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Modulation of the CXC Chemokine Receptor 4 Agonist Activity of Ubiquitin through C-Terminal Protein Modification

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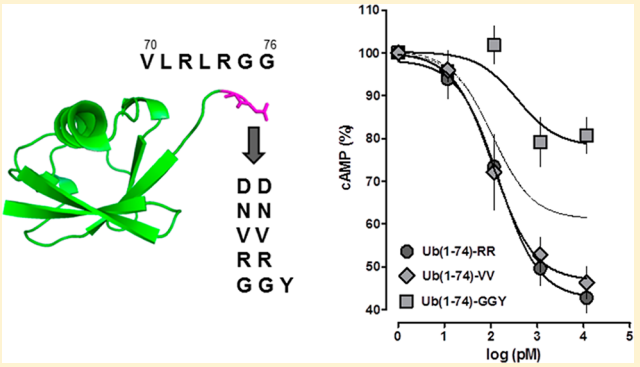
ABSTRACT: Extracellular ubiquitin has recently been described as a CXC chemokine receptor (CXCR) 4 agonist. Studies on the structure−function relationship suggested that the C-terminus of ubiquitin facilitates CXCR4 activation. It remains unknown, however, whether C-terminal processing of ubiquitin could be biologically relevant and whether modifications of the ubiquitin C-terminus can modulate CXCR4 activation. We show that C-terminal truncated ubiquitin antagonizes ubiquitin and stromal cell-derived factor (SDF)-1α induced effects on cell signaling and function. Reduction of cell surface expression of insulin degrading enzyme (IDE), which cleaves the C-terminal di-Gly of ubiquitin, enhances ubiquitin induced reduction of cAMP levels in BV2 and THP-1 cells, but does not influence changes in cAMP levels in response to SDF-1α. Reduction of cell surface IDE expression in THP-1 cells also increases the chemotactic activity of ubiquitin. As compared with native ubiquitin, C-terminal Tyr extension of ubiquitin results in reduced CXCR4 mediated effects on cellular cAMP levels and abolishes chemotactic activity. Replacement of C-terminal di-Gly of ubiquitin with di-Val or di-Arg enhances CXCR4 mediated effects on cAMP levels and the di-Arg substitution exerts increased chemotactic activity, when compared with wild type ubiquitin. The chemotactic activities of the di-Val and di-Arg mutants and their effects on cAMP levels can be antagonized with C-terminal truncated ubiquitin. These data suggest that the development of CXCR4 ligands with enhanced agonist activities is possible and that C-terminal processing of ubiquitin could constitute a biological mechanism, which regulates termination of receptor signaling.



Ubiquitin is a small (8.6 kDa) and highly conserved protein which fulfills important biological roles as a post-translational protein modifier inside the cell.[1] Ubiquitin is also detectable in normal plasma, and various diseases are associated with increased ubiquitin levels in the systemic circulation.[2] We have previously reported that extracellular ubiquitin functions as an immune modulator in patients.[3] Subsequently, it was shown that administration of exogenous ubiquitin has anti-inflammatory and therapeutically relevant effects in various animal models.[4−11] More recently, we provided evidence that extracellular ubiquitin acts as another natural agonist of CXC chemokine receptor (CXCR) 4 and utilizes a binding site on the receptor, which is distinct from the binding site of stromal cell-derived factor (SDF)-1α (chemokine (C-X-C motif) ligand 12).[12−14]

CXCR4 plays important roles in biology and is also involved in the pathophysiology of various disease processes, such as cancer metastasis, human immunodeficiency virus infection, ischemic myocardial injury, stroke, and neurovascular repair.[15−24] Thus, a better understanding of the mechanisms that regulate CXCR4 activation is of biological relevance and will help to define its role as a therapeutic target.

Studies on the structure−function relationship of ubiquitin suggested that ubiquitin contains separate receptor binding and activation sites.[14] While deletion of the C-terminal di-Gly of ubiquitin did not affect binding to CXCR4, truncated ubiquitin displayed significantly reduced agonist activity.[14] It remains unknown, however, whether truncation of the ubiquitin C-terminus could be of biological relevance and whether the ubiquitin C-terminus can be modified to enhance CXCR4 signaling. Here, we provide evidence that ubiquitin lacking the C-terminal di-Gly antagonizes the effects of the native CXCR4 ligands and that the expression level of cell surface insulin degrading enzyme (IDE), which has been shown to cleave the C-terminal di-Gly of ubiquitin,[25] influences the effects of ubiquitin on cell signaling and function. Furthermore, we show that subtle size and charge modifications of the ubiquitin C-terminus affect cell signaling and function upon CXCR4 activation. Our findings suggest a mechanism through which the activation of CXCR4

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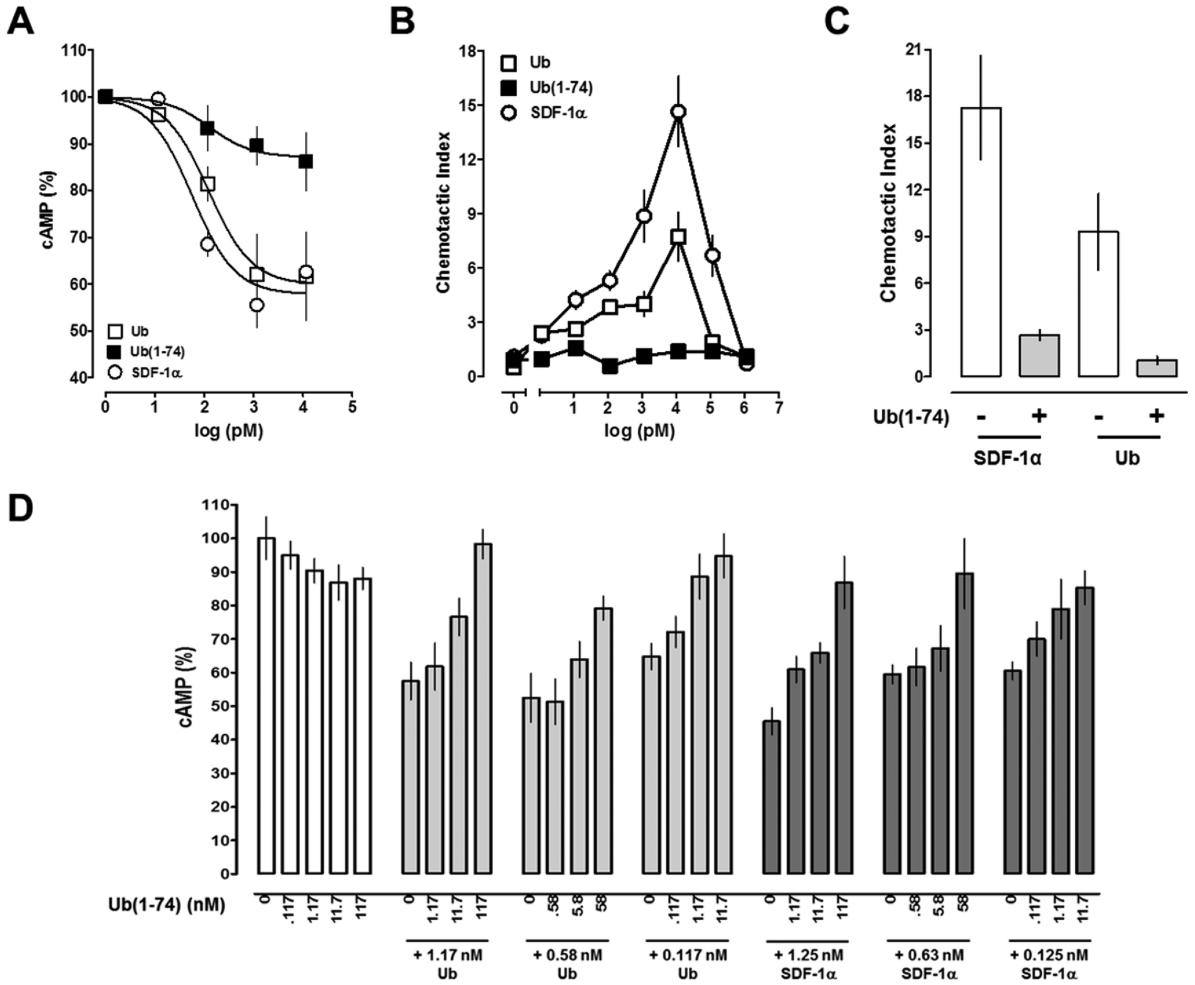

Figure 1. C-Terminal truncated ubiquitin functions as a CXCR4 antagonist. (A) Forskolin-stimulated THP-1 cells were incubated with wild type ubiquitin, SDF-1α, or C-terminal truncated ubiquitin (Ub(1−74)) and cAMP levels were measured (n = 3). Data are expressed as % of untreated cells (= 100%). (B) Dose-dependent migration of THP-1 cells toward native ubiquitin, SDF-1α, and Ub(1−74), n = 4−5. (C) Dose-dependent migration of THP-1 cells toward 10 nM native ubiquitin or SDF-1α in the absence (−) or presence (+) of 1 μM Ub(1−74), n = 4−5. (D) Forskolin-stimulated THP-1 cells were incubated with ubiquitin, SDF-1α, and Ub(1−74) as indicated and cAMP levels were measured. Data are expressed as % of untreated cells (= 100%), n = 4−8.

could be regulated and demonstrate that the development of CXCR4 ligands with enhanced agonist activities is possible.

EXPERIMENTAL PROCEDURES

Cell Lines. THP-1 cells (human monocytic leukemia cells), BV-2 cells (murine microglial cells), and BV-2 cells, in which insulin degrading enzyme was silenced by lentivirus-mediated shRNA interference (BV-2 shRNA-IDE), were as described previously.[12−14,26]

Proteins and Reagents. Ubiquitin was purchased from R&D Systems. AMD3100, BSA, and forskolin were purchased from Sigma. N-Terminal FITC labeled ubiquitin (FITC-ubiquitin) and a C-terminal ubiquitin deletion mutant that lacks Gly-75 and Gly-76 (Ub(1−74) were obtained from Boston Biochem. SDF-1 α was expressed in *Escherichia coli* and purified as previously described.[27]

Ubiquitin and ubiquitin mutants, in which the C-terminal diglycine was replaced against Asp-Asp (Ub(1−74)DD), Asn-Asn (Ub(1−74)NN), Arg-Arg (Ub(1−74)RR), or Val-Val (Ub(1−74)VV) and a ubiquitin mutant, in which the C-terminus of ubiquitin was extended by Tyr (Ub(1−74)GGY), were expressed in *E. coli* and purified to homogeneity. The pGEX-6P-1 expression vector containing the GST-ubiquitin sequence between EcoR I and Xho I restriction sites was kindly provided by Dr. Harald Stenmark,[28] and plasmids containing the sequences for Ub(1−74)GGY, Ub(1−74)DD, Ub(1−74)NN, Ub(1−74)RR, and Ub(1−74)VV were generated by GenScript. The DNA sequences were verified by dideoxysequencing. Plasmids were transformed into One Shot TOP Chemically Competent *E. coli* (Invitrogen), protein expression was induced with 0.5 mM isopropyl β−α-thiogalactoside, and cells were lysed with bacterial protein extraction reagent (Pierce) as per manufacturers' instructions. GST-fusion proteins were purified on glutathione spin columns (Thermo Scientific), followed by PreScission protease (2 units/0.1 mg of GST-fusion protein) digestion in 20 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, pH 8 at 5 °C for 4 h. Mutant proteins were then purified to homogeneity on a Superose 12 HR column (inner diameter: 10 mm, lengths: 300 mm; GE Healthcare) in 20 mM Tris/HCl, 150 mM NaCl, pH 7.4 at 5 °C using a computer-controlled fast protein liquid chromatography system. Purity of the proteins was confirmed by SDS-PAGE. Endotoxin was removed from purified proteins using the Toxin Eraser Endotoxin Removal kit (GenScript) and confirmed with the PyroGene Recombinant Factor C Endotoxin Detection assay (Lonza) according to the manufacturer's instructions.

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IDE Gene Silencing by RNA Interference. 1×10^5 THP-1 cells in 1 mL of Accell siRNA delivery media (Thermo Scientific Dharmacon) were cultured per well in a 12-well tissue culture plate (Nunc). Commercially available Accell IDE siRNA (Thermo Scientific Dharmacon) was reconstituted with 1× siRNA buffer (Thermo Scientific Dharmacon) to a stock concentration of 100 μM. Cells were then transfected with 10 nmol of IDE siRNA and incubated for 72 h at 37 °C, 5% CO_2. Accell nontargeting siRNA pool was used as a negative control. After 72 h, the cells were assayed for IDE cell surface expression by FACS analyses and used for further analyses.

FACS Analyses. FACS was used to analyze cell surface expression of IDE. Cells were labeled with polyclonal rabbit anti-IDE (ab32216, Abcam) in combination with antirabbit FITC conjugated goat IgG (Abcam). Rabbit IgG (R&D Systems) in combination with FITC-conjugated antirabbit goat IgG (Abcam) was used as a negative control. The fluorescence intensities of at least 3×10^4 cells were recorded and analyzed using the FlowJo software (Tree Star).

Ubiquitin Binding Assays. Ubiquitin binding assays were performed with BV-2 and BV-2 shRNA-IDE cells, as described.[12,13] In brief, cells were washed with ice cold PBS, and 10^5 cells were suspended in 100 μL of cold (4 °C) PBS, 1% BSA, 0.01% sodium azide. FITC-ubiquitin was added and incubated for 1 min at 4 °C. Cells were washed twice, and the fluorescence intensities were measured ($\lambda_{excitation/emission}$: 485/528 nm). Nonspecific binding was assessed as binding of FITC-ubiquitin in the presence of 300 μM native ubiquitin.

cAMP Assay. Quantitative determination of cAMP levels was performed in forskolin (10 μM, 10 min, 37 °C) treated cells using the cAMP complete enzyme immunoassay kit (Assay Designs), acetylated format, as described.[12,13]

Chemotaxis Assays. Cell migration was assessed using the ChemoTx 96-well cell migration system (29 μL well plate, 8 μm filter pore size; Neuroprobe), as described.[13] In brief, the bottom wells were filled with 29 μL of test solutions. The microplate was then covered with the ChemoTX filter, and 25 μL of cell suspension containing 2×10^5 THP-1 cells in PBS was pipetted onto the filter over each well and incubated for 3 h at 37 °C, 5% CO_2. After incubation, cells that transmigrated through the filter were stained on the lower filter surface with Accustain Wright-Giemsa stain (Sigma). The average number of cells on the lower filter surface was determined by counting the number of cells in three random nonoverlapping high power (400×) fields by light microscopy. The chemotactic index (CI) was calculated as the ratio of cells that transmigrated through the filter in the presence versus the absence (= PBS/control) of the test solutions.

Data Analysis. Data are expressed as mean ± SE from duplicate to triplicate measurements of n independent experiments that were performed on different days. Data were analyzed with Student's t test or analysis of variance with Dunett's post-test to control for multiple testing. Best-fit values were compared with the extra sum-of-squares F test. Data were analyzed using the GraphPad-Prism 5 software.

RESULTS

We first studied whether Ub(1−74) could function as an antagonist at CXCR4. Ub(1−74) showed minimal effects on cAMP levels in forskolin stimulated THP-1 cells, whereas wild type ubiquitin and SDF-1α reduced cAMP levels by 45−65% (Figure 1A). In contrast to ubiquitin and SDF-1α, which induced chemotaxis with typical bell-shaped dose responses in THP-1 cells, chemotactic activity was not detectable for Ub(1−74) (Figure 1B). However, the effects of the native CXCR4 agonists on cAMP levels and chemotaxis could be antagonized with Ub(1−74) (Figure 1C,D).

We then evaluated whether C-terminal processing of ubiquitin by IDE may contribute to the regulation of its CXCR4 agonist activity utilizing BV-2 shRNA IDE cells. As determined by FACS analyses, IDE was detectable on the cell surface of BV-2 cells, and IDE cell surface expression was reduced by 75 ± 3% in BV-2 shRNA IDE cells (n = 3; Figure 2A). The K_d for FITC-ubiquitin binding to BV-2 and BV-2 shRNA IDE cells was 119 ± 67 nM and 154 ± 85 nM, respectively (Figure 2B). As shown in Figure 2C, ubiquitin reduced cellular cAMP levels in BV-2 shRNA IDE cells at concentrations that were lower than required for a significant reduction of cAMP levels in wildtype cells. In contrast, IDE silencing did not affect cellular cAMP levels in response to SDF-1α (Figure 2D). To confirm these observations in another cell line, we silenced IDE with siRNA in THP-1 cells (Figure 2E). In line with our observations in BV-2 cells, IDE was also expressed on the cell surface of THP-1 cells and could be reduced by 63 ± 11% (n = 3) after transfection with IDE siRNA. Reduction of cell surface IDE expression on THP-1 cells also enhanced the potency of ubiquitin to reduce cAMP levels (Figure 2F). To evaluate whether enhanced signaling of ubiquitin after cell surface IDE knockdown is associated with enhanced effects on cell function, we then determined the chemotactic activity of ubiquitin at a concentration that results in maximal chemotactic responses of THP-1 cells. As shown in Figure 2G, the CI was 4.8 ± 1.6 in THP-1 cells after transfection with nontargeting siRNA and significantly increased to 13 ± 3.9 in THP-1 cells after IDE silencing.

Next, we studied whether charge and size modifications of the ubiquitin C terminus influence its CXCR4 agonist activity. The effects of the various ubiquitin mutants (Figure 3A) on cAMP levels in forskolin-stimulated THP-1 cells are shown in Figure 3B,C. Ub(1−74)NN and Ub(1−74)DD were indistinguishable from native ubiquitin (Figure 3B). Whereas Ub(1−74)GGY showed reduced effects on cAMP levels, we observed enhanced efficacy of Ub(1−74)VV and Ub(1−74)RR to reduce cellular cAMP levels (Figure 3C). As for native ubiquitin and SDF-1α, the effects of all mutants on cellular cAMP levels could be neutralized with the CXCR4 antagonist AMD3100 (Figure 3D). Ub(1−74) also antagonized the effects of Ub(1−74)RR and Ub(1−74)VV on cAMP levels in THP-1 cells (Figure 3E).

To further evaluate whether the observed differences in activating CXCR4 mediated cell signaling among the ubiquitin mutants are also reflected by differences in their ability to activate CXCR4 mediated cell functions, we used the chemotactic response of THP-1 cells as a functionally relevant read-out. The chemotactic activities of Ub(1−74)DD, Ub(1−74)NN, and Ub(1−74)VV were indistinguishable from native ubiquitin, when compared in parallel experiments (Figure 4A,B). Cell migration toward Ub(1−74)GGY was reduced and increased toward Ub(1−74)RR, as compared with native ubiquitin (Figure 4C). Furthermore, the lower range of concentrations that induced maximal cell movements was extended by 1 order of magnitude for Ub(1−74)RR (CI at 100 pM: native ubiquitin −3.8 ± 0.8, Ub(1−74)RR − 9.1 ± 1.2). AMD3100 antagonized the chemotactic activities of native ubiquitin and the ubiquitin mutants (Figure 4D). As observed for their effects on cAMP levels, the chemotactic activities of Ub(1−74)RR and Ub(1−74)VV could be antagonized with Ub(1−74) (Figure 4E). The properties of

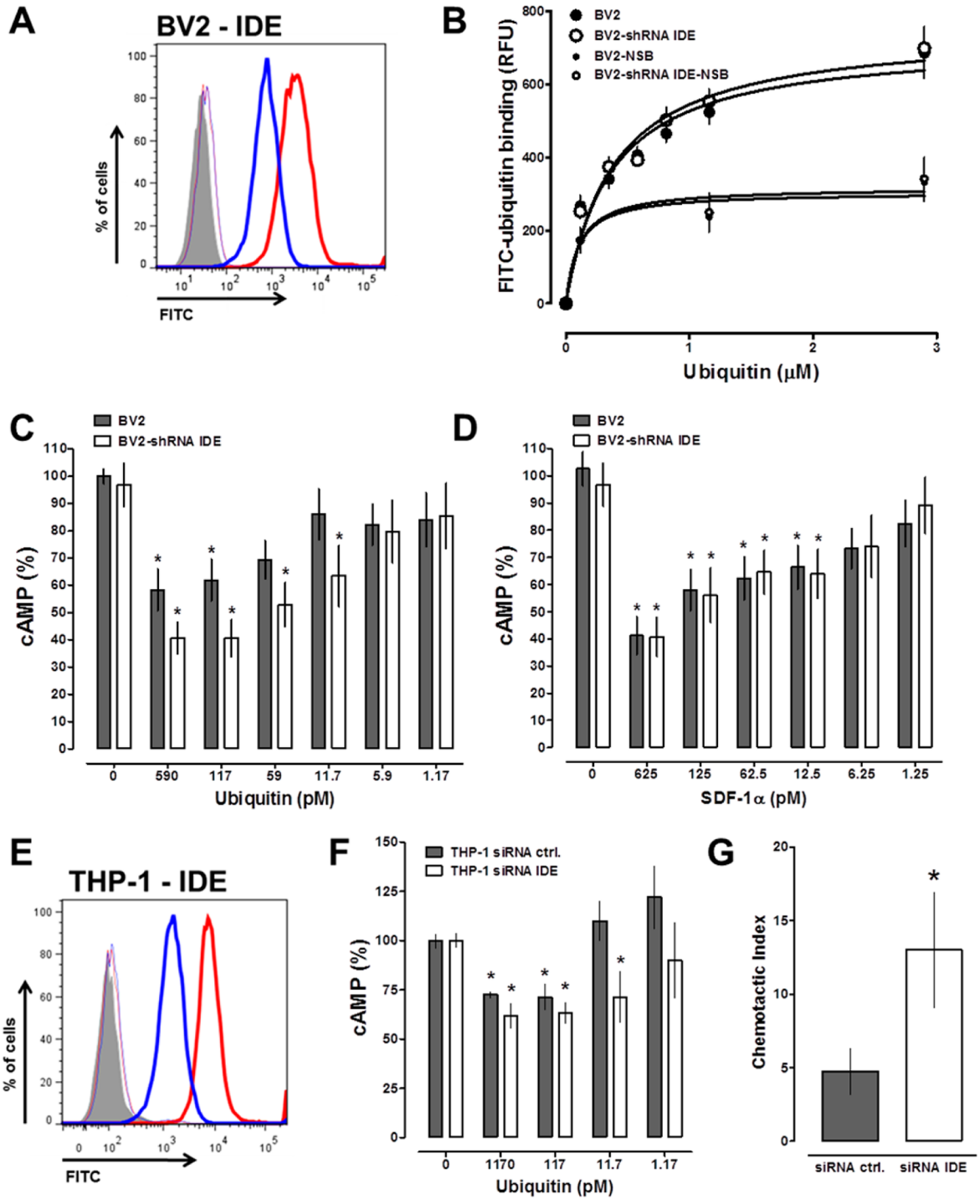

Figure 2. Cell surface expression of insulin degrading enzyme (IDE) modulates ubiquitin-induced effects on cell signaling and function. (A) Quantification of IDE cell surface expression by flow cytometry. Thick lines: Cells labeled with rabbit anti-IDE/antirabbit FITC goat IgG. Thin lines: Control; cells labeled with rabbit IgG/antirabbit FITC goat IgG. Gray: Unstained cells. Red: BV-2 cells. Blue: BV-2 shRNA IDE cells. (B) FITC-ubiquitin binding (1 min, 4 °C) to BV-2 (●) and BV-2 shRNA IDE (○) cells. ●: nonspecific binding (NSB) - BV-2 cells. ○: nonspecific binding (NSB) - BV-2 shRNA cells. RFU: relative fluorescence units. (C) Forskolin stimulated BV-2 (gray bars) and BV-2 shRNA cells (open bars) were incubated with ubiquitin and cAMP levels were measured (n = 6). *: $p < 0.05$ vs untreated BV-2 cells. Data are expressed as % of untreated BV-2 cells (= 100%). (D) Forskolin-stimulated BV-2 (gray bars) and BV-2 shRNA cells (open bars) were incubated with SDF-1α and cAMP levels were measured (n = 6). *: $p < 0.05$ vs untreated BV-2 cells. Data are expressed as % of untreated BV-2 cells (= 100%). (E) Quantification of IDE cell surface expression on THP-1 cells by flow cytometry after transfection with IDE siRNA (blue) or nontargeting siRNA (red). Thick lines: Cells labeled with rabbit anti-IDE/antirabbit FITC goat IgG. Thin lines: Control; cells labeled with rabbit IgG/antirabbit FITC goat IgG. Gray: Unstained cells. (F) Forskolin-stimulated THP-1 cells after transfection with nontargeting siRNA (ctrl.; gray bars) and IDE siRNA (open bars) were incubated with ubiquitin and cAMP levels were measured (n = 4). *: $p < 0.05$ vs untreated BV-2 cells. Data are expressed as % of untreated BV-2 cells (= 100%). (G) Migration of THP-1 cells toward ubiquitin (10 nM). Gray bar: Cells after transfection with nontargeting siRNA (ctrl.). Open bar: Cells after transfection with IDE siRNA. *: $p < 0.05$ vs ctrl.

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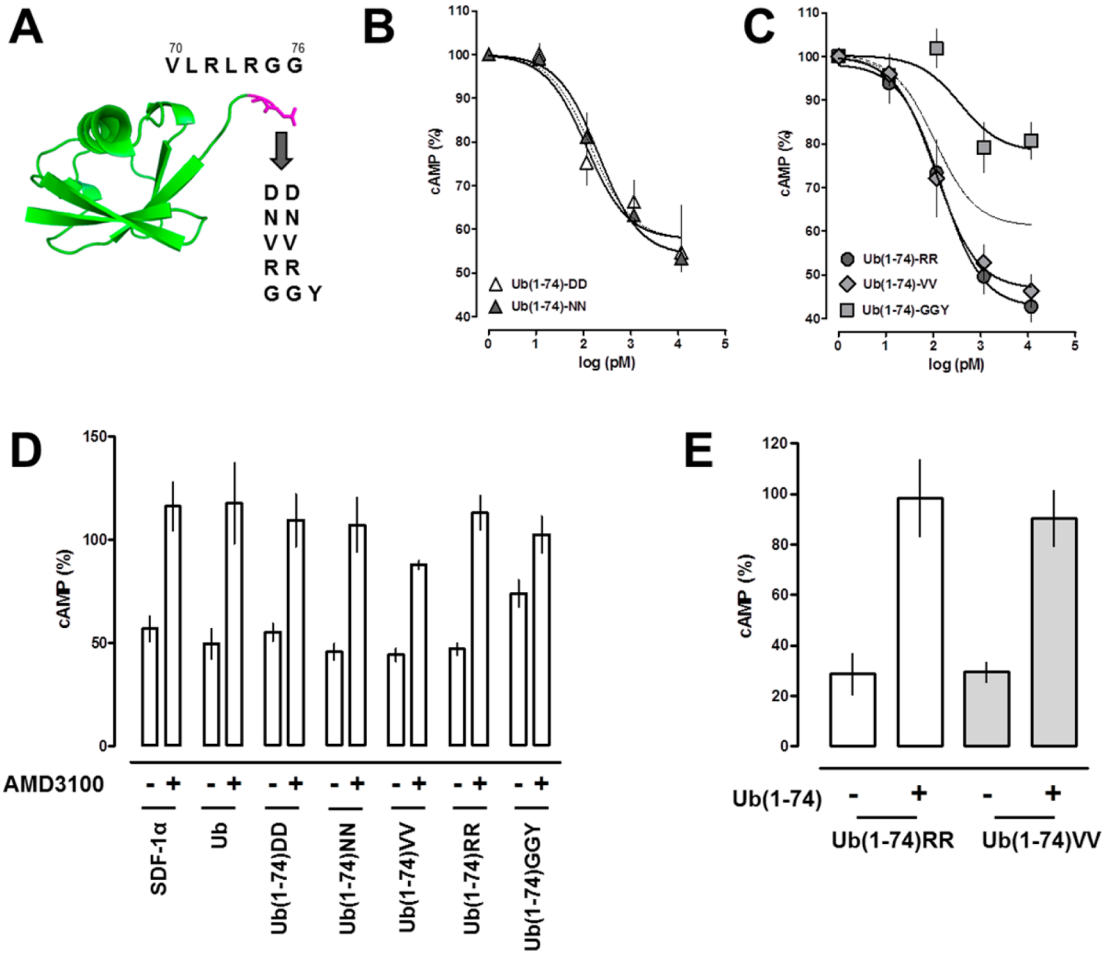

Figure 3. The ubiquitin C-terminus regulates signaling upon CXCR4 activation. (A) Ribbon diagram of ubiquitin. Gly-75 and Gly-76 are highlighted in magenta. The generated mutations of the C-terminus are shown. (B, C) Forskolin-stimulated THP-1 cells were incubated with the ubiquitin mutants and cAMP levels were measured (n = 3−5). Data are expressed as % of untreated cells (= 100%). The dashed lines show the dose−response curve for wild type ubiquitin from Figure 1A, which were measured in parallel experiments. (B) Open triangles: Ub(1−74)DD; gray solid triangles: Ub(1−74)NN. (C) ● Ub(1−74)RR; gray solid diamonds: Ub(1−74)VV; gray solid squares: Ub(1−74)GGY. (D) Forskoli-stimulated THP-1 cells were incubated with wild type ubiquitin, SDF-1α, or the ubiquitin mutants (10 nM each) in the absence (−) or presence (+) of AMD3100 (10 μM) as in B−D and cAMP levels were measured (n = 3). Data are expressed as % of untreated cells (= 100%). (E) Forskolin-stimulated THP-1 cells were incubated with Ub(1−74)RR or Ub(1−74)VV (10 nM each) in the absence (−) or presence (+) of Ub(1−74) (1 μM) and cAMP levels were measured (n = 3). Data are expressed as % of untreated cells (= 100%).

native ubiquitin and the various ubiquitin mutants are summarized in Table 1.

DISCUSSION

Previously, we provided evidence suggesting that ubiquitin contains separate receptor binding (Phe-4 and Val-70) and activation sites (C-terminal di-Gly),[14] and thus mimics the structure function relationship of chemokines.[29−31] We have shown that deletion of the C-terminal di-Gly of ubiquitin significantly reduces the ability to reduce cAMP levels in THP-1 cells but does not influence receptor binding.[14] The findings of the present study confirm this observation and further document that Ub(1−74) does not affect cell migration. Because Ub(1−74) also neutralized the effects of native ubiquitin and SDF-1α on cellular cAMP levels and cell migration, these data imply that Ub(1−74) acts as an antagonist at CXCR4.

AMD3100, SDF-1α, and Ub(1−74) have been shown to displace ubiquitin from the receptor.[12,14] Thus, the antagonistic actions of Ub(1−74) on effects mediated by wild type ubiquitin can be explained by competition binding. It is, however, currently unknown whether ubiquitin or Ub(1−74) displace SDF-1α from the receptor. Whereas SDF-1α binding to CXCR4 requires the receptor N-terminus, ubiquitin and AMD3100 binding to CXCR4 do not.[13,32,33] Moreover, AMD3100 has been shown to displace the SDF-1α N-terminus from the receptor binding pocket without interfering with binding of the SDF-1α core domain to the CXCR4 N-terminus.[31] Thus, in analogy to AMD3100, the antagonistic actions of Ub(1−74) on SDF-1α mediated effects on cell signaling and function could be explained through partial or complete displacement from the receptor binding sites. Alternatively, as SDF-1α and ubiquitin appear not share the same binding sites on CXCR4,[14] Ub(1−74) binding to CXCR4 may result in allosteric inhibition of SDF-1α mediated effects without SDF-1α displacement.

Many chemokines, including SDF-1α, can be inactivated or activated by proteolytic processing, which is thought to contribute to the regulation of inflammation.[34−37] Ubiquitin was initially isolated as a 74 amino acid protein lacking the C-terminal di-Gly.[38,39] Subsequently, it was shown that the complete amino acid sequence contains Gly-75 and Gly-76 and suggested that ubiquitin

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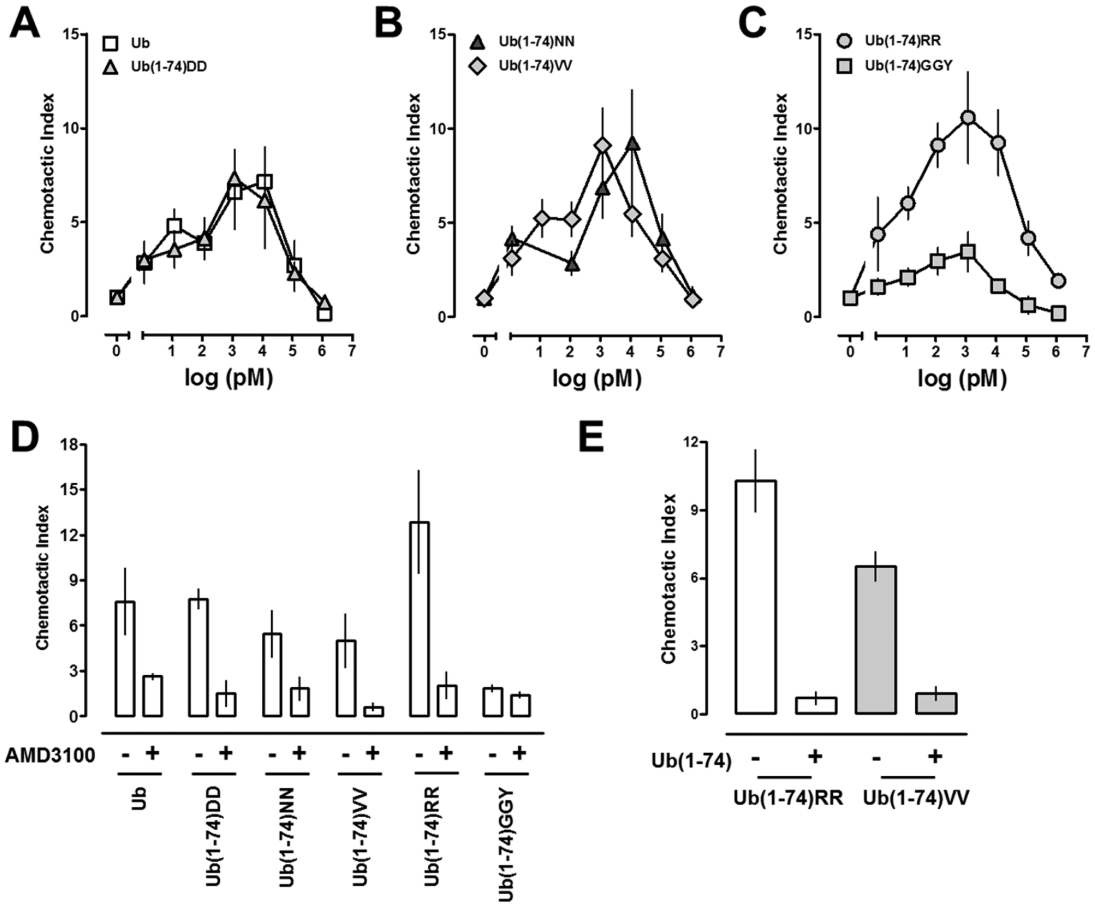

Figure 4. The ubiquitin C-terminus regulates cell function upon CXCR4 activation. (A) Dose-dependent migration of THP-1 cells toward native ubiquitin (Ub) and Ub(1−74)DD, $n = 7$. (B) Dose-dependent migration of THP-1 cells toward Ub(1−74)NN and Ub(1−74)VV, $n = 7$. (C) Dose-dependent migration of THP-1 cells toward Ub(1−74)RR and Ub(1−74)GGY, $n = 7$. (D) Migration of THP-1 cells toward ubiquitin and the ubiquitin mutants (10 nM each) in the absence (−) or presence (+) of AMD3100 (10 μM), $n = 5$. (E) Migration of THP-1 cells toward Ub(1−74)RR and Ub(1−74)VV (10 nM each) in the absence (−) or presence (+) of Ub(1−74) (1 μM), $n = 3$.

cleavage occurs during the isolation from most tissues.[40] IDE has recently been identified as an enzyme that can truncate the ubiquitin C-terminus.[25] IDE is a highly conserved zinc metallopeptidase that is ubiquitously expressed in tissues and can degrade a variety of proteins, including the chemokine macrophage inflammatory protein-1.[26,41−43] Besides its intracellular localization, IDE is also expressed on the cell surface and can be released into the extracellular space.[44,45] IDE rapidly removes the C-terminal di-Gly of ubiquitin, which is followed by a slower cleavage between Arg-72 and Leu-73 after several minutes.[25] To assess whether IDE could be involved in the regulation of CXCR4 activation by ubiquitin, we first utilized a gene knockdown cell system that has previously been employed to dissect physiological functions of IDE.[26] We detected that shRNA silencing of IDE resulted in the reduction of cell surface IDE expression in BV-2 cells. The degree of cell surface IDE knockdown is in agreement with the previously described reduction of IDE expression that was assessed by Western blotting with whole cell extracts.[26] The affinity of FITC-ubiquitin binding to BV-2 and BV2 shRNA IDE cells is consistent with the affinity of the binding of FITC-ubiquitin to various other cells, including THP-1 cells and primary leukocytes.[12−14,46,47] This suggests that cell surface IDE knockdown does not affect ubiquitin binding to CXCR4. We detected, however, that the potency of ubiquitin to reduce cAMP levels was higher after IDE knockdown in BV-2 cells and further confirmed this observation in THP-1 cells. Because knockdown of cell surface IDE in THP-1 cells was also accompanied by enhanced effects of ubiquitin on cell migration, our findings suggest that cell surface IDE may regulate CXCR4 activation and convert ubiquitin into an antagonist at CXCR4 through processing of its C-terminus. In contrast, SDF-1α mediated effects on cAMP levels were not influenced by modulation of cell surface IDE expression, indicating substrate specificity of IDE.

As these findings further support the concept that the ubiquitin C-terminus functions as receptor activation site, we then evaluated how C-terminal charge and size modifications modulate CXCR4 activation. We have reported a computational model of the ubiquitin−CXCR4 complex, in which the ubiquitin C-terminus is near the extracellular loop 3 of CXCR4, and thus, through interaction with Lys-271, may alter the conformation of helix 6 and induce a switch of intracellular loop 3 to activate the downstream G protein.[14,48−50] When applied to this computational model, charge neutral modifications of the C-terminus (Ub(1−74)NN) and introduction of negative charges (Ub(1−74)DD) would not interfere with the salt-bridge formation between the C-terminal carboxyl group of the ubiquitin mutants and Lys-271 of CXCR4. Thus, these mutations would not be expected to alter CXCR4 activation and were indistinguishable from native ubiquitin. Our observation from previous studies that Ub(1−74)AA was also undistinguishable from native ubiquitin in cAMP assays with THP-1 cells is consistent with this assumption.[14]

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Table 1. Properties of Native and C-Terminal Modified Ubiquitin[a]

	reduction of cAMP levels		chemotaxis	
protein	$LogIC_{50}$	bottom plateau	CI	$CI_{AMD3100}$
Ub	2.0 ± 0.4	59.9 ± 5.7	6.2 ± 0.6	2.6 ± 0.2
Ub(1−74)	2.1 ± 0.8	87.1 ± 4.2	1.2 ± 0.2	n.d.
Ub(1−74)DD	2.0 ± 0.3	58.7 ± 3.6	6.8 ± 1.0	1.5 ± 0.8
Ub(1−74)NN	2.3 ± 0.45	54.1 ± 7.7	7.1 ± 1.2	2.3 ± 1.6
Ub(1−74)RR	2.1 ± 0.16	42.8 ± 3.2	10.4 ± 1.3	2.0 ± 0.8
Ub(1−74)VV	2.1 ± 0.52	46.7 ± 3.2	6.8 ± 1.0	0.6 ± 0.3
Ub(1−74)GGY	2.6 ± 0.52	78.2 ± 4.2	2.4 ± 0.4	1.4 ± 0.2

[a]Data are mean ± S.E. LogIC50 (pM) and bottom plateaus (%) were calculated by nonlinear regression analyses (log inhibitor vs. response, top plateau was constrained to 100%) from Figures 1A and 3B,C. CI: Average chemotactic indices measured at concentrations (1 and 10 nM) which resulted in maximal responses for all proteins. $CI_{AMD3100}$: CI in the presence of 10 μM AMD3100.

The stronger $G\alpha_i$ signaling activity of Ub(1−74)VV could relate to enhanced conformational changes of helix 6 through hydrophobic interactions with Ile-269, Ile-270, and the methylene side chain of Lys-271 of CXCR4 when the charged ε-NH_3 is salt bridged by the C-terminal carboxyl group of Val-76.[51] Furthermore, the higher efficacy of Ub(1−74)RR could be explained by a salt bridge formed between CXCR4 Glu-268 and the C-terminal Arg of the ubiquitin mutant. It has been described that small charge changes in the extracellular loop 3 of secretin receptor modulate G protein signaling and that the overall net amount of positive charges in intracellular loop 2 of prokineticin receptor 2 determines signaling efficacy and potency.[52,53] Therefore, it appears possible that the strongly positive C-terminus of Ub(1−74)RR may lead to more pronounced conformational changes in extracellular loop 3 of CXCR4 and subsequently results in enhanced signaling. The reduced agonist activity of Ub(1−74)GGY could reflect that the bulky C-terminal extension constitutes a steric hindrance that prevents sufficient contact with the receptor domain surrounding Lys-271. It should be noted, however, that we did not determine the equilibrium dissociation constants for receptor binding for each mutant in saturation or competition binding experiments. We abstained from such extensive measurements because all mutants showed some degree of biological activity which could be blocked with AMD3100, and the effects of Ub(1−74)VV and Ub(1−74)RR could be antagonized with Ub(1−74), which provides indirect evidence for their binding to CXCR4. Furthermore, we showed previously that deletion of the C-terminal di-Gly as well as the di-Ala mutation does not influence receptor binding.[14] Nevertheless, we cannot exclude with certainty at this time that the C-terminal Tyr extension could impair the interaction between the ubiquitin surface domains surrounding Phe-4 and Val-70 and the receptor docking domain.[14] The observation that AMD3100 showed slightly weaker effects on Ub(1−74)VV induced reduction of cellular cAMP levels, as compared with all other mutants, appears insignificant as AMD3100 was able to fully neutralize its chemotactic activity.

Studies with mutated CXCR4, in which the intracellular loops were replaced by a modified intracellular loop 1 that is unable to transduce a signal, suggested that intracellular loop 3 is required for $G\alpha_i$ signaling in response to SDF-1α, whereas intracellular loop 2 is dispensable.[54] In contrast, intracellular loops 2/3 and the intracellular receptor C-terminus were required for SDF-1α mediated chemotaxis.[54] Thus, the discrepancy that Ub(1−74)VV and Ub(1−74)RR resulted in enhanced $G\alpha_i$ signaling, whereas Ub(1−74)RR alone showed increased chemotactic activity, suggests that activation of CXCR4 with Ub(1−74)VV enhances conformational changes only in intracellular loop 3, while CXCR4 activation with Ub(1−74)RR may also result in more pronounced conformational alterations of the other intracellular domains, when compared with wild type ubiquitin.

The findings that ubiquitin and SDF-1α show similar efficacy and potency to reduce cAMP levels, whereas the efficacy of ubiquitin to induce chemotaxis is lower than that of SDF-1α, is consistent with our previous reports.[12−14] In combination with the enhanced chemotactic response to Ub(1−74)RR in the present study, these data imply that native ubiquitin does not induce a full conformational switch of CXCR4 upon binding and thus may function as a partial agonist. Whereas our hypotheses on the possible mechanisms through which the generated mutants interact with CXCR4 remain to be tested, the findings of the present study demonstrate that charge and size modifications of the ubiquitin C-terminus modulate its ability to activate CXCR4.

In conclusion, our observations suggest that cleavage of ubiquitin by IDE could be of biological relevance. IDE could contribute to the fine-tuning of CXCR4 mediated cell functions through ligand processing and regulate termination of receptor signaling via generation of a CXCR4 antagonist. The mutational studies support the concept that the ubiquitin C-terminus regulates receptor activation and provide proof of principle that molecules with enhanced agonist activity on CXCR4 can be developed. As beneficial and therapeutically relevant effects of exogenous ubiquitin during inflammation have been reported in multiple animal models and species,[4−11] such molecules may lead to an engineered CXCR4 agonist with improved therapeutic properties.

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Notes

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■ ABBREVIATIONS

cAMP, 3′-5′-cyclic adenosine monophosphate; CXCR, CXC chemokine receptor; FITC, fluorescein isothiocyanate; IDE, insulin degrading enzyme (EC 3.4.24.56); SDF-1α, stromal cell-derived factor 1α

■ REFERENCES

(1) Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem. 67*, 425−479.

(2) Majetschak, M. (2011) Extracellular ubiquitin: immune modulator and endogenous opponent of damage-associated molecular pattern molecules. *J. Leukoc. Biol. 89*, 205−219.

(3) Majetschak, M., Krehmeier, U., Bardenheuer, M., Denz, C., Quintel, M., Voggenreiter, G., and Obertacke, U. (2003) Extracellular ubiquitin inhibits the TNF-alpha response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. *Blood 101*, 1882−1890.

(4) Baker, T. A., Romero, J., Bach, H. H. t., Strom, J. A., Gamelli, R. L., and Majetschak, M. (2012) Effects of exogenous ubiquitin in a polytrauma model with blunt chest trauma. *Crit. Care Med. 40*, 2376−2384.

(5) Earle, S. A., El-Haddad, A., Patel, M. B., Ruiz, P., Pham, S. M., and Majetschak, M. (2006) Prolongation of skin graft survival by exogenous ubiquitin. *Transplantation 82*, 1544−1546.

(6) Earle, S. A., Proctor, K. G., Patel, M. B., and Majetschak, M. (2005) Ubiquitin reduces fluid shifts after traumatic brain injury. *Surgery 138*, 431−438.

(7) Garcia-Covarrubias, L., Manning, E. W., 3rd, Sorell, L. T., Pham, S. M., and Majetschak, M. (2008) Ubiquitin enhances the Th2 cytokine response and attenuates ischemia-reperfusion injury in the lung. *Crit. Care Med. 36*, 979−982.

(8) Majetschak, M., Cohn, S. M., Nelson, J. A., Burton, E. H., Obertacke, U., and Proctor, K. G. (2004) Effects of exogenous ubiquitin in lethal endotoxemia. *Surgery 135*, 536−543.

(9) Majetschak, M., Cohn, S. M., Obertacke, U., and Proctor, K. G. (2004) Therapeutic potential of exogenous ubiquitin during resuscitation from severe trauma. *J. Trauma 56*, 991−999.

(10) Griebenow, M., Casalis, P., Woiciechowsky, C., Majetschak, M., and Thomale, U. W. (2007) Ubiquitin reduces contusion volume after controlled cortical impact injury in rats. *J. Neurotrauma 24*, 1529−1535.

(11) Ahn, H. C., Yoo, K. Y., Hwang, I. K., Cho, J. H., Lee, C. H., Choi, J. H., Li, H., Cho, B. R., Kim, Y. M., and Won, M. H. (2009) Ischemia-related changes in naive and mutant forms of ubiquitin and neuroprotective effects of ubiquitin in the hippocampus following experimental transient ischemic damage. *Exp. Neurol. 220*, 120−132.

(12) Saini, V., Marchese, A., and Majetschak, M. (2010) CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin. *J. Biol. Chem. 285*, 15566−15576.

(13) Saini, V., Staren, D. M., Ziarek, J. J., Nashaat, Z. N., Campbell, E. M., Volkman, B. F., Marchese, A., and Majetschak, M. (2011) The CXC Chemokine Receptor 4 Ligands Ubiquitin and Stromal Cell-derived Factor-1{alpha} Function through Distinct Receptor Interactions. *J. Biol. Chem. 286*, 33466−33477.

(14) Saini, V., Marchese, A., Tang, W. J., and Majetschak, M. (2011) Structural Determinants of Ubiquitin-CXC Chemokine Receptor 4 Interaction. *J. Biol. Chem. 286*, 44145−44152.

(15) Busillo, J. M., and Benovic, J. L. (2007) Regulation of CXCR4 signaling. *Biochim. Biophys. Acta 1768*, 952−963.

(16) Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature 382*, 635−638.

(17) Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature 393*, 591−594.

(18) Karin, N. (2010) The multiple faces of CXCL12 (SDF-1alpha) in the regulation of immunity during health and disease. *J. Leukoc. Biol. 88*, 463−473.

(19) Zaruba, M. M., and Franz, W. M. (2010) Role of the SDF-1-CXCR4 axis in stem cell-based therapies for ischemic cardiomyopathy. *Expert Opin. Biol. Ther. 10*, 321−335.

(20) Li, M., Yu, J., Li, Y., Li, D., Yan, D., and Ruan, Q. (2010) CXCR4+ progenitors derived from bone mesenchymal stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. *Cell Reprogram 12*, 405−415.

(21) Nagasawa, T., Tachibana, K., and Kishimoto, T. (1998) A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin. Immunol. 10*, 179−185.

(22) Veldkamp, C. T., Ziarek, J. J., Peterson, F. C., Chen, Y., and Volkman, B. F. (2010) Targeting SDF-1/CXCL12 with a ligand that prevents activation of CXCR4 through structure-based drug design. *J. Am. Chem. Soc. 132*, 7242−7243.

(23) Teicher, B. A., and Fricker, S. P. (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin. Cancer Res. 16*, 2927−2931.

(24) Wang, Y., Huang, J., Li, Y., and Yang, G. Y. (2012) Roles of chemokine CXCL12 and its receptors in ischemic stroke. *Curr. Drug Targets 13*, 166−172.

(25) Ralat, L. A., Kalas, V., Zheng, Z., Goldman, R. D., Sosnick, T. R., and Tang, W. J. (2011) Ubiquitin is a novel substrate for human insulin-degrading enzyme. *J. Mol. Biol. 406*, 454−466.

(26) Ren, M., Guo, Q., Guo, L., Lenz, M., Qian, F., Koenen, R. R., Xu, H., Schilling, A. B., Weber, C., Ye, R. D., Dinner, A. R., and Tang, W. J. (2010) Polymerization of MIP-1 chemokine (CCL3 and CCL4) and clearance of MIP-1 by insulin-degrading enzyme. *EMBO J. 29*, 3952−3966.

(27) Veldkamp, C. T., Seibert, C., Peterson, F. C., Sakmar, T. P., and Volkman, B. F. (2006) Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12). *J. Mol. Biol. 359*, 1400−1409.

(28) Slagsvold, T., Aasland, R., Hirano, S., Bache, K. G., Raiborg, C., Trambaiolo, D., Wakatsuki, S., and Stenmark, H. (2005) Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. *J. Biol. Chem. 280*, 19600−19606.

(29) Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J. 16*, 6996−7007.

(30) Gupta, S. K., Pillarisetti, K., Thomas, R. A., and Aiyar, N. (2001) Pharmacological evidence for complex and multiple site interaction of CXCR4 with SDF-1alpha: implications for development of selective CXCR4 antagonists. *Immunol. Lett. 78*, 29−34.

(31) Kofuku, Y., Yoshiura, C., Ueda, T., Terasawa, H., Hirai, T., Tominaga, S., Hirose, M., Maeda, Y., Takahashi, H., Terashima, Y., Matsushima, K., and Shimada, I. (2009) Structural basis of the interaction between chemokine stromal cell-derived factor-1/CXCL12 and its G-protein-coupled receptor CXCR4. *J. Biol. Chem. 284*, 35240−35250.

(32) Rosenkilde, M. M., Gerlach, L. O., Jakobsen, J. S., Skerlj, R. T., Bridger, G. J., and Schwartz, T. W. (2004) Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J. Biol. Chem. 279*, 3033−3041.

(33) Veldkamp, C. T., Seibert, C., Peterson, F. C., De la Cruz, N. B., Haugner, J. C., 3rd, Basnet, H., Sakmar, T. P., and Volkman, B. F.

(2008) Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. *Sci. Signal 1*, ra4.
(34) McQuibban, G. A., Butler, G. S., Gong, J. H., Bendall, L., Power, C., Clark-Lewis, I., and Overall, C. M. (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J. Biol. Chem. 276*, 43503−43508.
(35) McQuibban, G. A., Gong, J. H., Wong, J. P., Wallace, J. L., Clark-Lewis, I., and Overall, C. M. (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood 100*, 1160−1167.
(36) Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., and Opdenakker, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood 96*, 2673−2681.
(37) Wolf, M., Clark-Lewis, I., Buri, C., Langen, H., Lis, M., and Mazzucchelli, L. (2003) Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer. *Am. J. Pathol. 162*, 1183−1190.
(38) Schlesinger, D. H., Goldstein, G., and Niall, H. D. (1975) The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry 14*, 2214−2218.
(39) Schlesinger, D. H., and Goldstein, G. (1975) Molecular conservation of 74 amino acid sequence of ubiquitin between cattle and man. *Nature 255*, 42304.
(40) Wilkinson, K. D., and Audhya, T. K. (1981) Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly. *J. Biol. Chem. 256*, 9235−9241.
(41) Shen, Y., Joachimiak, A., Rosner, M. R., and Tang, W. J. (2006) Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature 443*, 870−874.
(42) Guo, Q., Manolopoulou, M., Bian, Y., Schilling, A. B., and Tang, W. J. (2010) Molecular basis for the recognition and cleavages of IGF-II, TGF-alpha, and amylin by human insulin-degrading enzyme. *J. Mol. Biol. 395*, 430−443.
(43) Ralat, L. A., Guo, Q., Ren, M., Funke, T., Dickey, D. M., Potter, L. R., and Tang, W. J. (2011) Insulin-degrading enzyme modulates the natriuretic peptide-mediated signaling response. *J. Biol. Chem. 286*, 4670−4679.
(44) Lynch, J. A., George, A. M., Eisenhauer, P. B., Conn, K., Gao, W., Carreras, I., Wells, J. M., McKee, A., Ullman, M. D., and Fine, R. E. (2006) Insulin degrading enzyme is localized predominantly at the cell surface of polarized and unpolarized human cerebrovascular endothelial cell cultures. *J. Neurosci. Res. 83*, 1262−1270.
(45) Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *J. Biol. Chem. 273*, 32730−32738.
(46) Saini, V., Romero, J., Marchese, A., and Majetschak, M. (2010) Ubiquitin receptor binding and signaling in primary human leukocytes. *Commun. Integr. Biol. 3*, 608−610.
(47) Bach, H. H., 4th, Saini, V., Baker, T. A., Tripathi, A., Gamelli, R. L., and Majetschak, M. (2012) Initial assessment of the role of CXC chemokine receptor 4 after polytrauma. *Mol. Med. 18*, 1056−1066.
(48) Rasmussen, S. G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, J., Weis, W. I., and Kobilka, B. K. (2011) Structure of a nanobody-stabilized active state of the $\beta(2)$ adrenoceptor. *Nature 469*, 175−180.
(49) Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature 477*, 549−555.
(50) Standfuss, J., Edwards, P. C., D'antona, A., Fransen, M., Xie, G., Oprian, D. D., and Schertler, G. F. X. (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature 471*, 656−660.
(51) Dyson, H. J., Wright, P. E., and Scheraga, H. A. (2006) The role of hydrophobic interactions in initiation and propagation of protein folding. *Proc. Natl. Acad. Sci. U. S. A. 103*, 13057−13061.
(52) Di Paolo, E., Vilardaga, J. P., Petry, H., Moguilevsky, N., Bollen, A., Robberecht, P., and Waelbroeck, M. (1999) Role of charged amino acids conserved in the vasoactive intestinal polypeptide/secretin family of receptors on the secretin receptor functionality. *Peptides 20*, 1187−1193.
(53) Peng, Z., Tang, Y., Luo, H., Jiang, F., Yang, J., Sun, L., and Li, J. D. (2011) Disease-causing mutation in PKR2 receptor reveals a critical role of positive charges in the second intracellular loop for G-protein coupling and receptor trafficking. *J. Biol. Chem. 286*, 16615−16622.
(54) Roland, J., Murphy, B. J., Ahr, B., Robert-Hebmann, V., Delauzun, V., Nye, K. E., Devaux, C., and Biard-Piechaczyk, M. (2003) Role of the intracellular domains of CXCR4 in SDF-1-mediated signaling. *Blood 101*, 399−406.