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A lyso-platelet activating factor phospholipase C, originally suggested to be a neutral-sphingomyelinase, is located in the endoplasmic reticulum

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Abstract Recently a putative mammalian neutral-sphingomyelinase was cloned [Tomiuk et al. (1998) Proc. Natl. Acad. Sci. USA 95, 3638–3643; GenBank accession number AJ222801]. We have overexpressed this enzyme in cultured cells and demonstrate, using four different tagged constructs, that it is localized at the endoplasmic reticulum and not at the plasma membrane. This localization precludes a role for enzyme AJ222801 in the sphingomyelin cycle. Furthermore, a recent publication demonstrated that this enzyme has lyso-platelet activating factor (PAF) phospholipase C activity [Sawai et al. (1999) J. Biol. Chem. 274, 38131–38139]. Together, these data suggest a role for enzyme AJ222801 in the regulation of PAF metabolism.

Key words: Sphingomyelinase; Platelet activating factor; Phospholipase C; Sphingomyelin

1. Introduction

Over the past decade, the sphingomyelin (SM) cycle has received much attention due to its apparent role in a variety of intracellular signalling pathways, including apoptosis [1]. In this cycle, SM is hydrolyzed to ceramide either by acid-sphingomyelinase, located in lysosomes, or by neutral-sphingomyelinase (N-SMase), presumably located at the plasma membrane (PM) [1]. A variety of N-SMase activities have been reported, and within the past year, the first two sequences of putative N-SMases have become available [2,3]. Whereas one of these purified proteins appears to play a role as a regulator in the SM cycle [3], the other appeared inactive in signal transduction pathways [2], raising concerns about the validity of the SM cycle in signalling events.

In the current study, we have overexpressed the enzyme cloned by Tomiuk et al. [2] (GenBank accession number AJ222801) in a variety of cultured mammalian cells. The protein was localized in the endoplasmic reticulum (ER), irrespective of whether it was tagged at the N- or C-terminus, or in the loop between the putative transmembrane domains. This localization is inconsistent with enzyme AJ222801 being involved in the SM cycle. However, a recent biochemical study has demonstrated that the purified enzyme shows no SMase activity when overexpressed in cultured cells, but is rather a lyso-platelet activating factor (lyso-PAF) phospholipase C [4], and our data are consistent with this suggestion.

2. Materials and methods

An expressed sequence tag (EST) clone with an identical 5′ sequence to that of Tomiuk et al. [2] was obtained from the American Tissue Culture Collection (ATCC clone #921318; GenBank accession number AA028477). Sub-cloning into pCDNA3/*Bgl*II/*Bam*HI and repeat sequencing of 5′ and 3′ ends confirmed its identity. However, an in vitro transcription/translation reaction revealed a translated protein of only 22 kDa. Sequencing of the entire clone revealed a point mutation relative to the originally published sequence, specifically a deletion of C_{495}. This deletion, and the subsequent frame shift, created a stop codon at nucleotide 571. In order to restore the desired reading frame, PCR primers overlapping and flanking the mutation site were designed, containing the missing C_{495}. These primers were used versus plasmid primers to amplify both segments of the clone. The two PCR products were then mixed in an equimolar ratio, annealed and filled in by DNA polymerase. The final product was digested with *Kpn*I and *Hin*dIII to yield a 187 bp fragment containing the mutated region, which was ligated back into the original clone. Correction of the mutation was verified by both sequencing and in vitro translation.

The corrected cDNA clone in pcDNA3 was fused in frame with four different tag sequences, as follows. C-terminal green fluorescent protein (GFP) (C-GFP–AJ222801), created by deleting the enzyme's stop codon and inserting a GFP coding sequence in *Bam*HI/*Not*I with an eight residue linker between the two open reading frames. Hemagglutinin (HA) epitope tags (HA–AJ222801) were inserted via encoding the sequence YPYDVPYA in three separate locations, either N-terminal in *Bgl*II (N-HA–AJ222801), C-terminal in *Bam*HI (C-HA–AJ222801), or inside the open reading frame in *Pst*I at amino acid residue Ala_{346} (A_{346}-HA–AJ222801) (see Fig. 1). Full details of the cloning strategies and the oligonucleotide sequences can be obtained from mike.fainzilber@weizmann.ac.il.

COS, HEK-293 and NIH-3T3 cells were grown on glass coverslips in DMEM and 10% fetal calf serum. One day after plating, COS cells were transfected using DEAE–dextran [5]. Two days after transfection, cells were fixed with 4% formaldehyde (20 min, 37°C), and in some cases, subsequently permeabilized using 0.5% Triton X-100 in phosphate-buffered saline (5 min, room temperature). Transfections of HEK-293 were performed using calcium phosphate, and NIH-3T3 cells were transfected with the Fugene reagent (Boehringer Mannheim) according to the manufacturers' instructions. The following antibodies were used for immunofluorescence localization: anti-HA (Berkley Antibody Company); anti-p75 (mc192) (Boehringer Mannheim), and anti-protein disulfide isomerase (PDI) (Stressgen Biotechnologies Corp.). A rhodamine-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) was used for detection. Cells were examined using a Plan Apochromat 63×/1.4 n.a. objective of a Zeiss Axiovert 35 microscope equipped with a filter for rhodamine or FITC (for GFP) fluorescence, and photographed using a Contax 167MT camera and Kodak Tmax p3200 film.

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3. Results

Original analysis [2] of enzyme AJ222801 by hydropathy plots suggested that the protein has two adjacent membrane-spanning domains at the C-terminus separated by a loop of eight amino acid residues, with the catalytic domain facing the cytosol (Fig. 1). However, analysis of the sequence by three different prediction programs suggested the presence of an additional transmembrane domain (Fig. 1), and moreover, the previously proposed topology was not favored by any of the prediction programs. We therefore tagged the enzyme at the C-terminus (HA tag and GFP tag), N-terminus (HA tag), and in the inter-membrane loop (HA tag), in such a way that at least one of the tags should be exposed on the membrane surface if the enzyme is located at the PM (Fig. 1).

C-GFP–AJ222801 (Fig. 2A,B), C-HA–AJ222801 (Fig. 2C,D), and N-HA–AJ222801 (Fig. 2E,F) were all localized to an intracellular reticular network in COS cells. Likewise, A_{346}-HA–AJ222801 was detected at a similar location (Fig. 3A,B), although the resolution of the reticular labeling was lower, probably due to the inaccessibility of the antibody to the HA epitope. When cells were fixed, but not permeabilized, no labeling with an anti-HA antibody could be detected in cells transfected with A_{346}-HA–AJ222801 (Fig. 3C,D) or with either of the other two HA-tagged proteins (not shown). Thus, irrespective of the position of the tag on the transfected protein, labeling of the PM was never detected, strongly suggesting that enzyme AJ222801 is an intracellular protein. This was confirmed by co-transfection experiments, in which COS cells were transfected with the p75 neurotrophin receptor (p75NTR), which was localized to the PM (Fig. 4A,B), and by co-localization with an ER marker, PDI (Fig. 4C,D). A similar localization was obtained in HEK-293 cells and in NIH-3T3 fibroblasts (not shown). Finally, we examined whether enzyme AJ222801 is translocated to the PM upon activation of the SM signalling pathway. No difference in the intracellular localization of C-GFP–AJ222801 was detected when 3T3 cells were co-transfected with p75NTR in the absence or presence of nerve growth factor (data not shown), a natural ligand of p75NTR that activates SM signalling [6,7].

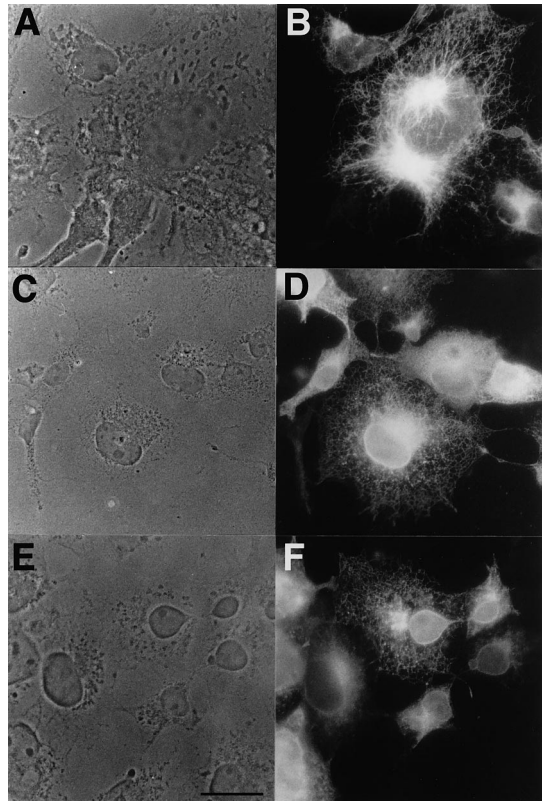

Fig. 2. Distribution of enzyme AJ222801 in COS cells. COS cells were transfected with C-GFP–AJ222801 (A, B), C-HA–AJ222801 (C, D), or N-HA–AJ222801 (E, F). The left-hand panels are phase contrast micrographs, and the right-hand panels are immunofluorescence micrographs. Bar = 20 μm.

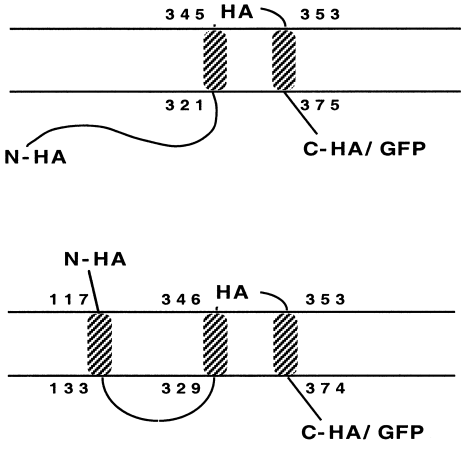

Fig. 1. Putative topology of enzyme AJ222801. The upper scheme shows the topology as originally suggested [2], and the lower scheme shows a topology prediction based on three other prediction programs that each give essentially the same result. Note that neither of these schemes specifies the orientation of the protein with respect to the inner or outer leaflet of the membrane bilayer.

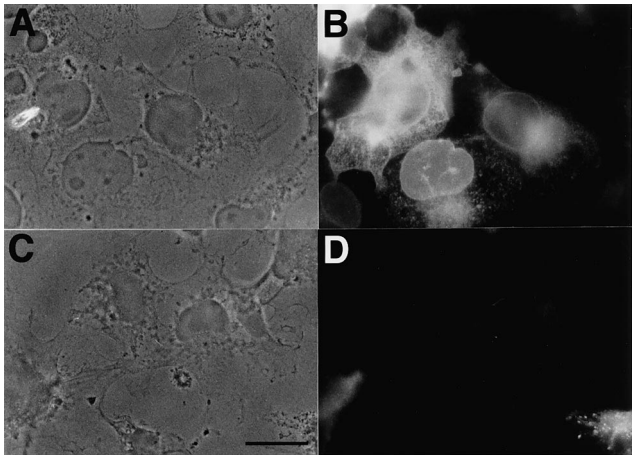

Fig. 3. Distribution of A_{346}-HA–AJ222801 in COS cells. Cells were transfected with A_{346}-HA–AJ222801 and localization was analyzed in permeabilized (A, B) and non-permeabilized (C, D) cells. The left-hand panels are phase contrast micrographs, and the right-hand panels are immunofluorescence micrographs. Bar = 20 μm.

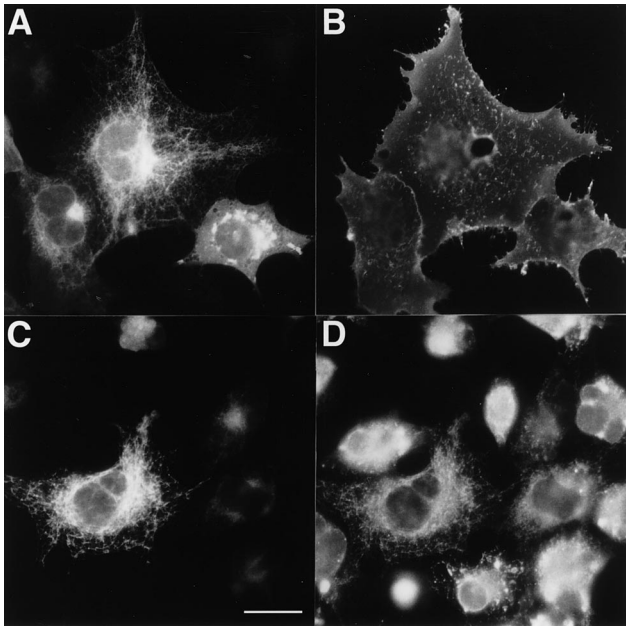

Fig. 4. Analysis of C-GFP–AJ222801 localization by comparison with PM and ER markers. COS cells were transfected with C-GFP–AJ222801 (A) and p75NTR (B); note the complete lack of overlap of immunofluorescence labeling. The localization of PDI (D) was compared with that of C-GFP–AJ222801 (C); note that these two proteins display identical intracellular localizations in the transfected cells. Bar = 20 μm.

4. Discussion

In the current study, we have demonstrated that enzyme AJ222801, originally suggested to be a N-SMase, is localized to the ER in a variety of cultured cells. This conclusion is different from that reached by Tomiuk et al. [2], who suggested that the protein is located at the PM. However, this conclusion was based solely on cell fractionation experiments, in which a PM-enriched fraction was obtained by centrifugation at $2500 \times g$. Clearly, the PM cannot be resolved from other membrane fractions by such low-speed fractionation. Interestingly, amino acid residues 405–413 of the mouse homolog of enzyme AJ222801 (EPHLAYCL) are 75% identical to residues 78–85 (EPYLFYCL) of human UDP-glucuronosyltransferase (UGT1A6). This sequence is part of a domain that acts as an internal signal retention sequence and an ER-targeting domain for UDP-glucuronosyltransferase [8].

Our data are also consistent with the recent suggestion that the natural substrate for enzyme AJ222801 is lyso-PAF, not SM, since little if any SM is believed to be associated with the ER [9]. PAF is deacylated to lyso-PAF by the activity of an acetylhydrolase, and it is generally thought that the next steps in the pathway of PAF inactivation are sequential degradation by a lyso-phospholipase D, yielding alkylglycerol phosphate, and a phosphohydrolase, to yield alkylglycerol [10]. A lyso-PAF phospholipase C alleviates the need for two enzymatic steps to produce alkylglycerol. Further study will be required to determine the role of this reaction in lipid metabolism and/or signalling in the ER.

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