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Comparative processing of bovine leukemia virus envelope glycoprotein gp72 by subtilisin/kexin-like mammalian convertases

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Abstract Intracellular proteolytic processing of bovine leukemia virus (BLV) envelope glycoprotein precursor (gp72) at the C-terminal end of the RVRR^{268}↓ site is an essential step for virus infectivity. Subtilisin/kexin-like convertases cleave proproteins at preferred RX(K/R)R↓ sites, including those commonly found in viral envelope glycoprotein precursors. We first demonstrated that gp72 is processed into gp51/gp30 in both CV1 cells and the furin-deficient LoVo cells, leading us to compare the ability of mammalian convertases to cleave BLV gp72 in vitro. In contrast to the inability of the neuroendocrine PC1 to cleave gp72, the convertases furin, PACE4, PC5-A and PC5-B, which process constitutively secreted precursors, can effectively cleave gp72 into gp51/gp30. N-terminal sequence analysis of the convertase-generated gp30 demonstrated that cleavage occurs at the in vivo-utilized RVRR↓SPV site. Such furin-, PACE4- and PC5-mediated processing was completely inhibited by the α_1-antitrypsin variant α1-PDX. Mutagenesis of the gp72 cleavage site into RVRG-TPV resulted in complete abrogation of gp72 processing by endogenous CV-1 cells and by convertases in vitro. Since our in vitro data suggest a redundancy in the ability of the convertases to cleave gp72, RT-PCR analysis was used to define the convertases expressed in B-lymphocytes, representing one of the major targets of BLV infection. Our data revealed that only furin and the newly discovered PC7 mRNAs are expressed in Raji, B-Jab and LG2 cell lines.

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Key words: Furin-like; Bovine leukemia virus; Envelope glycoprotein; Dibasic residue; gp72; gp51; gp30; Convertase; Processing; B-lymphocytes

1. Introduction

Bovine leukemia virus (BLV), a retrovirus structurally and functionally related to the human T-lymphotropic viruses HTLV-I and II [1], is the etiological agent of bovine leukosis. It infects B-lymphocytes and induces lymphoid tumors in ruminants (for reviews see [2,3]). The type-I membrane-bound BLV envelope glycoprotein is synthesized as a 72 kDa (gp72) precursor that is cleaved by cellular proteases into 51 (gp51) and 30 (gp30) kDa mature forms. The exposed gp51 contains the cellular receptor-binding domain, and the membrane-bound gp30 is responsible for anchoring the gp51/gp30 complex. The N-terminal domain of gp30 is involved in membrane fusion [4]. Systematic comparison of the cleavage site of different viruses revealed the presence of a consensus cleavage sequence RX(R/K)R↓ identical to that recognized by mammalian subtilisin/kexin-like proteinases [5]. Accordingly, intracellular proteolytic cleavage of gp72 occurs at the proposed internal sequence RVRR↓SPV, a process crucial for the infectivity of the virus [4]. So far, a total of seven mammalian subtilisin-like proteases have been identified: furin (also called PACE), PC1 (PC3), PC2, PACE4, PC4, PC5-A (PC6-A) and its isoform PC5-B (PC6-B) and the newly discovered PC7 (PC8, LPC or SPC7) (for reviews and updates [6–9]). While furin is ubiquitously expressed, PC7, PACE4 and PC5 exhibit a widespread tissue distribution, and all catalyze the processing of precursors within the constitutive secretory pathway [6–11]. In addition, we recently showed that immune cells express large amounts of both furin and PC7 [9–11]. In contrast, PC1, PC2 [9,12,13] and possibly PC5-A [14] are responsible for precursor processing within the regulated secretory pathway, mainly in endocrine and neural cells. PC4 is specific to testicular germ cells [15].

Several reports implicated the mammalian subtilisin/kexin convertases in viral envelope precursor cleavage. Thus, in cellular coexpression experiments, furin was shown to catalyze the proteolytic processing of Newcastle disease virus F protein [16], parainfluenza virus type 3 HA protein [17] and cytomegalovirus gpUL55 protein [18], and furin, PACE4 [19,20] and PC7 [10] cleave HIV-1 gp160. In vitro experiments have demonstrated that furin, PACE4, PC5 as well as PC1 can cleave the HIV-1 gp160 into gp120/gp41 [10,11,20,21]. Although these results suggest that a certain redundancy exists in the ability of mammalian convertases to process viral envelope glycoproteins, quantitation of the level of expression of the PCs in virus-infectable host cells should be considered. Thus, in the case of CD4$^+$ T-cells, the major targets of HIV-1 infection, furin and PC7 were reported to be the major PC-like gp160 convertases expressed in human primary T-lymphocytes [10].

In this report, we present the first experimental evidence that the BLV surface glycoprotein gp72 is processed into gp51/gp30 in LoVo cells which are devoid of furin activity [22], suggesting that more than one convertase can cleave this precursor. This led to the in vitro demonstration that furin, PACE4, PC5, but not PC1, can correctly process gp72 into gp51/gp30. N-terminal sequence analysis of gp30 generated by each convertase conclusively demonstrated that PC-preferred processing occurs at the physiological cleavage

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Abbreviations: VV, vaccinia virus; PC, precursor convertase; RT-PCR, reverse transcriptase polymerase chain reaction; BLV, bovine leukemia virus; gp, glycoprotein; WT, wild type; PBS, phosphate buffer saline; BSA, bovine serum albumin

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site RVRR↓SPV[271] [23]. Furthermore, we could not demonstrate a PC-mediated cleavage of mutated gp72 in which the processing site is replaced by RVRG-TPV. Finally, we also show that the PC-mediated processing of gp72 is not inhibited by the serpin variant α_1-antitrypsin Pittsburgh (α1-PIT), but is completely blocked by α_1-antitrypsin Portland (α1-PDX) [24], an inhibitor whose active site sequence R-X-X-R mimics sites recognized by most convertases [7,9,13,20,24].

2. Materials and methods

2.1. Cell culture

CV1 [11] and LoVo [20] cells were cultivated in DMEM (Gibco Life technologies, Inc.) supplemented with 10% fetal calf serum (FCS), non-essential amino acids and kanamycin (Gibco). The cell lines Raji, B-Jab and LG2 were a gift of Dr. R. Sekaly (IRCM, Montreal, Canada). At 80% confluence, the CV1 and LoVo cells were infected for 1 h with a recombinant vaccinia virus carrying the *env* gene of BLV (a gift of Dr. D. Portetelle, Facultés Agronomiques de Gembloux, Belgium) at a multiplicity of infection of one plaque forming unit/cell. Following infection, the cells were grown for 6 h and then lysed in RIPA buffer [20] and the lysate run on 10% SDS-PAGE, blotted onto a nitrocellulose membrane which was then analyzed by Western blotting using a cocktail of monoclonal anti-gp72 antibodies (a gift of Dr. D. Portetelle). Immunoreactive proteins were reacted with a secondary antibody coupled to alkaline phosphatase, followed by coloration with the substrate nitroblue tetrazolium 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP; Boehringer Mannheim).

2.2. Purification of gp72

The procedure used to purify BLV gp72 was described by Decroly et al. [21]. Briefly, CV1 cells were infected with recombinant vaccinia virus carrying the *env* gene of BLV at a multiplicity of infection of one plaque forming unit/cell. Twenty hours post infection, CV1 cells were starved of cysteine or leucine for 1 h in a DMEM cysteine- or leucine-free medium containing 5% FCS. The cells were then labeled in the same medium supplemented with [^{35}S]Cys or [^{3}H]Leu (ICN Flow). After 1 h labeling, the cells were lysed in 30 mM Tris, 150 mM NaCl, 1% Triton X-100 pH 7.4. The clarified lysate was then purified on a lentil lectin agarose column (Pharmacia). After several washes with lysis buffer, gp72 was eluted in the same buffer containing 0.5M α-methylmannopyranoside (Sigma).

2.3. Enzyme production and fluorimetric assays

Shed-furin, BCRD-furin, PACE4, PC1, PC5-A and shed-PC5-B were isolated from the medium of GH4C1 cells infected with vaccinia virus recombinants expressing each protease gene as described [11,21,25,26]. Enzyme activity was tested on 200 μM of the fluorogenic peptide pGluArgThrLysArg-AMC (Peptide Institute, Inc., Japan) [26]. The reactions were carried out in 50 mM Tris-acetate buffer, 1% TX-100, 4 mM $CaCl_2$ pH 6 for PC1 and 1 mM $CaCl_2$ pH 7 for the other enzymes. After overnight incubation at 25°C, the reactions were stopped by the addition of 25 μl of glacial acetic acid. Fluorescence of free 7-amino-4-methylcoumarin (AMC) was measured with a spectrofluorimeter using 370 nm as excitation wavelength and 460 nm as emission wavelength.

2.4. Endoproteolytic cleavage of gp72

Enzymes (50 nmol AMC cleaved/h) were added to gp72 in 50 mM Tris-acetate, 1% Triton X-100, 4 mM $CaCl_2$ pH 6 for PC1 and 1 mM $CaCl_2$ pH 7 for shed-furin, BCRD-furin, PACE4, PC5-A or shed-PC5-B. After overnight digestion at 25°C, samples were run on a 10% SDS-polyacrylamide gel. The gel was then dried and autoradiographed on Kodak X-omat AR films. A 200-fold concentrated supernatant of GH4C1 cells infected with recombinant vaccinia virus expressing either α1-PDX or α1-PIT [11] was used as a source of inhibitors. Inhibition experiments of the in vitro PC-mediated processing of radiolabeled gp72 using either α1-PDX or α1-PIT were done as described [11].

2.5. Microsequencing of the [^{3}H]Leu-labeled gp30

For microsequencing purposes, [^{3}H]leucine-labeled gp72 was incubated with the soluble shed-furin, BCRD-furin, PACE4, PC5-A or shed-PC5-B and the products separated on a 10% polyacrylamide SDS-PAGE. The gel was then blotted on a PVDF membrane, autoradiographed and the dissected gp30 containing band was layered over a protein filter (Beckman Instruments, Inc.). Microsequencing was performed on a Beckman LF3400 protein sequencer (Beckman Instruments, Inc.). All samples were sequenced using a standard Beckman sequencer procedure 4. The radioactivity recovered in each cycle was estimated by scintillation counting using liquid scintillation analyzer Tri-Carb model 1600CA.

2.6. Convertases detection by semi-quantitative RT-PCR

The RNAs were isolated from 10^7 B-lymphocytic cells (Raji, B-Jab and LG2) using the TRIAzol (Gibco) procedure. RT-PCR and cDNA quantitation were performed as described by Decroly et al. [11].

3. Results and discussion

3.1. Cellular processing of BLV gp72

The ubiquitously expressed convertase furin has been reported to be a major intracellular processing enzyme of most precursors transiting through the constitutive secretory pathway and exhibiting the consensus RX(K/R)R sequence [6,7,9]. Since such a motif is found at the putative processing site of gp72 [4], we first tested the cellular processing of this precursor in LoVo cells which are devoid of furin activity [22]. Fig. 1 shows the results of Western blot analysis of cell lysates obtained 6 h following vaccinia virus infection with VV:gp72 of either human colon carcinoma LoVo cells or monkey kid-

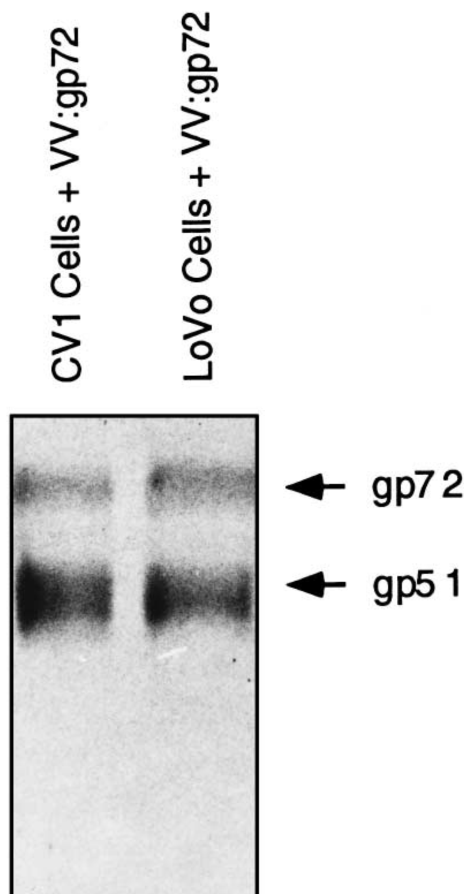

Fig. 1. gp72 Western blot of CV-1 and LoVo cell lysates. The cells were lysed 6 h post-infection by VV:gp72, and run on 10% SDS-PAGE. The cocktail of monoclonal antibodies used recognizes gp72 and gp52 but not gp30.

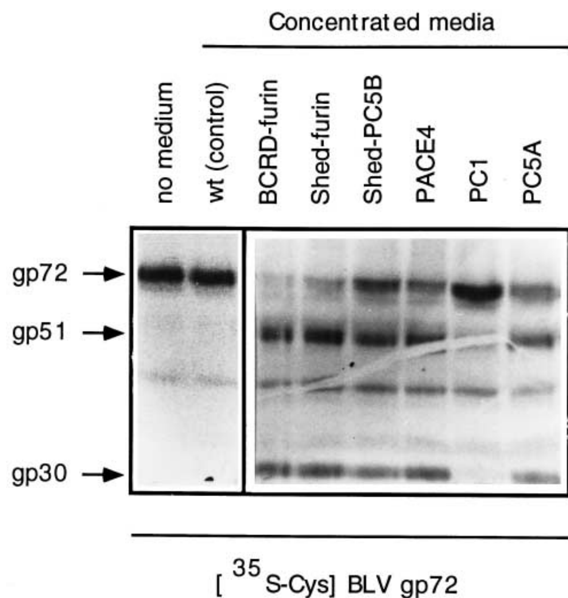

Fig. 2. In vitro processing of gp72 by convertases. Autoradiography of the 10% SDS-PAGE sepration of the in vitro cleavage products of the [35S]cysteine-labeled gp72 (5000 cpm) digested overnight with 50 nmol AMC/h equivalent convertase activities, based on the digestion of pERTKR-MCA. The soluble tested enzymes are shown. The negative control represents digestion with the equivalent volume of concentrated medium obtained from GH4C1 cells infected with wild-type vaccinia virus. The position of undigested gp72 is also shown.

ney CV1 cells. The data reveal that processing of gp72 into gp51 occurs to about the same extent in both cell lines, suggesting that endogenous enzymes in both cells can process gp72. No gp30 is detected in this system as it is not recognized by our monoclonal antibody cocktail (D. Portetelle, personal communication). Since LoVo cells are defective in furin activity, these results clearly demonstrate that in these cells, enzymes distinct from furin can process gp72 into gp51/gp30. However, furin may also participate in this processing since co-expression of VV:furin and VV:gp72 in CV-1 cells increased the level of gp51 production (not shown). Because mammalian PCs are likely candidates for such processing, it is thus possible that apart from furin, either PACE4, PC5 or PC7, which are expressed in LoVo cells [6,8,14], could also process gp72.

3.2. In vitro cleavage of gp72 by mammalian convertases

In an effort to identify the candidate convertases implicated in the cleavage of the BLV precursor, gp72 was incubated in vitro with several mammalian subtilisin-like endoproteases. These included the shed form of furin which is secreted into the medium [27], its isoform lacking the Cys-rich domain called BCRD-furin [11], PACE4 [20], PC1 [26], PC5-A and its isoform shed PC5-B [11,14]. Since membrane-bound PC7 ([8]; Munzer and Seidah, unpublished data) is not secreted into the medium [11], we have not studied this enzyme in vitro.

The glycoprotein gp72 was produced in CV1 cells following 1 h infection with VV:gp72 and 20 h growth. After 1 h of metabolic labeling with [35S]cysteine and purification on a lentil lectin column, the affinity purified gp72 was used for in vitro assays. As a source of enzymes, we used a 20-fold concentrated medium of GH4C1 cells infected with vaccinia virus recombinants that express each protease [11,21]. The

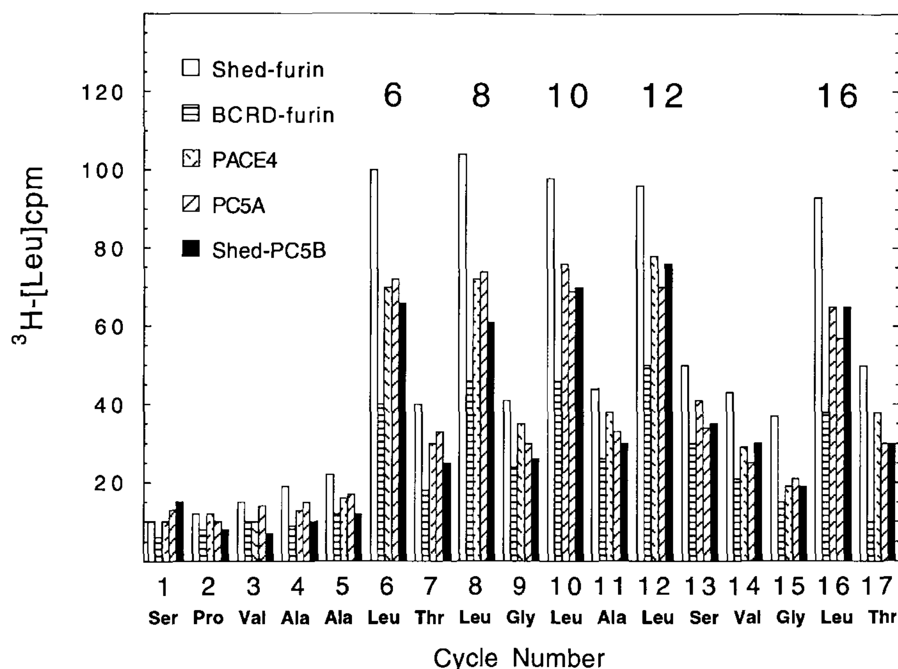

Fig. 3. Microsequence identification of gp72 processing site recognized by convertases. N-terminal microsequencing of [3H]Leu-labeled gp30 derived from the processing of gp72 by either shed-furin, BCRD-furin, PACE4, PC5-A or shed-PC5-B. The deduced Leu positions are emphasized, together with the primary sequence surrounding the gp51/gp30 cleavage site [23].

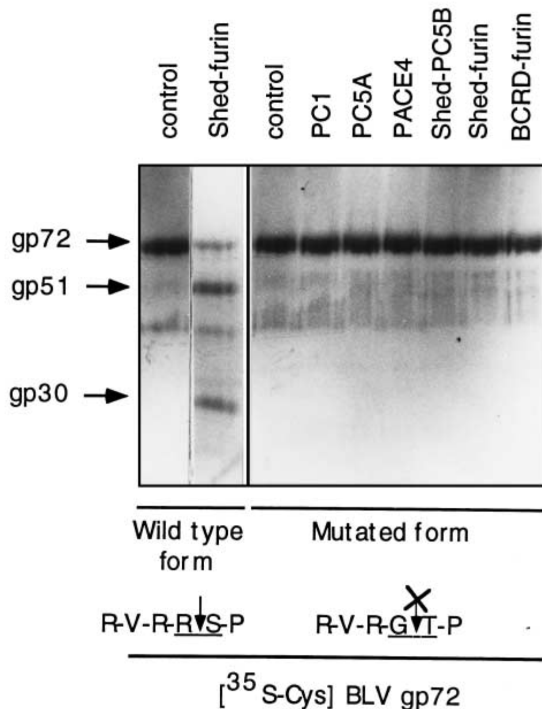

Fig. 4. Site-directed mutagenesis of the gp72 processing site. This figure shows the effect of substitution of the gp72 cleavage site RVRR^{268}↓SP [23] by RVRG-TP [4] on its proteolytic cleavage by convertases. The data show that all tested PCs do not process the mutant, as compared with shed-furin which processes the wild-type sequence. The experimental conditions are the same as in Fig. 2.

level of active enzyme was evaluated on a pentapeptide fluorogenic substrate pERTKR-MCA [11,21,26], whereby we used in all incubations with gp72 (Fig. 2) an enzymatic activity equivalent to 50 nmol AMC cleaved/h.

The data in Fig. 2 demonstrate that except for PC1, the convertases BCRD-furin, shed-furin, shed-PC5-B, PACE4 and PC5-A cleave gp72 in vitro, generating gp51 and gp30. The concentrated medium of GH4C1 cells infected with the wild-type vaccinia virus, used as negative control, did not reveal appreciable pentapeptide or gp72 converting activity (Fig. 2). To locate the BLV cleavage site recognized by each convertase, gp72 protein was labeled with [^{3}H]leucine and incubated with shed-furin, BCRD-furin, PACE4, PC5-A and shed-PC5-B. The digestion products of [^{3}H]leucine-labeled gp72 were separated on SDS-PAGE and transferred onto a PVDF membrane. The band corresponding to gp30 was excised and the protein microsequenced. The relative distribution of leucines along the sequence (at positions 6,8,10,12 and 16) (Fig. 3) demonstrate unambiguously that cleavage occurs at the Arg^{268} in the sequence ArgValArgArg^{268}↓SerProValAlaAla**Leu**Thr**Leu**Gly**Leu**Ala**Leu**SerValGly**Leu**ThrGly, as observed in the mature virus [23]. Thus, the in vitro data suggest that more than one PC can process gp72. The physiological enzyme(s) responsible for the processing of gp72 in vivo will depend on other parameters such as their co-localization in the same cells infected by BLV, and within the same intracellular secretory pathway.

Interestingly, PC1, which cleaved HIV gp160 in vitro [21], has no activity on gp72 at 50 nmol AMC/h (Fig. 2) and only weak activity on BLV gp72 at 250 nmol AMC/h (not shown). Gotoh et al. [16] also observed a lack of in vitro cleavage of the NDV F0 precursor when PC1 was used. This result may be rationalized by the presence of a proline residue at position P2′ from the processing site RVRR↓SPV of gp72. Indeed, it was previously observed that unlike PC2, the convertase PC1 does not process ACTH 1–39 into ACTH 1–17 involving cleavage at the VGKKR↓RPV where proline occupies the P2′ position [28].

Vonèche et al. demonstrated that replacement of the gp72 cleavage site by RVRG^{268}-TP prevented the processing of this envelope glycoprotein by cellular convertases and abolished BLV-induced syncytia formation [4]. In accordance with these cellular data, the results of Fig. 4 demonstrate that no convertase can process this gp72 mutant, suggesting that a PC-like enzyme(s) could be implicated in the processing of gp72 in vivo. These data emphasize the critical importance of Arg^{268} in gp72 processing. Similarly, a point mutation (Arg^{511} to Ser) at the HIV-1 gp160 cleavage site (REKR^{511}↓) completely prevented its processing [29]. It is unlikely that the lack of cleavage of the mutated gp72 is due to the substitution of serine (S; at P1′) by threonine (T). Indeed, systematic site-directed mutagenesis of hemagglutinin obtained from a virulent avian influenza virus revealed that the substitution of the cleavage site RKKR↓GL by RKKR↓TL does not significantly affect the maturation of the HA precursor by furin or PC5 [30].

3.3. Inhibition of gp72 processing by α1-PDX

In order to identify inhibitors of the gp72 cleavage we tested in vitro either the α1-PIT or the α1-PDX [24] obtained from the 20-fold concentrated medium of GH4C1 cells infected with their respective recombinant vaccinia viruses. Our data show that like the HIV-1 gp160 processing [10,20,24], α1-PIT does not affect the processing of gp72 by any of the enzymes tested (not shown). In contrast, the serpin variant α1-PDX completely inhibits the in vitro shed-furin, BCRD-furin, PACE4, PC5-A and shed-PC5-B cleavage of gp72 (Fig. 5). This finding confirms that α1-PDX is not a specific inhibitor of a single convertase at the exclusion of

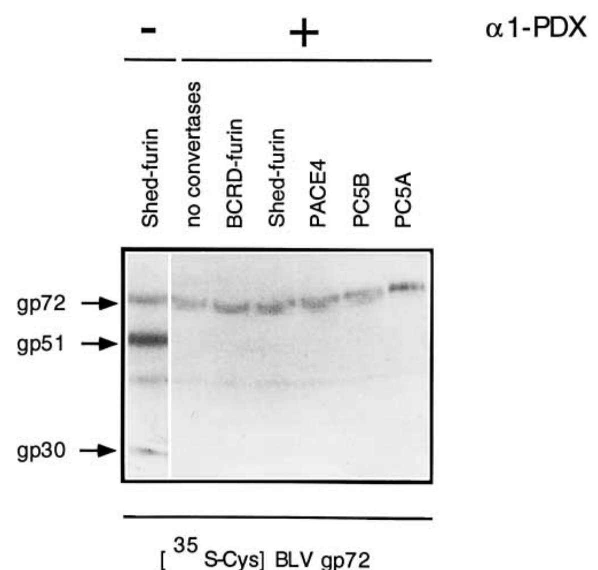

Fig. 5. Inhibition of gp72 processing by α1-PDX. Autoradiogram of a 10% SDS-PAGE separation of the processing products of [^{35}S]Cys gp72 by shed-furin (control) and by all other convertases tested in the presence of α1-PDX, as described [11].

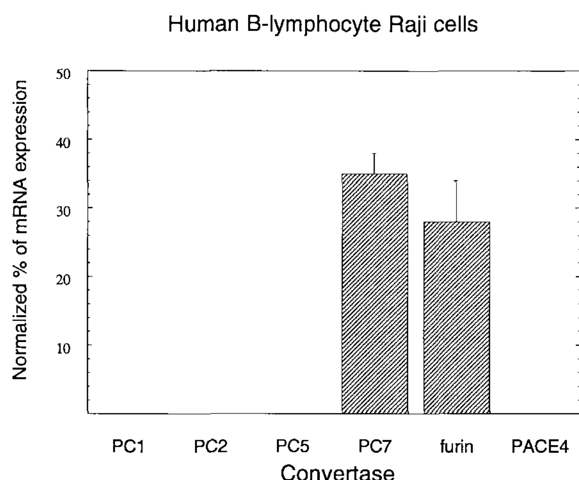

Fig. 6. Identification of precursor convertases mRNA in human B-lymphocyte-derived Raji cells. Each RT-PCR amplification (28 cycles) was performed using convertase specific primers together with two others specific for the control ribosomal L27 protein, as described [11]. RT-PCR products were separated on agarose gel (1.8%) electrophoresis and the relative amounts of each convertase in the tested lymphocyte cells were quantitated by densitometric analysis. The bar graphs represent the quantitation of the relative amount of each convertase normalized to the control L27. The data are an average (±standard deviation) RT-PCR experiment performed on RNA isolated from quintuple values obtained from five different RT-PCR amplifications.

the others. Indeed, in other systems, α1-PDX has been shown to inhibit furin [24] but also PACE4 and PC5 both ex vivo [20] and in vitro [10]. These observations provide proof of function of the PCs in the processing of viral glycoproteins and suggest that wide-specificity inhibitors could have potential applications in antiviral therapy.

3.4. Expression of the convertases in B-lymphocyte-derived cell lines

Our in vitro data suggested a certain redundancy in the ability of the convertases to process the BLV gp72. Since B-lymphocytes represent one of the major targets of BLV infection, we used a semi-quantitative RT-PCR assay to detect the mRNA corresponding to each convertase [11] in the B-lymphocyte-derived Raji cell line. Under resting conditions, in these cells, furin and PC7 were the only significantly transcribed convertases (Fig. 6). In contrast, we did not detect significant mRNA levels of PC1, PC2, PACE4 or PC5 in this cell line, as opposed to a positive control which consists of a mixture of human pituitary and HT-29 cells [11] which expresses all of them (not shown). In order to assess the generality of this relative mRNA distribution, we confirmed that furin and PC7 are also the only PCs expressed in two other B-lymphocyte-derived cell lines B-Jab and LG2 (data not shown). These data suggest that the convertases mediating the gp72 cleavage in infected B-lymphocytes may be restricted to furin and the newly discovered enzyme PC7, both of which are highly enriched in lymphoid-associated tissues [8–10].

The absence or the low expression levels of PACE4 and PC5 in the tested B-lymphocyte-derived cell lines suggests that these convertases cleaving in vitro the BLV gp72 protein may not play a significant role in BLV-infected B-cells. Nevertheless, in view of the plasticity of cellular expression of the convertases in lymphocytes before and after immune cell activation [10,21], the determination of the convertases expressed in BLV-infected B-cells could be helpful to identify the enzymes implicated in gp72 processing in vivo. In conclusion, our data demonstrate that at least furin is one of the gp72 convertases and that the newly discovered PC7 is also a plausible physiological candidate in B-lymphocytes.

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