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Selective labelling of stromal cell-derived factor 1α with carboxyfluorescein to study receptor internalisation‡

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SDF1α plays an important role in the regeneration of injured tissue after ischemia or stroke by inducing the migration of progenitor cells. In order to study the function of this therapeutically relevant chemokine site-specific protein labelling is of great interest. However, modification of SDF1α is complicated because of its complex tertiary structure. Here, we describe the first site-specific fluorescent modification of SDF1α by EPL. We recombinantly expressed SDF1α (1–49) by intein-mediated protein expression. The *C*-terminal peptide SDF1α (50–68) was synthesised by SPPS and selectively labelled with carboxyfluorescein at Lys[56]. In a cell migration assay, M-[K[56](CF)]SDF1α showed a clear potency to induce chemotaxis of human T-cell leukaemia cells. Microscopic analysis on HEK293 cells transfected with the CXCR4 revealed specific binding of the fluorescent ligand. Furthermore, receptor-induced internalisation of the ligand could be visualised. These results show that site-specific modification of SDF1α yields in a biologically functional molecule that allows the characterisation of CXCR4 production of cells on a molecular level. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: stromal cell-derived factor 1α; SDF1α; chemokine; expressed protein ligation; internalisation; CXC receptor 4; fluorescence labelling

Introduction

Chemokines are small proteins that consist of 70–130 amino acids, show similar structural properties and bind to G-protein-coupled receptors. Most of them contain four cysteine residues that form two disulfide bridges. According to the position of the first two cysteine side chains, chemokines are classified into CXC, CC, C and CX_3C chemokines [1,2]. Usually, chemokines are responsible for receptor-induced cell migration by the formation of a gradient. For this purpose, they need to bind to glycosaminoglycans on cell surfaces or to the extracellular matrix [3,4]. In general, constitutively expressed chemokines that play a major role in organogenesis and cell homing [5] can be distinguished from chemokines that are expressed in response to inflammation and that induce an immune response [2]. SDF1α (or CXCL12), the ligand of the CXCR4 [6], displays an exception within this class of proteins, since it is responsible for the formation of the bone marrow during embryogenesis and the homing of bone marrow cells during adult life [7] on the one hand, and on the other hand it is also expressed during inflammation or after ischemia and stroke [8–10]. So it is mainly involved in the formation and migration of neuronal progenitor cells and neuroblasts, resulting in the regeneration of the damaged or injured tissue. Accordingly, SDF1α constitutes an interesting molecule for therapeutic engineering. In this approach, we aimed to investigate the possibility of selectively modifying SDF1α. Due to the complex structure of SDF1α consisting of a flexible *N*-terminus and a three-stranded β-sheet core overlaid by a *C*-terminal α-helix and stabilised by two disulfide bridges (Figure 1) [11,12], selective modification is challenging. Here, we chose the approach of EPL [13–15] by using an *N*-terminal recombinantly expressed thioester and a *C*-terminus carrying an *N*-terminal cysteine residue and the specific modification. The sulfhydryl group of the cysteine then attacks the thioester leading to a transthioesterification followed by an S-N-

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Abbreviations used: *ACN, acetonitril; CF, (5,6)carboxyfluorescein; CXCL, CXC ligand; CXCR, CXC receptor; DIC, N,N′-diisopropylcarbodiimide; DMEM, Dulbecco's minimal essential medium; DMF, N, N′-dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; EPL, expressed protein ligation; ESI-FTICR MS, electrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry; FCS, fetal calf serum; HATU, O-(7-azabenzotriazol-1-yl)-N, N, N′, N′-tetramethyluronium hexafluorophosphate; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; IMPACT, intein-mediated purification with a affinity chitin binding tag; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria Bertani; MALDI-TOFTOF-MS, matrix assisted laser desorption ionisation time of flight time of flight mass spectrometry; MEM, minimun essential medium; MESNa, mercaptoethane sulfonic acid sodium salt; Mxe, mycobacterium xenopi; NaSPh, sodium thiophenolate; NCL, native chemical ligation; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl; PMSF, phenylmethanesulfonyl fluoride; SDF, stromal cell-derived factor; SPPS, solid phase peptide synthesis; tBu, tert-butyl; tBuO, tert-butoxy; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.*

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Figure 1. Sequence of human stromal cell-derived factor SDF1α.

acyl shift that yields the intact peptide bond. For this purpose, we used the IMPACT™ system (intein-mediated purification with an affinity chitin-binding tag) [16] that provides the possibility to generate a reactive thioester recombinantly. The *C*-terminus was synthesised by SPPS yielding in a peptide that is site-specifically modified. As an initial modification we chose CF to investigate selective binding, to visualise receptor-expressing cells and to follow receptor-mediated internalisation.

Experimental

Materials

For automated SPPS, N^{α}-Fmoc-protected amino acids and N^{α}-Boc-protected cysteine as well as HOBT and the 4-benzyloxybenzyl alcohol polystyrene resin (Wang resin) coupled to Fmoc-Lys(Boc) were purchased from Novabiochem (Schwalbach, Germany). DIC was obtained from Sigma–Aldrich (Taufkirchen, Germany). Protection groups were *t*BuO for aspartate, Boc or Dde for lysine, *t*Bu for threonine and serine, Pbf for arginine, and trityl for asparagine and histidine. All amino acids were purchased from Novabiochem or Iris Biotech GmbH (Markneukirchen, Germany). Ampicillin, L-arginine, HATU, *t*Bu alcohol, CF, L-cystine, DIPEA, DTT, 1,2-ethanedithiol, EDTA concentrate, hydrazine hydrate solution, Luria Bertani (LB) medium, MESNa, magnesium chloride ($MgCl_2$), PMSF, piperidine, sodium chloride (NaCl), NaSPh, thioanisole, TFA, TCEP and urea were from Fluka (Taufkirchen, Germany). ACN was from VWR International (Darmstadt, Germany). Diethyl ether and DMF, and ethanole were obtained from Biosolve (Valkenswaard, The Netherlands). Agarose was obtained from Invitrogen (Karlsruhe, Germany). Materials for cloning, DNase I and IPTG were from Fermentas (St. Leon-Roth, Germany). Glycerol and Tris were purchased from Roth (Karlsruhe, Germany). Chitin beads and the vector pTXB1 were from New England Biolabs (Frankfurt, Germany). L-cysteine was from SERVA Feinbiochemica (Heidelberg, Germany). All materials for tissue culture were supplied by PAA Laboratories GmbH (Pasching, Austria). Hoechst 33 342 was from Roche Applied Science (Mannheim, Germany).

DNA Constructs

The cDNAs encoding the human M-SDF1α (1–49) and M-SDF1α were cloned into the pTXB1 vector using the *Nde*I and *Sap*I restriction sites. Correct insertion was verified by sequencing of the entire coding region. Methionine (M) was added at the *N*-terminus to allow bacterial expression.

Peptide Synthesis of [K^{56}(CF)]SDF1α (50–68)

The peptide was synthesised on a Wang resin with a manual coupling of Fmoc-Lys(Boc)-OH before automated multiple SPPS (Syro; MultiSynTech, Bochum, Germany) using the Fmoc/*t*Bu strategy. The Fmoc-protected amino acids were introduced in tenfold excess by double-coupling procedures (twice for 40 min) by using *in situ* activation with DIC and HOBT. The Fmoc removal was carried out with 40% (v/v) piperidine in DMF for 3 min and 20% (v/v) piperidine for 10 min. Fmoc-Lys(Dde)-OH on position 56 was introduced by manual coupling by using a fivefold excess of N^{α}-Fmoc-protected amino acid and *in situ* activation with 5 eq DIC and 5 eq HOBT. The Dde protection group was removed by washing 10 times for 10 min with 2% (v/v) hydrazine in DMF. Success of removal was examined by measuring the absorption of the washing solution at 301 nm.

CF was coupled by using 1.5 eq of CF, HATU and DIPEA in DMF. Overall coupling time was 1 h. CF polymers were cleaved by applying a solution of 20% (v/v) piperidine in DMF for 40 min. Hydroxyl groups of CF were then trityl protected by using 4 eq of trityl chloride and DIPEA in DCM for 16 h [17].

The peptide was then cleaved with a mixture of TFA/thioanisole/ethanedithiole [90 : 3 : 7 (v/v/v)] within 3 h. The peptide was precipitated from cold diethyl ether, collected by centrifugation and lyophilised from water/*t*Bu alcohol [3 : 1 (v/v)].

Purification of the peptides was achieved by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Phenomenex Jupiter 10 U Proteo column (250 × 21.20 mm, 90 Å, Aschaffenburg, Germany) by using a linear gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN] of 20–60% of B in 40 min with a flow rate of 10 ml/min. Identification was performed by MALDI-TOFTOF-MS (Ultraflex III MALDI-TOFTOF; Bruker Daltonics, Bremen, Germany) and purity was confirmed by analytical RP-HPLC on a Vydac RP18-column (4.6 × 250 mm; 5 µm/300 Å; Grace, Deerfield, IL, USA) using a linear gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN] of 10–60% of B in 30 min with a flow rate of 0.6 ml/min.

Recombinant Expression of M-SDF1α (1–49) and M-SDF1α

50 ng of plasmid DNA M-SDF1α (1–49)_pTXB1 and M-SDF1α was transformed into *Escherichia coli* ER2566. The bacteria were plated on agarose plates containing 100 µg/ml ampicillin and grown at 37 °C for 16 h. For expression, a clone was picked and precultured in 100 ml LB medium containing 100 µg/ml ampicillin at 37 °C overnight. Glycerol stocks of the cells were prepared by the addition of 150 µl glycerol to 850 µl of cell suspension. Stocks were kept at −70 °C. Cells of the preculture were then centrifuged at 5000*g* for 10 min and resuspended in 10 ml LB medium. For main cultures, cell suspension of the preculture was added to LB medium containing 100 µg/ml ampicillin until the $OD_{600\ nm}$ was 0.1. Cells were grown at 37 °C until an $OD_{600\ nm}$ of 0.6. Then IPTG was added to a final concentration of 1 mM, and the cells were left at 37 °C for at least 6 h. Cultures were centrifuged at 10, 000*g* and 4 °C for 10 min. Next, cells were lysed by a French Pressure Cell after resuspension in standard buffer (20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 8.0) and addition of 250 µM PMSF. DNA was digested by addition of 3 mM $MgCl_2$, 125 µM PMSF and 1 U DNase I and by shaking for 1 h at room temperature. Suspensions were centrifuged at 4 °C for 30 min at 15, 000*g*. Pellets were washed with washing buffer 1 [standard buffer with 2 M urea and 1%

(v/v) Tween 20], washing buffer 2 [standard buffer with 2 M urea and 2% (v/v) Tween 20] and washing buffer 3 (standard buffer with 2 M urea). Protein in inclusion bodies was solubilised by resuspension of the pellets in standard buffer containing 8 M urea and by shaking at 4 °C. Solubilisates were centrifuged at 15,000*g* at 4 °C for 30 min. The supernatant was diluted with standard buffer containing 200 mM L-arginine to a final urea concentration of 1.5 M. The solution was passed twice over chitin beads with a bed volume of 10 ml pre-equilibrated with column buffer (1.5 M urea, 20 mM Tris, 500 mM NaCl, 200 mM L-arginine, 1 mM EDTA, pH 8.0). The column was washed with column buffer containing 0.1% Tween 20 and pure column buffer. Cleavage of the final protein was induced by the addition of 20 ml of a 200 mM MESNa solution in column buffer, pH 8.5. The column was left at room temperature for 24 h. The MESNa fraction was then collected and protein content was determined by a Bradford assay. Identification was performed by MALDI-TOFTOF-MS (Ultraflex III MALDI-TOFTOF; Bruker Daltonics) and purity was confirmed by analytical RP-HPLC on a Vydac RP18-column (4.6 × 250 mm; 5 µm/300 Å; Deerfield) using a linear gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN] of 20–70% of B in 40 min with a flow rate of 0.6 ml/min.

M-SDF1α was prepared by the same method. The fusion protein was immobilised on the chitin column and was cleaved by the addition of 0.1 M DTT for 48 h at room temperature. The gained thioester was hydrolysed by increasing the pH to 9.5 and incubation at 4 °C for 16 h. After adjusting the pH to 8.0 again, the product was refolded by dialysis as described in the following section.

Ligation of M-SDF1α (1–49)_MESNa and [K^{56}(CF)]SDF1α (50–68)

Urea was added to the M-SDF1α (1–49)_MESNa solution to a final concentration of 3 M. [K^{56}(CF)]SDF1α (50–68) was used in a fivefold excess and dissolved in a TCEP solution in water adjusted to pH 7 (final TCEP concentration 2.5 mM). NaSPh was added to the solution yielding in a final concentration of 75 mM. Dissolved [K^{56}(CF)]SDF1α (50–68) solution was then added to the M-SDF1α (1–49)_MESNa solution. pH was adjusted to 8.5 and the mixture was incubated at room temperature for at least 24 h. The ligation reaction was dialysed against 3 M urea in dialysis buffer (20 mM Tris, 500 mM NaCl, 200 mM L-arginine, 1 mM EDTA, 10 mM L-cysteine and 1 mM L-cystine, pH 8.0), 1.5 M urea in dialysis buffer, dialysis buffer and dialysis buffer without L-cysteine and L-cystine. Finally, the dialysed product was purified by semipreparative RP-HPLC on a Phenomenex Jupiter 5u C18 column (250 × 10 mm, 300 Å) using a linear gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN] of 20–60% of B in 50 min with a flow rate of 4 ml/min. The product was analysed by analytical RP-HPLC using a linear gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN] of 20–70% of B in 40 min with a flow rate of 0.6 ml/min, by MALDI-TOFTOF-MS and ESI-FTICR MS (Bruker Daltonics).

Cell Migration Assay

Induction of chemotaxis was investigated in tissue culture plates with transwell inserts that allow free migration of cells. The human T-cell leukaemia cell line Jurkat, a suspension cell line that endogenously expresses CXCR4, was applied. Cells were cultured in RPMI 1640 medium with 10% (v/v) heat-inactivated FCS. For migration assays, peptide dilutions in a range of 10^{-9} to 10^{-7} M in RPMI 1640 medium with 1% (w/v) bovine serum albumin were prepared. Seven hundred microlitres of each peptide dilution was pipetted into a well of a 24-well cell culture plate; 200 µl of a Jurkat cell suspension in RPMI 1640 with 1% (w/v) bovine serum albumin, concentrated to 6 Mio cells/ml, was pipetted into Maxicell cell culture inserts (TPP, Trasadingen, Switzerland) with a pore size of 8 µm. Each insert was immersed into one well with a peptide dilution and incubated for 2 h at 37 °C and 5% CO_2. Then, inserts were removed and cells in the peptide solutions were counted with a cell counter and analyser system (CASY® model TTC, Schärfe System GmbH). Analysis by CASY distinguishes living cells from dead cells or cell debris by size differentiation. Since only living cells are able to migrate and in order to ensure that only the migrated cells are counted, solely the number of viable cells was taken into account for analysis. All data were obtained as duplicates and analysed by Graphpad Prism.

Microscopic Studies

Specificity and internalisation studies were performed on HEK293 cells transfected with the CXCR4 receptor and stimulated with the indicated ligand concentrations. HEK293 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 without L-glutamine containing 15% (v/v) heat-inactivated FCS. For microscopic studies, cells were seeded into sterile μ-slides eight-well plates (ibidi GmbH, Martinsried/Munich, Germany) and transfected with 1 µg CXCR4_pVitro2 and CXCR4_EYFP_pVitro2 or no DNA and 1 µl lipofectamin 2000 in OPTI®-MEM I Reduced Serum Medium. After successful transfection, cells were starved for 20 min in OPTI®-MEM I Reduced Serum Medium. In order to visualise nuclei, 0.5 µl of 1 µg/ml Hoechst 33 342 was added and incubated for additional 20 min. Next, medium was removed, and 100 µl of a 1 µM solution of M-[K^{56}(CF)]SDF1α and, where indicated, of 10 µM M-SDF1α were added and incubated for 5 min in case of specificity studies and for indicated time points in case of internalisation studies. The solution was removed, and the cells were washed once with 200 µl HBSS. For microscopic studies, cells were kept in 100 µl OPTI® -MEM I Reduced Serum Medium. ApoTome Imaging System with an Axio Observer microscope fitted with a Plan-Apochromat 63×/1.40 Oil DIC objective and an AxioCam MRmcamera (Zeiss, Jena, Germany) was used. The fluorescence was detected using filter set 38 (green). Apotome images were taken at 512 × 512 resolution with 10× optical zoom using the AxioVision software (Release 4.6.3) at room temperature. The final composite image was also created using the AxioVision software (Release 4.6.3) [18].

Results

Recombinant Expression of M-SDF1α (1–49)_MESNa and Peptide Synthesis of [K^{56}(CF)]SDF1α (50–68)

Usually, human SDF1α is expressed biosynthetically with an *N*-terminal signalling sequence that contains 21 amino acids. Since we only used the processed form of SDF1α, we started amino acid numbering of the sequence with the first amino acid of the mature SDF1α, lysine1, and only left the methionine residue for expression. Accordingly, this led to the abbreviation M-SDF1α (1–49) or M-SDF1α, respectively. Mass spectrometry of the expression products revealed masses that are increased by 130.5 Da compared with the calculated mass of SDF1α or SDF1α (1–49)_MESNa, which is consistent with the *N*-terminally extended methionine residue. This is necessary for the bacterial expression that uses the

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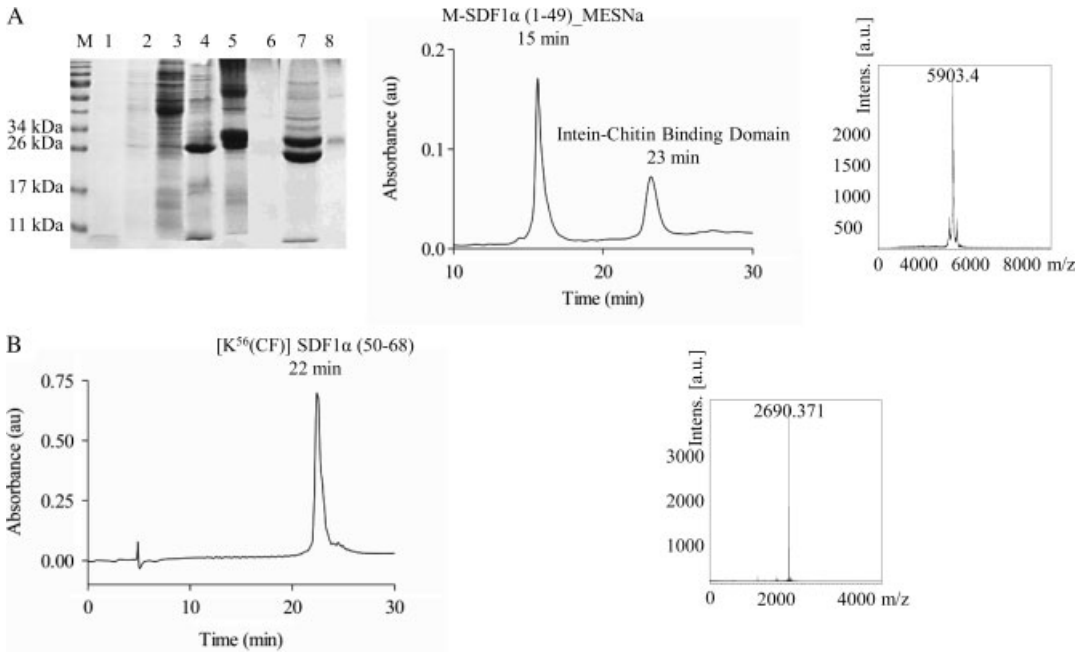

Figure 2. Analytical data of (A) M-SDF1α (1–49)_MESNa and (B) [K[56](CF)]SDF1α (50–68). (A) SDS-PAGE of protein expression, RP-HPLC {gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN]: 20–70% of B in 40 min} and MALDI-TOFTOF mass spectrum of M-SDF1α (1–49)_MESNa. Lane M, Fermentas protein standard; lane 1, main culture before IPTG induction; lane 2, main culture 6 h after IPTG induction; lane 3, lysis supernatant; lane 4, lysis pellet; lane 5, solubilised protein; lane 6, flow through after column loading; lane 7, loaded chitin beads; lane 8, chitin beads after cleavage. (B) RP-HPLC {gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN]: 10–60% of B in 30 min} and MALDI-TOFTOF mass spectrum of [K[56](CF)]SDF1α (50–68).

codon of methionine as a starting signal. Sometimes methionine is cleaved off enzymatically after synthesis; however, since we used inclusion bodies to obtain the products, methionine cleavage cannot occur. In functional studies, however, it could be verified that this methionine does not influence the functionality of the molecules (data not shown). Therefore, no artificial methionine cleavage was applied, and the expression products are referred to as M-SDF1α and M-SDF1α (1–49)_MESNa.

In order to gain a reactive thioester at position 49 of the SDF1α molecule, we applied the IMPACT™ system from New England Biolabs. The M-SDF1α (1–49) fragment was cloned at the *N*-terminus of an *Mxe* intein that was *C*-terminally fused to a chitin-binding domain (CBD). The M-SDF1α (1–49)-intein-CBD fusion protein could be purified by binding to a chitin column. Cleavage from the column then was induced by addition of a thiol, which mimicked the actions of the *C*-terminal cysteine of the *Mxe* intein that had been removed. This led to the formation of a thioester bond at the *C*-terminus of M-SDF1α (1–49). Expression and purification of the thioester M-SDF1α (1–49)_MESNa are illustrated in Figure 2A. The fusion protein has a size of 27 kDa with the construct of intein and CBD at 21 kDa. Lane 2 indicates expression of the fusion protein by a weak band at 27 kDa. After lysis, the whole protein was sedimented in the lysis pellet (lane 4) and needed to be solubilised by the application of 8 M urea. Solubilised protein is shown in lane 5. Lane 6 shows the flow through after column loading. No band could be detected which indicates a full loading of the chitin beads. This could be further demonstrated in lane 7 which shows the loaded chitin beads. Herein, the band at 27 kDa represents the fusion protein, while the bands at 21 and 11 kDa demonstrate intein CBD and the thioester, respectively. After cleavage, only a small amount of fusion protein could be detected on the column (lane 8). The HPLC chromatogram showed two peaks at 15 min which represented M-SDF1α (1–49)_MESNa and at 23 min, indicating intein CBD (Figure 2A). The MALDI-TOFTOF mass spectrum revealed a mass of *m/z* 5903.4 Da $[M+H]^+$ (calc. *m/z* 5904.1 Da $[M+H]^+$). The protein content of the final solution after cleavage was determined to be 10 μM (Figure 2A). Peptide synthesis of [K[56](CF)]SDF1α (50–68) provided a >95% pure peptide with a mass of *m/z* 2690.37 Da $[M+H]^+$ (calc. *m/z* 2689.31 Da $[M+H]^+$) (Figure 2B). These results indicate that both fragments could be obtained in sufficient yield and purities.

EPL of M-SDF1α (1–49)_MESNa and [K[56](CF)]SDF1α (50–68)

The peptides were dissolved in a ratio of 1:5 for M-SDF1α (1–49)_MESNa and [K[56](CF)]SDF1α (50–68), respectively. In order to keep the protein in solution, ligation was carried out in a 3 M urea buffer. pH was adjusted to 8.5, since ligation appeared to be most effective at these conditions. TCEP was added as a reducing agent in order to prevent oligomerisation of the *C*-terminal peptide. The thioester was eluted for 15 min and [K[56](CF)]SDF1α (50–68) for 21 min under the chosen HPLC conditions. Already after 24 h of ligation at room temperature, a new peak could be detected at 22 min, indicating the product, as concluded from the RP-HPLC in Figure 3A. Mass spectrometry revealed a mixture of the dimer of [K[56](CF)]SDF1α (50–68) and the product. Since SDF1α contains two disulfide bridges, the product needed to be refolded and oxidised after successful ligation. This was achieved by the application of cysteine and cystine as a redox system. The retention time of the refolded product shifted on the HPLC from 21 to 19 min (Figure 3B) and yielded 15%. MALDI-TOFTOF-MS showed a pure product at the expected average mass of *m/z* 8448.2 Da $[M+H]^+$ (calc. *m/z* 8448.2 Da $[M+H]^+$) (Figure 3C). Purity was also confirmed by RP-HPLC to be

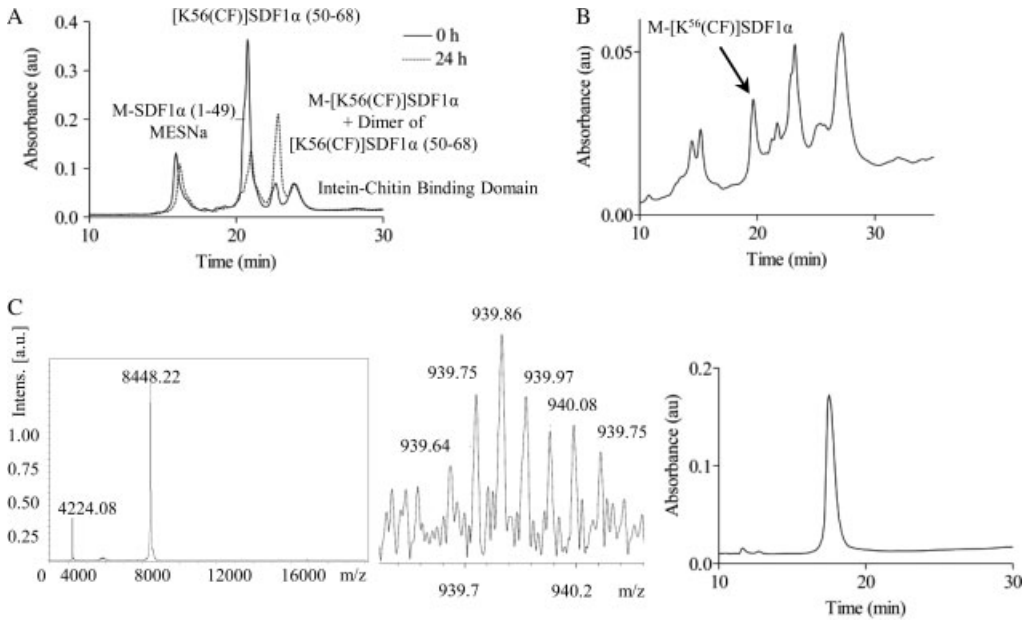

Figure 3. Ligation and refolding of M-[K^{56}(CF)]SDF1α. (A) RP-HPLC analysis {gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN]: 20–70% of B in 40 min} of ligation mixture after 0 and 24 h. (B) RP-HPLC analysis {gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN]: 20–70% of B in 40 min} of ligation mixture after refolding. (C) MALDI-TOFTOF mass spectrum (left), ESI-FTICR mass spectrum (middle) and RP-HPLC (right) {gradient A [0.1% (v/v) TFA in water] → B [0.08% (v/v) in ACN]: 20–70% of B in 40 min} of refolded M-[K^{56}(CF)]SDF1α.

>95% (Figure 3C). High-resolution mass spectrometry (ESI-FTICR) confirmed the correct formation of the disulfide bonds with a signal at *m/z* 939.86 $[M+9H]^{9+}$ (calc. *m/z* 8448.22 Da $[M+H]^{+}$) (Figure 3C).

Cell Migration Assay

Cell migration was performed with Jurkat cells, a human T-cell leukaemia cell line that grows in suspension and that endogenously expresses CXCR4. Migration was induced by several concentrations of M-[K^{56}(CF)]SDF1α or M-SDF1α as a positive control. For analysis, only viable cells were counted since migration is only performed by living cells. We found that M-[K^{56}(CF)]SDF1α significantly is not as potent as M-SDF1α, but still is able to activate the receptor CXCR4 and induce cell migration (Figure 4). While M-SDF1α leads to the migration of about 60% of the viable cells at the highest concentration of 1 µM, M-[K^{56}(CF)]SDF1α still activates about 25%.

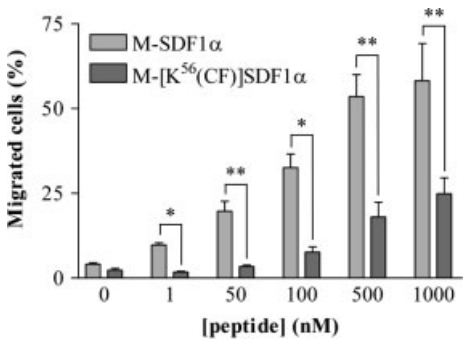

Figure 4. Biological activity of M-SDF1α and M-[K^{56}(CF)]SDF1α. Cell migration assay of M-SDF1α and M-[K^{56}(CF)]SDF1α with Jurkat cells was performed with transwell inserts with a pore size of 8 µm. The results are shown as mean ± standard deviation of three independent experiments each performed in duplicate. $^{*}p < 0.01$, $^{**}p < 0.001$ *versus* control.

Microscopic Studies of M-[K^{56}(CF)]SDF1α

In order to investigate the specificity of binding and internalisation, we transfected HEK293 cells with the CXCR4_pVitro2 vector, added 1 µM of M-[K^{56}(CF)]SDF1α and incubated the cells for several time points. For specificity studies, the ligand was removed after 5 min, while for internalisation the reaction was stopped immediately, after 30 or 90 min, respectively.

The results of the specificity studies are shown in Figure 5A. On the left side of Figure 5A, clearly fluorescence is visible around a blue cell nucleus, indicating binding to a living cell. In the middle part of Figure 5A, microscopy was performed with 1 µM M-[K^{56}(CF)]SDF1α and a tenfold excess of non-labelled M-SDF1α. Since there is no fluorescence detectable, it can be concluded that M-SDF1α efficiently displaces M-[K^{56}(CF)]SDF1α, which indicates specific binding. On the right side of Figure 5A, the same assay is performed as on the left side, but with cells that have not been transfected with CXCR4. Here again, no fluorescence can be detected, emphasising the specific binding of M-[K^{56}(CF)]SDF1α to CXCR4 on the cell surface.

In Figure 5B, the results of the internalisation studies are illustrated. While at the beginning and after 30 min, a lot of the fluorescence is found near the membrane, after 90 min the ligand is completely located in the cytoplasm, indicating that the ligand is internalised upon receptor binding.

Discussion

Modification of polypeptides larger than 50 amino acids implies several difficulties. Usually, peptide synthesis in this size is not easily feasible because of long sequences and a loss of product, and requires specific protocols. Recombinant expres-

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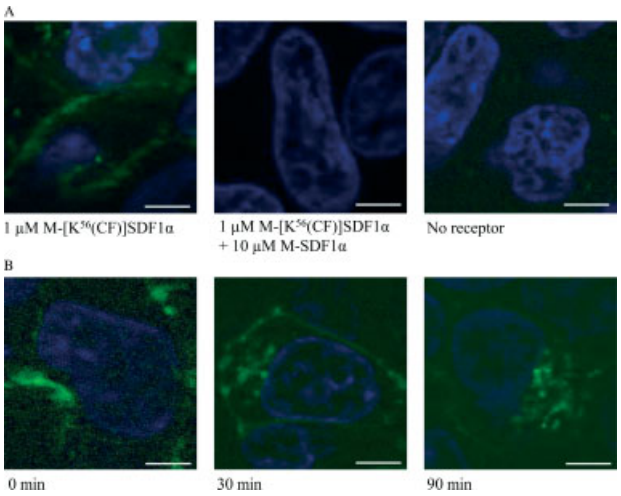

Figure 5. Microscopic studies of M-[K^{56}(CF)]SDF1α on HEK293 cells transfected with the CXCR4_pVitro2 vector. (A) Specificity of binding. Left, HEK293 cells transfected with CXCR4_pVitro2 and stimulated with 1 μM M-[K^{56}(CF)]SDF1α; middle, HEK293 cells transfected with CXCR4_pVitro2 and stimulated with 1 μM M-[K^{56}(CF)]SDF1α and 10 μM M-SDF1α; right, untransfected HEK293 cells stimulated with 1 μM M [K^{56}(CF)]SDF1α. (B) Internalisation of M-[K^{56}(CF)]SDF1α: left, 0 min; middle, 30 min; right, 90 min. Scale bar = 5 μm.

sion, however, usually prevents site-specific modification with unnatural substances. With the NCL approach [14] and its further development of EPL [15], protein chemistry can circumvent these problems. This has been shown to work perfectly for other chemokines, e.g. interleukin 8 (IL-8) [19,20]. However, SDF1α shows only 27% sequence identity to other CXC chemokines [11] due to its early development during evolution [21] which questions the synthetic pathway of CF-IL-8 to be directly transferable to SDF1α.

One of the most critical issues is the position, at which the modification should be introduced. Since carboxyfluorescein is functionalised as a carboxy acid, amino side chains are of interest. SDF1α contains a number of lysine residues. Lysine at position 1 is known to be crucial for binding to the receptor as well as for receptor activation [22,23]. Modification of amino groups in the middle of the protein might disturb the formation of the β-sheet structure [11]. Therefore, we approached the amphiphillic α-helix at the *C*-terminus for modification, believing that this would not interfere too much with the overall structure of the protein. Here, lysine residues at positions 54, 56, 64 and 68 were of interest. As an initial approach, we chose position 56. Peptide synthesis of the *C*-terminus occurred without any problems, while the production of the *N*-terminus yielded in the thioester and the intein CBD. This is due to the fact that loading of the chitin column as well as cleavage had to be performed under slightly denaturating conditions in order to keep the protein in solution. This partly led to a misfolding of the CBD and therefore to a loss of binding affinity to the chitin beads.

While the ligation of the fragments occurred rather rapidly, refolding and formation of the disulfide bridges happened to be the most critical step during the whole synthesis procedure. Since SDF1α showed a high tendency to form aggregates and to precipitate as soon as misfolding occurred, we chose L-arginine as a dissolving agent [24]. Only partial success was obtained, since still some precipitation occurred, which led to the rather low yield of the reaction. However, the disulfide bridges as well as the three-dimensional structure at least partly could be formed and yielded in soluble protein. In the migration assay, we could confirm that the fluorescently modified variant M-[K^{56}(CF)]SDF1α was still able to induce cell migration, although to a less extent than M-SDF1α. Cell migration upon binding to CXCR4 is triggered by several signalling pathways within the cell. One way is the activation of mitogen-activated protein kinase, which occurs either after dissociation of the G-protein trimer from the receptor or after arrestin binding to the receptor. Other pathways include the formation of inositol 3-phosphate and calcium mobilisation [25]. Therefore, the migration assay provided the best and most natural assay with respect to receptor activation by SDF1α. The question, however, remained why M-[K^{56}(CF)]SDF1α is not as potent as M-SDF1α. Since the control M-SDF1α also was prepared recombinantly and refolding occurred under the same conditions as for the ligation product, activity loss due to wrong refolding can be excluded. In 2004, Cai *et al*. succeeded in the production of a full CXCR4 antagonist by eliminating residues 55–68, which suggests that the *C*-terminal α-helix plays a critical role especially in receptor activation [26,27]. Obviously, modification at position 56 could eliminate a crucial positive charge required for an effective signalling or the carboxyfluorescein modification simply is too voluminous and blocks critical interaction sites with the receptor. Furthermore, an additional negative charge is introduced by carboxyfluorescein which could lead to unfavorable side chain interactions. Therefore, modification of lysine side chains approaching the *C*-terminus like Lys^{64} or Lys^{68} could be preferable in the future.

However, specific binding of M-[K^{56}(CF)]SDF1α to its receptor could be demonstrated by fluorescence microscopy on transfected HEK293 cells. Here, M-SDF1α could efficiently displace the initial fluorescence caused by binding of M-[K^{56}(CF)]SDF1α to the cells. Furthermore, internalisation of the ligand together with its receptor could be observed. Internalisation of CXCR4 usually happens after ligand attachment upon binding of arrestin followed by endocytosis and resulting in desensitisation of the receptor [28,29]. In our studies, the whole fluorescence could be found in the cytoplasm after 90 min, confirming a complete receptor endocytosis.

In conclusion, we worked out a method for the site-specific modification of the chemokine SDF1α by a semisynthetic approach. The obtained protein shows biological functionality, which confirms its correctly folded state. This study opens the way to new modification of the chemokine with other moieties like PEGylation yielding in therapeutically interesting molecules with higher metabolic stability.

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