A human intracellular apyrase-like protein, LALP70, localizes to lysosomal/autophagic vacuoles

Annette Biederbick¹, Scott Rose² and Hans-Peter Elsäsßer¹,*

¹Department of Cell Biology, Robert-Koch Str. 5, 35033 Marburg, Germany
²UT Southwestern Medical Center, Department of Molecular Biology and Oncology, 5323 Harry Hines Blvd, Dallas, TX 75235-9140, USA

*Author for correspondence (e-mail: elsaesse@mailer.uni-marburg.de)

Accepted 26 May; published on WWW 7 July 1999

SUMMARY

Using antibodies against autophagic vacuole membrane proteins we identified a human cDNA with an open reading frame of 1848 bp, encoding a protein of 70 kDa, which we named lysosomal apyrase-like protein of 70 kDa (LALP70). Sequence analysis revealed that LALP70 belongs to the apyrase or GDA1/CD39 family and is almost identical to a human uridine diphosphatase, with the exception of nine extra amino acids in LALP70. Members of this family were originally described as ectoenzymes, with some intracellular exceptions. Transfected LALP70 fused to the green fluorescent protein localized in the cytoplasm with a punctate pattern in the perinuclear space. These structures colocalized with the autophagic marker monodansylcadaverine and the lysosomal protein lam1.

Hydrophobicity analysis of the encoded protein revealed a transmembrane region at the N and C termini. Most of the sequence is arranged between these transmembrane domains, and contains four apyrase conserved regions. In vitro transcription/translation in the presence of microsomes showed that no signal sequence is cleaved off and that the translation product is protected from trypsin treatment. Our data indicate that LALP70 is a type III lysosomal/autophagic vacuole membrane protein with the apyrase conserved regions facing the luminal space of the vacuoles.

Key words: Apyrase, Lysosome, Autophagy, Membrane protein

INTRODUCTION

Macroautophagy is a widespread phenomenon in the degradation of cellular components in the lysosomal compartment (Dunn, 1994). The structural correlate in macroautophagy is the autophagic vacuole, the formation of which has been subdivided into two consecutive steps: formation of autophagosomes, which lack lysosomal hydrolases and are delineated by two membranes, and their subsequent development into autophagolysosomes, which contain lysosomal hydrolases and are delineated by a single membrane (Dunn, 1990a,b; Yokota, 1993; Yokota et al., 1993). Autophagosomes develop from endomembranes, most likely from ribosome-free cisternae of the endoplasmic reticulum (Furuno et al., 1990), which sequester cytoplasmic domains or intracellular organelles addressed for degradation (Dunn, 1990a). In a subsequent step the endomembranes fuse and constitute a closed vacuole lined by a double membrane (Dunn, 1990b).

The crucial event in the transition from an autophagosome to an autophagolysosome is the fusion with lysosomes. Classification of vacuoles as mature autophagic vacuoles is predominantly based on their appearance in the electron microscope. The ultrastructural hallmarks of fully developed autophagic vacuoles are myelin-like membrane whirls filling the lumen of these organelles (Seglen, 1987; Papadopoulos and Pfeiffer, 1987). This membrane material is thought to represent remnants from degraded organelles. However, it cannot be ruled out that parts of the plasma membrane are also present in autophagic vacuoles, since a connection between the endocytic and autophagic pathway has been demonstrated (Tooze et al., 1990; Punnonen et al., 1993). It has recently been shown that autophagic vacuoles can be stained with the autofluorescent substance monodansylcadaverine (MDC; Biederbick et al., 1995), due to an interaction of MDC with the highly concentrated lipids in these organelles (A. Niemann et al., unpublished).

Studies on the structure, function and turnover of autophagic vacuoles in higher eukaryotic cells have been mostly descriptive. However, in yeast remarkable progress has been achieved in describing the mechanism of autophagy on the molecular level. An autophagy-like process in yeast can be induced by starving the cells in culture and it is assumed that the subsequent delivery of cytoplasmic domains to the vacuole is at least in part comparable to autophagy in higher eukaryotes (Takeshige et al., 1992; Baba et al., 1994). Applying a genetic approach, a variety of mutants defective in autophagy have been described: apg 1-15 (Tsukada and Ohsumi, 1993), aut 1-8...
MATERIALS AND METHODS

Cell lines and culture conditions

The cell line PaTu 8902 was established from a human primary pancreatic adenocarcinoma and characterized in detail as described previously (Elsässer, 1993). Cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 g/l Hepes, 5% fetal calf serum, 5% adult calf serum, 50 μg/ml gentamicin (Gibco, Karlsruhe, Germany) and incubated at 37°C in a humidified chamber equilibrated with 5% CO₂.

Antibodies

The polyclonal AV1-serum was raised against a mixture of purified membrane proteins from autophagic vacuoles. Isolation of autophagic vacuoles by subcellular fractionation from PaTu 8902 cells was described elsewhere (Biederbick, 1995). For one preparation cells from 6x55 cm² plates were used. The intact organelles were biotinylated with the membrane-impermeable sulfo-nitrohydroxysuccinimidobiotin (Pierce Chemical Corp., USA) according to the cell surface biotinylation techniques of Zurrzolo et al. (1994). In brief, organelles were washed twice with ice-cold PBS-CM (PBS, pH 7.2, supplemented with 1 mM MgCl₂, 0.1 mM CaCl₂, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A (Sigma, Deisenhofen, Germany) and 5 μl/ml Trasylol (Bayer, Leverkusen, Germany)), and incubated on ice for at least 60 minutes with 0.25 mg/ml Sulfo-NHS-Biotin in PBS-CM in a final volume of 1 ml. The reaction was stopped adding NH₄Cl at a final concentration of 50 mM. After 10 minutes on ice vacuoles were centrifuged at 4°C at 13,000 g for 30 minutes. The pellet was resuspended in 250 μl lysis buffer (20 mM Tris/HCl, pH 5.0, 150 mM NaCl, 1% Triton X-100 and proteinase inhibitors as described above) and free biotin was removed with a Fast Desalting column HR 10/10 equipped with Sephadex G-25 Superfine (Pharmacia, Uppsala, Sweden). The biotinylated membrane proteins were incubated overnight at 4°C with streptavidin-agarose beads (Sigma Chemical Co., USA) equilibrated in lysis buffer. The agarose beads with the bound biotinylated autophagic vacuole membrane proteins were washed twice with PBS-CM supplemented with proteinase inhibitors and resuspended in 100 μl PBS-CM. Material from six preparations was pooled. 200 μl were mixed with 300 μl Gerbu adjuvans (Gerbu Biotechnik, Germany) and injected subcutaneously into rabbits to raise polyclonal antibodies against the membrane proteins.

Other antibodies used for immunofluorescence microscopy were a mouse monoclonal antibody against purified anti-human CD107a (LAMP-1) (Pharmingen, Germany), a Cy2-labelled goat anti-rabbit IgG polyclonal antibody and a Cy3-labelled goat anti-mouse IgG antibody (both from Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

cDNA expression library and cloning

AV1-serum was used for immunoscreening a Uni-ZAP XR cDNA Library (Stratagene, LJ, USA) derived from human pancreatic adenocarcinoma cell line CF Pac-1. AV-1 serum was cleared from antibodies against bacterial antigens by the method of Gruber and Zigang (1995). Immunoscreening was performed according to the manufacturer’s instructions (picoBlue Immunoscreening Kit, Stratagene, USA). Three rounds of expression and immunoscreening yielded a single clone insert of 2.4 kb in a pBluescript SK vector. The insert was sequenced on both strands using the Perkin Elmer Applied Biosystems 377 DNA Sequencer (Applied Biosystems, USA). The nucleotide and deduced protein sequences were screened against the GenBank/EMBL database using the FASTA/BLAST programs as implemented on the internet resource. Sequence alignments were performed using the CLUSTAL W (1.74) Multiple Sequence Alignment software as implemented on the internet resource by the ExPasy server.

Expression plasmids

A 1960 bp SmaI fragment from the LALP70 cDNA clone, containing the full-length cDNA without the stop codon and the last three predicted amino acids, was cloned in-frame into the SmaI site of the mamalian expression vector pEGFP-N3 (Clontech Laboratories Inc.,...
Living cells were incubated on a thermostated and CO2-perfused stage confocal laser scanning microscope LSM 410 (Zeiss, Köln, LALP70-EGFP or EGFP in living or paraformaldehyde-fixed cells at a wave length, 530-560 nm; barrier filter, 580 nm). For detection of 340-380 nm; barrier filter, 430 nm) and a filter system N2 (excitation 515 nm filter. When Cy3-conjugated secondary antibodies were used for immunofluorescence staining of PaTu 8902 cells, a perinuclear pattern was observed, reminiscent of the structures stained by MDC (Fig. 1A). Double staining using an antibody against the lysosomal membrane protein lamp1 (Biederbick et al., 1995). The protein residues exposed on the outer membrane were biotinylated and separated from proteins of the inner membranes with streptavidin-agarose beads. Proteins enriched by this procedure were used to raise a polyclonal antisera (AV1). When AV1 was used for immunofluorescence staining of PaTu 8902 cells, a perinuclear and punctate pattern was observed, reminiscent of the structures stained by MDC (Fig. 1A), Double staining using an antibody against the lysosomal membrane protein lamp1 revealed that AV1-positive and lamp1-positive structures were mainly colocalized (Fig. 1A,B). Furthermore, lamp1-positive organelles were also mainly colocalized with MDC-positive structures (Fig. 1C,D), indicating that AV1 recognizes lysosomal/autophagic vacuoles.

RESULTS

Isolation and characterization of the LALP70 cDNA clone

Autophagic vacuoles are characterized by myelin-like membrane whirls filling the lumen of these organelles. In order to obtain an antisera against membrane proteins located in the membrane delineating the autophagic vacuole, we isolated these organelles from human pancreatic adenocarcinoma cells as MDC-positive vacuoles with a density of about 1.098 g/cm3 (Biederbick et al., 1995). The protein residues exposed on the outer membrane were biotinylated and separated from proteins of the inner membranes with streptavidin-agarose beads. Proteins enriched by this procedure were used to raise a polyclonal antisera (AV1). When AV1 was used for immunofluorescence staining of PaTu 8902 cells, a perinuclear and punctate pattern was observed, reminiscent of the structures stained by MDC (Fig. 1A). Double staining using an antibody against the lysosomal membrane protein lamp1 revealed that AV1-positive and lamp1-positive structures were mainly colocalized (Fig. 1A,B). Furthermore, lamp1-positive organelles were also mainly colocalized with MDC-positive structures (Fig. 1C,D), indicating that AV1 recognizes lysosomal/autophagic vacuoles.
A V1 serum was used to screen a commercially available human expression library derived from pancreatic adenocarcinoma cells. After three rounds of screening the isolated clones were sequenced and we obtained eight independent clones. One contained a 2.4 kb insert, which was fully sequenced from both the 3′ and 5′ directions, revealing an open reading frame between nucleotides 170 and 2018. The amino acid sequence deduced from this cDNA is a 616-amino-acid protein (Fig. 2). We designated this protein LALP70.

Sequence analysis
The methionine codon in position 170 is the first ATG in the sequence and conforms to the consensus eukaryotic translation sequence (Kozak, 1989) as a strong initiator codon with the purines A at position −3 and G at position +4 of the coding sequence (Fig. 2). Sequence comparison using the BLASTN program revealed high sequence similarity to the human mRNA for a human uridine diphosphatase located in the Golgi apparatus (Wang and Guidotti, 1998; accession number AF016032). Comparing with the uridine diphosphatase sequence, the LALP70 sequence had an extra stretch of 24 bp (1028-1052 bp) encoding eight amino acids. Furthermore, the uridine diphosphatase sequence had an extra triplet between 299 and 300 bp of the LALP70 sequence. In particular the additional eight amino acids in the LALP70 sequence indicate that there are possibly several tissue-specific isoforms of LALP70, because the uridine diphosphatase cDNA was isolated from a human brain expression library and the LALP70 cDNA shown here was isolated by screening a pancreas cDNA library. The encoded protein had a calculated molecular mass of 70255 Da and a theoretical pI of 8.55. Analysis of the deduced amino acid sequence using BLASTP and multiple sequence alignment indicates that the LALP70 protein is almost identical (see above) to the Golgi-located human uridine diphosphatase (AF016032), and homologous to the human apyrase CD39 as well as to other 13 known apyrases from different species, including plants (human CD 39, P49961; mouse CD39, P55772; mouse ecto-ATPase, AF042811; human CD39-like 1 gene, U91510; human brain ecto-apyrase, AF034840; rat brain ecto-ATPase, Y11835; Drosophila apyrase, AF041048; yeast GDPase, P32621; C. elegans hypothetical 63 kDa protein, Q21815; chicken ATPase, U74467; pea NTPase, P52914; potato ATPase, P80595; yeast hypothetical 71 kDa protein, P40009). Although the overall similarity to the homolog sequences is only 19-29%, the relatedness to the apyrase family is evident in four sequence clusters in the N-terminal half (boxed regions in Fig. 3), which have been described by Handa and Guidotti (1996) as apyrase conserved regions (ACR). Amino acid alignments of the LALP70 and hUDPase (Wang and Guidotti, 1998) amino acid sequences with the predicted sequences of the other known human members of this family, CD39 (Maliszewski et al., 1994), CD39-like genes (Chadwick and Frischau, 1997, 1998), and
The human apyrase-like protein LALP70 is lysosomal
potential transmembrane regions, one near the N terminus (amino acids 37-53) and one near the C terminus (amino acids 566-582). This has also been shown for most other apyrases described so far, especially for the hUDPase (Wang and Guidotti, 1998). Exceptions are a yeast GDPase with only one transmembrane region at the N terminus (Abeijon et al., 1993), and a potato ATPase, which is soluble (Handa and Guidotti, 1996). A third possible membrane-associated region at the N terminus (amino acids 3-25) is consistent with the in vitro translation experiments in the presence of microsomal membranes (Fig. 5) discussed below. Hence, the hydrophobicity plot suggests that the LALP70 protein is a type III integral membrane protein (Singer, 1990). In vitro transcription/translation of the LALP70 cDNA produced a single major protein chain of approximately 70 kDa when analyzed by SDS-PAGE (Fig. 5, lane 1), in good agreement with the predicted molecular mass of 70255 Da. Translation in the presence of microsomal membranes did not alter the apparent size of the protein product (Fig. 5, lane 3), indicating that no signal peptide cleavage and no glycosylation events had occurred.

The main and the smaller polypeptide chains synthesized in the absence of microsomes were completely digested by trypsin (Fig. 5, lane 2). In contrast, the 70 kDa protein synthesized in the presence of microsomes was protected from trypsin digestion, indicating that it was cotranslationally translocated into the microsomal vesicles. Nevertheless, treatment of the microsomal preparation with trypsin caused a faint band with a slightly reduced molecular mass of about 67 kDa (Fig. 5, lane 4). These bands were only digested with trypsin, when Triton X-100 was added (Fig. 5, lane 5). In relation to our sequence analysis data discussed above and to the model of apyrase integration into membranes deduced by others, we propose that the 67 kDa band represents a LALP70 degradation product where the 33 C-terminal amino acid residues have been proteolytically cleaved. The main part of the protein flanked by the transmembrane domains is orientated into the lumen of the microsomal vesicles, while the N terminus is associated with the membrane and not accessible to trypsin.

### Subcellular distribution of LALP70

To study the subcellular localisation of the LALP70 protein we cloned the cDNA lacking only the stop codon and the last three amino acid codons in-frame into the mammalian expression vector pEGFP-N1, with the green fluorescent protein (EGFP) fused to the C terminus of LALP70. PaTu 8902 cells were transfected with this construct to express a LALP70-EGFP fusion protein under the control of the cytomegalovirus promotor. As soon as 12 hours after transfection the expression of the LALP70-EGFP fusion protein could be observed by confocal fluorescence microscopy as a punctate pattern around the nucleus (Fig. 6A). Control cells transfected with the expression vector missing the LALP70 domain were uniformly fluorescent in the cytoplasm and nuclei (Fig. 6B). LALP70-EGFP fluorescence became more intense when cells were incubated for up to 72 hours and in some of the transfected cells a diffuse cytoplasmic background was observed. However, under no conditions and at no time after transfection did fluorescent staining of the plasma membrane occur. These results indicate that the LALP70-EGFP fusion protein was associated with endomembranes and not with the plasma membrane. To further characterize organelles associated with the LALP70-EGFP fusion protein, transfected PaTu 8902 cells were fixed and immunostained with various antibodies against known marker proteins of subcellular compartments. Analysis with a confocal laser scanning microscope revealed a colocalization of LALP70-EGFP with lamp1, a membrane protein enriched in lysosomal organelles (Fig. 6C-F). However, in all transfected cells examined (>50) there were more LALP70-EGFP positive organelles than lamp1-labeled organelles. Furthermore, the degree of colocalization differed from cell to cell, with some intracellular compartments containing only lamp1 or LALP70 or containing both, occurring in different ratios. Some of the LALP70-EGFP positive organelles, which were not stained by the lamp1
The human apyrase-like protein LALP70 is lysosomal.

![Fig. 3.](image)

### Table 1: Apyrase conserved regions (ACR) defined by Handa and Guidotti (1996)

<table>
<thead>
<tr>
<th>ACR</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR1</td>
<td>hUDPase, mouse-ecto, chicken-ecto, human-brain</td>
</tr>
<tr>
<td>ACR2</td>
<td>yeast-hyp71, yeast-GDPase, potato-ATPase, yeast-GDPase</td>
</tr>
<tr>
<td>ACR3</td>
<td>human-UDPase</td>
</tr>
<tr>
<td>ACR4</td>
<td>human-ecto, chicken-ecto, human-CD39</td>
</tr>
</tbody>
</table>

### Table 2: Amino acid sequence alignment of LALP70

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUDPase</td>
<td>P52914</td>
<td>YGKRLRTKDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>human-CD39</td>
<td>P55772</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>mouse-ecto</td>
<td>P52914</td>
<td>YGKRLRTKDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>chicken-ecto</td>
<td>P52914</td>
<td>YGKRLRTKDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>human-brain</td>
<td>P52914</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>C. el.-hyp63</td>
<td>P52914</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>drosophila</td>
<td>P52914</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>pea-ATPase</td>
<td>P52914</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
<tr>
<td>potato-ATPase</td>
<td>P52914</td>
<td>GL-98YRATLDFQKYARVTIDIAETDTNNVNVIGIVDVCCGSGGIVTYCVCFHRGNNPHD</td>
</tr>
</tbody>
</table>

### Table 3: Amino acid sequence alignment of LALP70 with other known apyrases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pea-NTPase</td>
<td>P52914</td>
<td>VSKK------TAKNAPKVADGDDPYIKKVVLKGIPYDLYVHSYLHFGREASRAEILKLTP</td>
</tr>
<tr>
<td>chicken-ecto</td>
<td>P52914</td>
<td>TSD--------AIEDPKNEVMLKLYGQP-------YKVYTHSFLCYGRDQVLKRLLSKVL</td>
</tr>
<tr>
<td>rat-ecto</td>
<td>P52914</td>
<td>TTS--------PSEDPGNEVHLRLYGQH-------YRVYTHSFLCYGRDQILLRLLASAL</td>
</tr>
<tr>
<td>hum-CD39</td>
<td>P52914</td>
<td>PQNQ-------TIESPDNALQFRLYGKD-------YNVYTHSFLCYGKDQALWQKLAKDI</td>
</tr>
<tr>
<td>hum-UDPase</td>
<td>P52914</td>
<td>VPKT--------EEVAKNLLAEFNLGCDVHQTEHVYRVYVATFLGCGGNARQAERDIF</td>
</tr>
</tbody>
</table>
organelles (Fig. 1D), which were previously shown to be autophagic vacuoles (Biederbick et al., 1995).

**Tissue distribution of LALP70 RNA**

To examine the distribution of LALP70 mRNA in various human organs, a cDNA fragment of the C-terminal half of the LALP70 sequence with lowest homologies to other human members of the apyrase family was hybridized to a northern dot blot of poly(A)+ RNAs from 50 different organs. LALP70 mRNA was detected in all the human tissues including fetal tissues, although mRNA expression levels differed up to sixfold, with the highest expression in testis (Fig. 7A, D1) and the lowest expression in bladder (Fig. 7A, C5). Control RNA or DNA from yeast and *E. coli* (Fig. 7A, H1-H4) as well as polyadenylic acid (Fig. 7A, H5) and human repetitive DNA (Fig. 7A, H6) were negative.

A single mRNA of about 7.4 kb was also detected in RNA prepared from PaTu 8902 cells (Fig. 7B). This long message indicates the presence of a very long 3’ untranslated region, which is not present in the LALP70 clone. However, a partial coding sequence which had been published as an EST sequence (KIAA0392) before (AB002390, Nagase et al., 1997), contained a 3’-UTR of about 3.8 kb.

**DISCUSSION**

We have cloned a 2330 bp cDNA encoding the lysosomal/autophagolysosomal specific protein LALP70 by immunoscreening an expression library derived from a human pancreatic adenocarcinoma cell line. The antibody used was raised against the biotinylated proteins of the outer membrane of autophagic vacuoles (AVs) and recognized subcellular structures that were also positive for the lysosomal/autophagic vacuole markers lamp1 or monodansylcadaverine (Fig. 1). The sequence is almost identical to a human Golgi UDPase (Wang and Guidotti, 1998), with the exception of an additional 24 bp stretch (from 1028 to 1052 bp) in the LALP70 sequence. Furthermore, the hUDPase contained an extra base pair triplet between 299 and 300 bp of our sequence. In particular the additional 24 bp encoding eight more amino acids raise the possibility that LALP70 may occur in differently spliced isoforms. Since LALP70 was cloned from a pancreatic...
expression library and hUDPase from a brain expression library, splice variants may be tissue-specific. Splice variants were also proposed for a rat apyrase for which multiple bands in northern blots from various tissues had been observed (Kegel et al., 1997).

The LALP70 sequence as well as the hUDPase sequence showed striking homologies to diphosphate phosphohydrolases (E.C. 3.6.1.15), known as apyrases (Plesner, 1995). There are five further human homolog proteins known: the cell surface protein CD39 (21% homology; Maliszewski et al., 1994), which has also been cloned from mice, three CD39-like genes (CD39L1, 22% homology; Chadwick and Frischauf, 1997; CD39L2, 20% homology and CD39L4, 22% homology; Chadwick and Frischauf, 1998) and a brain ecto-apyrase identical with CD39L3 (23% homology; Smith and Kirley, 1998). Other apyrases have been cloned from rat, chicken, *Caenorhabditis elegans, Toxoplasma gondii*, yeast, potato and pea, indicating that this protein family appeared early in phylogeny and is spread over all kingdoms of eukaryotes.

Most apyrases are plasma membrane proteins with ecto-NTP/NDPase activity. They are anchored in the membrane with two transmembrane domains which are located at the N and C terminus, respectively, constituting a type III membrane protein (Singer, 1990). While two short amino acid sequences point to the cytosol, the sequence between the transmembrane domains faces the extracellular space. This extracellular part of the protein contains four sequence stretches, highly conserved between all known apyrases. They were described as apyrase conserved regions (ACRs) by Handa and Guidotti (1996). The LALP70 sequence also contains the four ACRs (boxes in Fig. 3) as well as the N- and C-terminal transmembrane domains. Computer analysis predicted two possible N-terminal transmembrane sequences, one between +3 and +25, and one between +37 and +53. In the in vitro

Fig. 6. Localization of the LALP70/EGFP-fusion protein in PaTu 8902. Cells were transiently transfected with an expression plasmid containing either LALP70/EGFP cDNA (A) or the cDNA for EGFP alone (B). LALP70/EGFP was located in perinuclear granular structures (A), while EGFP alone was homogenously dispersed throughout the cytoplasm and the nucleus (B). In colocalization experiments using an antibody against lamp1 (D,F), LALP70/EGFP occurred in granular structures which were also positive for lamp1 (C, LALP70/EGFP; D, lamp1). However, in some cells the amount of LALP70/EGFP positive structures exceeded those positive for lamp1 (E, LALP70/EGFP; F, lamp1). LALP70/EGFP also colocalized with the autophagic vacule marker monodansylcadaverine (MDC), as shown in (G) (LALP70/EGFP) and (H) (MDC). Arrowheads in H indicate the transfected cells shown in G. Cells were analysed 24 hours after transfection, either by in vivo microscopy (A,B) or after fixation (C-H). Analysis was performed either by confocal microscopy (A-F) or by conventional fluorescence microscopy (G,H). Bar, 10 μm (A,B,G,H); 5 μm (C-F).
translation experiment in the presence of microsomal membranes no signal peptide was cleaved off, indicating that both N-terminal transmembrane domains are maintained in the mature protein. This is in accordance with reports about other apyrases (Kegel et al., 1997). We assume that only the C-terminal end of the protein is facing the cytoplasmic site, while there are two membrane-spanning domains at the N terminus with the end of the protein pointing to the extracytosolic space. Thus, LALP70 would be a type IIIb membrane protein (Singer, 1990; Fig. 8).

The molecular function of the ACRs are only partly understood (Handa and Guidotti, 1996; Wang and Guidotti, 1998). The ACR1 and ACR4 domains are homologous to the β- and γ-phosphate binding motif found in such different proteins as actin, hsp70 and hexokinase (Flaherty et al., 1991). Neither the ACRs nor other parts of the sequence are homologous to the glycine-rich Walker consensus sequence for ATP binding described for other ATPases (Walker et al., 1982), indicating that the ACRs are critical for the enzymatic function of apyrases. From Toxoplasma gondii an isoform was cloned missing the ACR1 without alteration of the NTPase activity. Moreover, an even more truncated apyrase was characterized in pig pancreas containing only the ACR4 domain, but still active as an apyrase (Kaczmarek et al., 1996). This indicates that ACR4 harbors all capabilities necessary for a basal apyrase function, while ACR1-3 might be involved in further regulatory items of these enzymes. This was proposed by Wang and Guidotti (1998), comparing amino acid sequence and nucleotide substrate specificity of the ACR1 domain from different apyrases. However, isoforms from Toxoplasma gondii (NTP I and NTP II; Bermudes et al., 1994) differing in only 16 amino acids which are not located in the ACRs, differed markedly in their ability to cleave ATP and ADP (75:1 in NTP I versus 1:1 in NTP II). Although the LALP70 sequence differs from the hUDPase sequence in only nine amino acids, enzymatic activity of the LALP70 protein has still to be shown. It will be of special interest whether the 9-amino-acid insert influences the enzyme activity and/or substrate specificity.

Although apyrases were originally described as ecto-enzymes being involved in the extracellular nucleotide metabolism, the localisation at the plasma membrane has been explicitly shown for only a few members of this family, i.e. for CD39 (Kaczmarek et al., 1996), the rat and the human brain apyrases (Kegel et al., 1997; Smith and Kirley, 1998). However, six types of apyrases were associated with intracellular compartments. The three apyrases NTP1-3 from T. gondii and the apyrase from potato were located in intracellular vacuoles, which have not been characterized in detail. The apyrase from yeast (GDA1) and the hUDPase were located in the Golgi apparatus, where they cleave UDP molecules accumulating during protein glycosylation.

We used a cDNA for a LALP70/EGFP-fusion protein to...
Fig. 8. Model of the insertion of LALP70 into the lysosomal membrane as deduced from sequence alignment analysis, hydrophobicity plot and in vitro transcription/translation experiments. ACR1-4, apyrase-like regions in the N-terminal half of the protein; green, C-terminal intraluminal part of the protein; blue, transmembrane regions; red, 8 amino acid stretch unique for the LALP70 protein. Arrows indicate possible trypsin cleavage sites in the cytosolic domains. There are six possible cleavage sites which are clustered near the transmembrane domains.

reveal the cellular localisation of this member of the apyrase family in transient transfection experiments. The LALP70/EGFP-fusion protein gave rise to an intracellular punctate pattern resembling vacuolar structures. These structures were concentrated in the perinuclear region in a similar pattern to autophagic vacuoles stained with monodansylcadaverine (MDC). When transfected cells were counterstained with MDC, a partial colocalisation was observed, indicating that the LALP70/EGFP-fusion protein is located in a subset of the vacuoles of the lysosomal/autophagic compartment. This was further confirmed in a colocalisation study using an antibody against the lysosomal marker protein lamp1 (Kornfeld and Mellmann, 1989). Using confocal microscopy only a subpopulation of vacuoles were positive for both the LALP70/EGFP-fusion protein and lamp1, while there were also vacuoles either positive only for the LALP70/EGFP-fusion protein or for lamp1. Localisation of the LALP70/EGFP-fusion protein to the plasma membrane was not observed under any conditions. Thus, LALP70 is located in a subfraction of lysosomal/autophagic vacuoles. Since the hUDPase described by Wang and Guidotti (1998) is almost identical to LALP70, the structures that are LALP70/EGFP-positive and lamp1-negative might belong to the Golgi apparatus. However, a localisation to the endoplasmatic reticulum (ER) cannot be completely ruled out, especially since the formation of autophagosomes originates most likely from ER membranes (Dunn, 1990a). The difference between the intracellular localisations of LALP70 and hUDPase might depend on the nine different amino acids between these proteins, which, as an alternative to their possible role for the enzymatic activity (see above), could also act as a sorting signal.

An intracellular apyrase function has only been shown for the yeast apyrase GDA1 (Abeijon et al., 1993) and the hUDPase (Wang and Guidotti, 1998). These Golgi proteins are capable of cleaving GDP/UDP to GMP/UMP. GDP/UDP accumulation from activated sugars used during protein glycosylation. However, diphosphate nucleotides cannot cross the Golgi membrane. In order to reutilize diphosphate nucleotides they have to be converted to monophosphate nucleotides, which then can be transported into the cytoplasm across the Golgi membrane. Functional mutation of GDA1 results in accumulation of GDP in the Golgi cisternae and an abrogation of protein glycosylation (Abeijon et al., 1993). The lysosomal system receives material for degradation from different sources like endocytosis, phagocytosis and autophagocytosis. Especially during autophagocytosis, large amounts of cytoplasm containing high concentrations of tri- and diphosphate nucleotides are sequestered. However, tri- and diphosphate nucleotides cannot cross the lysosomal membrane and have to be converted to nucleosides (Pisoni and Thoene, 1991). The lysosomal membrane protein acid phosphatase is only capable of cleaving monophosphate nucleotides to nucleosides, and cannot cleave higher phosphorylated nucleotides (Pisoni, 1996). We propose that LALP70 participates in this part of nucleotide metabolism and is critical for the salvage of nucleotides from the lysosomal/autophagic vacuole lumen.

The lysosomal compartment is an endomembrane system present in all eukaryotic cells. However, the total quantity of this compartment and the ratio of subcompartments like late endosome, phagolysosome or autophagolysosome differ between cell types or in one cell type under different functional conditions. The expression of LALP70 was analysed using a dot blot northern hybridisation and also revealed a ubiquitous distribution in 50 different human tissues, with only a sixfold difference between the tissues with the highest and the lowest expression. Similar results were obtained by Wang and Guidotti (1998), analysing eight different human tissues. This points to a general function presented in all cells, such as turnover of nucleotides, and is compatible with our finding that LALP70 is a lysosomal/autophagolysosomal membrane protein.

We gratefully acknowledge the technical assistance of Uschi Lehr and the preparation of the photographic reprints by Volkwin Kramer. We thank R. MacDonald, H. F. Kern and T. Möröy for helpful and critical discussions. This work was supported by Deutsche Forschungsgemeinschaft, grant EL 125/1.

REFERENCES


