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STRUCTURAL INVESTIGATION AND KINETIC CHARACTERIZATION OF POTENTIAL CLEAVAGE SITES OF HIV GP160 BY HUMAN FURIN AND PC1

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Summary: A key event in the biosynthesis of the human immunodeficiency virus is the maturation of the gp160 precursor generating gp120 and gp41, two proteins that are fundamental for the infective process. *In vivo*, gp160 is specifically cleaved at the 515-519 site (REKR↓A), in spite of the presence in its sequence of another consensus sequence KAKR↓R (residues 507-511). Comparative kinetic studies on synthetic peptides reproducing different sequences of gp160 by the enzymes PC1 and furin are reported in this paper. The data demonstrate the higher efficiency of furin in the cleavage of peptidic substrates with respect to PC1 and its preference for REKR↓A vs. KAKR↓R. Furthermore, furin and PC1 are unable to process peptides patterned on the sequence 307-330 of specific viral strains of the gp120 V3 loop.

The precursor envelope glycoprotein gp160 of HIV, is normally processed within the *trans* Golgi network in mammalian cells to give the mature glycoproteins gp120 and gp41. Such processing is essential for infectivity [1], and cleavage occurs at one or two closely linked sites with the amino acid consensus sequence Basic-X-Basic-Basic found at gp120-gp41 junction [2]. The cleavage recognition sequence of HIV type 1 (HIV-1) gp160 is shared with HIV-2 and also with a number of other viral glycoproteins and represents a preferred substrate for the cellular enzyme furin. Recent studies have provided evidence for additional cleavage of gp120 within a variable region of the molecule, named V3 loop, that does not contain sequences that match the preferred substrate specificity of furin [3].

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Six processing enzymes generally referred to as the precursor convertases (PCs), have been recently identified in mammalian species: these Ca^{+} dependent serine proteinases belonging to the subtilisin/kexin family are PC1/3, PC2, furin, PC4, PACE4 and PC5/6 [4-7] and their cleavage preference has been studied by their coexpression with a number of pro-proteins and prohormones [7,8]. The concept that furin is responsible for processing of HIV-1 gp160 and other viral and cellular proproteins, is primarily based upon the observation that furin, coexpressed mostly by vaccinia virus vectors, can enhanced the maturation of the respective precursors in normal cells possessing a basal level of processing activity. There is now evidence for involvement of furin and PC1 in the cleavage of gp160 into gp120 and gp41 at the REKR↓A site [9]. Moreover, the observation by Morikawa et al. [3] that furin could be involved *in vivo* in the cleavage of gp160 into gp120 and gp41, has been confirmed by recent studies showing that both furin and, with a reduced efficiency, PC1 are able to generate gp120 and gp41 from gp160 as a substrate [9], however, no comparative kinetic data are available to date. Since a detailed knowledge of the specificity and the mode of action is necessary in order to elucidate the kinetic and thermodynamic significance of the cleavage accomplished by furin and PC1 on the gp160 precursor, we present in this report a study of substrate specificity of furin and PC1 towards synthetic peptides reproducing the different potential cleavage sites of gp160, namely KAKR↓R and REKR↓A (Table I). The present kinetic data clearly demonstrate the preference of furin for the REKR↓A site, while PC1 cleaves at both sites.

Moreover, since furin has been shown to cleave gp120 within the V3 loop region [10], three model peptides derived from the HIV-1-MN, RF and IIIB strains have also been studied. It is confirmed that furin and PC1 failed to cleave all the V3 loop derived synthetic peptides.

A correlation between the K_m values for both enzymes and substrate structures evidenced by CD and FT-IR studies suggests that the substrate-enzyme binding could be related to the propensity of peptidic substrates to adopt a secondary structure.

METHODS

Peptide Synthesis

Peptides were synthesized by solid phase methods using an Applied Biosystems mod.431-A apparatus. The peptide 1-19 was obtained by Fmoc chemistry using HBTU as *in situ* activating agent, whereas the other peptides were synthesized by Boc chemistry using activation *via* active esters of N-hydroxybenzotriazole. The peptides derived from the V3 loop of different strains, were obtained as carboxy-terminal peptides by Fmoc chemistry

using the pre-activation of Fmoc-protected amino acids as active esters. Purification was achieved by using RP-HPLC. All peptides were characterized by analytical HPLC, capillary electrophoresis, amino acids composition and mass spectrometry. The purity grade exceeded 95%.

Enzyme Assay

The cleavage of each of described peptides was tested using purified preparations of PC1 and furin, obtained from vaccinia virus infected GH4C1 cells, purified using DEAE-biogel A column as described from Jean et al. [11]. The standard assay was as follows: the incubation with synthetic peptide substrates were conducted for various time intervals with 10 µL of purified human (h) hPC1 containing an average processivity of 30 pmol of synthetic peptide 1-19 / min, at 37 °C in a total volume of 50 µL; the final incubation mixture contained 50 mM sodium acetate and 5 mM $CaCl_2$ at pH 6. In the case of furin the incubations were made with 2.5 µL of purified hfurin fraction giving an average processivity of 100 pmol of synthetic peptide 1-19 / min at 37 °C, in a total volume of 50 µL; the final mixture contained 50 mM sodium acetate and 1 mM $CaCl_2$ at a pH adjusted to 7. Following incubation, aliquots were acidified using 5 µL of acetic acid and analyzed on Vydac C_{18} column. Elution was carried out with a linear gradient of 0%-25% acetonitrile in 0.05% TFA over 100 min at a flow rate of 1 mL/min. Emerging peaks were collected and the identity of the corresponding peptide fragments was determined to identify the cleavage site by comparison with the retention times of shorter peptides (Table I) or by amino acids analysis. K_m and V_{max} values were determined under the following conditions: peptide substrates at final concentrations ranging from 50 to 800 µM with furin and from 100 to 1400 µM with PC1. Incubations were stopped when less than 10% of the substrate was digested The substrates and products were separated by HPLC. K_m and V_{max} were determined from initial velocity measurements plotted versus various substrate concentrations using the Lineweaver - Burk representation.

RESULTS AND DISCUSSION

Enzymatic cleavage of the inactive viral proprotein gp160 generates gp120 and gp41 that are essential for the binding and penetration of the HIV-1 virus into the target cells. Thus, understanding the details and requirements of the recognition process between the enzyme and the substrate in the processing of gp160 is of fundamental importance in order to develop specific inhibitors capable to block this key-event in infective virion biosynthesis. Furin has been recently indicated by different authors [3,9,10,12] as the endogenous enzyme responsible of the maturation process. The kinetic data in Table I, on the cleavage by furin or

TABLE I

Peptide	Derivation	Sequence
1-19	505-523 HIV-1 gp160	P T K A K R R V V Q R E K R A V G I G
1-10	505-514 HIV-1 gp160	P T K A K R R V V Q
7-19	511-523 HIV-1 gp160	R V V Q R E K R A V G I G
8-19	512-523 HIV-1 gp160	V V Q R E K R A V G I G
14-19	518-523 HIV-1 gp160	R A V G I G
15-19	519-523 HIV-1 gp160	A V G I G
DB1	307-330 HIV-1-IIIB gp120	N N T R K S I R I Q R G P G R A F V T I G K I
DB2	307-330 HIV-1-RF gp120	N N T R K S I T K G P G R V I Y A T G Q I I
DB3	307-330 HIV-1 MN gp120	Y N K R K R I H I G P G R A F Y T T K N I I

PC1 of a model synthetic peptide (peptide 1-19), spanning the 505-523 sequence of gp160 which contains two consensus sequences Basic-X-Basic-Basic, add confirmatory evidence to this hypothesis. Furthermore, in order to better clarify the specificity of furin and PC1, the kinetic studies have been extended to two peptides with shortened sequence containing one consensus sequence at the time, namely peptides 1-10 and 8-19. The data presented in Table II clearly demonstrate that furin cleaves more efficiently the processing site located in the C-terminal portion of the 1-19 sequence, while PC1 cleaves equally well both sites. Interestingly, furin shows a higher specificity for the REKR↓A site, i.e. the one which is recognized and processed *in vivo*; whereas PC1 is less specific. These data support the hypothesis of a specific role played by furin in the maturation of gp160 into gp120/gp41. Furthermore, the V_{max}/K_m data indicate a clear preference of furin for more extended substrates, at variance with the behavior of PC1, as originally observed by comparing the gp160 versus tetrapeptide flurogenic substrates [9]. Since this characteristic could be due to a higher affinity of furin for more structured substrates, CD and FT-IR studies have been performed (data not shown) in different solvents on the synthetic substrates used in this study. Based on the Lincomb method [13], these conformational analyses indeed suggest a more ordered structure of the 1-19 peptide which in 90% TFE/H_2O (v/v), presents a 35% α-helical content, a value which is much higher than that estimated for the shorter 1-10 (16%) and 8-19 (0%) analogues.

TABLE II

Enzyme	Peptide	K_m (μM)	V_{max} (pmols/min)	V_{max}/K_m (x 10^{-9} L/min)
Furin	1-19	64	96	1500
Furin	8-19	134	120	896
Furin	1-10	256	20	78
Furin	DB3	92	2	22
PC1	1-19	176	13	74
PC1	8-19	251	25	100
PC1	1-10	703	91	130

On the other hand, it has been recently proposed that the same proteases involved in the maturation of gp160 could be involved also in the cleavage of the V3 loop [3,10]. This region has been the subject of numerous investigations, not only for the ability to induce neutralizing antibodies formation, but also for its key-role in the fusion process, in determining viral tropism, in speeding-up viral replication, and finally, as a candidate for a cooperative interaction in gp120-CD4 binding [14-18]. In order to evaluate the feasibility of a cleavage at this site, kinetic studies were performed on synthetic substrates reproducing the 307-330 sequence of the V3 loop. Since this region is known as one most variable of gp120, the peptide substrates utilized in the present study reproduced the V3 loop of three different viral strains. In our system, both enzymes were found to be unable to process the synthetic substrates. Therefore, provided that this cleavage may play an important role in infection, one should assume that other factors such as intracellular environment and/or other structural determinants would be required in order to allow for this cleavage *in vivo*.

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