potters Bar, UK). The CCL3 isoform used is the CCL3 D26A form and has been described in [24] and was a generous gift from British Biotech (Oxford, UK). Anti CCR5 antibodies HEK/1/85a/7a and anti CD4 antibodies have been described previously [22,23]. The following anti-G protein antibodies were used: antibody C-10 (Santa Cruz) (recognises G_{i1}, G_{i2}, G_{i3}), anti G_q (E-17) (Santa Cruz), anti G_o (MAB 3073), anti-G_{i2} (MAB 3077) (Chemicon), and anti G_{i3} (SA-129) (Biomol). Anti-phosphotyrosine antibody (PY99) and anti-JAK2 antibodies were obtained from Santa Cruz. Anti-phospho-STAT1 and anti-STAT1 antibodies were purchased from New England Biolabs. The JAK2 inhibitor AG490 and the phospholipase C inhibitor U73122 (Calbiochem) were used at 100 and 5 μM, respectively, for 1 h before an experiment. [^{35}S]GTPγS (37–55 TBq mmol^{-1}) was from Amersham Pharmacia. All other chemicals were from Sigma.

Immunoprecipitation, SDS–PAGE and Western blot. Cells were harvested and then resuspended in lysis buffer (1% Brij97, 5 mM iodoacetamide, added immediately before use, 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, phosphatase inhibitor cocktail (Sigma) and protease inhibitors cocktail (Roche)) at 4 °C for 40 min with gentle mixing. The immunoprecipitation and analysis of the proteins on SDS–PAGE

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Abbreviations: CHO cells, Chinese hamster ovary cells; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GPCR, G protein coupled receptor; HEK cells, human embryonic kidney cells; HIV, human immunodeficiency virus; IP, immunoprecipitation; JAK, Janus kinase; PBS, phosphate-buffered saline; PTX, pertussis toxin; STAT, signal transducer and activator of transcription

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was done as described [22]. Antibodies were removed from the membrane before a second stain by incubation with 100 mM mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.8, at 50 °C for 30 min.

Pertussis toxin treatment. Pertussis toxin (PTX) (Sigma) was used to uncouple the G proteins from the receptor at 100 ng/ml PTX for 2 h before immunoprecipitation and for 18 h before calcium assays. For [^{35}S]GTPγS binding assays, cells were treated with PTX overnight before the membranes were prepared.

Analysis of intracellular calcium ion concentration. Chemokine-induced intracellular calcium mobilisation was determined by spectrofluorometry as described previously [22].

Internalisation assay and flow cytometry analysis. Internalisation and recycling assays were performed as described [23]. Where an inhibitor such as AG490 was used in the experiments, it was present in the three sets of cells used, i.e., negative control, treated with medium without serum and chemokine-treated. G protein levels were quantified using a buffer containing 1% saponin to permeabilise cell membranes followed by flow cytometry using specific antibodies.

Membrane preparation. Membranes were prepared from confluent cells as described previously [22].

[^{35}S]GTPγS-binding assays. The [^{35}S]GTPγS-binding assays were carried out essentially as described in [25]. 30 μg cell membranes were incubated in [^{35}S]GTPγS-binding buffer containing 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$ and 0.1% BSA, pH 7.4, and 10 μM GDP in a final volume of 0.9 ml in the absence or presence of an agonist (50 nM) to measure the basal and stimulated nucleotide exchange, respectively. The reaction was initiated by the addition of cell membranes and the tubes were incubated at 30 °C for 30 min before addition of 100 μl of [^{35}S]GTPγS to give a final [^{35}S]GTPγS concentration of 100 pM. The assay was incubated for a further 30 min before termination by rapid filtration through GF/C filters and bound radioactivity was determined by liquid scintillation counting. Alternatively, the assay was terminated by collecting the membranes by centrifugation (10 000 × *g*) and cooling them to 4 °C. The membranes were then resuspended in ice cold lysis buffer and incubated with the corresponding antibodies for 1 h at 4 °C. The antibodies had been precoupled to Protein G–Sepharose (PGS) overnight. Samples were washed five times in ice-cold lysis buffer and bound radioactivity was determined by liquid scintillation counting.

Data analysis. Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t* test with a $P < 0.05$.

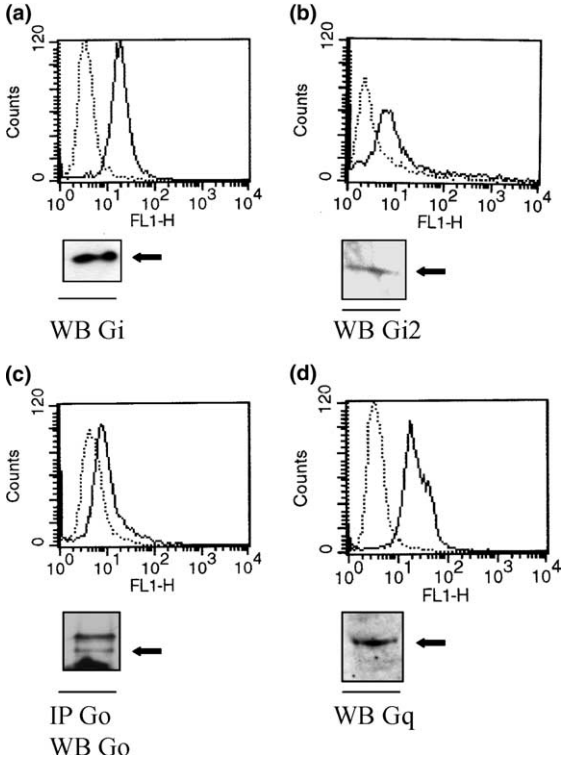

Fig. 1. Detection of G protein expression in CHO.CCR5 cells using flow cytometry analysis and Western blot. CHO.CCR5 cells were permeabilised and stained with unspecific antibody (thin line) or anti-G protein antibody (bold line) and analysed by flow cytometry. Inset: for Western blots 20 μg of membranes was separated on a SDS–PAGE. G_i and G_q were detected on a Western blot using specific antibodies. For detection of G_o, cells were subjected to an immunoprecipitation with an anti G_o antibody and G_o was detected on a Western blot using a specific antibody. Arrows indicate G protein. (a) Detection of $G_{\alpha i}$ with antibody selective for G_{i1}, G_{i2}, and G_{i3}, (b) $G_{\alpha i2}$, (c) $G_{\alpha o}$ and (d) $G_{\alpha q}$. In (c) the upper band is the heavy chain of IgG.

3. Results

3.1. CCR5 interaction with G proteins

Using flow cytometry it was possible to demonstrate the presence of α-subunits of G_o, G_q and G_{i2} proteins in CHO.CCR5 cells using specific antibodies (Fig. 1). Expression of the same G_q and G_{i2} G protein subunits could also be demonstrated using the antibodies in Western blots on cell membranes or using immunoprecipitation (Fig. 1). G_o expression was very low and could only be detected after

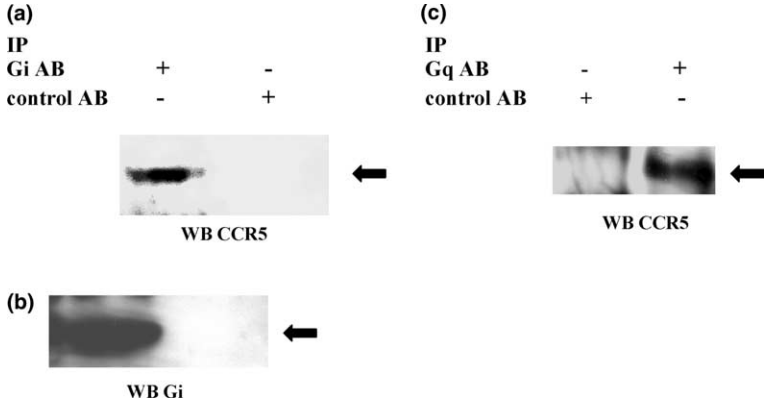

Fig. 2. CCR5 co-precipitates with G_i and G_q. CHO.CCR5 cells were lysed and an immunoprecipitation with G protein specific antibodies or control antibodies was performed. (a) Interaction with G_i: Lane 1 shows co-immunoprecipitation with antibody specific for G_{i1}, G_{i2}, and G_{i3}. Lane 2 shows control immunoprecipitation with antibody unrelated to G proteins and CCR5 Western blot was performed with anti CCR5 antibody. (b) Blot in a was stripped and re-probed with antibody specific for $G_{i1}/G_{i2}/G_{i3}$. (c) Interaction with G_q: Lane 1 shows control immunoprecipitation with antibody unrelated to G proteins and CCR5. Lane 2 shows immunoprecipitation with G_q specific antibody. Western blot was performed with anti CCR5 antibody. Blots shown are representatives of three independent experiments.

immunoprecipitation with G_o-specific antibodies followed by Western blotting. Immunoreactivity in flow cytometry and Western blotting was also seen with an antibody that cross reacts with G_{i1}, G_{i2} and G_{i3}. G_{i3} could be detected at low levels in Western blots (data not shown), but the antibodies used were unsuitable for immunoprecipitation. These data are in agreement with previous studies [26,27] where it was shown that CHO cells express G_{i2} and G_{i3} (in smaller amounts) but only low levels of G_{i1} and G_o. In several experiments described below, the antibody that cross-reacts with G_{i1}, G_{i2} and G_{i3} has been used. Immunoreactivity detected with this antibody will be referred to as G_i, but we may assume that it is largely G_{i2} that is being assessed.

We then investigated the association of CCR5 with different G proteins. Co-immunoprecipitation studies revealed that CCR5 associates with G_i, G_o and also G_q (Fig. 2). Owing to the low amount of G_o in CHO cells, only a very faint CCR5-specific band was observed in the co-precipitation experiments (data not shown).

CCR5, therefore, interacts with several members of the G protein family in CHO cells. In order to determine if these interactions are functional, we performed a [^{35}S]GTPγS binding assay followed by immunoprecipitation with specific anti G protein antibodies. This approach has been used to demonstrate functional interaction between muscarinic acetylcholine receptors and $G_i/G_o/G_q$ proteins and between Edg receptors and G_i/G_o proteins [28,29]. Application of this technique showed that CCR5 is able to activate G_i proteins as bound [^{35}S]GTPγS was detected following immunoprecipitation with antibodies specific for G_{i1-3} and G_{i2} species after stimulation with CCL3 (Fig. 3(a)). However, we did not observe a specific activation of G_q or G_o proteins in this assay. The stimulation of G_i proteins was prevented by PTX treatment (Fig. 3(b)). In complementary experiments, PTX treatment completely abolished the CCL3-induced increase in intracellular calcium ions showing that the interaction of CCR5 with G_i proteins is functionally relevant (Fig. 3(b)). Further evidence that the interaction of CCR5 with G_i proteins is functionally relevant comes from the observation that chemokine-induced internalisation of CCR5 is sensitive to PTX treatment implying a role for CCR5/G_i interaction in the internalisation process [23].

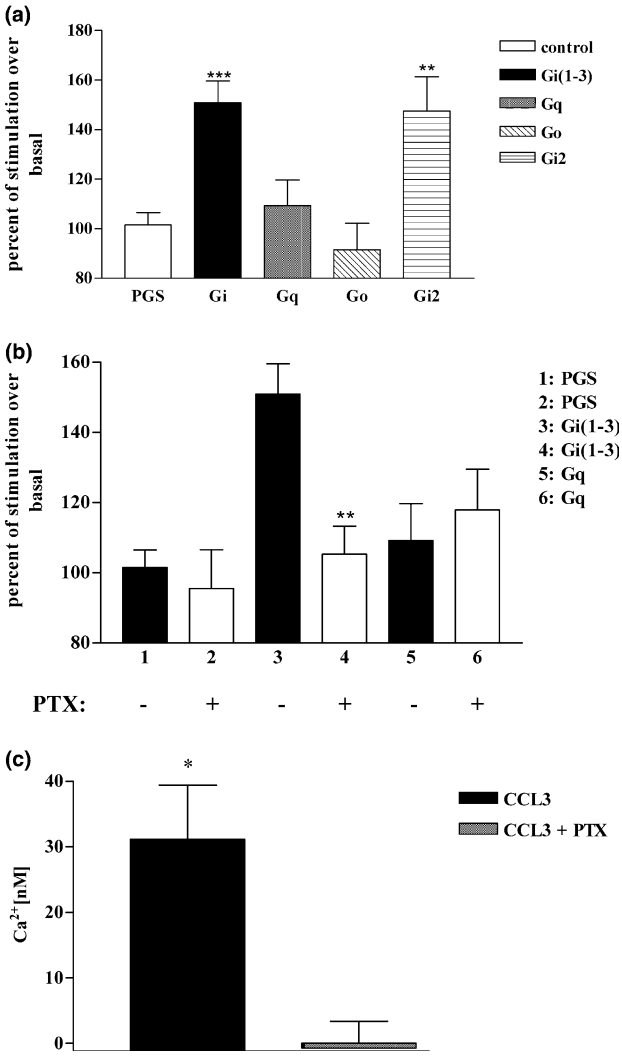

Fig. 3. Activation of G proteins by chemokines via CCR5. [^{35}S]GTPγS binding was determined after 30 min incubation in the presence or absence of 50 nM CCL3. G proteins were precipitated using specific antibodies and [^{35}S]GTPγ S binding determined as described in Section 2. Results are expressed as a percentage of the unstimulated basal level of [^{35}S]GTPγS binding. Data shown are the means and standard errors of at least three separate experiments. (a) shows immunoprecipitation with antibodies specific for different G proteins (***$P < 0.001$, **$P < 0.01$), control experiments were performed with PGS alone. (b) shows immunoprecipitation without antibody or with G_i and G_q specific antibodies for membranes with or without PTX treatment. As a negative control immunoprecipitation with PGS was performed. (c) Intracellular calcium ion concentration was determined as described with CCL3 (50 nM). Data are shown with control cells and cells treated with PTX (100 ng/ml, 18 h).

3.2. CCR5 interaction with JAK2

CHO.CCR5 cells were treated with CCL3 and proteins exhibiting enhanced tyrosine phosphorylation were detected using immunoprecipitation with an antibody directed against phosphotyrosine residues. The phosphorylation of one of the bands was prevented by AG490, a JAK1/JAK2 inhibitor [30] (Fig. 4(a)), whereas a phospholipase C inhibitor U73122 had no effect on the amount of phosphorylation after CCR5 activation. We suspected that this band might be JAK2 as other GPCRs have been reported to alter JAK2 phosphorylation [16,21,32]. We were unable to prove this as the available JAK2 antibody was specific for the human protein and did not react with CHO cell JAK2 (data not shown).

The experiments were, therefore, repeated using a HEK cell line expressing CCR5. Treatment of the HEK.CCR5 cells with CCL3 leads to enhanced tyrosine phosphorylation of proteins in these cells and this was prevented by AG490 (Fig. 4(b)) in agreement with the data obtained in CHO.CCR5 cells. In order to examine the role of JAK2 in these observations, we performed an immunoprecipitation with an anti-JAK2 antibody followed by a Western blot probed with an anti-phosphotyrosine antibody. These experiments showed that JAK2 phosphorylation was enhanced by CCL3 activation of CCR5 and this occurred in a transient manner, reaching a peak after 1 min and returning to baseline by 15 min (Fig. 5(a)). Treatment of cells overnight with PTX or for 1 h with U73122 did not affect the amount of phosphotyrosine phosphorylation that was observed, indicating that JAK2 activation is independent of $G_{i/o}$ protein activation and also independent of phospholipase C activation (Fig. 5(b) and (c)).

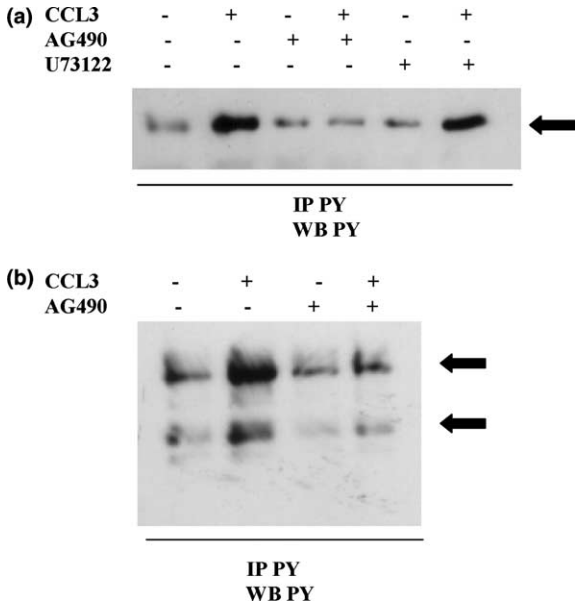

Fig. 4. Ligand-induced CCR5 activation leads to a JAK-inhibitor dependent increase in tyrosine-phosphorylation in CHO and HEK cells. (a) CHO.CCR5.CD4 cells were treated with AG490 (100 M) and U73122 (5 μM) when indicated for 1 h before addition of the agonist. Immunoprecipitation and Western blot analysis were performed with an anti-phosphotyrosine antibody. (b) HEK.CCR5 cells were treated with AG490 (100 μM) for 1 h before addition of CCL3. Immunoprecipitation and Western blot analysis were performed with an anti-phosphotyrosine antibody. Arrows indicate the staining pattern after CCR5 activation, upper arrow corresponds to JAK2.

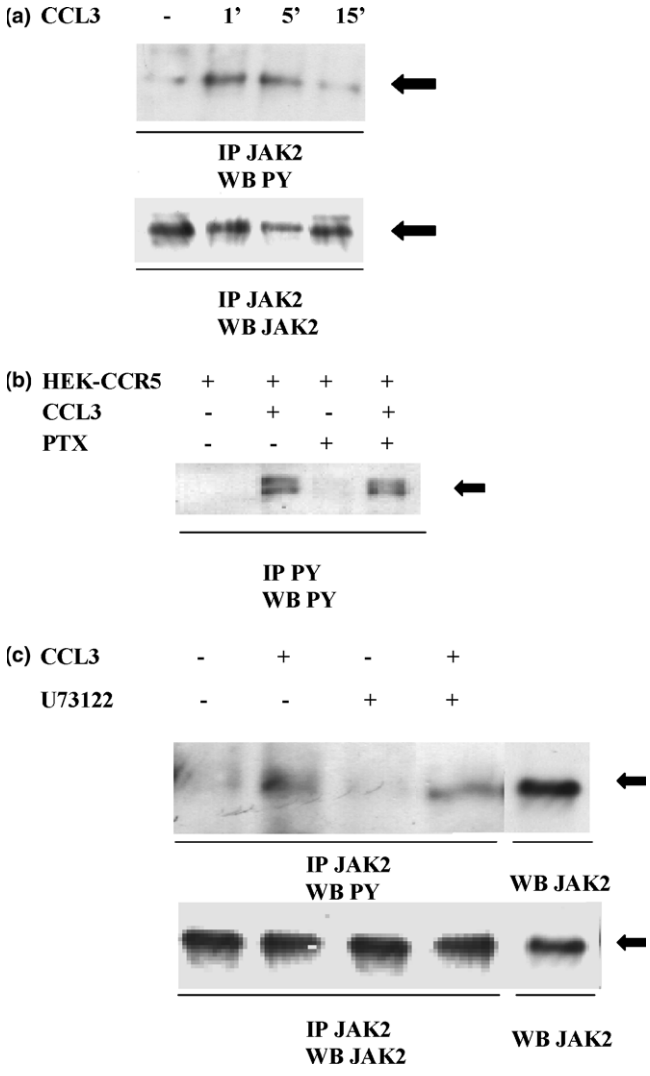

Fig. 5. Transient activation of JAK2 is independent of heterotrimeric G protein activation. (a) HEK.CCR5 cells were treated with 50 nM CCL3 for the times indicated and then subjected to immunoprecipitation with JAK2 antibody. Upper panel: Western blot analysis was performed with an anti-phosphotyrosine antibody. Arrow indicates JAK2, as shown in experiments with the JAK2 antibody. Lower panel: membranes were reprobed with an anti-JAK2 antibody. Arrow indicates JAK2. (b) HEK.CCR5 cells were treated with PTX (100 ng/ml) for 18 h and treated with CCL3 (50 nM, 10 min) when indicated. Immunoprecipitation and Western blot analysis were performed with anti-phosphotyrosine antibody. Arrow indicates position of JAK2. (c) HEK.CCR5 cells were treated with U73122 (5 μM) for 1 h and then treated with CCL3 (50 nM, 10 min) when indicated and then subjected to immunoprecipitation with an JAK2 antibody. Upper panel: Western blot analysis was performed with an anti-phosphotyrosine antibody. Arrow indicates JAK2. Lower panel: membranes were reprobed with an anti-JAK2 antibody. Arrow indicates position of JAK2. Control Western blots are also shown identifying the position of JAK2.

In order to investigate whether JAK2 activation is necessary for G protein activation, we examined the stimulation of [^{35}S]GTPγS binding induced by CCL3 in membranes of CHO.CCR5 cells (Fig. 6(a)). This has been shown to be a reflection of the activation of $G_{i/o}$ proteins by CCR5 [22]. Stimulation of [^{35}S]GTPγS binding by CCL3 was unaffected by the presence of AG490 (100 μM) the JAK1/JAK2 inhibitor [30]. This concentration of AG490 was sufficient to prevent JAK2 phosphorylation stimulated by CCL3 (see above). In order to investigate the effects of JAK2 on G protein activation in whole cells, we examined CCL3 stimulation of intracellular calcium ion release. This has been shown to be a reflection of activation of $G_{i/o}$ proteins (Fig. 3(b)). AG490 did not affect the CCL3-induced increase in intracellular calcium ion concentration (Fig. 6(b)). These data suggest that activation of JAK1 or JAK2 is not a prerequisite for the stimulation of G proteins by CCR5 in these cells.

Since activation of CCR5 leads to activation of JAK2, we examined whether the two proteins interacted. Immunoprecipitation studies clearly showed that CCR5 interacts directly with JAK2 (Fig. 7). CCL3-induced receptor activation did not affect the interaction of JAK2 and CCR5. Similar interaction of CCR5 and JAK2 was seen, using co-precipitation, in the monocytic THP-1 cell line (Fig. 7), suggesting that this is not a phenomenon restricted to recombinant systems.

In order to examine the importance of the interaction of CCR5 and JAK2 further, we examined the effect of JAK2 inhibition on CCL3-induced internalisation and recycling of CCR5. Inhibition of JAK1/JAK2 with AG490 did not affect CCR5 internalisation, but in the two cell lines used (CHO.CCR5, CHO.CCR5.CD4), there was some inhibition of receptor recycling to the cell surface (Fig. 8) compared to untreated cells. This most likely reflects the effects on JAK2, but owing to the specificity of AG 490 we cannot rule out the effects via JAK1.

It has been shown recently that CCR5 is shed from CHO cells into the supernatant in microvesicles [31]. In the present

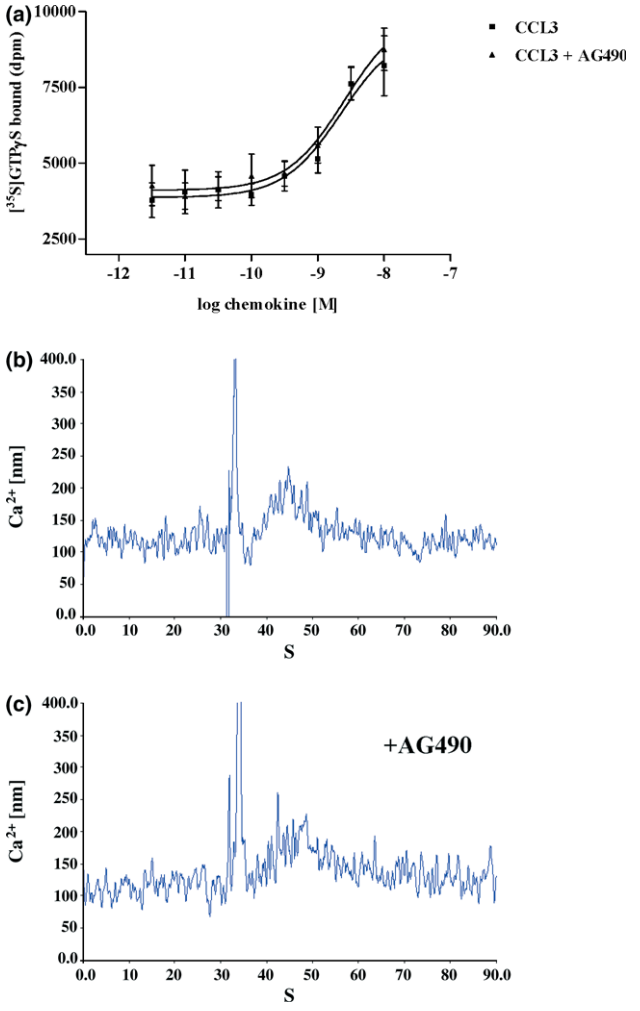

Fig. 6. Effects of JAK inhibitor AG490 on heterotrimeric G protein activation. (a) Stimulation of [^{35}S]GTPγS binding in CHO.CCR5.CD4 membranes by CCL3: effect of JAK inhibitor AG490. [^{35}S]GTPγS binding was determined after 30 min incubation in the presence of increasing concentrations of CCL3 as described in Section 2. Where indicated, membranes were pre-treated with 100 μM AG490 for 1 h before determination of [^{35}S]GTPγS binding. Data shown are the means of triplicate determinations from a representative of at least three separate experiments. (b) CHO.CCR5.CD4 cells were prepared for calcium flux as described and then stimulated with 100 nM CCL3. (c) CHO.CCR5.CD4 cells were incubated with AG490 (100 μM) for 1 h and then prepared for calcium flux. Cells were stimulated with 100 nM CCL3.

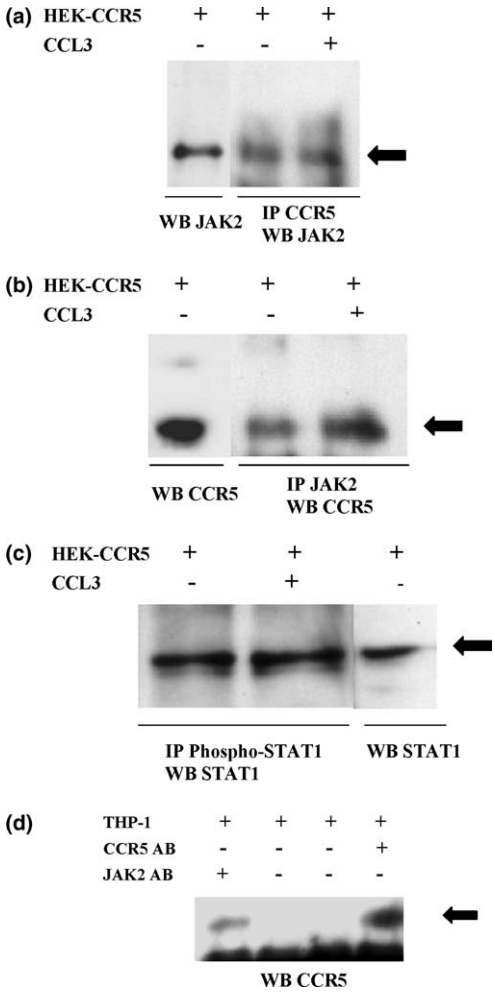

Fig. 7. Co-immunoprecipitation of JAK2 with CCR5 is independent of ligand-induced receptor activation. HEK.CCR5 cells were treated with 50 nM CCL3 for 10 min when indicated. (a) Cells were then subjected to immunoprecipitation with an anti-CCR5 antibody, Western blot analysis was performed with an anti-JAK2 antibody. Arrow indicates position of JAK2 verified by control Western blot. (b) Cells were subjected to immunoprecipitation with an anti-JAK2 antibody, Western blot analysis was performed with an anti-CCR5 antibody. Arrow indicates position of CCR5 verified by control Western blot. (c) Activation of CCR5 does not lead to activation of STAT1 in HEK.CCR5 cells. HEK.CCR5 cells were activated with CCL3 and then subjected to immunoprecipitation with an anti-phospho-STAT1 antibody, Western blot analysis was performed with an anti-STAT1 antibody. Arrow indicates position of STAT1 verified by control Western blot. (d) CCR5 and JAK2 interact in THP-1 cells. Immunoprecipitation was performed with anti-JAK2 or anti-CCR5 antibody where indicated and Western blot was performed with anti-CCR5 antibody. The non-specific bands are immunoglobulins.

study, shedding of CCR5 was detected from CHO.CCR5.CD4 cells using specific anti-CCR5 antibodies. We also detected G_i proteins in the same supernatant (Fig. 9). It, therefore, seems that CCR5 is coupled very tightly to G_i proteins even in the inactive state of the receptor and the two proteins are shed together from the cells. In the same experiments, CD4 was found only in the fraction containing whole cells, not in the fraction containing shed vesicular material. This shows that the shedding of CCR5 is a specific process and is not due to cell lysis during the experiment. It is possible that CCR5 and G_i are being shed as part of a complex of signalling proteins. We, therefore, repeated the experiments in HEK.CCR5 and untransfected HEK cells. In the supernatant of cells that were stably transfected with CCR5 we detected CCR5 in addition to JAK2. The supernatant of the cells was passed through a 0.45 μm filter to eliminate any cells in the supernatant. Even after filtration, we were still able to detect CCR5 and JAK2 in the supernatant. We then used parental HEK cells to investigate whether JAK2 is leaking non-specifically from cells, or whether the shedding of the protein is dependent on the presence of CCR5. In parental HEK cells we were unable to detect any JAK2 in the supernatant of the cells confirming the tight association between CCR5 and signalling proteins such as JAK2 and G_i (data not shown).

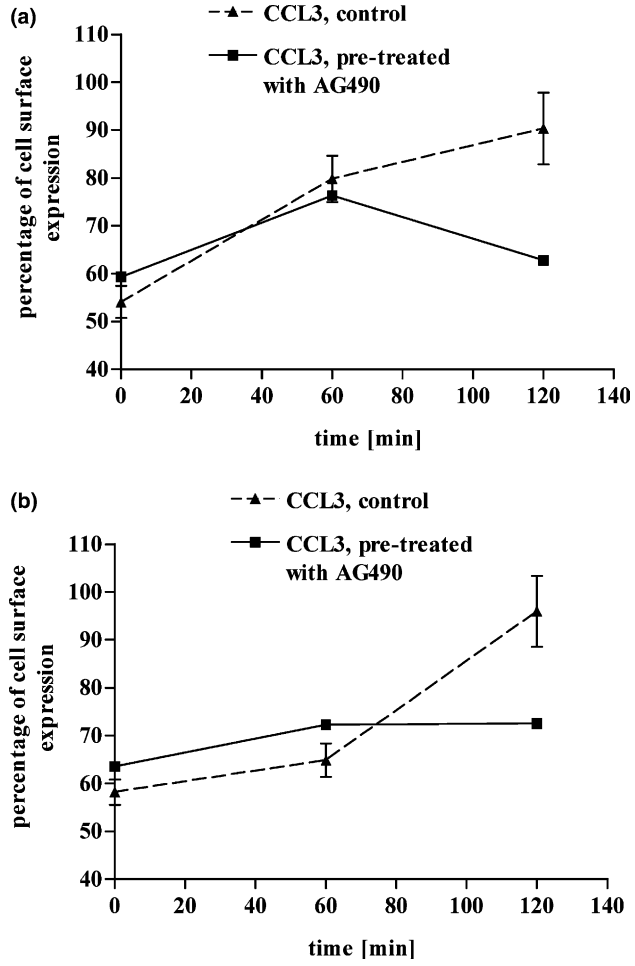

Fig. 8. Internalisation and recycling of CCR5. (a) CHO.CCR5.CD4 cells were treated with CCL3 as in [23] and cell surface expression of CCR5 was determined by flow cytometry. The zero time point in the graph represents the CCR5 level after internalisation. Cells were then washed to remove CCL3 and recycling of CCR5 determined after different times as in [23]. Cells were pre-treated with AG490 where indicated (100 μM) for 1 h. (b) CHO.CCR5 cells were treated as in (a). Data represent means ± S.E.M. of at least three independent experiments.

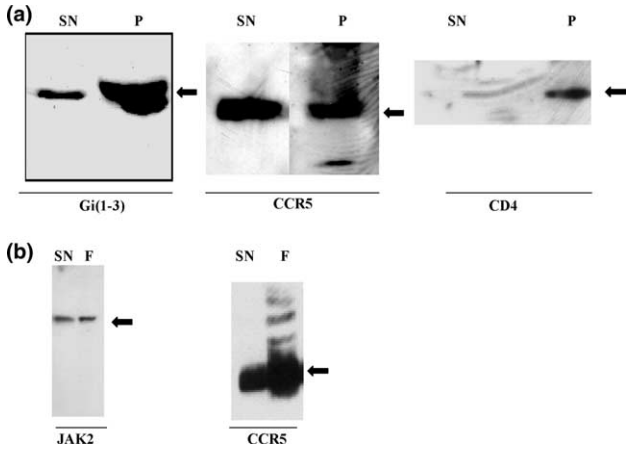

Fig. 9. Detection of CCR5 and G protein in supernatant of cells expressing CCR5. (a) CHO.CCR5.CD4 cells were incubated in PBS for 15 min and then centrifuged for 10 min at 1000 × *g*. Supernatant was collected, diluted in 2× sample buffer and boiled for 5 min. Pellets were lysed using an ultra-turrax, then 2× sample buffer was added and the samples were boiled for 5 min. Both sets of samples were then separated on SDS–PAGE. SN is supernatant, *P* is pellet fraction. (b) HEK.CCR5 cells were treated as described in (a). SN is supernatant, *F* is supernatant fraction after filtration through 0.45 μm filter. Western blot analyses were performed with antibodies specific for CCR5, CD4, G proteins or JAK2 as indicated.

4. Discussion

In this study, we examined the effects of stimulation of the chemokine receptor, CCR5, on two signalling systems, heterotrimeric G proteins and JAK/STAT proteins. We provide the first evidence that CCR5 can couple independently to G protein-coupled and non-G protein-coupled pathways.

Recently, evidence has emerged that the JAK/STAT pathways may be activated by GPCRs, e.g., angiotensin AT1 receptor [16], α_1-adrenergic receptors [32], and CCR5 [21,33,34]. Interaction of JAK2 with CCR5 after activation with CCL5 has also been described [21]. These effects of GPCRs on JAK/STAT pathways raise questions about how one receptor can signal to multiple signalling pathways. In the case of CCR5, it has even been suggested that activation of heterotrimeric G proteins is dependent on JAK activation [19].

In this study, therefore, we have investigated the activation of different signalling pathways linked to CCR5 using recombinant cells expressing CCR5. Activation of CCR5 by CCL3 leads to activation of the G protein G_{i2} as shown by increased [^{35}S]GTPγS binding. CCL3 activation of CCR5 also leads to activation of JAK2 as shown by increased tyrosine phosphorylation. The two signalling events are independent, as inhibition of one event (PTX to inhibit G_i proteins AG490 to inhibit JAK2) has no effect on the other event. Co-immunoprecipitation studies showed that CCR5 interacted with the two signalling proteins (G_i and JAK2) in support of these observations. Additionally, use of the phospholipase C inhibitor, U73122, showed that JAK2 activation by CCR5 was not dependent on activation of phospholipase C. This also eliminates the effects of CCR5 via G_q proteins. Although we did observe activation of JAK2 in our system, we were not able to detect any downstream activation of STAT 1 following activation of CCR5 (Fig. 7). Other downstream effectors of JAK2 (see [35] for review) must, therefore, be involved in these responses.

Recently, Mack et al. [31] reported that CCR5 is shed from cells into the supernatant without lysis of the cells. This process may be involved in movement of CCR5 from one cell to another. CCR5 has also been shown to be located on microvilli, an observation that strengthens the possibility of shedding the receptor in microvesicles from cells [36]. We used these observations to examine the interaction of CCR5 with signalling proteins. Both CCR5 and G_i proteins could be detected in the supernatant of CHO.CCR5.CD4 cells, whereas CD4 was not found in the supernatant. In HEK.CCR5 cells, we could detect JAK2 together with CCR5 in the supernatant, whereas in parental HEK cells, no JAK2 could be detected in the supernatant. This suggests that G proteins and JAK2 are either tightly associated with the receptor even in an inactive state, so that they are shed from the cells together with CCR5 or that these proteins are in close proximity to CCR5, when the receptor is shed in microvesicles. Whether this phenomenon is of physiological importance remains to be clarified. It does,

however, strengthen the theory that both G proteins and JAK2 are directly associated with CCR5.

This study therefore shows that CCR5 is able to activate G_i proteins as well as JAK2 proteins in an independent manner, providing new insights into the complex signalling pathways that are activated by CCR5. Thus, as well as having short term effects on cell activity via changes in G protein activation, CCR5 may lead to changes in cell growth via alterations in JAK activity [35].

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