Heparin enhances the furin cleavage of HIV-1 gp160 peptides

A. Pasquatoa,b, M. Dettinb, A. Basakc, R. Gambarettoa, L. Toninb, N.G. Seida,*,1, C. Di Bellob,1

a Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, Montreal, Quebec, Canada H2W 1R7
b Department of Chemical Process Engineering, University of Padova, 35131 Padova, Italy
c Regional Protein Chemistry Center, Diseases of Aging Program, Ottawa Health Research Institute, Ottawa, Ontario, Canada K1Y 4E9

Received 19 July 2007; revised 1 November 2007; accepted 16 November 2007
Available online 26 November 2007

Edited online by Hans-Dieter Klenk

© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: HIV-1; Furin; Heparin; Glycosaminoglycan; gp160; Processing

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent for the acquired immunodeficiency syndrome (AIDS). The envelope glycoprotein gp160 is processed by host proteases into the gp120/gp41 heterodimer. This allows the virus to infect target cells following the cell surface binding of the trimeric complex gp120/gp41 to the human CD4 receptor [1], and subsequently to the CXCR4/CCR5 co-receptors [2,3]. This interaction induces conformational change, which leads to the dissociation of gp120 from gp41, allowing the N-terminal gp41 fusion peptide to be inserted into the host cell membrane [4]. Though the structures of a monomeric gp120 core in complex with the CD4 receptor/Fab 17b [5] and that of a post-fusion form of gp41 were solved [6], little is known about the entire Env conformation. In fact, individually

2. Materials and methods

2.1. Peptide synthesis

The synthesis of the 14mer and 19mer was reported [10]. The 18mer, 41mer, and 51mer were synthesized on a semi-automatic synthesizer (Applied Biosystems, Mod. 431A) using a Rink amide MBHA resin
(NovaBiochem, La Jolla, 0.48 mmol/g, 0.25 mmol), Boc chemistry and HBTU/HOBt activation. Detachment from the solid support and removal of the side chain protecting groups were achieved treating with HF:anisole:DMSO:2-mercaptothiophene:10:1:1 (1 h, 0 °C). Crude products were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Delta Pak HR C18 column (Waters, 5 μm, 60A, 7.8 × 300 mm). Homogeneity grade was evaluated by RP-HPLC on a Vydac C18 column (Waters, 5 μm, 5 μm, 300 A, 4.6 × 250 mm).

Molecular mass was checked by electrospray-time of flight (TOF) mass spectrometry (MS, Mariner 5120 API-TOF). Molecular mass was checked by electrospray-time of flight (TOF) mass spectrometry (MS, Mariner 5120 API-TOF).

### 3. Results

#### 3.1. Peptides design

Four peptides, 51mer, 41mer, 19mer and 13mer, spanning the gp160 cleavage sequence, were synthesized (Table 1). The 13mer containing site1, and the 19mer, which includes site1 and site2, were chosen as Ref. [10]. The extended 41mer and 51mer were synthesized to investigate the influence of the regions surrounding the physiological cleavage site on furin processing. It was reported that a cell-permeable 22mer sequence KIEPLGVAPTAKRRVQREKR [23], which does not contain P residues, interferes with gp160 processing [24]. Thus, to test for a possible in vitro inhibitory function we also synthesized a 18mer peptide (LGVAPTAKRRVQVREKR [31]), mimicking the furin-processing product of the 41mer (Table 1).

#### 3.2. Circular dichroism

The spectra in phosphate buffer pH 7.0 and water showed a diagnostic band with a minimum at 198 nm, suggesting that the 51mer, 41mer and 19mer are unstructured (Fig. 1A–C). This indicates a likely SDS interaction that could be due to the insertion of the hydrophobic C-terminus into micelles and/or could result from the electrostatic binding of the positively charged site1 and/or site2 to the negatively charged micellar surface.

### Table 1

| Name | Sequence |
|------|----------|
| 51mer | 484YKYKKVIEPLGVAPTKAKRRVQREKR AVGIGALFLGGLAGSTMGAA534 |
| 41mer | 495LGVAPTKAKRRVQREKR AVGIGALFLGGLAGSTMGAA534 |
| 18mer | 495LGVAPTKAKRRVQREKR |
| 19mer | 498PTAKRRVQREKR AVGIG516 |
| 13mer | 506RVQREKR AVGIG516 |
Since CD profiles in negatively charged SDS micellar solutions showed a transition of conformers towards a more structured population, and gp160 cleavage site is positively charged, further conformational investigations were performed. The CD profile of 20 μM heparin is similar to that of water (Fig. 1D) and that of the 19mer does change in the presence of heparin (Fig. 2A). In contrast, the CD spectra of the 41mer and the 51mer were significantly modified in the presence versus absence of heparin (Fig. 2B,C). Similar results were obtained with higher heparin concentrations (100 μM).

### 3.3. Enzymatic assays

The 13mer and 19mer peptides were digested equally well by furin at site1 (Tables 2 and 3), showing complete processing at 5 h (Fig. 3A,B). In contrast, the 41mer and 51mer peptides were either barely or unprocessed, respectively, even after
24 h digestion at pH 7 (Fig. 4A,B; Tables 1–3). Since in vitro gp160 cleavage was reported to be optimal at pHs 6–7 [25], further assays were performed on the 41mer and 51mer at acidic conditions (pH 6.3, 6.7), again revealing no differences with respect to the results obtained at pH 7. Furthermore, similar data were observed in presence of low levels of denaturants (0.05% TX-100 or SDS) (not shown).

We first hypothesized that product inhibition could explain these results. We thus tested the in vitro ability of the 18mer peptide, representing the furin-derived product of the 41mer (Table 1), to inhibit the processing of either the fluorogenic Pyr-RTKR-MCA or the 19mer peptides. While the 18mer peptide effectively reduced the release of free AMC with an estimated IC50 of 1.6 μM (Fig. 5A), it could only partially

### Table 2
Percent cleavage of the 13mer, 19mer, 41mer, and 51mer upon 2 h and overnight incubation in presence or absence of heparin

| Time incubation [h] | 13mer | 19mer | 41mer | 51mer |
|---------------------|-------|-------|-------|-------|
| 0                   |       |       |       |       |
| 2                   | 53    | 54    | 59    | 52    |
| 6                   | 100   | 100   | 100   | 100   |
| Overnight           | Traces| 40    | 0     | 60    |

### Table 3
Fragments sequences derived from cleavage at site1 or site2 and their corresponding theoretical and experimental masses

| Precursor | Fragments sequence | Theoretical mass (Da) | Experimental mass (Da) |
|-----------|-------------------|-----------------------|------------------------|
| 13mer     | RVVQREKR          | 1069.65               | 1070.26                |
|           | AVGIG             | 415.24                | 415.27                 |
| 18mer     | LGVAPTKAKR        | 1069.65               | Undetectable          |
|           | RVVQREKR          | 1039.65               | Undetectable          |
|           | PTKAKR            | 1751.08               | 1751.16                |
| 19mer     | AVGIG             | 415.24                | 415.27                 |
|           | PTKAKR            | 699.44                | Undetectable          |
|           | RVVQREKRAGVGIG    | 1466.88               | Undetectable          |
|           | LGVAPTKAKRRVVQREKR | 2091.29              | 2091.30                |
| 41mer     | AVIGALFLGFLGAGSTMAAS | 2039.06            | Undetectable          |
|           | LGVAPTKAKR        | 1039.65               | Undetectable          |
|           | RVVQREKRAGVGIGALFLGFLGAGSTMAAS | 3090.69 | Undetectable |
|           | YKYKVKKIEPLGVAPTKAKR| 3339.01              | Undetectable          |
| 51mer     | AVIGALFLGFLGAGSTMAAS | 2039.06            | Undetectable          |
|           | YKYKVKKIEPLGVAPTKAKR | 2287.38            | Undetectable          |
|           | RVVQREKRAGVGIGALFLGFLGAGSTMAAS | 3090.69 | Undetectable |

Fig. 3. RP-HPLC profiles of: (A) 13mer digestion and (B) 19mer digestion obtained on a Varian C18 column with UV detector (214 nm). Arrows indicate the fragments.
inhibit the 19mer processing with an IC$_{50}$ > 100 μM (Fig. 5B). We conclude that product inhibition cannot explain the inability of furin to process the 41mer and 51mer peptides. Because CD investigations showed a likely binding between heparin and the 41mer or 51mer (Fig. 2B,C), all four gp160-derived analogues were digested overnight in the absence or presence of 2.5 μM heparin. Under these conditions, the 13mer and 19mer were digested at site1 with similar rates independent of the presence of heparin. In contrast, while no significant processing occurred in the absence of heparin, 40% and 60% processing at the REKR site of the 41mer (into an 18mer product with identical retention time on RP-HPLC to the synthetic version) and 51mer peptides, respectively, were observed in the presence of 2.5 μM heparin (Fig. 6). As control, we confirmed that the 41mer peptide is not cleaved by the recombinant VV:WT-infected culture supernatant (Fig. 6, upper center panel). Furthermore, cleavage was inhibited by adding a well known PC-inhibitor, dec-RVKR-cmk (Fig. 6, upper right panel) [23]. At 25 μM heparin we obtained a more extensive processing, but also noticed precipitation of the peptides (not shown). Finally, in a separate 6 h furin incubation experiment, the processing of the 41mer and 51mer peptides also showed a similar enhancement effect of heparin (not shown). In conclusion, these data indicate a likely heparin-peptide interaction that may better expose site1, and hence allow more effective furin cleavage.

**4. Discussion**

Five peptides (Table 1) spanning the gp120/gp41 junction were investigated to better define the gp160 glycoprotein cleavage. The 19mer and its shorter analogue 13mer were processed at site1 (REKR$^{511}$), while site2 (KAKR$^{503}$), which is included only in the 19mer, was uncleaved (Fig. 3). The lack of processing at site2 may be rationalized on the basis of structural motifs. In fact, the 19mer NMR molecular model in TFE revealed that site2 is embedded in a helical segment, whereas site1 is in a exposed loop at the C-terminus of the peptide [12]. In contrast, the 41mer and 51mer, spanning extensive sequence of the gp160 cleavage region, were shown to represent very poor furin substrates. This suggests that the generated fragments could either act as inhibitors or that the more extended regions surrounding the physiological cleavage site prevents effective processing.

Since the possibility of product inhibition by the 18mer was excluded, we turned our attention towards structural restrictions and/or the need of other factors to rationalize the non-cleavability of the 41mer and 51mer peptides. CD analysis on the 19mer, 41mer and 51mer in aqueous solution revealed that the three analogues are unstructured, and yet only the 19mer is digested by furin. Thus, some structural constraints must exist in the 41mer and 51mer, at least around site1. The same argument may explain the inability of furin to cleave...
at site2 in any substrates used. In an attempt to increase the 41mer and 51mer processing, we added some detergents to enhance the peptides backbone flexibility without affecting enzyme activity. However, neither TX-100 nor SDS had any effect.

Therefore, we suspected that cellular/extracellular factors may influence the cleavability of gp160 by furin. Indeed, surface proteins containing heparin-binding motifs processed by furin were reported [16,26]. In particular, Sindbis virus attachment to target cells was enhanced in the presence of heparan sulfate (HS) via the furin recognition motif of the unprocessed envelope glycoprotein PE2 [16]. Similarly, peptides derived from the cleavage site of the human respiratory syncytial virus (RSV) fusion glycoprotein bind heparin and cellular GAGs [26]. Since the gp120/gp41 does not form stable trimers, while unprocessed gp160 does, it was hypothesized that gp160 oligomer attachment to the plasma membrane heparin sulfate occurs via its furin cleavage site [27]. Indeed, the KAKR305RVVQREKR511 sequence exhibits a basic region, which contains two potential inverted consensus HS-binding domains. Thus, it was shown that the affinity of gp160 for heparin is about 3-times higher than that observed for gp120, implying that gp120 and/or hidden motifs in the mature gp120 may be involved in heparin binding [22].

CD spectra of the 41mer and 51mer suggest these peptides could interact with heparin (Fig. 2) and undergo structural reorganization. In fact, in presence of heparin, the profiles change with respect to those of the peptides alone. The negative band at 198 nm, diagnostic for aperiodic structures, is replaced by a positive one. In contrast, it is noteworthy that the shorter 19mer does not change its CD profile in the presence of heparin. Since the difference between the 41mer and the 19mer lies in 22 hydrophobic residues and the interaction between heparin and polypeptides is supposed to be electrostatic, these additional residues may support a favorable peptide conformation that optimally orients the positively charged side chains towards the negatively charged sulfate moieties. Our results agree with a probable glycosaminoglycans-gp160 interaction, as proposed [22,27], and suggest that the residues spanning the gp120/gp41 junction may contribute in gp160-GAGs binding.

Moreover, given that heparin induces a change in the 41mer and 51mer conformation, which could play a key role in the enzyme-substrate recognition, we analyzed how it may influence their furin processing. Surprisingly, while up to 100 μM heparin did not influence furin activity on Pyr-RTKR-MCA processing (Fig. 6, upper panel), the 41mer and 51mer peptides were digested at site1 (Fig. 6). Therefore, we hypothesize that heparin induces conformational change, optimally exposing the furin-cleavage REKR511 site. This is the first time that heparin is shown to enhance the in vitro cleavage of precursors by furin.

In conclusion, this study has shown that, in the absence of heparin, the 41mer and 51mer gp160 derived peptides represent very poor furin substrates in vitro, in contrast to the shorter analogues (13mer and 19mer) that are efficiently processed.

Fig. 6. Effect of heparin on the processing of the 41mer and 51mer. Upper panel controls: (left) RFU released versus time upon Pyr-RTKR-MCA cleavage by furin in (black) the absence, or presence of 20 μM (dark gray) or 100 μM (light gray) heparin; (upper central panel) incubation of the 41mer peptide with recombinant vaccinia WT-infected culture supernatant (control); (upper right panel) effect of decanoyl-RVKR-cmk on the processing of the 41mer by furin. Processing. Lower panels: 20 μL of the enzymatic assay solution containing 2.5 μM heparin was taken (top) immediately after the addition of the substrate and (bottom) after overnight incubation. Arrows indicate the fragments, (*) being LGVAPTKAKRRVVQREKR for the 41mer peptide or YKYKVKIEPLGVAFTKAKRRVVQREKR for the 51mer peptide and (**) being AVGIGALFLGFLGAAGSTMGAAAS.
Heparin was shown to strongly interact with the 41mer and 51mer peptides, inducing conformational changes, thereby exposing site1 for cleavage. Since the 41mer and 51mer peptides may not faithfully mimic the conformation around the cleavage site within the complete gp160 precursor, more analyses are required to assess if heparin is essential in vivo during gp160 maturation and how GAGs modulate HIV-1 activity.

Acknowledgement: We would like to thank Brigitte Mary for her secretarial help. This work was supported by a CIHR grant (#MGP-44363), a Canada Chair (#201652) and by a generous gift from the Strauss Foundation.

References

[1] Lifson, J.D., Feinberg, M.B., Reyes, G.R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong-Staal, F., Steiner, K.S. and Engleman, E.G. (1986) Induction of CD4-dependent cell fusion by the HTLV-I/IIa envelope glycoprotein. Nature 323, 725–728.

[2] Feng, Y., Broder, C.C., Kennedy, P.E. and Berger, E.A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272, 872–877.

[3] Dimitrov, D.S. (1996) Fusin-a place for HIV-1 and T4 cells to meet. Nat. Med. 2, 640–641.

[4] Eckert, D.M. and Kim, P.S. (2001) Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70, 777–810.

[5] Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J. and Hendrickson, W.A. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393, 648–659.

[6] Chan, D.C., Fass, D., Berger, J.M. and Kim, P.S. (1997) Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263–273.

[7] Pinter, A., Honnen, W.J. and Tilley, S.A. (1993) Conformational changes affecting the V3 and CD4-binding domains of human immunodeficiency virus type 1 gp120 associated with env processing and with binding of ligands to these sites. J. Virol. 67, 5692–5697.

[8] Vollenweider, F., Benjannet, S., Decroly, E., Savaria, D., Lazure, C., Thomas, G., Chretien, M. and Seidah, N.G. (1996) Comparative cellular processing of the human immunodeficiency virus (HIV-1) envelope glycoprotein gp160 by the mammalian subtilisin/kexin-like convertases. Biochim. Biophys. Acta 1180, 123–129.

[9] Crim, R.L., Audet, S.A., Feldman, S.A., Mostowski, H.S. and Beeler, J.A. (2007) Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity. J. Virol. 81, 261–271.

[10] Lifson, J.D., Feinberg, M.B., Reyes, G.R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong-Staal, F., Steiner, K.S. and Engleman, E.G. (1986) Induction of CD4-dependent cell fusion by the HTLV-I/IIa envelope glycoprotein. Nature 323, 725–728.

[11] Rocha, B., Sabatier, J.M. and Fenouillret, E. (1998) An antihIV peptide construct derived from the cleavage region of the Env precursor acts on Env fusogenicity through the presence of a functional cleavage sequence. Virology 247, 137–143.

[12] Willey, R.L., Bonifacino, J.S., Potts, B.J., Martin, M.A. and Klausner, R.D. (1988) Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. J. Biol. Chem. 263, 39580–39584.

[13] Straupoli, L., Chanel, C., Girard, M. and Altmeyer, R. (2000) Processing, stability, and receptor binding properties of oligomer envelope glycoprotein from a primary HIV-1 isolate. J. Biol. Chem. 275, 35137–35145.