Furin and proprotein convertase 7 (PC7)/lymphoma PC endogenously expressed in rat liver can be resolved into distinct post-Golgi compartments

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The intracellular compartmentalization in rat liver of the membrane-associated convertases furin and proprotein convertase 7 (PC7)/lymphoma PC (LPC) was investigated by analytical subcellular fractionation. In control animals, both enzymes were found to localize in fractions depleted of endoplasmic reticulum, *cis*-Golgi and lysosomal markers, but to co-distribute with the Golgi marker galactosyltransferase and the *trans*-Golgi network (TGN) marker TGN38. After overloading Golgi-derived vesicles with very-low-density lipoproteins (VLDL) by feeding rats with ethanol, the distribution of PC7/LPC was shifted markedly towards lower densities, in contrast with those of furin and the TGN marker. This provides support for the TGN localization of endogenously expressed furin and indicates that, at steady state, a considerable proportion of PC7/LPC may be associated with vesicles derived from the TGN.

INTRODUCTION

Several proteins, including hormones, neuropeptides, growth factors, cell receptors and viral envelope glycoproteins, are synthesized as inactive proproteins and cleaved further at the C-terminal side of paired basic amino acid residues to yield mature forms [1,2]. Although the endoproteolytic maturation of these proproteins is initiated in the Golgi complex [3], processing is not restricted to this compartment. Many precursors are cleaved later: (i) in the regulated or constitutive exocytic pathways, (ii) in endosomal compartments, or (iii) at the cell surface [4].

A major step towards understanding proprotein processing has been the identification of a family of resident endoproteinases of the secretory pathway, the proprotein convertases (PCs), that catalyse cleavage at the C-terminal side of the consensus sequence Lys/Arg-Xaa-Lys/Arg-Arg↓. To date, seven mammalian PCs have been discovered, which exhibit significant similarities with both yeast kexin and bacterial subtilisin. These subtilisin/kexin-like convertases are called furin [5], PC1/PC3, PC2 [6–8], PC4 [9,10], PACE4 (where PACE is paired basic amino acids converting enzyme) [11], PC5/PC6 [12,13] and PC7/lymphoma PC (LPC) [14–16]. Whereas all share high identity in their catalytic domain and contain the Asp/His/Ser triad typical of serine proteinases, variations of PC sequences in their C-terminal domains determine their subcellular localization. For example, furin, PC5/PC6-B and PC7/LPC, three type I transmembrane proteins, are associated with the constitutive secretory pathway, while PC1/PC3 and PC2, containing membrane-associated amphipathic domains, as well as PC5/PC6-A, transit through the regulated secretory pathway [17].

Since a given cell generally expresses several convertases, the problem remains to clarify whether processing of a particular substrate is mediated by one or several of these proteinases. Furthermore, even if one convertase cleaves a given substrate *in vitro*, this is not sufficient proof of involvement of this particular convertase *in vivo*; it is also necessary that enzyme and substrate effectively co-localize along the regulated or constitutive secretory pathways.

Accordingly, there has been considerable interest in determining the subcellular localization of the proprotein convertases in comparison with their potential substrates. However, attempts to immunolocalize endogenous convertases have been hampered by their low level of expression in most cultured cells. It was therefore necessary to develop overexpression systems combined with an epitope tag-based strategy to study the biosynthesis and routing of convertases.

The first localization studies on convertases were performed on furin. An examination of epitope-tagged furin localization by immunofluorescence and immunoelectron microscopy revealed a prominent concentration in the *trans*-Golgi network (TGN). In addition, cytoplasmic tail motifs, as well as the phosphorylation state of furin, were shown to be crucial for its shuttling between the cell surface and the TGN [18–24]. More recently, studies in transfected AtT20 cells, which have both constitutive and regulated secretory pathways, demonstrated that two isoforms of PC5/PC6 containing different C-terminal sequences have different destinations along the secretory pathway [25]. The shorter soluble form, PC5/PC6-A, is sorted into secretory granules of the regulated pathway, while the C-terminally extended and membrane-bound form PC5/PC6-B remains in the Golgi stacks. Taken together, these observations suggest that C-terminal-specific motifs and the phosphorylation state of convertases may determine their subcellular localization. Since PC7/LPC is not phosphorylated [26] and does not contain the same localization motifs as furin and PC5/PC6 in its cytoplasmic tail, we decided to determine the extent to which the localization of PC7/LPC is distinct from that of furin.

The above-mentioned studies were performed using mainly

Abbreviations used: PC, proprotein convertase; LPC, lymphoma PC; TGN, *trans*-Golgi network; VLDL, very-low-density lipoproteins.

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immunolabelling in overexpressing cell systems. This powerful approach might nevertheless lead to artefacts, resulting from unequal accessibilities of protein epitopes in distinct subcellular compartments or to abnormal localization due to overexpression. In the present work, we compared the subcellular distributions of two membrane-anchored convertases, furin and PC7/LPC, using subcellular fractionation. We chose rat liver for this study, for two reasons: first, a method for analytical subcellular fractionation is well established; secondly, the convertases in this tissue are expressed at a basal level. In addition, very-low-density lipoproteins (VLDL) mark the constitutive secretory pathway in rat hepatocytes, and their abundance can be rapidly increased upon ethanol treatment.

The present study, based on such a fractionation analysis, supports the concentration in TGN of endogenously expressed furin and provides new information concerning PC7/LPC localization. In particular, we demonstrate that, upon ethanol treatment, PC7/LPC can be resolved from the TGN marker TGN38 and furin. PC7/LPC is shifted to markedly lower equilibrium densities, presumably in secretory vesicles that are enriched in VLDL particles. In contrast, furin co-localizes with TGN38 both in control and in ethanol-treated rats.

EXPERIMENTAL

Analytical subcellular fractionation

All experiments were performed using male Wistar rats weighing about 250–300 g and fasted overnight. Some rats were further given 0.6 g of ethanol/100 g body weight as a 50% (w/v) solution by stomach gavage 90 min before they were killed. The liver was perfused with PBS, cut into small pieces, dispersed with approx. 3 vol. of ice-cold 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole/HCl and transferred into a Potter–Elvehjem homogenizer. The homogenate was submitted to differential centrifugation [27] to successively pellet the N fraction (6000 $g \cdot$min; E4 rotor, GR 4.11 Jouan), the M fraction (33000 $g \cdot$min; Ti50 rotor, Beckman), the L fraction (275000 $g \cdot$min; same rotor) and the P fraction (3×10^6 $g \cdot$min); this last fraction included the fluffy layer and hence is referred to hereafter as the 'total microsomal fraction'. This final fraction was brought to a density of 1.27 g/ml by the slow addition of concentrated sucrose and layered below a linear sucrose gradient (1.05–1.23 g/ml) for flotation isopycnic centrifugation (192×10^6 $g \times$min; VTi 50 rotor, Beckman). Fourteen fractions were collected, weighed and analysed for density, enzyme activities and antigenic content by quantitative Western blotting. Recoveries ranged between 80 and 113%.

Assays

Protein was assayed by the procedure of Lowry et al. [28], using BSA as a standard. The following marker enzyme activities were measured using established procedures: 5′-nucleotidase [29], galactosyltransferase [30] and *N*-acetyl-β-hexosaminidase [31].

Western blotting

Equal volumes of each gradient fraction were boiled for 3 min in SDS/PAGE sample buffer [32] and resolved on SDS/7.5% polyacrylamide gels. Western blotting was performed using a monoclonal antibody (MON-139) specific for the cytoplasmic, C-terminal region of human furin [33] and a rabbit antiserum (MP1) directed against the N-terminus of mature human LPC and cross-reacting with rat PC7/LPC [26]. Rabbit antiserum anti-TGN38 (TGN marker) and anti-p58 (*cis*-Golgi network marker) were kindly provided by Dr. G. Banting (University of Bristol, Bristol, U.K.) [34] and Dr. J. Saraste (University of Bergen, Bergen, Norway) [35] respectively. Antigens were detected using chemiluminescent horseradish peroxidase substrate (ECL; Amersham).

Cell lines

The CHO-DHFR$^-$ cell line stably expressing human LPC has been described before [26]. Confluent CV-1 cell monolayers were infected with VV:hfur recombinant vaccinia virus, as described [36], at a multiplicity of infection of 1 plaque-forming unit/cell.

RESULTS

Subcellular distribution of furin and PC7/LPC in control rats

It is thought that the subcellular distributions of furin and LPC/PC7 are controlled by signals present in their cytoplasmic tails. We report here the first use of a subcellular fractionation strategy for comparison of the intracellular localization of naturally expressed furin and LPC/PC7. We selected as starting material rat liver, for which fractionation procedures have been well established [27,37], and in which Northern blot analysis has demonstrated the presence of furin and LPC/PC7 mRNA [16,38]. Liver homogenate was first submitted to subcellular fractionation by differential centrifugation. Three particulate fractions, N (nuclear), ML (large granules) and P (microsomes), and a final supernatant (S), were obtained. The distribution profiles of furin and PC7/LPC in these fractions were determined according to de Duve et al. [27] (Figure 1). Since essentially all furin and PC7/LPC were found in the total microsomal fraction (P) (97% and 90% respectively), this fraction was selected for further analysis. After isopycnic centrifugation of microsomes by flotation in linear density sucrose gradients, the density distributions of both convertases were determined by Western blotting and compared with those of known markers.

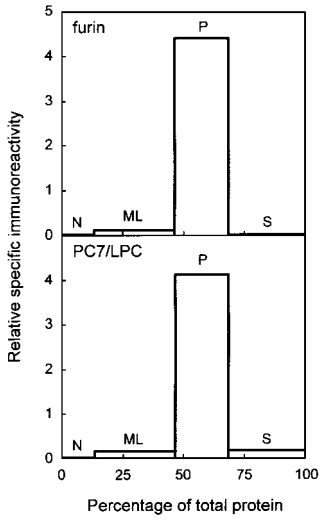

Figure 1 Patterns of distribution of furin and PC7/LPC after fractionation of liver by differential centrifugation

Fractions are plotted in order of the average coefficient of sedimentation of their subcellular components, i.e. from left to right: N (nuclear fraction), ML (large granules fraction), P (total microsomal fraction) and S (soluble fraction). Each fraction is represented separately in the ordinate scale by the relative specific immunoreactivity of the convertases (percentage of total amount/percentage of total protein). On the abscissa scale, each fraction is represented cumulatively by its percentage of protein.

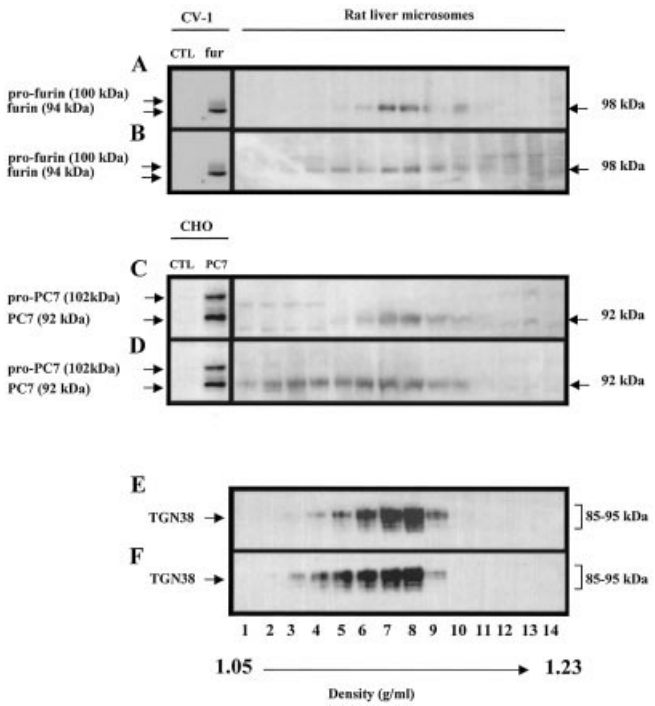

Figure 2 Immunoblot analyses of furin, PC7/LPC and TGN38 in isolated rat liver subcellular fractions

The microsomal fraction of control (**A**, **C**, **E**) or ethanol-treated (**B**, **D**, **F**) rat livers was fractionated by isopycnic centrifugation in a linear sucrose gradient. Fourteen fractions were isolated, and furin (**A**, **B**), PC7/LPC (**C**, **D**) and TGN38 (**E**, **F**) were detected therein by immunoblot analyses using specific monoclonal antibodies. Lane CTL, control immunoblot of untransfected CV-1 or CHO cells; lane CV1-fur, CV-1 cells transiently transfected with VV:hfur; lane CHO-PC7, CHO cells stably expressing PC7/LPC. The molecular masses of human (pro)-furin and -PC7/LPC in cell lines are indicated on the left, and those of endogenous convertases in rat liver are indicated on the right. The data are representative of one from a total of three independent experiments.

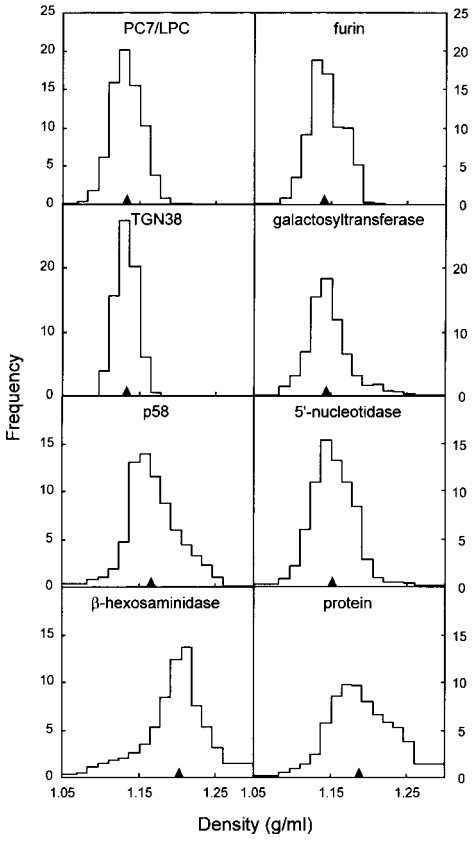

Figure 3 Density distributions of marker constituents and the convertases after isopycnic equilibration of microsomes

Liver was homogenized and a total microsomal fraction (P) was isolated and further equilibrated by flotation in a linear sucrose gradient (density 1.05–1.30 g/ml). Fourteen fractions were collected and subjected to densitometry and immunoblot analyses of furin and PC7/LPC. The subcellular markers are 5′-nucleotidase (plasma membrane), *N*-acetyl-β-hexosaminidase (lysosomes), galactosyltransferase (*trans*-Golgi cisternae), p58 (*cis*-Golgi network), TGN38 (TGN). Median equilibrium densities are indicated by arrowheads. The data are representative of one from a total of three independent experiments.

In CV_1 cells infected by VV:hfur, used as positive control, the monoclonal anti-furin antibody MON-139 recognized two bands at 100 kDa and 94 kDa, corresponding to the previously described pro- and mature forms of human furin respectively [33]. In rat liver microsomal fractions 6–10 (equilibrating from density 1.113–1.169 g/ml), the antibody recognized a protein migrating at $\sim$ 98 kDa (Figure 2A). This $\sim$ 98 kDa band corresponds to the mature form of rat furin lacking the propeptide. The molecular mass discrepancy of mature furins can be accounted for by differences in glycosylation between recombinant human and endogenous rat proteinases [39]. For PC7/LPC detection, CHO cells that stably express recombinant human PC7/LPC [26] were used as a positive control (Figure 2C). The polyclonal antiserum MP1 detected two major proteins, migrating at 102 kDa and 92 kDa [26]. Whereas the 102 kDa form was shown to correspond to pro-PC7/LPC, the 92 kDa form reflects mature PC7/LPC that is N-glycosylated [26]. In the linear sucrose gradient, the antiserum MP1 recognized only the 92 kDa protein in fractions 5–10 (density 1.101–1.169 g/ml). This band corresponds to mature PC7/LPC.

To define the subcellular localizations of furin and PC7/LPC, we compared their distribution patterns with those of established subcellular markers in the same sucrose gradient, using 5′-nucleotidase and *N*-acetyl-β-hexosaminidase as plasma membrane and lysosomal markers respectively. The Golgi complex markers were p58 (*cis*-Golgi network [35]), galactosyltransferase, a membrane-bound enzyme localized predominantly in the *trans*-most Golgi cisternae, and TGN38, a marker of the TGN [34]. After isopycnic flotation of the subcellular organelles, furin and PC7/LPC appeared as narrow peaks centred around a median density of $\sim$ 1.136 g/ml (Figure 3). Their density distributions were clearly distinct from those of the bulk of microsomal proteins (i.e. the rough endoplasmic reticulum) and that of the lysosomal marker enzyme *N*-acetyl-β-hexosaminidase (median density of 1.202 g/ml), and only partially overlapped the p58 *cis*-Golgi network marker (1.166 g/ml). In contrast, the two convertases largely co-distributed with galactosyltransferase (1.144 g/ml), TGN38 (1.133 g/ml) and 5′-nucleotidase (1.152 g/ml) in the same sucrose gradient. These observations exclude a primary localization of furin and PC7/LPC in lysosomes, the rough endoplasmic reticulum or the *cis*-Golgi compartment, and indicate that the active forms of both convertases are localized in one or several subcellular compartments distal to the *cis*-Golgi network. Nevertheless, this fractionation method does not resolve the *trans*-Golgi from the derived secretory vesicles.

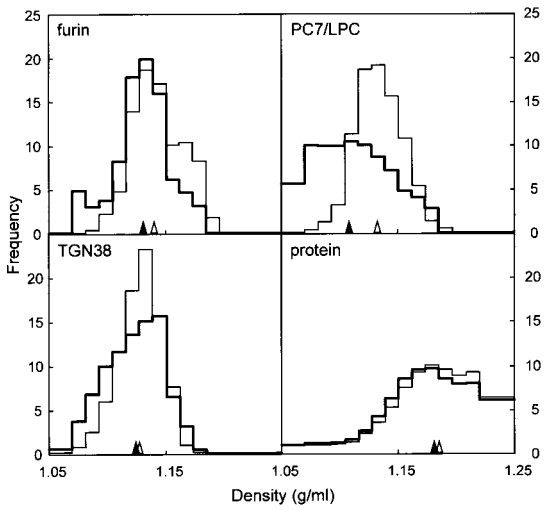

Figure 4 Comparison of density distributions of the convertases in control and ethanol-treated rats

The microsomal fraction (P) of livers from control or ethanol-treated rats was isolated, equilibrated in a linear sucrose gradient (density 1.05–1.23 g/ml) and analysed as described in the legend to Figure 2. The density distributions of furin, PC7/LPC and TGN38 in an ethanol-treated rat are represented by thick lines, and are superimposed on the corresponding density distributions in control rats (thin lines). Data are averaged distributions of two experiments in control rats and are representative of one from a total of three independent experiments in ethanol-treated rats.

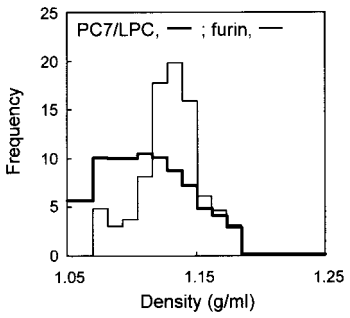

Figure 5 Resolution of PC7/LPC from furin upon ethanol treatment

Distributions of furin and PC7/LPC from the ethanol-treated rat shown at Figure 4 have been superimposed for convenience. The density distribution of PC7/LPC is here represented by a thick line, and that of furin by a thin line.

Partial resolution of Golgi/TGN and distal secretory compartments upon ethanol treatment

To further distinguish the subcellular distributions of furin and PC7/LPC, Golgi-derived elements were loaded with VLDL, by feeding rats with ethanol. Overloading by VLDL (0.950–1.006 g/ml) not only provides a morphological marker, but also decreases the buoyant density of Golgi-derived vesicles [40,41]. By electron microscopy (results not shown), the Golgi apparatus in hepatocytes of control rats generally contained separated VLDL, that usually occurred in large vacuoles and, to a lesser extent, in the dilated rims of the *trans*-Golgi cisternae and the TGN. Upon ethanol treatment, the structure of the TGN did not change appreciably, but VLDL partially accumulated in Golgi stacks and the TGN and were conspicuously packed in TGN-derived secretory vesicles, as already reported [40].

In the microsomes of ethanol-treated rats (Figure 4), the mature form of furin was detected in fractions 4–10 (at densities of 1.102–1.175 g/ml; Figure 2B) and its median density was only weakly displaced (from 1.141 to 1.131 g/ml). In contrast, PC7/LPC was displaced to much lower densities ($\sim$ 1.109 g/ml), i.e. by up to 5 fractions (cf. Figures 2C and 2D). Since ethanol treatment also provoked some accumulation of VLDL in the dilated rims of *trans*-Golgi cisternae and the TGN [40,41], we also determined the effect of ethanol on the density distribution of the TGN. Its marker TGN38 was marginally displaced by ethanol treatment (median density from 1.128 to 1.125 g/ml; see also Figures 2E and 2F), showing that the TGN distribution was only weakly modified by ethanol. Although the extent of ethanol-induced density shift varied between experiments, PC7/LPC was consistently displaced towards much lower buoyant densities than were TGN38 and furin. Thus, in ethanol-treated rats, PC7/LPC was clearly resolved from furin (Figure 5) and the TGN, suggesting that PC7/LPC may be localized in subcellular compartments distal to the TGN along the constitutive secretory pathway.

DISCUSSION

Available immunofluorescence and immunoelectron microscopy studies on the intracellular localization of furin and PC7/LPC were based on overexpression systems and did not disclose major differences: both convertases were predominantly associated with the TGN, with PC7/LPC being somewhat less concentrated than furin, and both were transported out of the TGN in clathrin-coated vesicles [19,22,26,42]. However, overexpression may alter protein distribution and fate by saturating retention/retrieval mechanisms [43]. This pitfall is well documented for furin: in cells expressing endogenous furin, the convertase was shown to be associated with membranes, whereas furin overexpression using the vaccinia system resulted in its secretion as a soluble protein, after proteolytic removal of the membrane anchor in the TGN [36,44].

The present study addresses the subcellular distribution of the endogenously expressed convertases in rat liver and demonstrates that, in the subcellular fractions derived from ethanol-treated rats, PC7/LPC can be clearly resolved from furin. Upon acute ethanol treatment, VLDL-enriched secretory vesicles, which represent the constitutive secretory pathway, were shifted towards lower equilibrium densities and thereby become well separated from Golgi and TGN compartments. In ethanol-treated rats, the bulk of furin still co-distributes with the TGN marker TGN38, in agreement with morphological results from overexpression systems [18,19], but PC7/LPC was shifted towards lower equilibrium densities, like secretory vesicles dispatched from the TGN. These results suggest that, at steady state, endogenously expressed furin and PC7/LPC may be present in different subcellular compartments. Alternatively, the possibility cannot be excluded that acute ethanol treatment could differentially affect membrane protein trafficking, so that PC7/LPC would be diverted to a new non-physiological compartment, whereas furin would not be disturbed.

Whereas endogenous furin was essentially co-distributed with the *trans*-Golgi cisternae marker galactosyltransferase and the TGN marker TGN38, it also largely overlapped with the density distribution of the (apical) plasma membrane marker 5′-nucleotidase and of early endosomes [45,46]. These findings are con-

sistent with previous morphological studies, showing a major localization of recombinant furin in the TGN and a minor component at the cell surface as well as in the endosomal/lysosomal system [18–24]. However, as furin was not detected in the N and ML fractions, which contain the large basolateral plasma membrane fragments, it is suggested that only a marginal proportion of furin is localized at the basolateral cell surface at steady state. In ethanol-treated rats, the minor component of the furin distribution that was shifted towards lower densities could correspond to the small proportion of furin escaping the TGN and transiting through secretion vesicles.

Distinct distributions of furin and PC7/LPC might result from differences in addressing motifs in their cytoplasmic tails or transmembrane domains. Several trafficking signals governing TGN localization have been defined in the cytoplasmic tail of furin [18–24]. These include: (i) a cluster of acidic amino acids with the sequence CPSDSEEDEG in which phosphorylation of the serine residues (bold) plays an important modulatory role in the return of furin to the TGN [23,24]; (ii) a tyrosine-containing motif (YXXL) which is a signal to return integral membrane proteins from the cell surface to the TGN [47]; and (iii) leucine and/or isoleucine residues, which are required for the internalization and/or lysosomal delivery of a large number of membrane proteins [48].

Interestingly, the cytoplasmic tail of PC7/LPC does not contain the acidic stretch or the retrieval motif YXXL, but includes two dileucine internalization motifs [25]. The absence of TGN localization and retrieval signals in the PC7/LPC C-terminal domain suggests that this convertase may indeed pass through the TGN and localize in TGN-derived vesicles. Furthermore, PC7/LPC is palmitoylated [26], and this lipid anchor has been suggested as a necessary step in membrane trafficking [49]. The extent to which these various motifs are responsible for a distinct localization of PC7/LPC in TGN-derived vesicles instead of in the TGN, or cause its redistribution thereto upon acute ethanol treatment, remains to be examined.

Cell fractionation followed by Western blot analysis further allows the various molecular forms associated with different subcellular compartments to be distinguished. Lindberg and colleagues [50] have discussed the respective subcellular distributions of the pro- and mature forms of PC1 and PC2 in AtT20 cell lines. Interestingly only the active, mature forms of the naturally expressed convertases were detected in rat liver, suggesting that, in the absence of overexpression, little if any of the proforms is present at steady state. Distributions shown in the present paper are thus in excellent agreement with pulse–chase experiments, which demonstrated that (i) removal of the propeptide is required for furin and PC7/LPC to leave the endoplasmic reticulum so as to reach their steady-state compartments [26,39]; and (ii) after a long chase period, only the mature form of the convertase is detected [26].

In conclusion, distinct subcellular distributions of the mature forms of furin and PC7/LPC, if confirmed, may have important physiological implications. Furin, being mainly present in the TGN, appears to be involved in both the constitutive and the regulated secretory pathways. In contrast, a localization of PC7/LPC in the vesicles of the constitutive secretory pathway could restrict its function to the proteolysis of protein precursors directed to this pathway. It has been proposed that both furin and PC7/LPC participate in the endoproteolytic processing of the HIV envelope glycoprotein gp160 into gp120 and gp41, an essential step for the infectivity of the virus [51,52]. The fractionation method reported in the present paper is currently being used to determine in which subcellular compartment (TGN or TGN-derived vesicles) the HIV gp160 is processed, by assessing the extent to which an endoproteolytic activity present in subcellular compartments enriched in furin and/or PC7/LPC cleaves gp160 into gp120 and gp41.

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