

A single nucleotide polymorphism in the CCR3 gene ablates receptor export to the plasma membrane

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Background: The chemokine receptor CCR3 orchestrates the migration of eosinophils, basophils, T_H2 lymphocytes, and mast cells during the allergic response, with CCR3 blockade a potential means of therapeutic intervention. Non-synonymous single nucleotide polymorphisms (SNPs) within the *ccr3* gene have previously been described, with little information regarding their effects on CCR3 function.

Objective: To characterize the effects of nonsynonymous SNPs within the *ccr3* gene.

Methods: Site-directed mutagenesis was used to generate N-terminally tagged mutant CCR3 constructs corresponding to reported SNPs. Cell transfectants expressing either wild-type or mutant CCR3 were studied by flow cytometry, Western blotting, and confocal microscopy and examined for their ability to migrate to the CC chemokine ligand CCL11/eotaxin.

Results: An L324P mutant CCR3 protein corresponding to the previously identified T971C SNP was not expressed at the cell surface, and cells remained unresponsive to CCL11 in chemotaxis assays. Confocal microscopy confirmed that L324P-CCR3 had a predominantly intracellular distribution compared with wild-type CCR3. A L324A variant of CCR3 had an identical phenotype to the L324P mutant, suggesting that L324 *per se* is critical for successful trafficking of nascent CCR3 to the cell membrane. The processes involved appear to be specific for CCR3, because an identical mutation in the homologous receptor CCR1 had minor effects.

Conclusion: Trafficking to the cell surface of nascent CCR3 is critically dependent on a C-terminal leucine residue, suggestive of specific mechanisms for CCR3 export. Manipulation of these mechanisms may suggest novel means of antagonizing CCR3 function in the treatment of allergy. (*J Allergy Clin Immunol* 2010;■■■:■■■-■■■.)

Key words: Chemokine receptor, chemokine, CCR3, single nucleotide polymorphism, protein trafficking

The accumulation of eosinophils within the bronchial wall is a characteristic feature of asthma, but the precise role of the

Abbreviations used

ER: Endoplasmic reticulum
GM130: Golgi matrix protein 130
GPCR: G protein-coupled receptor
HA: Hemagglutinin
LAMP-1: Lysosome-associated membrane protein 1
SNP: Single nucleotide polymorphism
WT: Wild-type

eosinophil in disease symptoms is complex and controversial. *In vivo* studies using either IL-5 neutralization via antibodies or gene deletion suggested a strong link between lung eosinophil activation and airway hyperresponsiveness.^{1,2} However, early studies of IL-5 neutralization in patients with asthma did not show an effect on airway function,³ casting doubt on the role of the eosinophil in asthma. Interest in the therapeutic targeting of the eosinophil has undergone a recent renaissance. The generation of mice deficient in eosinophils allowed the role of the cell to be probed further, with data from allergen challenge models implicating the eosinophil in both airway remodelling⁴ and airway hyperresponsiveness.⁵ These studies support work in man suggesting that eosinophils contribute to airway remodeling in asthma via TGF- β production.⁶ Recent clinical studies have also reported the efficacy of an anti-IL-5 mAb in the treatment of eosinophilic asthma, reducing the levels of circulating and sputum eosinophils and the frequency of asthma exacerbations.^{7,8}

Chemokines direct the spatial organization of immune cells under basal and inflammatory conditions by binding specific cell surface receptors on leukocytes.⁹ The first potent, selective eosinophil chemoattractant to be described was eotaxin-1/CCL11,¹⁰ which was subsequently followed by the discovery of eotaxin-2/CCL24 and eotaxin-3/CCL26.¹¹⁻¹⁴ These recruit eosinophils by signaling through the G protein-coupled receptor (GPCR) CCR3, which is highly expressed on human eosinophils,¹⁵ basophils,¹⁶ mast cells,¹⁷ and T_H2 lymphocytes.¹⁸ An extensive body of literature implicates the CCR3:eotaxin axis in asthma.¹⁹ Increased expression of CCL11 and CCL24 is observed in patients with asthma at sites of allergic inflammation²⁰ and in their sputum.²¹ Mice deficient in either CCR3 or both CCL11 and CCL26 exhibit reduced eosinophil recruitment to the lungs and skin after allergen challenge.²²⁻²⁵

Consequently, there is a compelling hypothesis that targeting CCR3 in allergic disease would be beneficial, especially in the Western world where allergic disease is endemic but parasitic infections relatively rare. Consistent with this, a recent clinical trial involving a combined antisense therapy against CCR3 and the common β -chain of the IL-3, IL-5, and GM-CSF receptors was found to attenuate the allergen-induced increase in target gene mRNA and airway responses in patients with mild asthma.²⁶

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Early *in vitro* studies of CCR3 were dogged by difficulties in expressing the CCR3 cDNA in mammalian cell lines. In our experience, functional expression of CCR3 is achieved only in cells of lymphoid origin such as the pre-B cells 4DE4 and L1.2,^{27,28} and truncation of the C-terminal region of CCR3 severely affected cell-surface trafficking.²⁹ Collectively, this suggests that nascent CCR3 is assembled and traffics to the membrane via a mechanism dependent on the CCR3 C-terminus interacting with additional molecules specifically expressed by leukocytes. Here we describe the functional consequences of previously described nonsynonymous single nucleotide polymorphisms (SNPs) within the *ccr3* gene, which give rise to a change in the CCR3 protein sequence. In particular, we show that the T971C-SNP, which corresponds to a L324P substitution in the CCR3 C-terminal region, gives rise to a protein that, although expressed, cannot traffic to the cell membrane and is efficiently degraded. This leads to a complete loss of chemotactic function and points the way to alternative ways of antagonizing CCR3 function.

METHODS

Generation of mutant CCR3 constructs

Mutant constructs were generated by PCR according to the manufacturer's instructions by using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) with pcDNA3 plasmid encoding wild-type (WT) WT-CCR3 tagged with influenza hemagglutinin epitope (HA) serving as a template.³⁰ The N-terminal HA tag enables cell surface expression to be readily monitored, independently of the CCR3 conformation recognized by mAbs. The sequence of constructs was verified by DNA sequencing before experimentation (Eurofins, Ebersberg, Germany).

Cell culture and transfection

The murine pre-B lymphoma cell line L1.2 was maintained in suspension at a density of no more than 1×10^6 cells/mL as described previously.³⁰ L1.2 cells were transiently transfected by electroporation with 1 μ g DNA/ 1×10^6 cells as described previously²⁸ and were incubated overnight in the presence of 10 mmol/L sodium butyrate (Sigma-Aldrich, Dorset, UK) to increase responsiveness.

Flow cytometry

L1.2 cell transfectants were stained with either the anti-HA antibody (Covance Research Products, Berkeley, Calif) or the corresponding mouse IgG₁ isotype control (Sigma-Aldrich) followed by the secondary fluorescein isothiocyanate-conjugated goat antimouse IgG antibody (Alere Ltd, Stockport, UK) as per previously published methodologies.³⁰ For intracellular staining, cells were fixed in 4% paraformaldehyde, and antibodies were diluted in fluorescence-activated cell sorting buffer containing 0.5% saponin before they were added to cells. The mean fluorescence of the isotype control was subtracted from that of the antibody-stained control, and data were expressed as a percentage of wild-type antibody staining. Data were analyzed by using CellQuestPro software on a FACScalibur flow cytometer (Becton Dickinson UK, Oxford, UK).

Cell migration assay

The chemotactic responsiveness of cells to concentrations of CCL11 (PeproTech EC Ltd, London, United Kingdom) was ascertained by using ChemoTx plates with a 5- μ m pore filter (NeuroProbe, Gaithersburg, Md), as described previously.³¹ After a 5-hour incubation, migrating cells were enumerated by using a hemocytometer, cell migration to buffer alone was subtracted from the data, and individual results were expressed as the percentage of total cells applied to the filter.

SDS-PAGE and Western blotting

CCR3 transfectants or naive L1.2 cells were resuspended at a concentration of 5×10^6 cells/mL. Cells were washed twice in ice-cold PBS and were resuspended in ice-cold lysis buffer containing 1% Igepal (Sigma-Aldrich, Dorset, UK), 1 μ g/mL aprotinin, 100 μ g/mL phenylmethylsulfonyl fluoride, 0.001 mol/L EDTA (pH 8), 20 mmol/L iodoacetamide. Equal quantities of lysates were separated on 4–12% SDS-PAGE gels and were electrophoretically transferred onto a nitrocellulose membrane which was subsequently blocked in 5% wt/vol nonfat milk in 0.01 mol/L PBS with 0.05% Tween. Blots were probed independently with either anti-HA or mouse anti- α -tubulin as a loading control (Abcam, Cambridge, UK) at a 1/1000 or 1/5000 dilution, respectively. After washing and probing with a secondary polyclonal horseradish peroxidase-conjugated goat antimouse IgG antibody blots were developed by ECL (GE Healthcare, Amersham, UK). To study the degradation of CCR3, transfectants were washed and resuspended in serum-free RPMI containing 10 μ g/mL cycloheximide, as previously described.³² Cells were incubated at 37°C for the indicated time before an aliquot was removed and cells analyzed by Western blotting as described. The relative OD of the major 43-kd HA-specific band to that of α -tubulin was determined by using ImageJ software (National Institutes of Health, Bethesda, Md, <http://rsb.info.nih.gov/ij/>). These values were then normalized to either WT-CCR3 as in Fig 1 or to WT-CCR3 and L324P-CCR3 as in Fig 4, B.

Subcellular fractionation

Cytosolic, cytoplasmic, and nuclear fractions were isolated from 5×10^6 CCR3 transfectants by using the Qiagen Q Proteome Cell Compartment kits (Qiagen, Crawley, UK) as per the manufacturer's instructions. Lysates were analyzed by Western blotting with anti-HA (as described) and antibodies to Golgi matrix protein 130 (GM130; Abcam, Cambridge, UK), calnexin (Stressgen, Enzo Life Sciences [UK] Ltd, Exeter, UK), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK), and lamin B (Santa Cruz, Heidelberg, Germany).

Confocal analysis

L1.2 transient transfectants (1×10^6) were taken for either antibody staining or control (isotype) tubes. Cells were fixed, stained, and mounted as described previously.³² Staining was carried out with the primary antibodies anti-HA, anticalnexin, anti-GM130, and anti-lysosome-associated membrane protein 1 (LAMP-1) by using equal concentrations of the antimouse IgG₁ or antirabbit IgG isotype control antibodies. Goat antimouse IgG Alexa Fluor 568 and goat antirabbit IgG Alexa Fluor 488 (1/100 dilution in 0.5% saponin buffer; Invitrogen Inc, Paisley, UK) were used as secondary antibodies for HA, calnexin, GM130, and LAMP-1 staining, as appropriate. Slides were analyzed by using a Leica TCS NT confocal microscope (Leica Microsystems [UK] Ltd, Milton Keynes, UK) with a 40x oil objective or a Leica SP2 upright confocal microscope with a 63x oil objective. Image analysis was conducted by using Leica LCS lite v2.61 software.

Data and statistical analysis

Data are expressed as the means $\pm$ SEMs of at least 3 experiments and were analyzed with 2-tailed paired *t* tests by using PRISM v4.03 software (GraphPad, San Diego, Calif). Where shown, *represents *P* < .05, **represents *P* < .01, and ***represents *P* < .001.

RESULTS

Expression and characterization of mutant CCR3 constructs

Six nonsynonymous SNPs were identified for study within the CCR3 coding region by using public access databases such as HapMap³³ and literature searches via PubMed (see this article's Fig E1, A, in the Online Repository at www.jacionline.org).^{34–36} The point mutations conferred on the primary sequence of

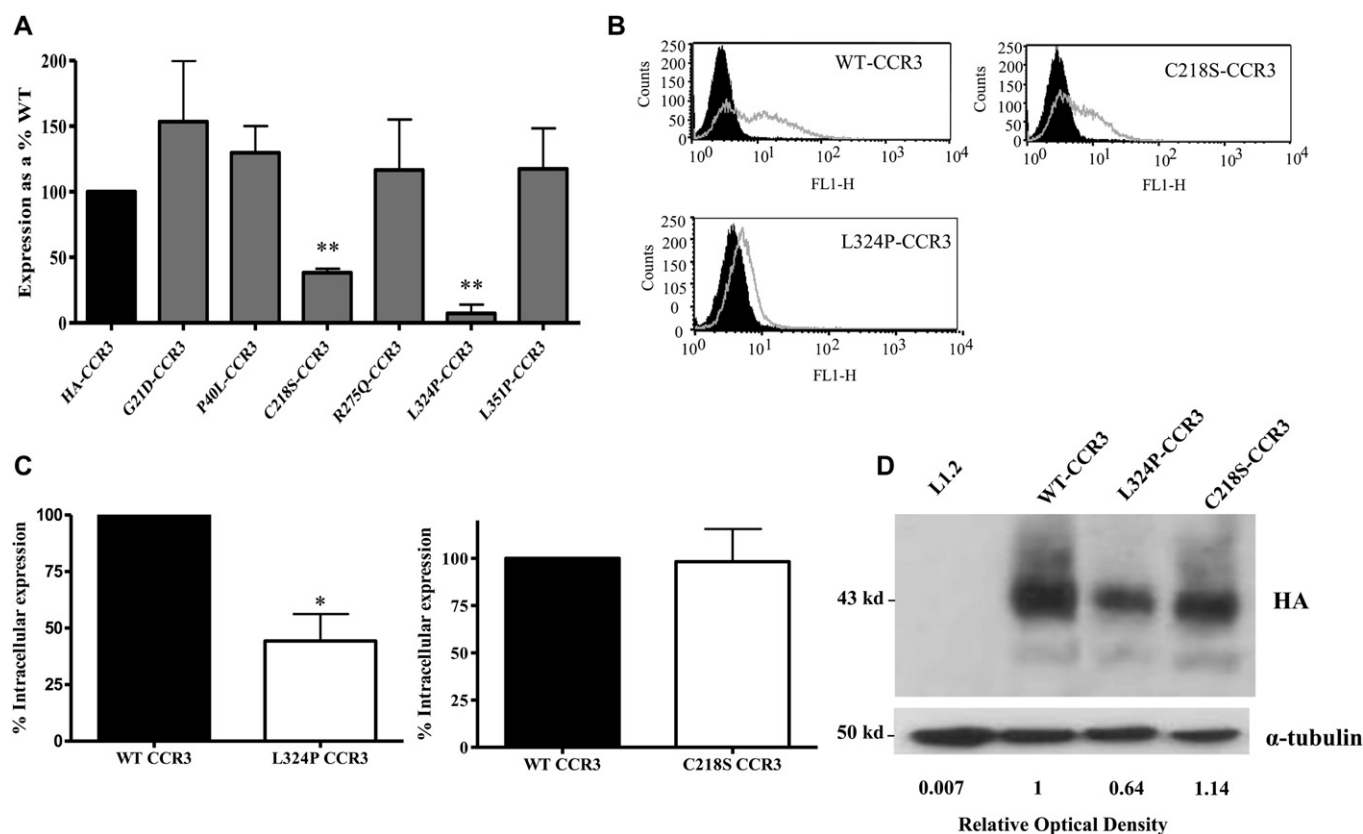


FIG 1. The L324P-CCR3 and C218S mutants exhibit reduced trafficking to the cell membrane but are detected intracellularly. **A and B**, Relative cell surface expression of CCR3 variants ($n = 3$) with representative examples of WT-CCR3, C218S-CCR3, and L324P-CCR3 staining. FL1-H, height of fluorescent intensity. **C**, Intracellular expression of L324P-CCR3 and C218S-CCR3 ($n = 3$). **D**, Western blot of WT-CCR3, L324P-CCR3, or C218S-CCR3 constructs with controls. Relative ODs are indicated. An α -tubulin loading control is indicated.

CCR3 were distributed throughout the protein (Fig E1, B). Plasmids encoding the CCR3 SNPs were generated and used to transfect L1.2 cells transiently to determine the functional consequences of mutation. The majority of constructs were expressed at levels comparable to WT-CCR3 with the exception of the C218S-CCR3 and L324P-CCR3 constructs, which were expressed at considerably lower levels (Fig 1, A and B).³⁶ A lack of extracellular expression may be envisaged to be a result of a defect in mRNA transcription or protein translation. To determine that the L324P-CCR3 and C218S-CCR3 protein products were expressed, we carried out intracellular staining for the HA tag in transfectants by using flow cytometry. Although L324P-CCR3 failed to traffic to the cell surface, L324P-CCR3 protein was detected intracellularly, albeit at approximately 50% of WT-CCR3 levels (Fig 1, C). In contrast, C218S-CCR3 was expressed at intracellular levels similar to WT-CCR3. The reduced levels of intracellular L324P-CCR3 were also confirmed by Western blotting (Fig 1, D). A band corresponding to approximately 40 kDa was apparent in lysates from WT-CCR3, L324P-CCR3, or C218S-CCR3 transfectants but not naive L1.2 cells. Densitometric analysis of the 40-kDa band found the L324P-CCR3 variant to be expressed at levels approaching 55% to 64% of the C218S-CCR3 and WT-CCR3 proteins, respectively. Collectively these data suggest that L324P-CCR3 and C218S-CCR3 are synthesized but do not traffic efficiently to the cell surface.

We subsequently assessed the responses of transfectants in cell migration assays to increasing concentrations of CCL11 (Fig 2, A–G). In agreement with cell surface expression, the majority of transfectants exhibited a similar bell-shaped chemotactic response to CCL11 as obtained with WT-CCR3 transfectants. However, CCL11 was much less efficacious in inducing the migration of transfectants expressing the C218S point mutation (Fig 2, D), in agreement with a previous report.³⁶ Maximal cell migration of the C218S-CCR3 transfectants (2.7% compared with 11.9% for WT-CCR3) occurred at a concentration of 1 nmol/L CCL11 compared with a concentration of 3 nmol/L CCL11 of WT-CCR3 transfectants, indicative of reduced receptor expression. In contrast, cells transfected with the L324P-CCR3 construct did not respond to CCL11 across the concentration range tested (Fig 2, F), which correlated with the severely reduced expression of this construct.

Specific requirement of L324 for CCR3 export

CCR3 shares a high degree of homology with the related CC chemokine receptor CCR1 (62% identity at the amino acid level) which is also expressed by eosinophils.³⁷ Alignment of the primary sequences of CCR1 and CCR3 revealed that CCR1 also contains a leucine residue at position 324 (see this article's Fig E2, A, in the Online Repository at www.jacionline.org). We therefore generated the analogous L324P mutation within

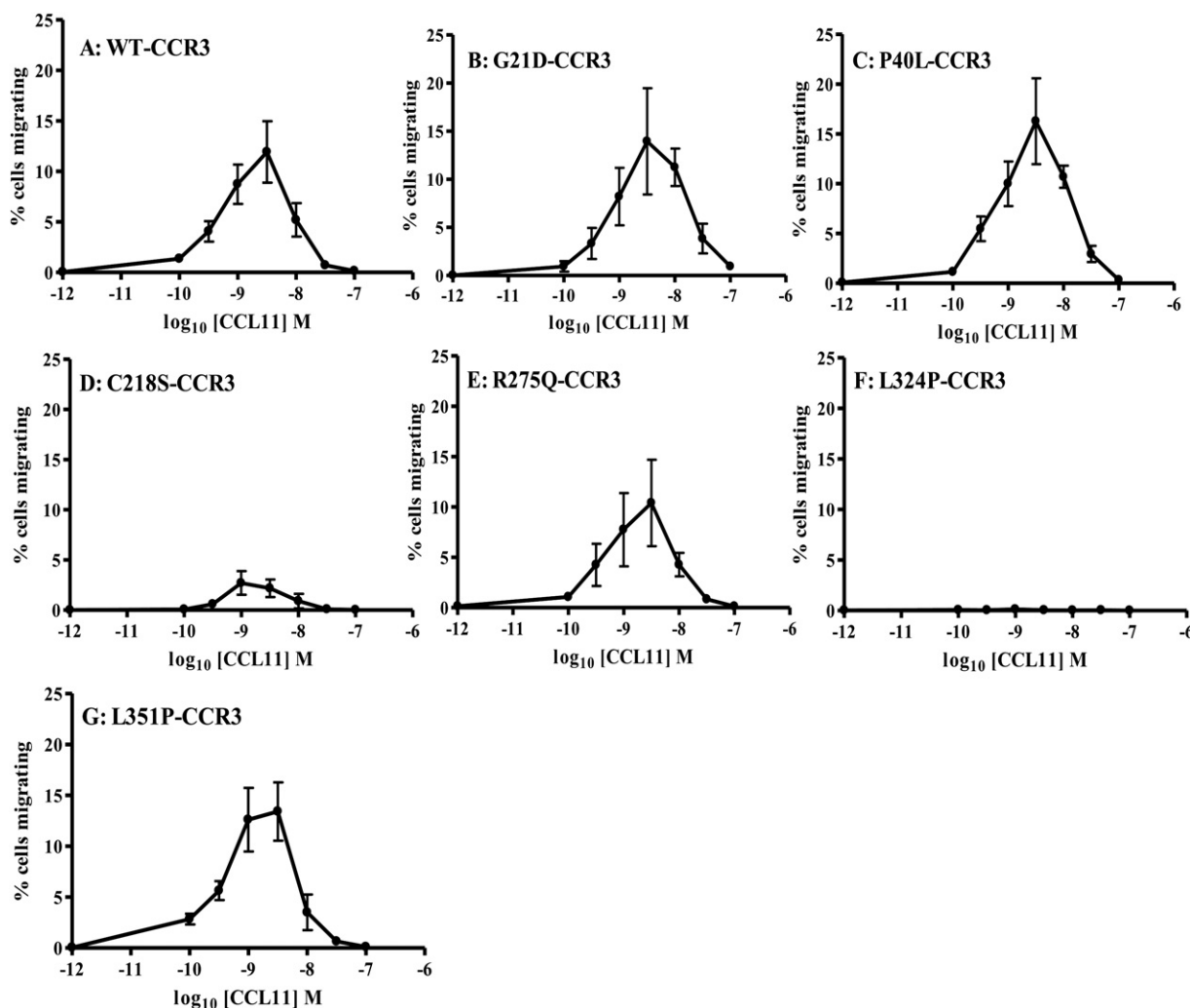


FIG 2. Migratory responses of L1.2 cells transiently transfected with either WT or CCR3 variant proteins as indicated in the panels A-G. Data are represented as means $\pm$ SEMs of 3 separate experiments. *M*, mol/L.

CCR1 to determine whether L324P-CCR1 transport to the cell surface was impaired. In contrast with the data obtained with CCR3 (Fig 1, *A and B*), the L324P-CCR1 construct was readily detected on the cell surface, although at reduced levels comparable to WT-CCR1 (Fig E2, *B*). In keeping with this finding, CCR1-L324P transfectants were functional in assays of chemotactic responsiveness, exhibiting similar dose response profiles to WT-CCR1 transfectants, with differences in efficacy observed at the optimal 3-nmol/L concentration of CCL1 (Fig E2, *C*). Therefore, although a minor effect was observed for L324P-CCR1, this was substantially lower than the loss-of-function phenotype conferred by the L324P mutation in CCR3.

L324 *per se* is critical for receptor trafficking

Studies of the crystal structure of bovine rhodopsin, one of the few GPCR structures solved to date, has shown that GPCRs possess an eighth α -helix within their C-terminus (Fig E2, *A*).³⁸ The introduction of proline in a helical region as afforded by the L324P mutation might be perceived to kink the helix with detrimental effects on receptor folding and expression. To test this hypothesis, we mutated L324 of CCR3 to alanine and examined

expression and function of the L324A-CCR3 construct. Transfectants expressing L324A-CCR3 exhibited the same phenotype as L324P-CCR3 transfectants with the L324A-CCR3 protein detected intracellularly but not at the cell surface (Fig 3, *A and B*). Unsurprisingly, L324A-CCR3 transfectants were unresponsive in chemotaxis assays to CCL11 (Fig 3, *C*), suggesting that L324 *per se* is critical for the trafficking of CCR3 to the membrane.

CCR3 is associated with membranes and undergoes constitutive degradation

We subsequently examined the cellular localization of WT-CCR3, L324P-CCR3, and C218S-CCR3. To accomplish this membrane (made up of plasma membrane and membrane-bound organelles), cytosolic and nuclear fractions were isolated by using subcellular fractionation (Fig 4, *A*). The presence of CCR3 within these compartments was detected by Western blotting using an antibody against the N-terminal HA tag, whereas antibodies against GAPDH (cytosolic protein), calnexin (endoplasmic reticulum [ER] membrane protein), GM130 (Golgi membrane protein), and lamin B (nuclear envelope component) were used as

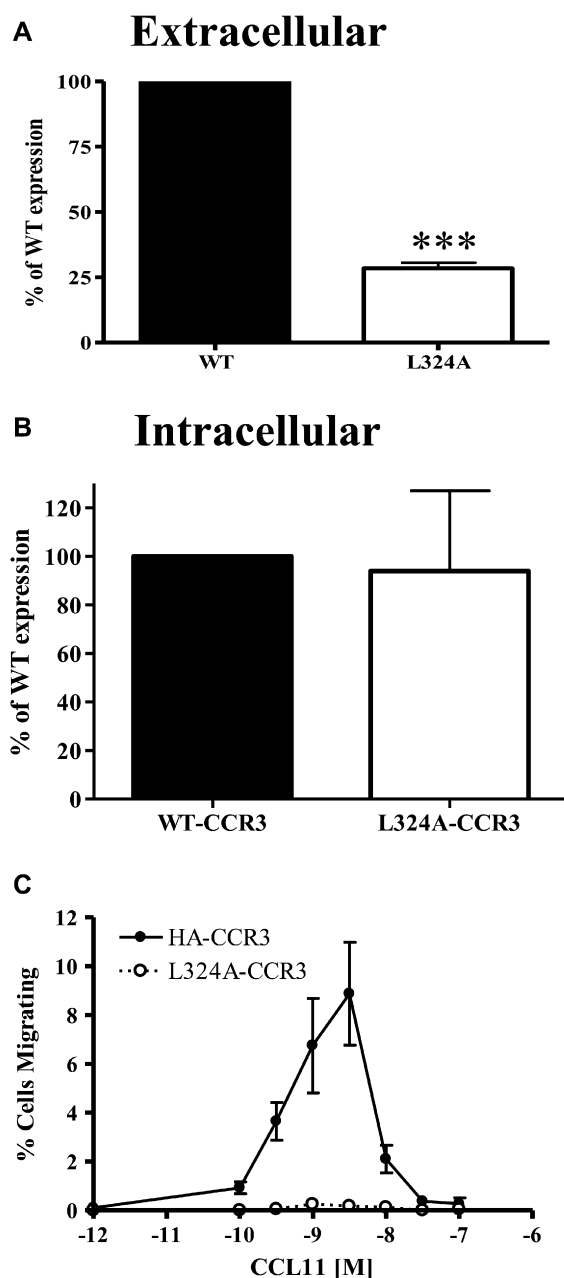


FIG 3. L324 *per se* is critical for CCR3 trafficking and function. **A**, Extracellular expression of L324A-CCR3 expressed as a percentage of WT-CCR3 staining ($n = 3$). **B**, Intracellular expression of L324A-CCR3 expressed as a percentage of WT staining ($n = 3$). **C**, Chemotaxis of WT-CCR3 and L324A-CCR3 transfectants in response to increasing doses of CCL11 ($n = 3$).

references. All 3 CCR3 variants were observed to reside in the membrane fraction, as was the case for calnexin and GM130. GAPDH and lamin B were observed in the cytosol and nucleus respectively, as anticipated. Thus, CCR3 is exclusively associated with the membrane fraction as befits an integral membrane protein. We subsequently addressed the previous finding that L324P-CCR3 protein was found at lower levels inside the cell than WT-CCR3 (Fig 1, C and D). The protein levels of CCR3 at any given time point will reflect both the rate of CCR3 synthesis and the rate of degradation. The accumulation of trafficking deficient proteins within the cell can have cytotoxic consequences

and consequently, misfolded proteins are often degraded by the cell via proteosomal or lysosomal pathways. To examine CCR3 degradation, transfectants were treated with cycloheximide to inhibit protein synthesis and lysates from cells at various time points subjected to Western blotting using α -tubulin as a loading control (Fig 4, B). Densitometric analysis was used to follow the relative loss of immunoreactivity. Both WT-CCR3 and L324P underwent constitutive degradation over time, with almost 50% of the original starting levels of WT-CCR3 remaining after a 3-hour incubation period. L324P-CCR3 underwent much more efficient degradation with only 20% of the original starting levels of protein detected after 2 hours. Thus, the apparent reduced protein synthesis of L324P compared with WT-CCR3 as detected by flow cytometry and Western blotting (Fig 1, C and D) is likely to reflect the enhanced degradation of the L324P-CCR3 variant. Previous studies have reported that WT-CCR3 is degraded after incubation with ligand,³⁹ but to our knowledge, the constitutive degradation of this receptor has not been previously reported.

WT and L324P-CCR3 traffic from the ER to the Golgi and lysosomes

A previous study has addressed the intracellular localization of CCR3 after ligand-induced internalization,³⁹ but to date, none have addressed the intracellular localization of CCR3 with respect to protein synthesis and maturation. Because L324P-CCR3 protein was detected intracellularly by flow cytometry and Western blotting, we hypothesized that L324P-CCR3 was synthesized but remains trapped at subcellular locations. Confocal microscopy was used with the HA antibody to detect CCR3 and antibodies to calnexin, GM130, and LAMP-1 as markers of the ER, Golgi, and lysosomes, respectively. WT-CCR3 appeared to be expressed at cell surface and intracellular locations, overlying with calnexin both in both a punctuate and a peripheral distribution (Fig 5, A) and with GM130 and LAMP-1 in a punctuate distribution (Fig 5, B and C). In contrast, the L324P-CCR3 showed no overlay with calnexin but did overlie with both GM130 and LAMP-1 (Fig 5, A-C).

DISCUSSION

We have described in this study the functional consequences of 6 nonsynonymous SNPs within the *ccr3* open reading frame. Although the majority of polymorphisms studied were without effect on CCR3 expression and function, we found that the T652A SNP, which encodes a C218S mutation in the fifth transmembrane helix, resulted in reduced levels of CCR3 on the cell surface and a more feeble chemotactic response. Eosinophils from a patient heterozygous for this variant allele have previously been reported to exhibit reduced surface staining for CCR3 and reduced chemotaxis to CCL11 compared with eosinophils from patients homozygous for the WT CCR3 allele.³⁶ The authors of that study hypothesized that the C218S mutation reduced the affinity of CCR3 for a specific antibody and the CCL11 ligand, because CCR3 mRNA levels were comparable in the eosinophils from both patients. With reference to the data generated from our transfectant system, we can now conclude that the phenomenon noted by Kato et al³⁶ is a result of reduced surface expression of C218S-CCR3, which we determined by means of an N-terminal HA epitope tag, independent of any effects of mutation on CCR3 conformation. Thus, in the case of the C218S-CCR3

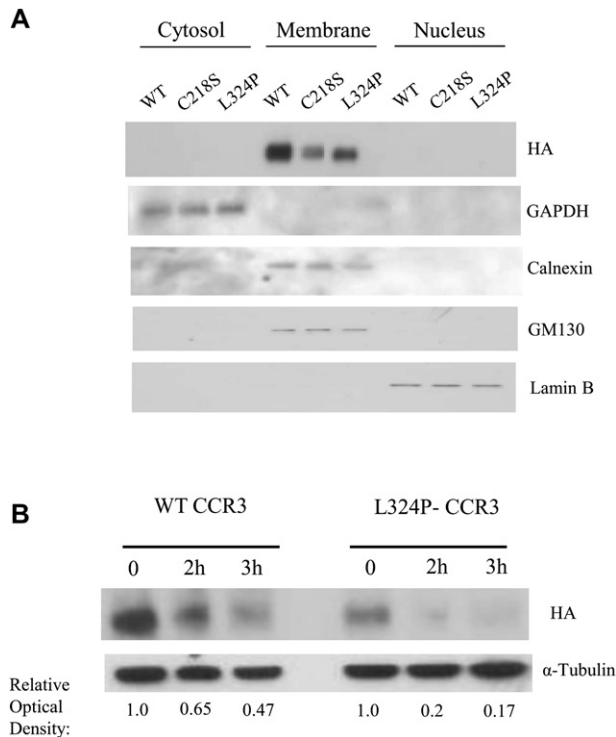


FIG 4. CCR3 is associated with the cell membrane fraction and undergoes constitutive degradation. **A**, Western analysis of CCR3 constructs after subcellular fractionation. Probing was with anti-HA (CCR3), anti-GAPDH (cytosol), anti-calnexin (ER), anti-GM130 (Golgi), and anti-lamin B (nuclear envelope). **B**, Western analysis of CCR3 constructs after cycloheximide treatment. Probing was with anti-HA or anti-tubulin- α antibodies. Relative ODs of CCR3 are shown.

construct, our *in vivo* transfection system accurately models *ex vivo* findings with eosinophils. In contrast with the T652A SNP, the previously uncharacterized T971C SNP,³⁵ which encodes the L324P-CCR3 mutant, completely fails to traffic to the cell surface, and transfected cells are consequently unresponsive to CCR3 ligands. Mutagenesis revealed that the loss of the leucine residue at this position was responsible for the observed phenotype, rather than its replacement with proline, and that the mutant L324P-CCR3 underwent efficient degradation.

Previous clinical studies investigating SNPs within the CCR3 introns have demonstrated that, although certain CCR3 haplotypes are associated with a reduced number of peripheral blood eosinophils and decreased levels of CCR3 expression, this has no bearing on asthma susceptibility.⁴⁰ The T971C SNP we describe here that ablates rather than reduces CCR3 expression leads us to hypothesize that patients homozygous for the mutant allele may be less predisposed to having asthma. Unfortunately, the T971C SNP has only been identified to date in the Japanese population at extremely low levels in both patients with asthma (1/210 heterozygotes) and controls without asthma (1/181 heterozygotes), making it extremely difficult for us to test such a hypothesis directly in human beings (K. Asano, personal communication, March 2008). Future studies would be worthwhile to examine whether the T971C SNP is apparent in any other populations and if so, at what frequency. Small molecule antagonists of CCR3 might be envisaged to have little efficacy in individuals heterozygous for the T971C SNP, although it may be that if the compounds penetrate the lipid bilayer, they may act as a

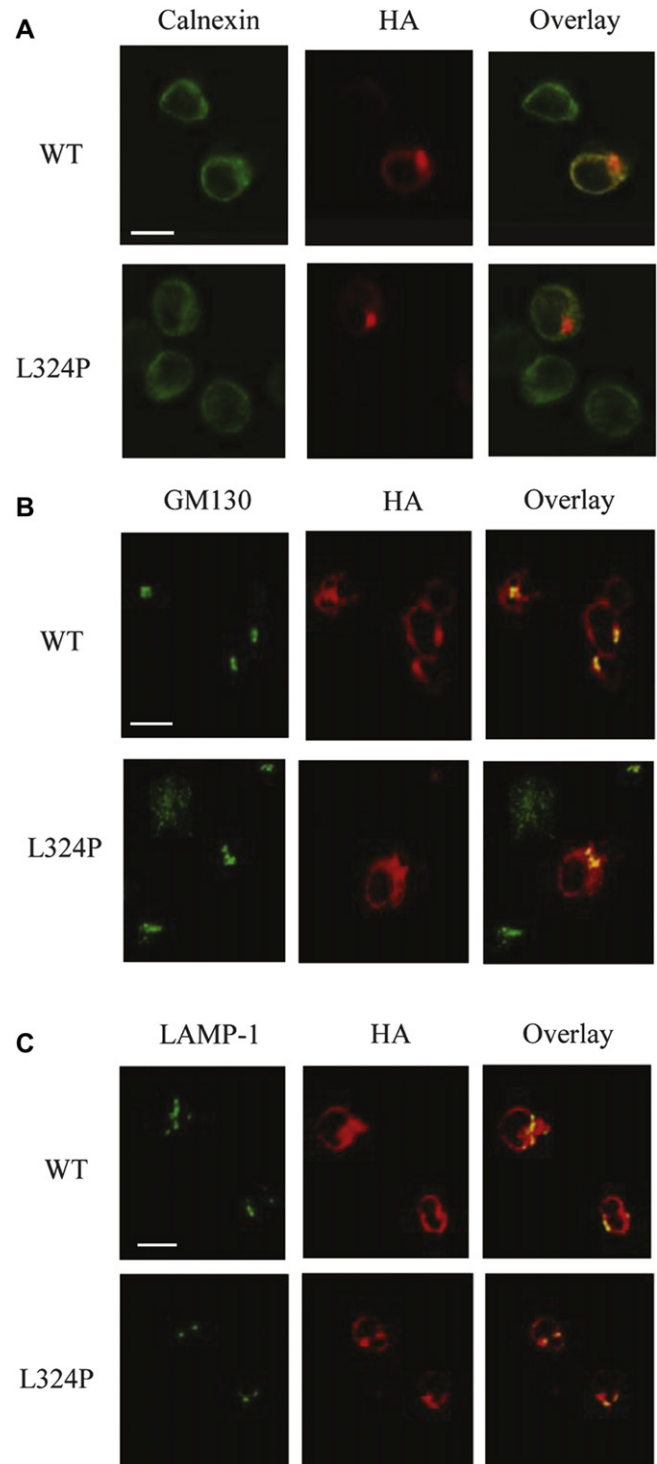


FIG 5. CCR3 is associated with the ER, Golgi, and lysosomes. L1.2 WT or L324P-CCR3 transfectants were analyzed by using confocal microscopy after staining with the anti-HA antibody and the ER marker calnexin (**A**), the Golgi marker GM130 (**B**), or the lysosome marker LAMP-1 (**C**). Data shown are representative of several independent experiments. Scale bars denote 10 μ m.

pharmacoperone and stabilize misfolded CCR3 and facilitate passage to the plasma membrane, as has been reported for the gonadotropin-releasing hormone receptor.⁴¹ Because an equivalent leucine residue is conserved in the C-terminus of murine

CCR3, a knock-in approach could be used to generate transgenic mice that which harbor a leucine to proline mutation similar to that found in human populations. Use of such mice in murine models of allergic airways disease would allow the effect of the mutation on disease susceptibility or disease severity to be examined directly.

Despite these limitations, the T971C SNP is highly informative with respect to the cell membrane trafficking of CCR3. Although the endocytosis of GPCRs after their exposure to ligand is reasonably well understood, the mechanisms by which nascent GPCRs traffic to the cell surface are poorly defined. Data from recent studies have resulted in a hypothesis in which GPCRs are synthesized, assembled, and folded in a conformation-dependent sorting process within the ER, allowing their continued trafficking to the cell surface as functional receptors.^{42,43} Confocal microscopy revealed that L324P-CCR3 trafficked to the Golgi apparatus and lysosomes in a similar fashion to WT-CCR3, although intriguingly, no coexpression of the mutant CCR3 was observed with the ER marker calnexin. In contrast, WT-CCR3 was coexpressed with calnexin both in a dense intracellular location, presumed to be the ER, and in a more even distribution at the cell membrane. Study of protein-protein interactions using the yeast-2-hybrid system has shown that GPCRs interact with a diverse range of chaperones while passing through the ER and Golgi apparatus.⁴³⁻⁴⁶ L324 of CCR3 is the ultimate amino acid of an LLXXL motif, common to several chemokine receptors. In the analogous sequence within the carboxyl tail of CXCR2, this region has been shown to interact directly with the chaperone Hsc70-interacting protein to regulate receptor function.⁴⁷ We hypothesize that during its synthesis, the LLXXL motif of the CCR3 C-terminus interacts with 1 or more chaperones, possibly including calnexin, and a lack of this motif means that the nascent protein does not fold correctly and is targeted for degradation. Supportive of this, C-terminal dileucine motifs have been shown to be important for the membrane targeting of other GPCRs such as the vasopressin V₂,⁴⁸ vasopressin V₃,⁴⁹ and 5-hydroxytryptophan (5-HT) 1AA receptors.⁵⁰

There are likely to be several quality control checkpoints during CCR3 synthesis. In the ER, misfolded proteins are targeted for proteosomal degradation via the ER associated degradation pathway,⁵¹ and further checkpoints may occur in the *cis*-Golgi whereby defective proteins may undergo retrograde transport to the ER and may be targeted to the ER associated degradation or may enter the endosomal system, with eventual degradation occurring via the lysosomes. The lack of coexpression of L324P and calnexin we observed, in contrast with WT-CCR3, may reflect extremely efficient degradation of L324P or a masking of the receptor's epitope tag on binding to a chaperone. Although calnexin contains an ER-retention signal, it has been shown to be on the cell surface of lymphocytes^{52,53} and is thought to traffic there by escorting proteins it first encountered in the ER. At the cell surface, calnexin undergoes constitutive endocytosis and targeting to the late endosomes and lysosomes for degradation.⁵⁴ It may be that the colocalization of both WT-CCR3 and L324P with LAMP-1 that we report here reflects such a mechanism and provides a molecular explanation for the observed constitutive degradation of both CCR3 variants.

In summary, we have identified a critical region of CCR3 that governs its export to the plasma membrane, most likely involving specific chaperones. A greater understanding of the regulatory mechanisms involved in CCR3 export may suggest alternative strategies for targeting this molecule in the allergic setting.

Key messages

- CCR3 is a chemokine receptor implicated in leukocyte recruitment to sites of allergic inflammation and a principal target for novel therapeutics.
- We describe a naturally occurring mutation that ablates the cell surface expression of CCR3.
- This mutation highlights the existence of novel export processes specific for CCR3 expression, which might provide alternative means of targeting this key receptor.

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<i>SNP</i>	<i>Amino Acid</i>	<i>Population</i>	<i>Allele Frequency</i>	<i>Reference</i>
G494A	G21D	African American	Not reported	33
C548T	P39L	Subsaharan Africa	0.005	33
T652A	C218S	Japanese	1/12 patients with Behçet	36
G894A	R275Q	US mixed asthma/nonasthma	0.01	34
T971C	L324P	Jap/Brit non-asth/asth	Not reported	35
T1052C	L351P	US mixed asthma/nonasthma	0.005	34

FIG E1. SNPs within the CCR3 gene and their corresponding protein product. **A**, Table showing non-synonymous SNPs of CCR3. *Asth*, asthmatics; *Jap*, Japanese; *Brit*, British. **B**, The CCR3 primary sequence with sites of point mutation shown in filled circles and annotated.

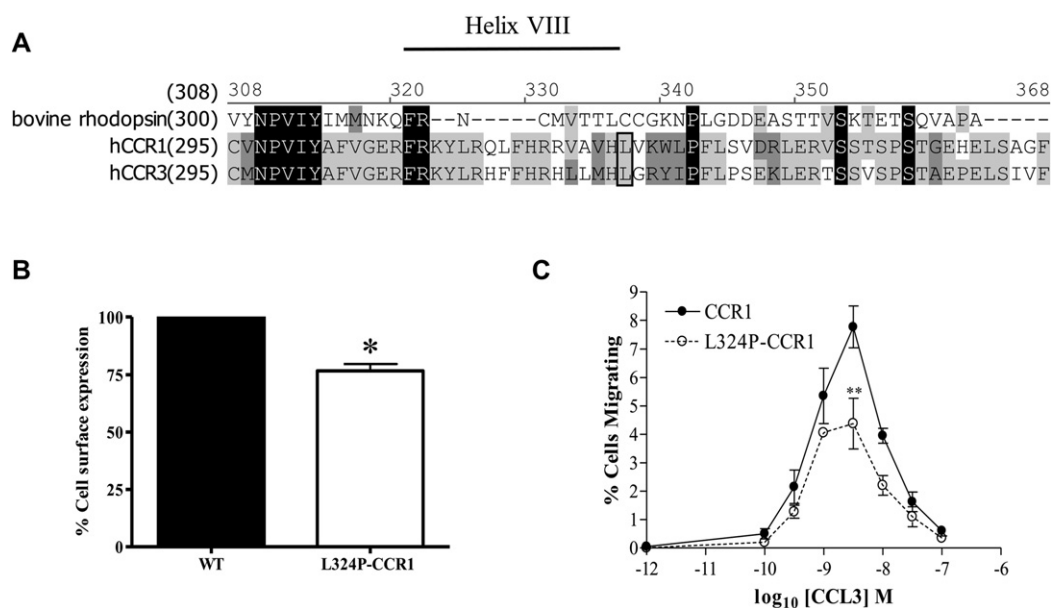


FIG E2. Mutation of L324 in CCR1 is without effect. **A**, Alignment of the C-termini of human (h) CCR1 and CCR3. The conserved L324 residue is boxed. **B**, Relative cell surface expression of the L324P-CCR1 construct ($n = 3$). **C**, Migration of L1.2 transfectants expressing either WT-CCR1 or L324P-CCR1 in response to increasing amounts of CCL3 ($n = 3$). M , mol/L.