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## The VSFASSQQ motif confers calcium sensitivity to the intracellular apyrase LALP70

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### Abstract

**Background:** Apyrases are divalent ion dependent tri- and dinucleotide phosphatases with different substrate specificity. The intracellular lysosomal apyrase LALP70 is also expressed as a splice variant (LALP70v) lacking a VSFASSQQ motif in the center of the molecule (aminoacids 287–294). However, the functional significance of this motif is unknown. In this report we used a thin layer chromatography approach to study separately the UTPase and UDPase activity of the two LALP-enzymes.

**Results:** We show, that LALP70 and LALP70v cleaved UTP to UDP in a calcium independent manner. In contrast, the cleavage of UDP to UMP was strongly calcium dependent for LALP70, but calcium independent for LALP70v.

**Conclusions:** The VSFASSQQ motif not only influences the substrate specificity of LALP70, but it confers calcium sensitivity to LALP70 during the UDP cleavage. Whether this is due to direct binding of calcium to this motif or to a conformational change of the enzyme, remains to be elucidated.

### Background

Apyrases, which are mostly ectoenzymes, are a protein family of enzymes capable of cleaving nucleotide tri- and diphosphates in a calcium or magnesium dependent manner, not being altered by P-type, F-type or V-type NTPase inhibitors [1]. We have previously identified the lysosomal apyrase of 70 kDa (LALP70, Acc. No. NP\_004892; NTPDase4 $\alpha$ , [2]) and its splice variant LALP70v (NTPDase4 $\beta$ , [2]), which are one of the first intracellular apyrases being described [3,4]. The difference between LALP70 and LALP70v is the 8 amino acid motif VSFASSQQ missing in the splice variant LALP70v [5]. This motif is encoded at the end of exon 7 and is located after the fourth apyrase conserved region of LALP 70 [3,5]. The

VSFASSQQ motif is also present in the mouse homologue of LALP70 (Acc. No. NP\_080450), which shows 93% identity with the human sequence. No other apyrase deposited in the common databases contains this motif.

In the original description LALP70v was located in the Golgi apparatus [4], while LALP70 was identified as a lysosomal membrane protein [3], implicating that the VSFASSQQ motif might be involved in the sorting of the LALP protein. However, analysis of the membrane topology of LALP70 indicated, that the spliced motif is on the site of the organelle lumen and not on the cytoplasmic site, making at least classical sorting mechanisms unlikely [6]. Furthermore, both the Golgi localisation of LALP70v

and the lysosomal localisation of LALP70 were proposed on evidences from transfection experiments using either myc-tagged or the GFP-tagged proteins, respectively. Thus, the intracellular localisation of the two endogenous splice variants is still uncertain and it is open whether the reported differences are based on technical reasons.

Apyrases are capable of cleaving NTPs and/or NDPs with different substrate specificities [1]. Using a variety of common NTPs and NDPs, LALP70 was found to be a UTPase or a TTPase [5], whereas LALP70v had a rather broad substrate specificity with preferences for CTP or UDP [4,5]. This points to a function of the VSFASSQQ motif concerning the preferred substrate of the enzyme. A further characteristic feature of apyrases is their dependence on calcium or magnesium ions [1]. Here we show, by dissecting the NTPase activity from the NDPase activity, that LALP70 has a calcium sensitive NDPase activity which is not present in LALP70v. Our data show, that the VSFASSQQ motif confers calcium sensitivity to LALP70 as a NDPase.

## Results and Discussion

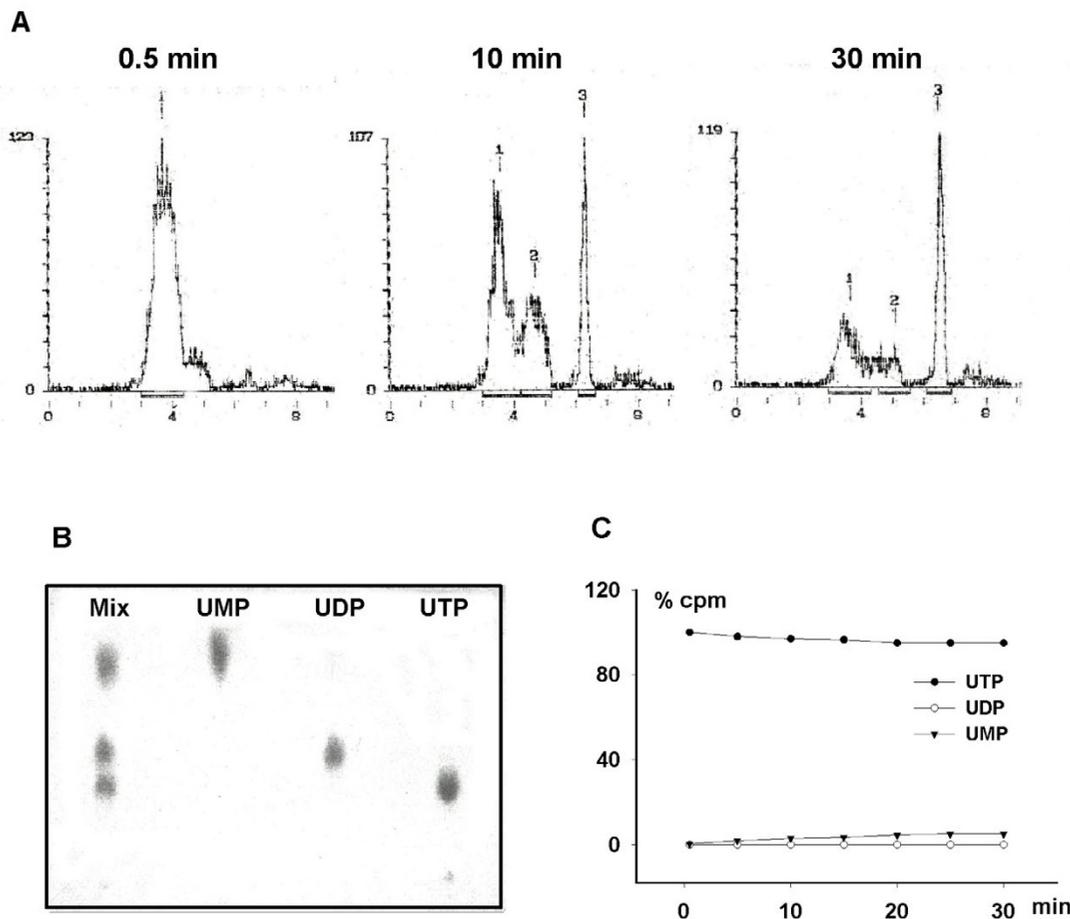
To investigate separately the cleavage of NTP to NDP and of NDP to NMP, UTP was chosen as a substrate, since both enzymes, LALP70 and LALP70v, revealed a similar substrate specificity for this nucleotide [5]. We used tritiated UTP, which together with its cleavage products  $^3\text{H-UDP}$  and  $^3\text{H-UMP}$ , could be detected by a quantitative thin layer chromatographic approach (Fig. 1A,1B). When crude membrane fractions from mock transfected cells were used in this assay, less than 5% of the tritiated UTP initially present was cleaved during a 30 minute incubation (Fig. 1C), when 5 mM calcium and 500  $\mu\text{M}$  magnesium were present. Moreover, when only 10  $\mu\text{M}$  calcium were used in the presence of 500  $\mu\text{M}$  magnesium, no cleavage of tritiated UTP could be detected (data not shown). This indicates that only a small amount of endogenous activity was present in the sample. Note that all experiments shown were done in triplicate. The standard deviation are not given graphically to keep the figures clearly arranged. The numerical mean values and standard deviations are given in table 1.

When membrane fractions were used from cells transfected with LALP70, tritiated UTP decreased rapidly and exponentially. This decrease was not dependent on the used calcium concentration (Fig. 2A). At lower calcium concentrations (1 – 100  $\mu\text{M}$ ) UDP increased rapidly until a steady state concentration. In contrast, at calcium concentrations higher than 100  $\mu\text{M}$  the UDPase activity was stimulated in a dose dependent manner (Fig. 2B). Accordingly, we observed a more rapid increase of UMP at these higher calcium concentrations (Fig. 2C). Thus, LALP70 has a UDPase activity which is calcium sensitive, while the

UTPase activity is independent on the calcium concentration. In contrast, when membrane fractions were used from cells transfected with LALP70v (Fig. 3A,3B,3C), the UDPase enzyme activity was almost identical for 10  $\mu\text{M}$  or 5 mM calcium, respectively (Fig. 3B), indicating that the loss of the VSFASSQQ motif abrogates the calcium sensitivity of the UDPase activity. Since in all experiments magnesium ions were present in a concentration of 500  $\mu\text{M}$ , the dependencies observed must be specific for calcium rather than for divalent cations in general.

It is not known how the VSFASSQQ motif influences the calcium binding abilities of LALP70. Three general types of calcium binding sites (class I to III) have been described, depending on the positioning of the ligands in the amino acid chain [12,13]. The best documented calcium binding protein domain is the EF-hand motif [14-16]. This motif provides, in a 12 amino acid stretch, 6 to 7 oxygen ligands to complex one  $\text{Ca}^{2+}$  ion [12,17]. However, using the PROSITE software, no EF-hand motif could be detected in the predicted LALP70 protein sequence. Other calcium binding motifs use amino acid side chains which are more or less scattered over the protein backbone (class II and III type) and which can hardly be detected by a sequence analysis *in silico*. Using CLUSTALW, alignments of the LALP70 amino acid sequence with sequences of different calcium binding proteins from the three classes mentioned did not result in significant homologies (data not shown). Recently, the calcium coordination of a soluble form of the ecto-nucleoside triphosphate diphosphorylase I (CD39), a typical membrane anchored apyrase [18,19], was investigated by electron paramagnetic resonance (EPR) spectroscopy [20]. Chen and Guidotti provided evidence that calcium is complexed by protein ligands as well as by oxygen atoms of the substrate molecule ATP or ADP, respectively. However, under our experimental conditions an excess of magnesium was present, which usually constitute strong complexes with NTPs or NDPs [12]. At least for lower calcium concentrations it is unlikely that magnesium is substituted by calcium according to the model proposed by Chen and Guidotti [20]. Although this model might describe correctly the involvement of divalent cations in the cleavage mechanism of NTPs and NDPs, our results indicate a further binding of calcium to LALP70. However, whether the VSFASSQQ motif contributes directly to the binding of calcium, or if it changes the structure of the protein, so that other potential calcium ligands are properly positioned for calcium binding, remains open.

The absence of the VSFASSQQ motif abrogates the calcium susceptibility of the UDPase activity (Fig. 2B and 3B). NTP/NDP binding in apyrases has been contributed to the apyrase conserved regions (ACRs) 1 and 4, which are homologous to the  $\beta$ - and  $\gamma$ -phosphate binding



**Figure 1**

Detection of mono- and diphosphate nucleotides generated by LALP70 or LALP70v apyrase activity. Crude membrane preparations containing expressed LALP70 were incubated with <sup>3</sup>H-UTP/UTP in the presence of 5 mM Ca<sup>2+</sup> and 500 μM Mg<sup>2+</sup>. After indicated time points, samples were separated by thin layer chromatography and tritiated nucleotides were detected with a thin layer radioactivity scanner (**A**). Peak 1 contains <sup>3</sup>H-UTP, peak 2 <sup>3</sup>H-UDP and peak 3 <sup>3</sup>H-UMP, and the integrated amount of counts per peak represents the amount of product generated by the enzyme in a certain time interval. The peak positions were calibrated using unlabeled nucleotides, which were separated onto fluorescence covered HPTLC plates and detected by UV-light (λ = 260 nm; **B**). When a crude membrane preparation from cells transfected with the expression vector only were used in the assay described in (A), less than 5% of the <sup>3</sup>H-UTP/UTP was cleaved during a 30 minute incubation (**C**).

domains of the actin/hsp70/hexokinase superfamily [21-23]. In accordance to this observation mutations in this regions led to changes in the substrate specificity of the apyrases tested [24,25]. However, deletions or mutations of the sequence outside the ACRs 1 and 4 were found to influence the substrate specificity, even if the mutated part

of the protein belongs to a membrane spanning region [26-28]. This suggests, that substrate binding and cleavage of NTPs/NDPs are related to the tertiary structure of the enzyme in a highly complex manner. The functional inter-relationship between calcium binding, substrate binding

**Table 1: Mean values and standard deviation in %cpm of  $^3\text{H}$ -UTP hydrolysis experiments, obtained with membrane fractions from COS cells transfected with LALP70 or LALP70v cDNA, respectively.**

			0	5	10	15	20	25	30
			mean $\pm$ S.E.M.						
Lalp70v	UTP	10 $\mu\text{M}$ Ca $^{2+}$	73 $\pm$ 7,5	67 $\pm$ 5,5	58 $\pm$ 4,9	48 $\pm$ 8,3	40 $\pm$ 2,0	32 $\pm$ 5,8	32 $\pm$ 2,0
		5 mM Ca $^{2+}$	91 $\pm$ 1,7	80 $\pm$ 4,0	70 $\pm$ 7,0	57 $\pm$ 4,1	51 $\pm$ 5,0	46 $\pm$ 5,7	36 $\pm$ 4,3
	UDP	10 $\mu\text{M}$ Ca $^{2+}$	27 $\pm$ 7,5	31 $\pm$ 4,9	36 $\pm$ 4,2	42 $\pm$ 6,6	45 $\pm$ 2,6	46 $\pm$ 4,7	40 $\pm$ 6,5
		5 mM Ca $^{2+}$	9 $\pm$ 1,7	18 $\pm$ 5,0	25 $\pm$ 7,0	36 $\pm$ 7,5	35 $\pm$ 5,5	37 $\pm$ 6,9	42 $\pm$ 8,5
	UMP	10 $\mu\text{M}$ Ca $^{2+}$	0	3 $\pm$ 1,2	6,3 $\pm$ 1,5	10 $\pm$ 1,7	16 $\pm$ 4,3	22 $\pm$ 4,3	28 $\pm$ 5,8
		5 mM Ca $^{2+}$	0	2,3 $\pm$ 1,1	5 $\pm$ 2,5	7 $\pm$ 3,5	14 $\pm$ 4,5	17 $\pm$ 4,9	21 $\pm$ 8,4
Lalp70	UTP	1 $\mu\text{M}$ Ca $^{2+}$	62 $\pm$ 12	40 $\pm$ 9,0	27 $\pm$ 1,7	21 $\pm$ 0,6	19 $\pm$ 4,5	19 $\pm$ 6,0	16 $\pm$ 5,0
		10 $\mu\text{M}$ Ca $^{2+}$	67 $\pm$ 2,0	41 $\pm$ 10	31 $\pm$ 2,1	27 $\pm$ 2,0	26 $\pm$ 2,0	22 $\pm$ 0,5	19 $\pm$ 1,0
		100 $\mu\text{M}$ Ca $^{2+}$	73 $\pm$ 8,0	44 $\pm$ 4,0	31 $\pm$ 8,0	26 $\pm$ 8,0	24 $\pm$ 5,0	24 $\pm$ 4,0	23 $\pm$ 5,0
		1 mM Ca $^{2+}$	62 $\pm$ 3,5	43 $\pm$ 5,0	23 $\pm$ 6,1	22 $\pm$ 1,4	17 $\pm$ 0,6	20 $\pm$ 2,8	19 $\pm$ 3,1
		5 mM Ca $^{2+}$	72 $\pm$ 0,7	40 $\pm$ 7,0	42 $\pm$ 8,0	28 $\pm$ 3,5	27 $\pm$ 2,1	22 $\pm$ 2,5	23 $\pm$ 3,5
	UDP	1 $\mu\text{M}$ Ca $^{2+}$	38 $\pm$ 10	58 $\pm$ 9,0	68 $\pm$ 2,0	72 $\pm$ 3,0	71 $\pm$ 2,5	70 $\pm$ 6,5	69 $\pm$ 5,0
		10 $\mu\text{M}$ Ca $^{2+}$	33 $\pm$ 2,0	58 $\pm$ 10	64 $\pm$ 0,5	65 $\pm$ 2,0	65 $\pm$ 5,4	67 $\pm$ 3,0	66 $\pm$ 4,5
		100 $\mu\text{M}$ Ca $^{2+}$	27 $\pm$ 8,5	53 $\pm$ 4,0	65 $\pm$ 7,0	65 $\pm$ 8,0	65 $\pm$ 0,5	61 $\pm$ 1,0	61 $\pm$ 0,5
		1 mM Ca $^{2+}$	35 $\pm$ 2,1	51 $\pm$ 5,0	59 $\pm$ 2,5	54 $\pm$ 2,1	49 $\pm$ 4,9	38 $\pm$ 3,9	19 $\pm$ 2,8
		5 mM Ca $^{2+}$	27 $\pm$ 0,7	44 $\pm$ 7,7	27 $\pm$ 8,5	25 $\pm$ 8,5	18 $\pm$ 6,4	15 $\pm$ 4,5	14 $\pm$ 7,0
	UMP	1 $\mu\text{M}$ Ca $^{2+}$	0,3 $\pm$ 0,7	2 $\pm$ 0,3	5 $\pm$ 2,0	7 $\pm$ 1,5	10 $\pm$ 1,7	11 $\pm$ 1,1	14 $\pm$ 1,0
		10 $\mu\text{M}$ Ca $^{2+}$	0,5 $\pm$ 0	2 $\pm$ 0,6	6 $\pm$ 2,0	8 $\pm$ 3,0	10 $\pm$ 4,0	12 $\pm$ 3,7	16 $\pm$ 5,6
		100 $\mu\text{M}$ Ca $^{2+}$	0	3 $\pm$ 0,6	5 $\pm$ 1,0	8 $\pm$ 1,0	12 $\pm$ 4,0	14 $\pm$ 5,0	17 $\pm$ 6,0
		1 mM Ca $^{2+}$	0	7 $\pm$ 1,8	18 $\pm$ 3,5	24 $\pm$ 3,9	34 $\pm$ 4,9	42 $\pm$ 1,0	51 $\pm$ 5,6
		5 mM Ca $^{2+}$	1,8 $\pm$ 0,4	16 $\pm$ 1,0	31 $\pm$ 0,5	48 $\pm$ 4,9	56 $\pm$ 3,9	63 $\pm$ 2,1	63 $\pm$ 3,5

and substrate cleavage has to be further elucidated by direct biochemical and structural approaches using the purified LALP70 and LALP70v proteins.

## Conclusions

The VSFASSQQ motif, which is lacking in the LALP70 splice variant LALP70v, plays an important function for enzymatic properties of this apyrase. This was shown by a new invented thin layer chromatographic approach which allows the simultaneous quantification of the UTPase and UDPase activity. Both apyrases were not calcium sensitive in their UTPase function. However, UDP cleavage was calcium sensitive in a concentration dependent manner in LALP70, but not in LALP70v. We hypothesize that the VSFASSQQ motif is a new calcium sensitive regulatory domain for the UDPase activity in LALP70. However, whether the calcium sensitivity depends on a direct calcium binding to the VSFASSQQ motif or is due to conformational changes of the protein induced through the VSFASSQQ motif remains to be elucidated.

## Methods

### Materials

All nucleotide phosphates and the 25-kDa polyethyleneimine were from Sigma-Aldrich (Taufkirchen, Germany), and the  $^3\text{H}$ -UTP (1  $\mu\text{Ci}/\mu\text{l}$ ; 25 Ci/mmol) were from Hartmann (Braunschweig, Germany). HPTLC plates Silica

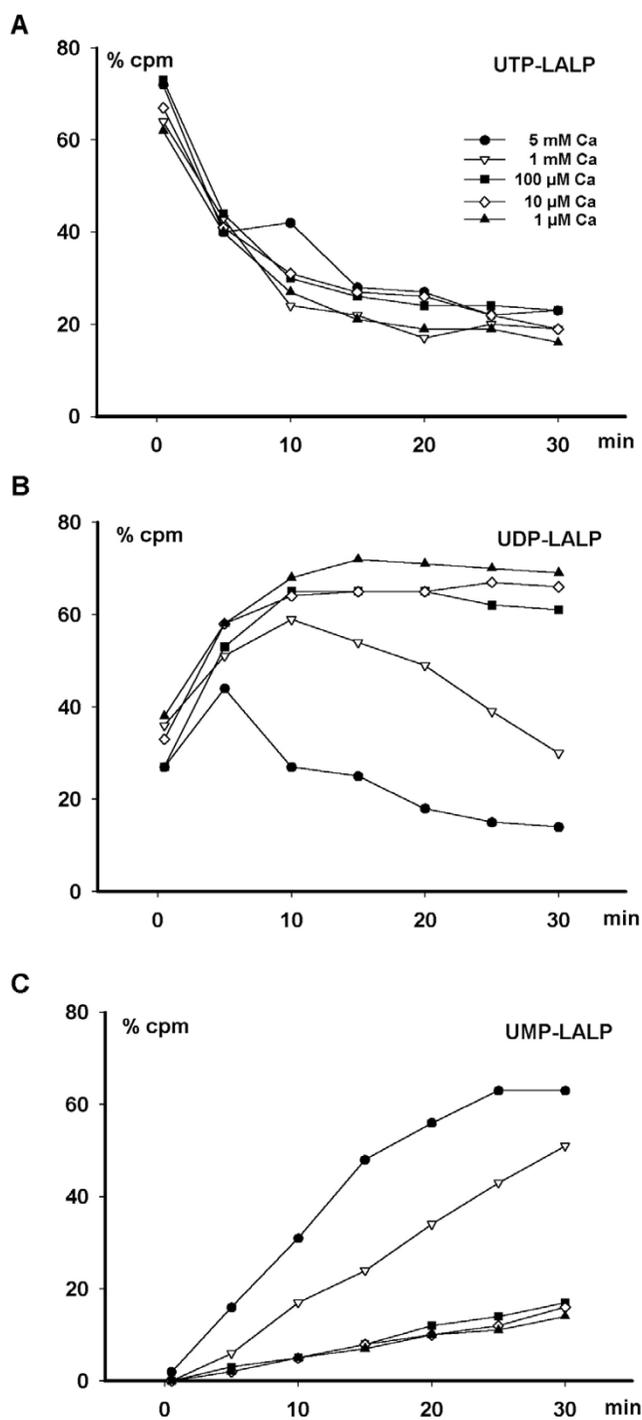
gel 60 (10  $\times$  10 cm) with concentrating zone and fluorescence covered HPTLC plates Silica gel 60 F $_{254}$  (10  $\times$  10 cm) were from Merck (Darmstadt, Germany).

### Expression vectors

Both the human full-length LALP70 cDNA and the LALP70v cDNA missing the 24 bp between nucleotides 1028–1051 were generated and cloned into the mammalian expression vector pCL-Neo (Promega) as described elsewhere [5].

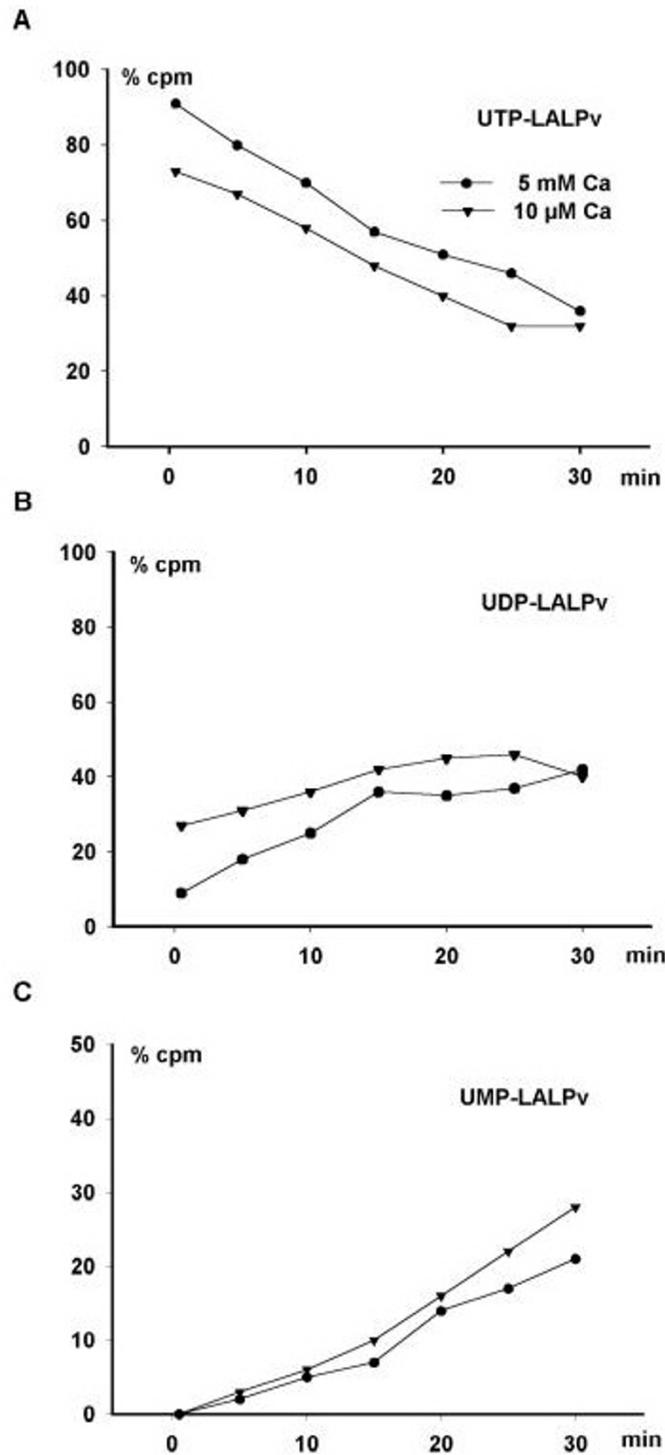
### Cell culture and transfection

COS-7 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 2 g/liter HEPES, 10% fetal calf serum, 50  $\mu\text{g}/\text{ml}$  gentamycin, and incubated at 37°C in a humidified chamber equilibrated with 5% CO $_2$ . COS-7 cells were transfected with the mammalian pCL-Neo expression vector alone or with this vector containing either the cDNA for LALP70 or for LALP70v, respectively, using a 25-kDa polyethyleneimine (Sigma-Aldrich) as transfection reagent [7]. Transfection procedure was performed as described elsewhere [3]. Transfected cells were harvested for nucleotide phosphatase assays 40 h after transfection.



**Figure 2**

Ca<sup>2+</sup> dependent generation of mono- and diphosphate nucleotides by LALP70 phosphatase activity. Crude membrane preparations containing expressed LALP70 were incubated with <sup>3</sup>H-UTP/UTP, 500 μM Mg<sup>2+</sup> and various concentration of Ca<sup>2+</sup>. Apyrase activity products were separated by thin layer chromatography and <sup>3</sup>H-labeled nucleotides were detected by a thin layer radioactivity scanner. At timepoints indicated cpm of each peak were counted and are given as percentage of all counted cpm. Mean values from three separate measurements are given. While dephosphorylation of <sup>3</sup>H-UTP by LALP70 is independent of the Ca<sup>2+</sup> concentration (A), dephosphorylation of <sup>3</sup>H-UDP (B) and thereby generation of <sup>3</sup>H-UMP (C) depends on the Ca<sup>2+</sup> concentration.



**Figure 3**

Ca<sup>2+</sup> independent generation of mono- and diphosphate nucleotides by LALP70v activity. Phosphatase products of LALP70v activity with <sup>3</sup>H-UTP/UTP as substrate and in the presence of 500 μM Mg<sup>2+</sup> and various concentration of Ca<sup>2+</sup> were detected and analysed as described in the legend to Fig.2. Mean values from three separate measurements are given.

### Preparation of COS cell crude membranes

Transfected COS-7 cells were homogenized with a Dounce homogenizer, and the crude membrane fraction were obtained by centrifugation as described elsewhere [8]. Briefly, after removal of cell detritus and cell nuclei, the postnuclear supernatant was centrifuged with 105,000 × g. The pellets, containing the crude membrane fraction, were resuspended in 400 µl of 20 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.2 mM EDTA containing 0,2 % Triton-X-100, and were stored at -20°C. Before use, the apyrase activity was measured using a standard nucleotide phosphatase assay as described elsewhere [5]. In all subsequent experiments membrane preparations were used in a dilution containing the same apyrase activity.

### Nucleotide phosphatase analysis using thin layer chromatography

To measure apyrase activity in the crude membrane fraction, 1–5 µl of the COS-7 membrane suspension were adjusted to 45 µl with reaction buffer containing 20 mM Hepes pH 7.4, 120 mM NaCl, 5 mM KCl, 0.2 mM EDTA, 1 mM Na<sub>3</sub>N, 0.5 mM Na<sub>3</sub>VO<sub>4</sub> and 500 µM MgCl<sub>2</sub>, with or without CaCl<sub>2</sub> in different concentrations. After preincubation of 5 minutes at 37°C, nucleotide phosphatase reactions were initiated by the addition of 4 µl of the same buffer containing 10 mM UTP and 1 µl <sup>3</sup>H-UTP. Samples were incubated between 0 and 30 minutes as indicated. After certain time points 5 µl aliquots were taken and the reaction was stopped by addition of 1 µl 50 mM EDTA. The aliquots were spotted onto HPTLC plates and the separation of mono-, di- and triphosphate nucleotides were achieved by using n-butanol, acetone, acetic acid, NH<sub>4</sub> (5%) and aqua dest. (35:25:15:15:10) as mobile phase. The distribution of <sup>3</sup>H-labeled nucleotides were analysed using a thin layer radioactivity scanner Berthold LB 2821 (Berthold, Wildbad, Germany). All measurements were done in triplicate.

As standards, 5 µl of 10 mM UTP, UDP, UMP or a mix of UTP/UDP/UMP in reaction buffer were spotted onto a fluorescence covered HPTLC plate (Silica gel 60 F<sub>254</sub>, Merck, Darmstadt, Germany) and separation of phosphate nucleotides were performed as described. The nucleotide spots were detected using 260 nm UV-light.

### Sequence analysis

The analysis of the VSFASSQQ motif was done with the software provided by PROSITE <http://hits.isb-sib.ch/cgi-bin/PFSCAN>[11]. Sequence alignment analysis was done with CLUSTALW <http://clustalw.genome.ad.jp/>.

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