Furin is important but not essential for the proteolytic maturation of gp160 of HIV-1

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Abstract The envelope glycoproteins of HIV are required for viral infectivity. Proteolysis of the precursor envelope glycoprotein gp160 results in the formation of gp120 and gp41. Cleavage occurs after the sequence Arg-Glu-Lys-Arg. This sequence is expected to be a substrate for the cellular protease furin. We examined whether furin is responsible for cleavage of gp160 by using a furin-deficient CHO cell line and the same cell line transfected with furin cDNA. Data obtained from viral transmission assays suggested that furin increased viral infectivity but was not essential for the maturation of gp160, implying that other proprotein processing enzymes also recognize this putative furin cleavage site.

Key words: Furin; HIV envelope; Furin-deficient CHO

1. Introduction

Human immunodeficiency virus type I (HIV-I) is the etiological agent of the acquired immunodeficiency syndrome [1,2]. The envelope glycoproteins of HIV are required to initiate the infection of human CD4+ cells by a high-affinity interaction between the viral envelope glycoprotein gp120 and the CD4 molecule on the T cell surface [1–5]. Cleavage of the HIV precursor envelope gp160 to form a large external glycoprotein (gp120) and a small transmembrane protein (gp41) occurs within the host cells in the rough endoplasmic reticulum-Golgi complex [6,7]. The endoproteolytic cleavage site of gp160 has been determined to be the amino acid sequence Arg-Glu-Lys-Arg[511] [8]. Site-directed mutagenesis of Arg[511] blocks the cleavage of gp160 as well as viral infectivity [9,10]. Several mammalian proprotein convertases that share structural and functional similarity with the yeast convertase Kex2 have been identified. One of these is furin, a calcium-dependent serine endoprotease that cleaves precursor proteins after the minimal consensus sequence Arg-X-X-Arg [11,12]. Early reports showed that furin cleaves the influenza virus hemagglutinin precursor and Newcastle disease virus glycoprotein [13,14]. Other proproteins requiring cleavage by furin include pro-von Willebrand factor [15], *Pseudomonas* exotoxin A (PE) [16], diphtheria toxin [17,18] and the protective antigen of anthrax toxin [19].

Hallenberger et al. reported that decanoyl-Arg-Glu-Lys-Arg-chloromethylketone, shown in vitro to inhibit furin,

greatly reduces the cleavage of HIV gp160 and consequently the infectivity of the virus [20]. However, it should be noted that this inhibitor would be expected to inhibit other proteases that cleave at Arg or Lys, and therefore cannot be considered a completely specific furin inhibitor. Anderson et al. [12] demonstrated inhibition of HIV gp160-dependent membrane fusion by a furin-directed α1-antitrypsin variant having the sequence Arg-Ile-Pro-Arg[358] at the active site. However, it recently was reported that in human T lymphocytes an endopeptidase differing from furin in either ion dependence or pH optimum appears to be able to process HIV gp160 [21]. Therefore, it has been uncertain whether furin is the only enzyme involved in the cleavage of gp160.

In this study, we provide evidence that furin contributes to, but is not essential for, HIV gp160 maturation by using furin-deficient CHO cells and the same cells bearing an exogenous furin cDNA expression construct. Co-cultivation of HIV generated from these two types of cells with human T lymphocytes suggested that furin and other endopeptidases may each process the viral envelope protein.

2. Materials and methods

2.1. Cell culture

CHO cells and H9 T cells were cultivated in α-MEM medium supplemented with 5% serum and RPMI 1640 medium with 10% serum, respectively, at 37°C with 5% CO_2. CHO transfectant cells were maintained in the same condition except G418 was added to the medium at a final concentration of 200 μg/ml.

2.2. Transfection of a mouse furin cDNA construct and selection of transfectants

pAGEFur DNA (2 μg), a mouse furin cDNA expression construct containing a neo gene [22], (a generous gift of Dr. K. Nakayama, U of Tsukuba, Japan), was transfected into furin-deficient CHO (FD11) cells (1×10^5) using Lipofectamine (BRL). The transfected cells were diluted into 100-mm dishes 48 h post-transfection and G418 was added to the medium at a final concentration of 400 μg/ml. Two weeks later clones were selected, expanded and tested for sensitivity to PE by MTT assay. The PE-sensitive cells were further analyzed by reverse transcription coupled polymerase chain reaction (RT-PCR) and used in later experiments involving HIV.

2.3. Reverse transcription coupled polymerase chain reaction (RT-PCR) assay

Total RNA from FD11 and FD11+furin cells was prepared with a RNA isolation kit (Promega). Plus and minus primers matched to the mouse furin cDNA gene from nt 2132 to 2167 and nt 2858 to 2887 [22], respectively, were synthesized on a DNA synthesizer (Applied Biosystems). A pair of primers corresponding to the mouse β-actin gene from nt 321 to 346 and from nt 735 to 760 was also prepared [23]. Furin and β-actin cDNA were made with a primer extension kit (Promega) following the manufacturer's protocol. Half of the reaction was used as DNA template in PCR which was carried out for 30 cycles at 94°C for 30 s, 55°C for 2 min, and 72°C for 2 min.

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2.4. Cocultivation and HIV p24 assay

FD11 and FD11 + furin cells (1×10^6) were seeded in T75 flasks. The next day cell culture medium was removed and the flasks were washed with serum-free α-MEM medium. A mixture of 5 μg of pHXB2gpt DNA [24] containing biologically active HIV-I genome (generous gift from Dr. Robert C. Gallo, NCI, NIH), 10 μl of Lipofectamine and 5 ml of serum-free medium was prepared and added to each flask. Five hours later 5 ml of medium containing 10% serum were transferred to each flask. Two days later, 3×10^6 H9 cells were added to the each flask for co-cultivation. The supernatant medium was collected and the p24 concentration was determined by an ELISA method performed by Advanced BioScience Laboratories.

3. Results and discussion

CHO FD11 is a furin-deficient cell line generated in this laboratory by ethyl methane sulfonate (EMS) mutagenesis of CHO cells [19,25]. This cell line is resistant to PE because intracellular cleavage of the toxin by furin is required for translocation to the cytosol. Cell fusion experiments showed that this cell line fails to complement the mutation in CHO RPE.40, a previously described furin-deficient line [26], indicating that both the FD11 and RPE.40 lines are mutated in the same gene (personal communication of Thomas and Joan Moehring, Univ. of Vermont).

To directly assess the role of furin in processing of gp160, we transfected CHO FD11 cells with pAGEFur. A number of cloned G418-resistant transfectants were assayed for sensitivity to PE. As expected, about 40% of the clones tested regained sensitivity to the toxin, suggesting that active furin was present in those cells. One of those clones, designated FD11 + furin, was shown to be sensitive to PE in a cytotoxicity assay (Fig. 1) and was used in the following cocultivation assay. To confirm that FD11 + furin cells contained the mouse furin gene, RT-PCR was performed. PCR results showed that mouse furin mRNA was present in FD11 + furin cells but not in FD11 cells, consistent with the results of toxin sensitivity assay of those two cell lines (Fig. 2).

Prior work in which the cleavage site in gp160 was mutagenized proved that infection of target cells by HIV absolutely
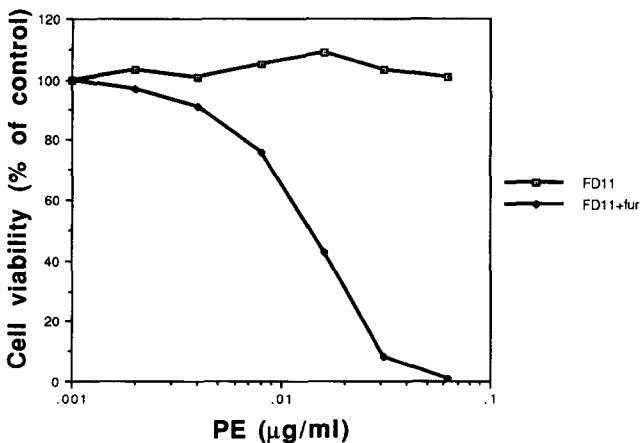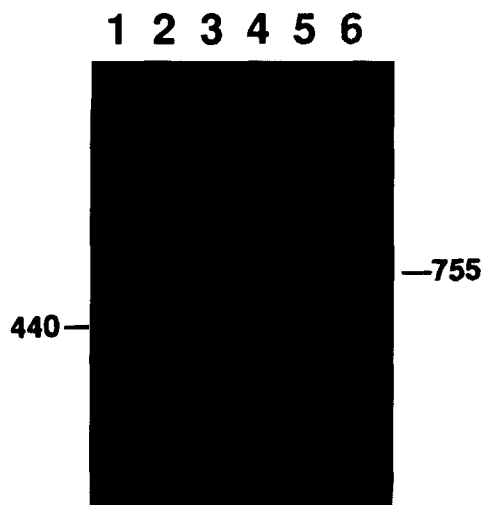

Fig. 2. RT-PCR of the mouse furin gene and the control β-actin gene. RNA prepared from FD11 and FD11 + furin cells was annealed with minus strand primers of either the mouse furin gene or the β-actin gene at 65°C for 20 min and cooled to room temperature. cDNA synthesis was performed in a 20 μl volume using a primer extension kit at 42°C for 30 min and 10 μl of each cDNA reaction were used as templates in the PCR. The PCR products were analyzed on a 1% agarose gel and stained with ethidium bromide. Samples are the β-actin DNA generated from RNA from either FD11 (lane 1) or from FD11 + furin (lane 2) cells; 100 bp DNA marker (lane 3); a positive control using mouse furin DNA plasmid as a template (lane 4). The mouse furin band marked to the right of the gel was absent in FD11 cells (lane 5) but present in FD11 + furin cells (lane 6).

requires processed gp120 and gp41 and that virus packaged with uncleaved gp160 is devoid of fusogenic activity [9]. To examine if HIV generated from FD11 cells would be less infective for human T cells than virus produced from FD11 + furin cells, the plasmid pHXB2gpt was transfected into these two cell types and H9 cells were added 2 days later. HIV replication in each cell mixture was measured by the p24 antigen concentration. The result indicated that p24 was present in both media but the concentration was higher in the medium from FD11 + furin cells than in the medium with FD11 cells (Fig. 3). In a separate experiment, HIV replication after co-cultivation was determined by assaying reverse transcriptase activity. Higher activity was found in the samples from FD11 + furin cells than from FD11 cells (data not shown). These results show that furin is a major protease involved in the processing of gp160. However, after incubation for 16 days, the p24 concentration in the FD11 cell co-cultivation reached a level equivalent to that present in the FD11 + furin cell co-cultivation. These virus transmission assays suggest that even in the cells without furin, infectious and cytopathic HIV virions were generated. The reduced, but not abolished, p24 concentration in the FD11 cell cocultivation medium indicated that other protease(s) in addition to furin can cleave at the viral envelope cleavage site.

Several members of the furin family of processing endoproteases are found in many tissues of mice and humans. PC1 was shown to possess endoproteolytic activity with specificity for cleavage sites that consist of paired basic amino acid residues [27]. Decroly et al. reported that PC1 and furin can cleave gp160 in cell-free system [28]. PACE4, like furin, is widespread in many cell types and therefore is anothercandidate protease

Fig. 1. Sensitivity of FD11 and FD11 + furin cells to PE. FD11 and FD11 + furin cells (0.2 ml of 1×10^5/ml) were transferred to wells of a 96-well plate one day before the assay. PE at different concentration was incubated with the cells for 36 h at 37°C. Cell viability was measured by adding (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma) to a final concentration of 0.4 mg/ml for 1 h at 37°C. The A_{540} of dissolved pigment was determined using a microplate reader. The data are presented as percentage of absorbance in the absence of the toxin.
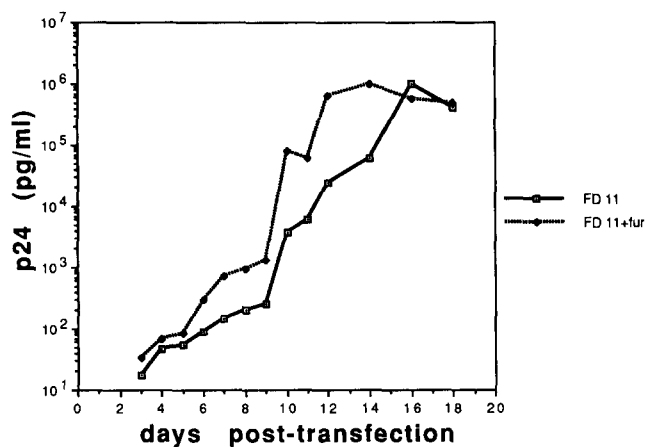
Fig. 3. Measurement of p24 concentration from the cell mixture of transfected CHO cells and H9 cells. FD11 and FD11 + furin cells were transfected with pHXB2gpt DNA using lipofectamine. Two days later, H9 cells were added to the each flask for co-cultivation. The supernatant medium was collected after further incubation for the time indicated. The p24 concentration was determined by an ELISA method.

that may be involved in cleavage of gp160 because it has a substrate specificity that is similar to that of furin [27].

The experiments described here depend on the transcription and translation of HIV-1 proteins in hamster cells. Previous workers have presented data suggesting that rodent cells do not support efficient replication of HIV-1 [29,30], presumably due to the absence of cellular cofactors needed for the activity of Rev and Tat [31]. However, the results shown here indicated that at least some gp160 is produced, processed to gp120 and gp41, and inserted in the CHO cell plasma membrane. Our results do not show that infective HIV-1 particles are produced, because the transfer of the HIV-1 genome to the T cells added subsequently may have occurred by fusion with CHO cells having surface-expressed gp120 and gp41. However, even in this case, infection of the T cells will depend on proteolytic processing of gp160.

The present study shows that furin plays a role in processing gp160 in CHO cells, but that other proteases can cleave gp160 when furin is not present. Our results are consistent with the observations of Ohnishi et al. [32], who showed that LoVo cells, in which furin is inactivated by a frameshift mutation in the homoB region, function normally with respect to gp160 processing and virus production. In addition, the evidence presented here mimics results seen with several bacterial toxins that are activated by cleavage at sites containing multiple basic residues [19]. In those cases also, other proteases can replace furin when it is absent or inhibited.

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