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The fusion of IGF I with stromal cell-derived factor I or α1 proteinase inhibitor alters their mitogenic or chemotactic activities while keeping their ability to inhibit HIV-1-gp120 binding

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Abstract

It has been previously reported that insulin-like growth factor I (IGF I) decreases in AIDS patients with wasting, a condition that is partially prevented by combined IGF I growth hormone therapy. By generating bifunctional proteins of IGF I and stromal cell-derived factor 1α (SDF-1α) or α1 proteinase inhibitor (API), two proteins known to prevent HIV infection, it may be possible to improve the therapeutic effectiveness of these compounds for the treatment of AIDS-mediated wasting. SDF-1α or the M351E-M358L mutant of API were attached at the C-terminal end of IGF I and synthesized by a stable insect cell expression technique. The IGF I-SDF-1α chimera reduced the enhancement of thymidine incorporation into bovine fetal erythroid cells observed in the presence of insect cell produced IGF I alone. It also decreased the SDF-1 and IGF I-stimulated hematopoietic cell migration, without losing the capacity to compete with the binding of HIV-1 (IIIB)-surface glycoprotein gp120. The IGF I-API chimera displayed the same mitogenic activity and a similar, but lower chemotactic activity than IGF I in the assays mentioned above. It had a comparable anti-elastase activity to that observed with a previously described IGF II-API fusion protein with the single mutation M351E. The binding of gp120 to a murine hematopoietic cell line was stimulated by human neutrophil elastase (25–100 nM) and inhibited by IGF I-API. In conclusion, the linkage of IGF I with SDF-1 or API can alter some biological functions of the single components of the chimera while keeping their ability to compete with HIV-1-gp120 binding.

Keywords: 32D cells; α1-Antitrypsin; Blasticidin; Bombyxin; CXCL12; Baculovirus

1. Introduction

IGFs are ubiquitous growth factors involved in essential cellular processes such as cell proliferation, differentiation, transformation, wound healing and apoptosis [1–3]. Although IGF I decreased in AIDS patients with wasting [4] and a combined IGF I growth hormone therapy in these patients increased lean body mass and decreased fat mass [5], it was concluded that the effects obtained did not justify the costly hormone treatment. Nevertheless, studies in azidothymidine-treated mice indicated that IGF I could have an additional advantage in AIDS patients undergoing antiretroviral therapy because it neutralized the azidothymidine-induced hematopoietic toxicity [6]. We developed a baculovirus-based technique for the synthesis of IGF II in insect cells in which the signal peptide and the first nine amino acids of IGF II were replaced by the corresponding sections of bombyxin, an insect insulin-like peptide [7]. The method was improved to allow the synthesis of IGF II-IL-3 fusion proteins. The linkage of the growth factor with the cytokine resulted in a synergistic effect in mitogenic and chemotactic activities [8,9]. Furthermore, the IGF II-IL-3 chimera increased body mass and reversed the hematopoietic toxicity associated with azidothymidine in mice [10]. Fusion proteins of IGF I and proteins known to inhibit HIV-1 infection such as SDF-1 [11] or API [12] could be advantageous as therapeutic agents in AIDS patients. Unfortunately, the baculovirus expression of IGF II-α1-proteinase

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Abbreviations: API, α1 proteinase inhibitor; BIGFI, fusion protein of bombyxin and human IGF I, as depicted in Fig. 1; BIGFI-SDF-1, fusion protein of BIGFI and stromal cell-derived factor 1α; BIGFI-API, fusion protein of BIGFI and α1 proteinase inhibitor with the mutations M351E and M358L; SDF-1, stromal cell-derived factor 1; TN, *Trichoplusia ni*.

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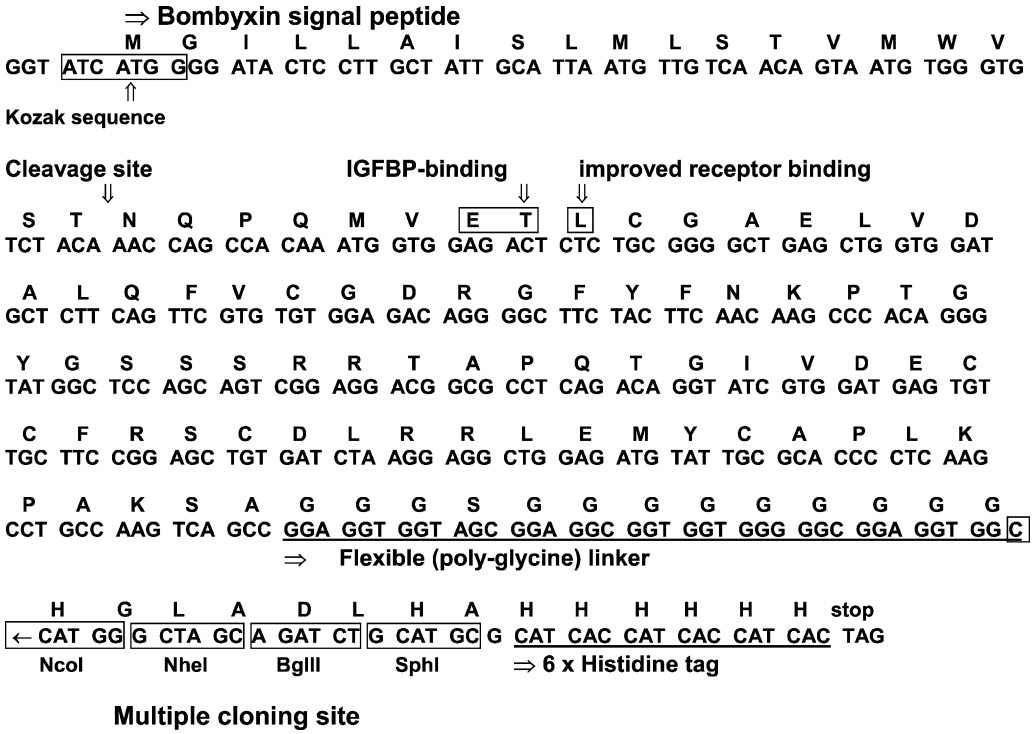

Fig. 1. Structure of the new bombyxin-IGF I chimera BIGFI.

inhibitor was associated with recombinant protein degradation at the time of optimal production [13] and the same technique was remarkably inefficient for the synthesis of the chimera IGF II-SDF-1β.[1] In this paper, we applied successfully a novel technique for the insect cell production of IGF I fusion proteins with SDF-1α and API. The large amounts of recombinant proteins produced allowed us to study some of their functions in hematopoietic cells.

2. Materials and methods

2.1. Construction of expression vectors

The synthetic cDNA coding for a bombyxin-IGF I chimera was produced by PCR using four overlapping oligonucleotides (Sheldon Biotechnology Centre) as previously described for the bombyxin-IGF II chimera [7]. The PCR product was integrated into the vector pIB/V5-His-TOPO (Invitrogen). The single *Nco*I and *Bgl*II sites originally present in the pIB/V5 vector were eliminated using the "QuikChange™" Site Directed Mutagenesis Kit (Stratagene). This allowed for the use of both restriction sites as components of the multiple cloning site of the new vector BIGFI-blasticidin depicted in Fig. 1 (B for bombyxin, and blasticidin to indicate the selection method).

The cDNA coding for SDF-1α was integrated into the vector BIGFI-blasticidin as follows. We incorporated first the SDF-1β cDNA (see Footnote 1) in the baculovirus transfer vector 265 [8], by amplifying the SDF-1-cDNA, kindly provided by Dr. Spotila (Thomas Jefferson University, Philadelphia). The portion of the gene coding for SDF-1α was then amplified by PCR using the Pfu polymerase (Stratagene) with two oligonucleotide primers containing restriction sites for *Nhe*I and *Sph*I for the in-frame integration into the multiple cloning site of BIGFI-blasticidin. The accuracy of the construct was confirmed by DNA sequencing (Sheldon Biotechnology Centre). We also constructed a second chimera of BIGFI linked to the M351E-M358L mutant of α1 proteinase inhibitor (API). The M358L mutation was introduced in our API vector already containing the M351E mutation [14] using the Site Directed Mutagenesis Kit indicated above. The new double mutant M351E-M358L was amplified by PCR and ligated into the multiple cloning site of BIGFI.

2.2. Protein expression and purification

The vectors coding for BIGFI and the chimeras with SDF-1 and API were transfected into *Trichoplusia ni* (TN) cells [15] using Insectin-Plus liposomes (Invitrogen) following the instructions of the manufacturer. Two days after transfection, blasticidin S (InvivoGen) was added at a concentration of 50–80 μg/mL in the serum-free medium 405 (JRH Biosciences). Surviving cells were cultured first in T-flasks and then transferred to suspension culture flasks, in which the blasticidin concentration was gradually reduced to 10 μg/mL.

BIGFI, BIGFI-SDF-1 and BIGF I-API were isolated from 160 mL cell culture supernatants using Ni-affinity

[1] DiFalco and Congote, unpublished results.

chromatography as previously described [13], with the modification of using a 16 mL column of affinity resin and an elution flow rate of 2 mL/min. The Ni-binding proteins were eluted with 0.45 M imidazol, concentrated and desalted using Centricon-20 tubes (Millipore, 5 kDa molecular weight limit), acidified with ice-cold 10% (v/v) triflouroacetic acid to a pH lower than 3 and separated with a semipreparative Vydac C4 column as previously indicated [8]. The fractions containing the recombinant proteins were identified with an ELISA method previously described for a similar chimera [13], using as primary antibody anti-IGF I (GroPep). They were analyzed for purity by SDS–PAGE and coomassie blue staining and quantitated by amino acid analysis (Sheldon Biotechnology Centre). The IGF II-API chimera containing the single mutation M351E was prepared as previously described [14].

2.3. Thymidine incorporation

The recombinant proteins were tested for the ability to stimulate thymidine incorporation into bovine liver erythroid cells (typical feature of IGFs) using the methods previously described [13,16].

2.4. Cell migration

The biological activity of the recombinant proteins was tested for the ability to stimulate cell migration (property of SDF-1 and IGFs) using Costar Transwells of 5 μm pore size (Fisher Scientific) as previously described [13]. Recombinant SDF-1α and β used as positive controls for the cell migration assay were from R&D Systems. Cell migration assays were done with murine 32D-I cells instead of TF-1 cells, which have shown a poor response towards IGFs in our previous studies. The 32D-I cell line was obtained from the hematopoietic cell line 32D (ATCC), transiently transfected with a plasmid containing the cDNA for the IGF I receptor [17] kindly provided by Dr. LeRoith (NIH) and selected for growth in RPMI 1640 medium supplemented with 2% (v/v) fetal bovine serum, 0.4% (v/v) WEHI cell-conditioned medium (as a source of murine interleukin-3, BD Biosciences), 10 mM HEPES, pH 7.5, 1 mM sodium pyruvate, 300 μg/mL bovine serum albumin (fatty acid free, tissue culture tested, Sigma), 30 μg/mL bovine transferrin (ICN) and 0.1–5 ng/mL human IGF I (GroPep). Before cell migration, the cells were starved for 3 hr in a serum-free medium (the composition is as indicated above but without IGF, serum and conditioned medium). The factors to be tested were dissolved in this medium. In some experiments the Costar Transwells were replaced with 96 Chemo-Tx plates (5 μm pore size, 5.7 mm diameter) from Neuro Probe. 2×10^5 cells in 50 μL serum-free medium were placed on the top of the membranes and the chemotactic factors were applied in 29 μL serum-free medium on the bottom wells.

2.5. Inhibition of neutrophil elastase

The capacity of the recombinant BIGFI-API chimera to inhibit human neutrophil elastase (EC 3.4.21.37; Calbiochem) was measured as previously described [14] by incubating the enzyme with increasing concentrations of the recombinant chimeras followed by a colorimetric determination of the enzymatic activity using the elastase substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma).

2.6. gp120 binding

32D-I cells were grown until reaching a cell density of $(3-4) \times 10^6$ cells/mL. The cells were washed twice with PBS supplemented with 10 mM HEPES (pH 7.5) and suspended in the serum-free incubation medium (the RPMI medium indicated above without fetal bovine serum, WEHI-conditioned medium or IGF I); $(2-4) \times 10^5$ cells were cultured in the serum-free medium (25 μL per 100,000 cells) with different concentrations of SDF-1α, BIGFI chimeras or neutrophil elastase for 1 hr at 37°. Recombinant HIV-1 (IIIB) gp120 conjugated with horseradish peroxidase (Immuno Diagnostics) were added (50 ng per 100,000 cells) and the incubation continued for 40 min at 37°. At the end of the incubation the cells were mixed with 1 mL of the ice-cold PBS-HEPES buffer indicated above, containing 0.1% (w/v) bovine serum albumin, centrifuged and washed two additional times with 750 μL buffer. Cells were suspended in 50 μL of the same buffer, mixed with 200 μL of the peroxidase substrate *o*-phenylenediamine (Sigma) and the absorbance measured as previously described [13]. Cells incubated in the absence of peroxidase-conjugated gp120 were used as controls. The non-conjugated gp120 used to compete in the elastase-mediated stimulation was from Intracel. The incubation conditions were adapted from binding studies with radiolabeled gp120 [18].

3. Results

Fig. 1 shows the structure of the coding region for the bombyxin-IGF I gene in the new expression vector BIGFI-blasticidin. The main features of the structure consist of (a) the introduction of a Kozak consensus sequence [19] at the initiation site of bombyxin, (b) the presence of the amino acids ETL preceding the first cysteine of IGF I, which were not present in our previous IGF II vector and are putative binding sites for the IGF I receptor and IGF-binding proteins [20,21], (c) the multiple cloning site containing the restriction sites for *Nco*I, *Nhe*I, *Bgl*II and *Sph*I, and (d) the C-terminal hexa-histidine peptide to facilitate purification of the recombinant proteins by Nickel affinity chromatography. The cDNA coding for SDF-1α and the M351E-M358L mutant of API were inserted using the

*Nhe*I and *Sph*I restriction sites of the BIGFI-blasticidin vector as indicated in Section 2.

TN cells were transfected with the vectors coding for BIGFI and BIGFI chimeras and cells containing the recombinant genes were selected with blasticidin. Unlike the earlier generation of baculovirus systems, the new pIB vectors do not secrete viral particles and do not kill the infected cells. Therefore, it should be expected that cell growth of the transfected cells is not impaired and that amount of proteases coming from the baculovirus or from lysed insect cells is negligible in the cell culture medium. This prediction was evaluated in the case of the API chimera, which is cleaved at the C-terminal end of API at a time coinciding with that of optimal protein production [13]. Supernatants from cells transfected with the BIGFI-API construct were separated by SDS–PAGE and analyzed by Western blots as previously described [13]. A single band corresponding to the intact chimera was detected. There were no signs of the typical 62 kDa degradation product resulting from the C-terminal cleavage of API, which can reach up to 50% of the total recombinant protein after 3 days of infection with the classical baculovirus expression system (results not shown). These results suggest that the protease activity in the culture medium of baculovirus-infected cells coming from lysed or infected cells or from the virus itself is absent in the new non-lytic expression system. The recombinant proteins were isolated from cell culture supernatants as indicated in Section 2. The amounts recovered per liter of culture medium were of 0.7 and 2.5 mg BIGFI-SDF-1 and BIGFI-API, respectively. This represents a remarkable improvement in the production of the SDF-1 chimera as compared to our previous baculovirus-based method, which delivered only a few micrograms under similar conditions (see Footnote 1).

Fig. 2A shows the dose-response curve for the stimulation of thymidine incorporation into bovine liver erythroid cells, a typical indication of IGF biological activity. SDF-1α had no thymidine incorporation stimulating activity (closed circles). BIGFI-SDF-1 (closed squares) was significantly less potent than BIGFI (triangles) or the equimolar mixture of SDF-1α and BIGFI (open squares). It is possible that the simple attachment of a protein at the C-terminal end of IGF I may reduce its capacity to stimulate cell proliferation, independent of the structure of SDF-1. Therefore, we did a control experiment with BIGFI-API, in which BIGFI is linked to the 52 kDa glycoprotein. The thymidine incorporation stimulating activity of BIGFI and BIGFI-API was identical (Fig. 2B).

The capacity of the recombinant proteins to affect cell migration was assessed using the murine hematopoietic cell line 32D-I. In preliminary experiments we found that SDF-1α-stimulated cell migration and the maximal stimulation was observed at a concentration of 6 nM. Using this concentration, the number of cells which migrated (starting with a total input of 150,000 cells) through the Costar Transwells coated with fibronectin [13] was 41600 ± 5800 (mean ± SEM, N = 6) in the presence of SDF-1α, 8300 ± 1600 (N = 4) in the presence of SDF-1β and 750 ± 180 (N = 6) for the control non-treated cells. The high activity of recombinant SDF-1α as compared with recombinant of SDF-1β corresponds to the higher chemotactic activity of SDF-1α in T-lymphocytes and CXCR4-transfected BaF/3 cells, as tested by the manufacturer (R&D systems, www.rndsystems.com). Furthermore, it was found that BIGFI or recombinant IGF I added together with SDF-1α did not increase any further the level of migration observed with SDF-1α alone. Fig. 3A shows the results of similar experiments carried out in the presence of 6 nM

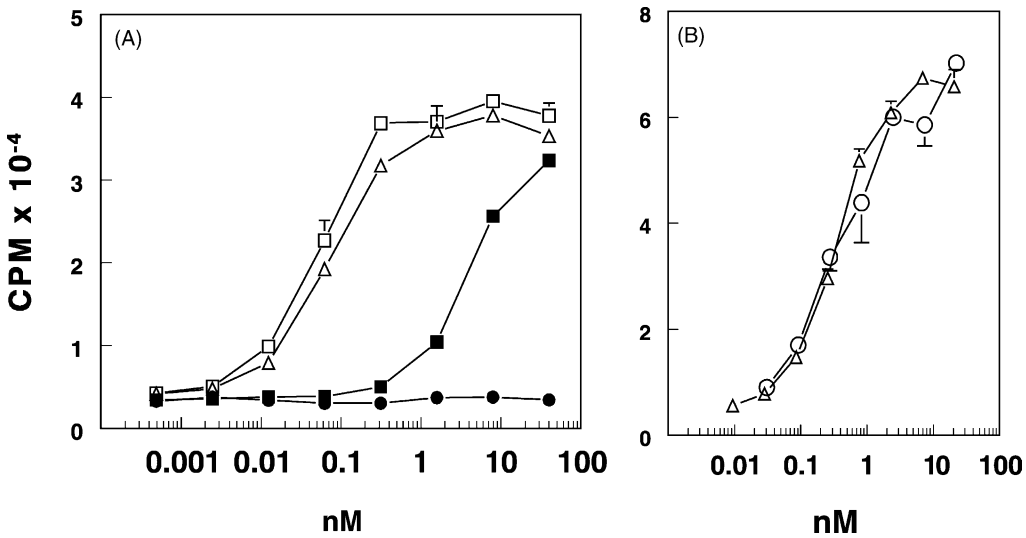

Fig. 2. Thymidine incorporation into bovine erythroid cells in the presence of the recombinant proteins. (A) Different concentrations of BIGFI (Δ), BIGFI-SDF-1 (■), SDF-1α (●) and equimolar mixtures of BIGFI and SDF-1α (□). Mean ± SEM (N = 3). The results of five independent experiments indicated that thymidine incorporation in the presence of BIGFI-SDF-1 was significantly lower than that observed with BIGFI ($P < 0.008$, Mann–Whitney U-test). (B) In separate experiments using a different batch of cells the effects of BIGFI (Δ) were compared with those of the BIGFI-API chimera (○). Mean ± SEM (N = 3). The figure is representative of three different experiments, each run in triplicates.

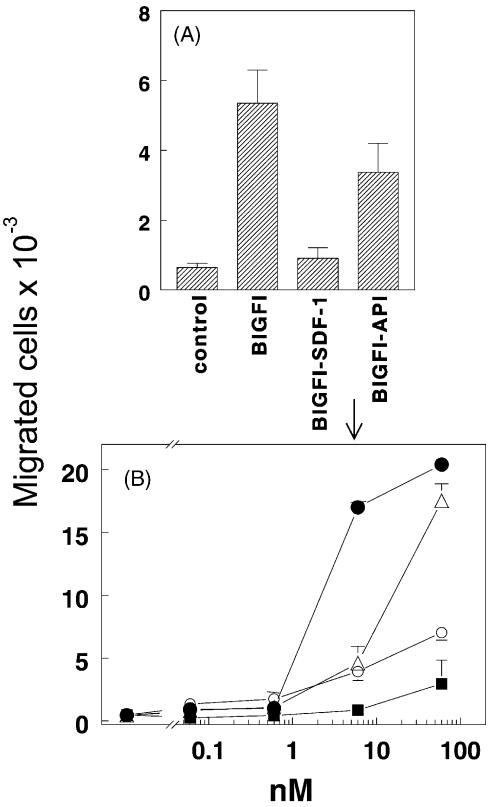

Fig. 3. Effect of recombinant chimeras on cell migration using the murine hematopoietic cell line 32D-I. (A) The recombinant chimeras (6 nM) were added to the bottom chamber of Costar Transwells previously coated with fibronectin and the number of cells migrating to the chamber were counted. The values are mean ± SEM from six to seven experiments. BIGFI and the chimera of BIGFI with α1 proteinase inhibitor (BIGFI-API) significantly stimulated cell migration as compared with control cells ($P < 0.01$ and <0.05, respectively). The difference in migration observed with BIGFI alone as compared to that in the presence of BIGFI-SDF-1 was significant ($P < 0.05$, Kruskal–Wallis non-parametric ANOVA). (B) Effect of different concentrations of the recombinant proteins (including 6 nM, arrow) on cell migration using Chemo-Tx plates, as indicated in Section 2.4. Values are mean ± SEM from four experiments (each done in triplicates) for all proteins except SDF-1α (●), which was done two times (in triplicates) and added to the figure as comparison. ANOVA and the Student–Newman–Keuls multiple comparisons test indicated that the effects of BIGFI-SDF-1(■) were not significantly different than control cell cultures. The stimulation observed with BIGFI (Δ) and BIGFI-API (○) at 60 nM over control cell cultures was highly significant ($P < 0.001$), as well as the difference between BIGFI and BIGFI-API (60 nM only, $P < 0.001$).

recombinant proteins. BIGFI significantly stimulated cell migration. The attachment of SDF-1α to BIGFI in the BIGFI-SDF-1 chimera drastically reduced cell migration to control levels, as has been similarly observed with the biological activity of mutants of the first two N-terminal amino acids of SDF-1 [22]. BIGFI attached to API still retained a significant chemotactic activity, although at a somewhat lower magnitude than that observed with BIGFI alone. Fig. 3B compares the chemotactic activity of the recombinant fusion proteins at concentrations ranging from 0.06 to 60 nM, using this time chemotactic plates (Neuro Probe). The results obtained at a 6 nM (B, arrow) are equivalent to using Transwells (A). At high concentrations (60 nM), the stimulation of cell migration was significantly higher than that observed with control cells for both BIGFI and BIGFI-API ($P < 0.001$) and BIGFI was significantly more effective than BIGFI-API ($P < 0.001$). In contrast, the BIGFI-SDF-1 chimera did not significantly increase cell migration as compared to control cultures at any of the concentrations tested. BIGFI-SDF-1α (60 nM) had a significantly lower chemotactic activity than the equivalent concentrations of BIGFI ($P < 0.001$) or BIGFI-API ($P < 0.01$). It can be concluded that the linkage of SDF-1 to IGF I remarkably decreases the chemotactic activities of both IGF I and SDF-1α.

It is known that SDF-1 binds to its receptor (CXCR4) at two different sites. The "binding site 2" is responsible for the chemotactic activity of SDF-1 and requires the association with the free-moving N-terminal amino acids of the chemokine [22]. The complete elimination of the chemotactic activity of the BIGFI-SDF-1 chimera suggests that the attachment of BIGFI to the N-terminal section of IGF I prevents its association with the binding site 2. However, it is known that HIV-1 binding to CXCR4 is independent of chemotactic activity and is likely to utilize the binding site 1. Therefore, we tested the capacity of BIGFI-SDF-1 to inhibit binding of the HIV-1 glycoprotein gp120, which is known to utilize CXCR4 as a coreceptor for viral entry. Fig. 4 shows that the chimera significantly inhibited gp120 binding to 32D-I cells. In fact, it seems to be a more effective inhibitor than SDF-1α alone, which could be explained by decreased proteolysis or decreased rate of internalization. However, the difference between the inhibition caused by the chemokine alone and the chimera was non-significant.

We have previously found that an IGF II-α1 proteinase inhibitor fusion protein with the mutation M351E was more efficient than the chimera with the wild-type inhibitor

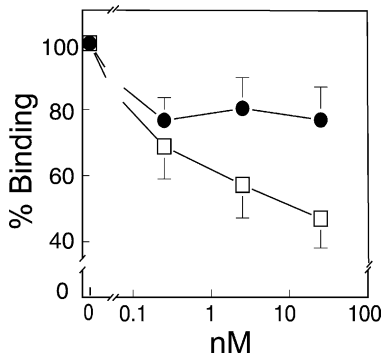

Fig. 4. Competitive inhibition of horseradish peroxidase-conjugated gp120 binding to 32D-I cells. Cells were incubated with increasing amounts of SDF-1α (●) or BIGFI-SDF-1 chimera (□) for 1 hr at 37° and gp120 binding was measured as indicated in Section 2.6. The values are expressed as % inhibition as compared to the binding of gp120 alone. Mean ± SEM from four to seven experiments. The chimera significantly decreased gp120 binding at 2.5 nM (N = 6, $P < 0.01$) and 25 nM (N = 7, $P < 0.001$) according to the Student–Newman–Keuls multiple comparisons test. The differences between the inhibition observed with SDF-1α and the chimera were not significant.

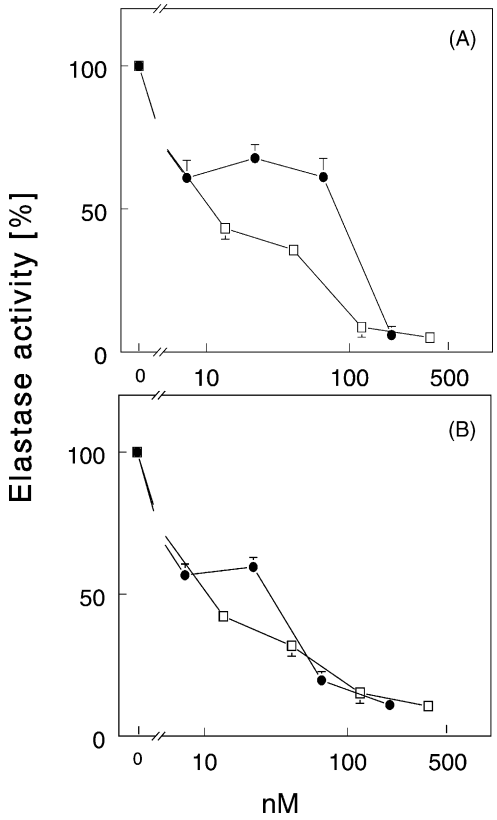

Fig. 5. Anti-elastase activity of the recombinant API chimeras. (A) Increasing concentrations of BIGFI-API (M351E-M358L mutant) (●) and IGF II-API (M351E mutant) (□) were incubated with human neutrophil elastase and the enzymatic activity (substrate conversion per minute) was calculated as described [14] and expressed as % of total elastase activity (the activity of the non-inhibited enzyme was in average 0.5 optical units increase (405 nm) per min per mL). (B) The mixtures of substrate–elastase and API recombinants used for the experiment A were sealed and kept in the dark for 2 days. Substrate conversion was measured at 405 nm and expressed as percent of the total amount measured with elastase alone (the absorbance of the non-inhibited enzyme was in average 5 optical units per mL). Mean ± SEM of triplicates. Similar experiments done with three preparations of the M351E mutant and two preparations of the M351E-M358L mutant indicated that there was no significant difference in the activity of both recombinants.

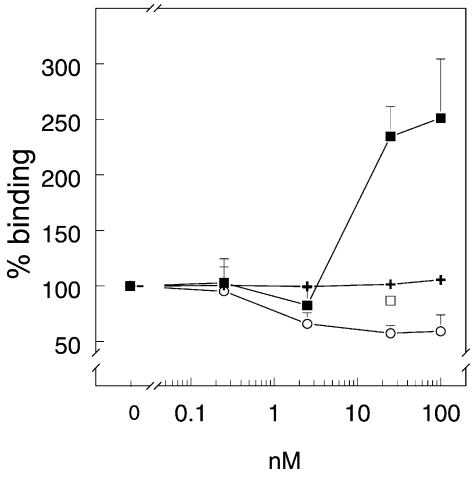

Fig. 6. Effect of BIGFI-API and neutrophil elastase on gp120 binding. 32D-I cells were treated with increasing concentrations of BIGFI-API (○) or neutrophil elastase (■) for 1 hr at 37° and gp120 binding was measured as indicated in Section 2.6. There was no increase in peroxidase activity in cells treated with elastase in the absence of gp120 (+) and a 40-fold excess of non-conjugated gp120 inhibited the binding of horseradish peroxidase-conjugated gp120 at a concentration of 25 nM elastase (□). ANOVA and Student–Newman–Keul multiple comparison test indicated that the stimulation of binding by 25 and 100 nM elastase was significant ($P < 0.01$, N = 7). The inhibition of binding caused by BIGFI-API at the concentrations 2.5, 25 and 100 nM was also significant ($P < 0.05$, N = 5). Values are mean ± SEM (N = 3 (+, □), 5 (○), 7 (■)).

in neutralizing the activity of elastase [14]. We introduced the additional mutation M358L, because it is well known that oxidation of methionine 358 is associated with the inactivation of the anti-elastase activity [23] and its replacement with leucine could in principle create a more stable or efficient inhibitor. Fig. 5A shows that the double mutant M351E-M358L was not a better elastase inhibitor than the original single mutant M351E using the conventional enzyme assay (substrate conversion per minute) as previously described [13]. For the experiments depicted in Fig. 5B, the plates containing the elastase–API mixtures and the elastase substrate were kept for 2 days in the dark at room temperature and then the absorbance at 405 nm, corresponding to the maximum absorbance of the reaction product, was measured and calculated as percent of the total absorbance measured with elastase alone. This experiment gives an idea of the long-term stability of the API–elastase complexes, which could be affected by denaturation or oxidation. Experiments with different preparations of the recombinant proteins similar to the one shown in Fig. 5 indicated that there were no significant differences in the anti-elastase activity between the double mutant and the single mutant M351E. Therefore, our assumption that the double mutant may eventually be more effective than the single mutant M351E due to the prevention of oxidation of the methionine at position 358 was not valid, at least under the experimental condition described in this paper.

Shapiro *et al.* [12] identified two mechanisms in the API-mediated HIV-1 inhibition, namely inhibition of HIV-1 production and reduction of HIV-1 infectivity. It is reasonable to assume that this reduction of infectivity may be due to an API-mediated competition of binding of the V3 loop of gp120 with membrane-bound serine proteases [24,25]. Fig. 6 shows that the IGF I-API chimera (○) significantly inhibited gp120 binding to 32D-I cells. Fig. 6 also shows that neutrophil elastase (25–100 nM) had the opposite effect (■), namely a large and significant increase of gp120 binding. This stimulation was blocked by addition of a 40-fold excess of non-HRP-conjugated gp120 (□) and was not due to the release or stimulation of peroxidase-like activity of 32D-I cells (+).

4. Discussion

We have previously found that a chimera consisting of the signal peptide and the N-terminal sequence of the

insect insulin-like peptide bombyxin attached to human IGF II was produced as a secreted, properly folded and biologically active IGF using the baculovirus expression system [5]. The linkage of the IGF II analog with proteins such as interleukin-3 or -α1 proteinase inhibitor resulted in a potentiation of biological activity [8,14]. However, the production of an IGF-SDF-1β chimera with the baculovirus expression system was extremely low and inefficient (see Footnote 1) and resulted in the considerable presence of degradation products in the case of the IGF-α1 proteinase inhibitor fusion protein [13]. Therefore, we decided to explore the use of non-lytic insect cell expression systems which do not require viral infection but use the immediate-early promoter Opie-2 from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus [26] for constitutive expression of foreign genes (Invitrogen). Stable cell lines are obtained by blasticidin selection, which is mediated by the expression of a blasticidin-resistance gene. We also introduced several modifications designed to improve the biological activity of the IGF moiety of the chimeras. We replaced IGF II with the more potent IGF I and changed portions of the bombyxin N-terminal sequence to include a Kozak consensus sequence to improve transcription and the amino acids ETL preceding the first cysteine of IGF I to improve binding to the IGF I receptor and IGF-binding proteins [20,21]. At least in theory, the presence of binding sites for IGF-binding proteins could be useful to improve the half-life of the recombinants, thanks to their newly acquired IGF-mediated affinity to the IGF-binding protein system. The first commercially available non-lytic insect cell expression system was based on zeocin selection (Invitrogen). We originally tried to produce an IGF II-SDF-1β chimera with this system with limited success, due to part to the time consuming and costly zeocin selection [27]. After developing the new BIGFI-blasticidin expression vector, we preferred to use SDF-1α as the first chemokine to be attached based on the observation that SDF-1α is more active than SDF-1β in T-lymphocytes and CXCR4-transfected BaF/3 cells (R&D Systems, www.rndsystems.com). Our results with 32D-I cells support this observation. This difference of activity should be further studied in other systems.

It is known that HIV infection of T-cells requires not only binding to CD4 but also to CXCR4, the receptor for SDF-1 (reviewed in [28]). In our laboratory, we have been investigating ways to treat another aspect of AIDS-related events, namely the elimination of the hematopoietic toxicity associated with antiretroviral drugs [10,29]. This is mediated as well by the HIV virus [30,31] and, more specifically, by the viral surface glycoprotein gp120 [32,33]. The 32D-I cell line is particularly attractive for studies on interactions between IGFs and SDF-1α, because it is extremely sensitive to the action of both agents. The most significant result, as far as the biological activity of the recombinant chimera BIGFI-SDF-1 is concerned, is the powerful elimination of cell migration in comparison to the substantial activity observed with BIGFI or SDF-1α. In an extensive study based on three-dimensional NMR structure and site-directed mutagenesis of SDF-1, Crump *et al*. [22] found that the activation of the receptor was not required for HIV-1 inhibition and opened the way for the design of powerful SDF-1 antagonists which could block HIV entry without having the biological effects of SDF-1. According to their model, amino acids 12–17 of SDF-1 are required for the initial docking of SDF-1 with CXCR4. This transient association then facilitates receptor interaction with the first two amino acids of SDF-1 within the highly flexible N-terminal end of the chemokine. Mutations of these two amino acids prevent receptor activation. We assume that the attachment of BIGFI to the N-terminal section of SDF-1 prevents the interaction of the two N-terminal amino acids of SDF-1 with CXCR4, without interfering with the initial docking to the receptor. This would explain the almost complete absence of chemotactic activity for 32D-I cells as compared with the powerful stimulation observed with SDF-1α. The chimera was nonetheless a very effective competitor to the binding of gp120 to the same cells (Fig. 4). What is surprising is that migration observed with BIGFI-SDF-1 was significantly lower than that observed with BIGFI alone (Fig. 3), suggesting that the binding of the SDF-1 component to the cells prevents as well the action of the BIGFI component of the chimera. Thymidine incorporation into bovine erythroid cells was also significantly lower in cells incubated with BIGFI-SDF-1 in comparison to BIGFI alone (Fig. 2), suggesting an interaction between the CXCR4 and IGF I receptors or their signal transduction pathways. An alternative explanation to the inhibitory action by SDF-1 component of the chimera is that internal folding of the different domains of the fusion protein may hinder receptor binding. Since the chimera was still able to inhibit gp120 binding, the suppression of mitogenic and chemotactic activities is not probably due to an internal molecular packing of the chimera.

We expected that the BIGF-API chimera with the double mutation M351E + M358L could improve the anti-elastase activity by preventing the inactivation of the inhibitor through oxidation of methionine 358. It is believed that this oxidation is accentuated by cigarette smoking and can lead to emphysema [23]. However, the double mutant did not have a higher anti-elastase activity than the IGF II chimera with the mutant M351E.

The BIGFI-API chimera inhibited binding of gp120 to the murine hematopoietic cell line 32D-I. This inhibition does not go beyond 50% and a similar behavior is observed with the BIGF-SDF-1 chimera. This is not surprising, because gp120 binding is a very complex phenomenon involving not only CD4 and CXCR4 but also a variety of membrane proteins [34], including membrane-bound serine proteases [24]. It is not known with certainty if the binding sites of gp120 are identical to those of SDF-1 [22].

The increase of gp120 binding observed with 25–100 nM neutrophil elastase is surprising. If the effect of added elastase is limited to a competitive inhibition of binding of membrane-bound elastase-like proteins, a small decrease of gp120 binding should be expected, as it seems to be the case at very low concentrations of the enzyme (2.5 nM). The significant increase of binding at 25–100 nM elastase suggests an independent, additional mechanism, which augments the number of available binding sites at the cellular membrane. This could take place by modifying the membrane domains involved in gp120 binding or through an increased expression/recycling of gp120 binding sites. Valenzuela-Fernandez *et al*. [35] observed a small increase of CXCR4 expression in jurkat cells with an elastase concentration of 200 nM, as measured with the monoclonal antibody 12G5, which recognizes the second extracellular loop of CXCR4. However, at high concentrations, the enzyme cleaves the N-terminal portion of CXCR4, which would effectively destroy the binding site 1 to SDF-1 [35]. The most important physiological function of elastase at low concentrations is most probably the specific degradation of the N-terminal portion of SDF-1, which destroys the chemotactic activity of the peptide [35,36]. These results emphasize the critical role played by elastase at multiple sites of action involving HIV-1 infection and explain the high degree of HIV-1 replication in blood from patients with hereditary deficiency of API, which is the natural inhibitor of elastase [12].

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