Identification of Allosteric Peptide Agonists of CXCR4*

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The chemokine receptor CXCR4 is a co-receptor for T-tropic strains of HIV-1. A number of small molecule antagonists of CXCR4 are in development but all are likely to lead to adverse effects due to the physiological function of CXCR4. To prevent these complications, allosteric agonists may be therapeutically useful as adjuvant therapy in combination with small molecule antagonists. A synthetic cDNA library coding for 160,000 different SDF-based peptides was screened for CXCR4 agonist activity in a yeast strain expressing a functional receptor. Peptides that activated CXCR4 in an autocrine manner induced colony formation. Two peptides, designated RSVM and ASLW, were identified as novel agonists that are insensitive to the CXCR4 antagonist AMD3100. In chemotaxis assays using the acute lymphoblastic leukemia cell line CCRF-CEM, RSVM behaves as a partial agonist and ASLW as a superagonist. The superagonist activity of ASLW may be related to its inability to induce receptor internalization. In CCRF-CEM cells, the two peptides are also not inhibited by another CXCR4 antagonist, T140, or the neutralizing monoclonal antibodies 12G5 and 44717.111. These results suggest that alternative agonist-binding sites are present on CXCR4 that could be screened to develop molecules for therapeutic use.

CXCR4 is a member of the chemokine receptor family of G protein-coupled receptors. A number of mechanisms for receptor activation of G protein-coupled receptors have been proposed (1, 2). For example, small molecule agonists bind within the transmembrane helices and cause activation. Larger molecules bind to specific sites on the extracellular surface of the receptor leading to a conformational change that is transmitted to an intracellular G protein complex. This results in an exchange of GTP for GDP in the G protein, dissociation of G protein from the G protein complex, and activation of downstream signal transduction pathways. Competitive receptor antagonists bind to the same or overlapping agonist site and prevent the physiological agonist from activating the receptor. In principle, allosteric modulators that bind to different sites on G protein-coupled receptors should have no effect on activity except in the presence of receptor agonists or antagonists (3). Allosteric agonists or antagonists could also bind at different sites and induce biological activity.

The first 7 or 8 N-terminal residues of SDF-1α that precede the first of four cysteines, which define the chemokine superfamily, are not evident in the three-dimensional structure (8, 9). In virtually all chemokine structures, the N-terminal sequence preceding the first cysteine is not observable in either NMR or X-ray structures and is presumed to be flexible. The flexibility of the N-terminal sequence in over a dozen known chemokine structures suggests that this mobility is important for the biological function of these proteins (10). Mutation or deletion of the residues at the N-terminal sequence of chemokines usually leads to a functional change from receptor agonism to antagonism (11, 12). Despite a large number of studies on chemokine structure and function, how these proteins interact with their G protein-coupled receptors remains to be elucidated. A two-step process has been proposed involving the binding of a chemokine to its receptor followed by placement of the N-terminal flexible loop of the chemokine on another site of the receptor, leading to its activation. In this model, the N-loop, consisting of the first 20–30 residues of chemokines, provides most of the specificity for the receptor. For SDF-1α, N-terminal peptides of 9–17 amino acids are weak agonists of CXCR4 (13, 57).

A number of mammalian receptors have been successfully expressed in Saccharomyces cerevisiae and shown to couple to the native or modified versions of G proteins leading to activation of the pheromone response pathway (14–22). To identify allosteric agonists or modulators of CXCR4 activity, we adapted a yeast expression system that produces functional

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1 The abbreviations used are: HIV-1, human immunodeficiency virus; FITC, fluorescein isothiocyanate.
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CXC4. *S. cerevisiae* is known to possess one of two G protein-coupled receptors, Ste2p and Ste3p, for each haploid cell type. In yeast Gßi activates a mitogen-activated protein kinase pathway resulting in transcription of genes containing a pheronome-responsive element in their promoter, growth arrest, and mating of the two different haploid yeast strains. We describe a genetically modified yeast strain that allowed us to identify variant Gßi and allosteric agonists. We focus on the characterization of two peptide agonists, designated RSVM and ASLW, that are resistant to the small molecule CXCR4 antagonists AMD3100 and T140 and the neutralizing antibodies ASLW, that are resistant to the small molecule CXCR4 antagonists AMD3100 and T140 and the neutralizing antibodies ASLW.

Materials and Methods

Vector Construction and Strains—The initial *S. cerevisiae* strain was CY12946 (MATa Fus1p his3 i2(5) GPA1GAP1125) and CY1141, reported in Klein et al. (18), with two differences. First, the two strains express different Gßi subunits. Specifically, CY12946 expresses a chimeric Gßi subunit (GAP1GAP1125) in which the C-terminal 5 amino acids of the yeast Gßi subunit, GPA1, are replaced by the C-terminal 5 residues of Gßi. In contrast, as described by Klein et al. (18), CY1141 expresses a chimeric Gßi subunit (GAP1125, Gßi) in which the N-terminal 33 residues of Gßi are replaced by the 41 N-terminal residues of GPA1. We have used CY12946 in the current report because GPA1GAP1125 is more efficient than GPA1125 in coupling CXCR4 to the phenornone response pathway. The use of GPA1GAP1125 necessitated deletion of SST2, which down-regulates the pheronome response pathway by accelerating the GTPase activity of GPA1. Thus, the second difference between CY12946 and CY1141 is the presence of an sst2 deletion allele in CY12946. All plasmids were 2 µm-derived and contained a REp3 element for autonomous replication in *S. cerevisiae* and Ampr for selection in *Escherichia coli*. The plasmid Cpi4181 contains the CXCR4 gene under the control of the constitutive phosphoglycerate kinase (PGK1) promoter and a LEu2 selectable marker. Plasmid Cpi584 possesses the Fus1p-1aZ construct that allows production of β-galactosidase when the pheronome pathway is stimulated. It also contains the TRP1 selectable marker. Cpi610 expresses full-length wild-type SDF-1a in yeast under the control of the constitutive alcohol dehydrogenase promoter. The β-factor signal sequence is cloned at the 5’-end of the SDF-1a sequence to allow secretion of the mature polypeptide. The vector also has the URA3 selectable marker allowing transformed cells to grow in media deficient in uracil. For expression of the 17-mer peptide library, Cpi6293 was used. This URA3+ plasmid was derived from Cpi610 and contains a frameshift in the SDF-1a sequence so that no functional protein is expressed unless an insert is appropriately cloned. The cDNA coding for 17-mer peptides was cloned using the restriction sites HindIII and Acc65, which also results in the secretion of the mature peptide. *S. cerevisiae* strains YAS1, YAS2, and YAS3 were constructed by transforming CY12946 with Cpi1584, Cpi1584/Cpi4181, and Cpi584/Cpi4181/Cpi610, respectively.

Media—All basic media were prepared essentially as described (24). In cases where plasmid selection was necessary, media that lacked histidine and contained 3-amino-1,2,4-triazole (Sigma), nutrients were employed. Library screening was performed in plates and mating of the two different haploid yeast strains. We describe a genetically modified yeast strain that allowed us to identify variant Gßi and allosteric agonists. We focus on the characterization of two peptide agonists, designated RSVM and ASLW, that are resistant to the small molecule CXCR4 antagonists AMD3100 and T140 and the neutralizing antibodies ASLW.

Peptide and Protein Production—Peptides were synthesized and purified by the Peptide Synthesis Facility at Yale. Me3SO was used when necessary to facilitate dissolution of the polyhistidine peptides. SDF-1a and its enantiomer were kind gifts of Glyphon Sciences (29). For expression of SDF-1a and its mutants, a mammalian vector pcDNA3.1 that coded for a fusion protein between SDF-1a and the Fc domain of antibodies was kindly provided by Drs. Qing Ma and Timothy Springer (Harvard University). HER-293 cells were transformed by the calcium phosphate method; the media were collected; iodoacetamide was added to prevent aggregation from the Fe portion, and the protein was purified using protein A-Sepharose affinity chromatography. For construction of mutants in the full-length protein, the Quikchange (Stratagene) site-directed mutagenesis kit was used, and each mutation was confirmed by DNA sequencing.

Antibodies and CXCR4 Antagonists—Neutralizing monoclonal antibodies 12G5 and its mutants, a mammalian vector containing an IgG2a constant region of *S. cerevisiae* and Ampr for selection in *E. coli*.

Chemotaxis Assay—The CCRF-CEM acute lymphoblastic leukemia cell line was shown previously to express CXCR4 and to migrate in response to SDF-1a (31). The procedure involved cells that were ~70–80% confluent and were centrifuged at 1000 g for 10 min. The cells were resuspended at a concentration of ~106 cells/ml. The transwell system (Corning) was used for quantifying the chemotactic response. Six hundred µl of solution with different concentrations of a CXCR4 agonist were added to the bottom of the wells of 24-well culture plates. One hundred µl of the cell suspension was aliquoted in the transwell cups and placed in the wells. At the bottom of each transwell, a filter membrane that has a 5-µm pore size and allows cell movement toward the higher concentration of chemotactant. The cells were incubated for 2 h at 37 °C, after which the migrated cells were collected and counted with an electron particle counter (Coulter). In cases where the effect of neutralization by CXCR4 monoclonal antibodies or antagonists was examined, the inhibitor was added to the upper compartment along with the cells.

2 R. Agatep, R, Kirkpatrick, D. Parchaliuk, R., Woods, and R. Gietz, Technical Tips Online (ttotrends.com).
Flow Cytometry—Flow cytometric analysis of cell subpopulations was performed using a FACS Vantage flow cytometer (BD Biosciences) for detection of CXCR4 surface expression with the 12G5 monoclonal antibody. An indirect staining protocol was used. Briefly, after a 90-min incubation with various agents, the cells were washed and resuspended in phosphate-buffered saline. Non-specific (Fc-mediated) binding was blocked using purified rat IgG. The primary antibody (12G5, a mouse IgG2a antibody) was added at 1 μg/ml for 45 min. After centrifugation the cells were resuspended in phosphate-buffered saline, and the secondary antibody (FITC-linked rat anti-mouse IgG2a) was added at 10 μg/ml for 45 min. The cells were fixed with 2% formaldehyde (in phosphate-buffered saline) and used within 2 days for data acquisition. Mouse IgG2a was used as an isotype control. For the fluorescence measurements the cells were excited at 488 nm. The FITC fluorescence was collected through a 530/30 nm bandpass filter. A minimum of 15,000 cells was examined for each sample. Analysis of data was performed using the CellQuest software (BD Biosciences).

RESULTS

Functional Expression of Human CXCR4 in Yeast—To identify allosteric peptide agonists or modulators of CXCR4, it was necessary to establish an assay that could isolate molecules with the desired properties from a large peptide library. The screen that was used consisted of two steps. The first step was to isolate peptide sequences from a synthetic cDNA library that would allow cells to grow only after activation of CXCR4. S. cerevisiae was deleted, since its gene product mediates the growth arrest that normally results from activation of the pheromone response pathway (32). As a result, deletion of FAR1 dissociated pathway stimulation from growth arrest while still allowing CXCR4-dependent induction of pheromone-responsive genes. Fourth, SST2, a negative regulator of GPA1 (the yeast Go subunit) was deleted in order to enhance responsiveness to activated CXCR4. Finally, STE14 was deleted to dampen background activity of the pheromone response pathway.

A number of genes for biosynthetic enzymes have been mutated to allow selection of the corresponding marker on a plasmid transformed into the yeast strain. The genomic HIS3 gene, coding for an enzyme essential for the biosynthesis of histidine, has been disrupted by an insertion. The wild-type HIS3 gene has been placed under the control of the pheromone-responsive FUS1 promoter; as a result activation of CXCR4 enables cells to grow in media lacking histidine. To reduce false positives due to any basal activity of FUS1-HIS3, 3-amino-1,2,4-triazole, an inhibitor of the enzyme coded by HIS3, is present at low levels in the media during agonist selection. Endogenously expressed SDF-1α stimulates the production of histidine and allows the strain expressing CXCR4 to grow in the absence of this amino acid due to the FUS1-HIS3 gene (Fig. 2A). In a strain where FUS1-lacZ is also present, endogenous or exogenous SDF-1α induces an increase of β-galactosidase activity (Fig. 2B). Similar effects were observed in response to a peptide agonist composed of the first 14 amino acids (1–14) of the SDF-1α sequence that had been shown in mammalian cells to act as a weak partial agonist (33). To show specificity, we tested the activity of the enantiomer of the chemokine, D-SDF-1α, which showed no effect on the β-galactosidase assay (Fig. 2B). Furthermore, the CXCR4 antagonist AMD3100 (34) inhibited YAS2 colony formation (data not shown) and β-galactosidase activity in response to SDF-1α (Fig. 2C).
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Library Screening and Agonist Isolation—The cDNA library encodes a 17-mer peptide with random amino acids at positions 1–4. At these positions the wild-type sequence is KPV. The amino acid sequence for the remaining peptide (positions 5–17) retains the SDF-1α wild-type sequence. Transformation of strain YAS2 with the plasmid library yielded ~10^6 transformants. Each possible peptide is expected to be represented by an average of approximately six times. The transformants were replica-plated en masse to histidine-deficient medium containing 1 mM 3-amino-1,2,4-triazole. Originally ~120 Ura "His" clones grew on these plates. The β-galactosidase activity of these colonies was measured and compared with strain YAS2, which expresses only CXCR4, or YAS3, which expresses SDF-1α with CXCR4. Forty one of these colonies displayed significant β-galactosidase activity. The active library plasmids were isolated, amplified in E. coli, and re-introduced into the YAS2 strain to confirm the CXCR4-mediated effects. From the 41 clones, 13 presented β-galactosidase activity that was plasmid-dependent. Two of these clones, predicted to express peptides with RSVM and ASLW sequences at their N termini, showed resistance to AMD3100 in β-galactosidase assays and were selected for further characterization using chemically synthesized peptides.

CXCR4 Dependence of RSVM and ASLW Effect—Initial experiments to test the effect of each exogenous peptide on the β-galactosidase activity of CXCR4expressing yeast cells that contained the pheromone-responsive FUS1-lacZ construct were hampered by the toxicity of the peptides during the overnight exposure at the high concentrations (≥50 μM) required for testing. Therefore, we tested the CXCR4 dependence by the in vivo expression of the peptides in two different yeast strains, one that contained CXCR4 and another one that lacked this receptor. The results of these experiments are shown in Fig. 3. Endogenous expression of the peptides leads to a significant increase of β-galactosidase activity only in the strain that co-expresses CXCR4. Consequently, CXCR4 is required for activation of the pheromone pathway by the RSVM and ASLW peptides in yeast.

Peptide Assays—To verify that yeast screening provided peptides that possessed biological activity on mammalian cells, chemotaxis of the human leukemic T cell line CCRF-CEM was measured. Fig. 4, A and B, display the concentration-response curves for the RSVM and ASLW peptides, respectively. The RSVM peptide has an apparent EC_{50} of greater than 100 μM (Fig. 4A). Due to poor solubility of the peptides we have not been able to test concentrations higher than 200 μM in order to observe the typical bell-shaped concentration-response curve of most chemotaxants. The second peptide, ASLW, has an apparent EC_{50} of greater than 60 μM (Fig. 4B) and displays superagonist activity, with a chemotactic index higher than the maximum observed with SDF-Fc. (SDF-Fc is a fusion protein between SDF-1α and the Fc region of an immunoglobulin that has similar agonist effects and is sometimes used instead of SDF-1α due to its stability.) Chemotactic effects induced by simultaneous stimulation of more than one agonist vary depending on which two agonists are used. The co-stimulation by SDF-Fc and RSVM leads to a chemotactic index that is about 30% lower than SDF-Fc alone (Fig. 4C). Thus, RSVM behaves as a weak partial agonist. However, the activities of SDF-Fc and ASLW are approximately additive (Fig. 4D). Chemotaxisin the presence of the two peptide agonists ASLW and RSVM is also additive (Fig. 4E). The relative chemotactic activities of all different CXCR4 agonists used in this study are shown in Fig. 4F at their optimal concentrations.

Effect of AMD3100, T140, 12G5, and 44717.111—The chemotactic activity of SDF-Fc or the wild-type 17-mer is sensitive...
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Fig. 3. CXCR4 dependence of each peptide activity established in yeast. The background strain (1st bar) expresses only the receptor and reporter plasmids. Its β-galactosidase activity is significantly increased when it is transformed with the RSVM- (A) or ASLW-expressing plasmid (2nd bar) (B). The presence of the reporter and peptide-expressing plasmids does not induce this effect in the absence of CXCR4 (3rd bar).

to AMD3100 (Fig. 5A). However, neither RSVM (Fig. 5B) nor ASLW (Fig. 5C) show any reduction in activity in the presence of AMD3100. The data suggest that AMD3100 and the peptides bind to different sites on the receptor. The unique agonist-binding site, however, continues to activate CXCR4. Strong resistance to inhibition remains even at very high AMD3100 concentrations (100 μg/ml, ~115 μm). This fact essentially excludes the possibility of a competitive interaction with the antagonist.

T140 is a 14-residue analogue of polyphemusin II from American horseshoe crabs that is also an antagonist of CXCR4 (35). The presence of this antagonist also does not inhibit CCRF-CEM chemotaxis in response to RSVM (Fig. 5D) or ASLW (Fig. 5E). Again, no significant reduction of the peptide activities is seen, even at T140 concentrations comparable with those of the agonists. Because AMD3100 and T134, a 14-residue analogue of T140, do not compete for binding to CXCR4 (36), it is reasonable to expect that AMD3100 and T140 also do not compete with each other and therefore bind to different sites on CXCR4. These sites must sufficiently overlap with SDF-1α or HIV-1 gp120 in order to exert their antagonist effects. The experiments here indicate that RSVM and ASLW bind to sites that differ from and do not overlap with the AMD3100 and T140 sites.

12G5 and 44717.111 are monoclonal antibodies to CXCR4 that prevent SDF-1α-mediated effects and HIV-1 infection of T-tropic cells (37, 38). We examined the effect of co-incubation of each antibody with CCRF-CEM cells on the chemotactic activity of the peptides. Fig. 6A illustrates a substantial decrease in the chemotactic index for SDF-Fc in the presence of 12G5 but a very small effect of the antibody on RSVM. The same is observed for the ASLW peptide mutant (Fig. 6B). 12G5 binds an epitope on the first and second extracellular loop (39, 40) that appears to overlap the AMD3100-binding site on the second extracellular loop and the adjacent transmembrane segment TM4 (41). The epitope for 44717.111 has not yet been determined. However, as can be seen in Fig. 6, C and D, neither RSVM- nor ASLW-mediated chemotactic activity is significantly affected by the presence of 44717.111, even at a concentration (50 μg/ml) 10-fold higher than the one that almost completely abolishes the activity of SDF-Fc.

Full-length Mutants—To determine the effect of the mutations in the context of the full-length SDF, we overexpressed and purified the two mutant SDFs from mammalian cells. The [ASLW]SDF-Fc mutant was inactive in chemotactic assays on CCRF-CEM cells and did not inhibit the activity of SDF-Fc on these cells at the concentrations tested (up to 2 μM) (data not shown). However, the [RSVM]SDF-Fc mutant displayed chemotactic activity on CCRF-CEM cells at ~500 nM-1 μM (Fig. 7A). The efficacy of this full-length mutant was substantially lower than wild type. Furthermore, this activity was completely inhibited by AMD3100, T140, 12G5, and 44717.111 (Fig. 7, B–E) indicating that the full-length mutant has a similar binding site to CXCR4 as the wild-type protein.

Flow Cytometry—After CXCR4 activation, SDF-1α induces receptor internalization (42). In order to gain insight on the differences in potency between the peptides and SDF-1α, we examined their effect on surface levels of CXCR4 as detected using flow cytometry (and a FITC/12G5-based indirect staining procedure). As can be seen in Fig. 8A, SDF-Fc (1 μM) caused a clear reduction of the CXCR4 levels on the cell surface. The RSVM peptide had a similar effect (Fig. 8B), whereas ASLW caused only a minor decrease (Fig. 8C). The effect of RSVM was concentration-dependent (Fig. 8D). No significant concentration-dependent response was observed for ASLW (Fig. 8E). These data show that RSVM causes down-regulation of CXCR4 in a manner similar to SDF-Fc. On the contrary, ASLW does not induce a marked reduction in CXCR4 surface levels after cell activation, which may partially explain its superagonist activity.

DISCUSSION

CXCR4 Activation—The activation of CXCR4 and most other chemokine receptors is believed to involve a two-step process. The first step involves the binding of SDF-1α to the CXCR4 N terminus followed by activation of the receptor through interactions with the second extracellular loop (43). Each extracellular region of chemokine receptors possesses a cysteine. The four cysteines form two disulfides that are believed to bring the extracellular regions together into a compact structure with a stable binding site (44). Accordingly, the binding and activation sites are likely to be in close proximity. The activation of the receptor by the N-terminal region of SDF-1α results in an intracellular conformational change leading to an exchange of

Fig. 7. Identification of Allosteric Peptide Agonists of CXCR4.
FIG. 4. Chemotactic properties of agonists on CCRF-CEM acute lymphoblastic leukemia cells. Concentration-response chemotactic activity of RSVM (A) and ASLW (B). A plateau in the chemotactic index of either peptide could not be observed due to insolubility of both peptides above the indicated concentrations. C, chemotactic activity of RSVM in the presence of SDF-Fc indicates that RSVM has properties of a partial agonist. Mixtures of agonists ASLW and SDF-Fc (D) or ASLW and RSVM (E) have additive chemotactic effects. F, chemotactic activities of the two mutant peptides, RSVM (200 μM) and ASLW (100 μM), compared with the activities of the wild-type agonists SDF-Fc (100 nM), SDF-1α (5 nM), and N-terminal 17-mer peptide (20 μM). All ligands were used at their optimal concentration. B = background chemotaxis.
FIG. 5. Effect of various antagonists on the chemotactic activity of CXCR4 agonists on CCRF-CEM cells. A, sensitivity of SDF-Fc and the wild-type 17-mer peptide to AMD3100. Resistance of RSVM (B) and ASLW (C) agonist activity to inhibition by AMD3100. Resistance of RSVM (D) and ASLW (E) to inhibition by the CXCR4 antagonist T140.
GDP for GTP in G proteins and dissociation of the G proteins from CXCR4 to initiate downstream signal transduction mechanisms.

**Allosteric CXCR4 Agonists**—The phenomenon of allosteric agonism is relatively uncommon in the literature for G protein-coupled receptors. Most of the available allosteric ligands are either antagonists or modulators/enhancers of the activity of the natural ligands without having intrinsic agonist activity. It has been suggested that the relative lack of allosteric agonists in the ligand repertoire of the G protein-coupled receptors may reflect the bias of most screens with radioligand binding assays for the native (orthosteric) site (45). With the advent of functional screens, the identification of allosteric agonists may become more widespread and thus lead to novel therapeutic agents.

Based on the differences of IC₅₀ values of AMD3100 on chemotactic and CXCR4 binding assays of SDF-1α as well as the biphasic binding behavior of ¹²⁵I-SDF-1α on CXCR4-expressing cell lines, it has been proposed that AMD3100 and SDF-1α can bind to CXCR4 simultaneously (46). Mutagenesis experiments suggest that the second extracellular loop, transmembrane IV, and transmembrane VI of CXCR4 are critical for its interaction with AMD3100 (47, 48). In principle, this allows novel agonists to bind to the N terminus or to other regions of the receptor that are not necessary for antagonist(s) interactions. Our results suggest that other binding sites exist for non-physiological agonists. These agonists were identified by screening a semi-randomized 17-mer library in a yeast strain expressing a functional CXCR4 receptor. The original, wild-type 17-mer has the same sequence as the first 17 amino acids of SDF-1α and has been shown previously to activate CXCR4 (13). Activation of CXCR4 in this yeast strain induces the pheromone-response pathway, with the exception that growth arrest is replaced with cell proliferation due to a number of genetic modifications. As a result of these changes, only cells that secrete an agonist peptide can grow on the appropriate selective media. In contrast to the wild-type 17-mer peptide and SDF-1α, two agonist peptides were identified that are insensitive to the CXCR4 small molecule antagonists AMD3100 and T140 and to the neutralizing anti-CXCR4 mono-
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Fig. 7. Effects of full-length mutant [RSVM]SDF-Fc. A, chemotactic activity of full-length, mutant [RSVM]SDF-Fc protein on CCRF-CEM cells. Sensitivity of chemotactic activity of [RSVM]SDF-Fc to the CXCR4 antagonists AMD3100 (B), T140 (C), and the CXCR4 neutralizing monoclonal antibodies 12G5 (D) and 44717.111 (E).
clonal antibodies 12G5 and 44717.111 in CCRF-CEM cells. One peptide with a sequence of RSVM for the first four amino acids behaves as a partial agonist. The other peptide, ASLW, is a superagonist, displaying a chemotactic index that is greater than the maximum observed with SDF-Fc or SDF-1α/H9251. SDF-Fc and RSVM peptide display a typical down-regulation of surface CXCR4 (Fig. 8, A–D). However, ASLW does not induce CXCR4 internalization (Fig. 8, C and E), which may explain the superagonist activity of this peptide due to continuous activation of the receptor. Although experiments with yeast strains clearly indicate that RSVM and ASLW activities are mediated by CXCR4 (Fig. 3), an alternative explanation in CCRF-CEM cells is that ASLW-induced chemotaxis may be mediated by another mechanism. This possibility remains to be further investigated.

When the same mutations are introduced into the full-length SDF-1α protein, one mutant, [ASLW]SDF-Fc, is inactive and the other, [RSVM]SDF-Fc, displays properties of a partial agonist that is sensitive to antibodies and small molecule antagonists (Fig. 7). The activity and pharmacological behavior of the [RSVM]SDF-Fc mutant indicates that it binds to the natural agonist site. Furthermore, the EC_{50} increases from 1 to 10 nM for SDF-1α (31) to >1 μM for the wild-type 17-mer peptide (13). Taken together these data indicate that the rest of the protein

Fig. 8. Effect of SDF-Fc and the RSVM and ASLW peptides on CXCR4 surface levels detected by flow cytometry. FL1-H on the x axis represents fluorescence intensity. A–C, the basal levels are indicated by heavy lines, the agent-treated cells by dashed lines, and the isotype and secondary antibody controls by dotted and straight lines, respectively. A, SDF-Fc (1 μM) causes a clear decrease of the basal CXCR4 surface levels. B, RSVM (200 μM) causes a similar effect. C, the effect is not shared by ASLW (100 μM). D, RSVM has a concentration-dependent response. E, the lack of a significant response for ASLW is evident at different concentrations.
that mimizes the toxic effects of AMD3100 and other CXCR4 antagonists. In this context, allosteric CXCR4 antagonists could maintain essential receptor function and allow the clinical combination of CXCR4 antagonists with other anti-HIV agents that have a different mechanism of action.

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