

NH₂-Terminal Targeting Motifs Direct Dual Specificity A-Kinase-anchoring Protein 1 (D-AKAP1) to Either Mitochondria or Endoplasmic Reticulum

Lily Jun-shen Huang,* Lin Wang,* Yuliang Ma,* Kyle Durick,* Guy Perkins,[‡] Thomas J. Deerinck,[‡] Mark H. Ellisman,[‡] and Susan S. Taylor*

*Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, and the [‡]National Center for Microscopy and Imaging Research, University of California, San Diego, La Jolla, California 92093-0654

Abstract. Subcellular localization directed by specific targeting motifs is an emerging theme for regulating signal transduction pathways. For cAMP-dependent protein kinase (PKA), this is achieved primarily by its association with A-kinase-anchoring proteins (AKAPs). Dual specificity AKAP1, (D-AKAP1) binds to both type I and type II regulatory subunits and has two NH₂-terminal (N0 and N1) and two COOH-terminal (C1 and C2) splice variants (Huang et al., 1997. *J. Biol. Chem.* 272:8057). Here we report that the splice variants of D-AKAP1 are expressed in a tissue-specific manner with the NH₂-terminal motifs serving as switches to localize D-AKAP1 at different sites. Northern blots showed that the N1 splice is expressed primarily in liver, while the C1 splice is predominant in testis. The C2 splice shows a general expression pattern. Microinjecting expression constructs of D-AKAP1(N0) epitope-tagged at either the NH₂ or the COOH terminus showed their localization to the mitochondria based on immunocytochemistry. Deletion of N0(1-30)

abolished mitochondrial targeting while N0(1-30)-GFP localized to mitochondria. Residues 1–30 of N0 are therefore necessary and sufficient for mitochondria targeting. Addition of the 33 residues of N1 targets D-AKAP1 to the ER and residues 1–63 fused to GFP are necessary and sufficient for ER targeting. Residues 14–33 of N1 are especially important for targeting to ER; however, residues 1–33 alone fused to GFP gave a diffuse distribution. N1(14-33) thus serves two functions: (a) it suppresses the mitochondrial-targeting motif located within residues 1–30 of N0 and (b) it exposes an ER-targeting motif that is at least partially contained within the N0(1-30) motif. This represents the first example of a differentially targeted AKAP and adds an additional level of complexity to the PKA signaling network.

Key words: cAMP-dependent protein kinase • AKAP • mitochondria • endoplasmic reticulum • subcellular localization

cAMP is an important second messenger that regulates multiple cellular processes by activating cAMP-dependent protein kinase (PKA)¹. PKA exists as a holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. cAMP binding cooperatively to the R-sub-

units causes the complex to dissociate, releasing two free and active C-subunits. Due to the broad substrate specificity of PKA, compartmentalization serves as an additional regulatory mechanism that can increase both the selectivity and intensity of a cAMP-mediated hormonal response. For the free C-subunit, subcellular localization also can be modulated by PKI which, in addition to serving as an inhibitor, also contains a nuclear export signal that actively exports C from the nucleus (Wen et al., 1995). Localization of the inactive holoenzyme, however, is achieved primarily through association with A-kinase-anchoring proteins (AKAPs; Rubin, 1994; Scott and McCartney, 1994; Pawson and Scott, 1997). AKAPs typically interact with the NH₂-terminal dimerization domain of the type II regulatory subunits and are tethered to structural proteins, membranes, or organelles (Leiser et al., 1986; Hausken et al., 1994; Lin, 1995; Lester et al., 1996). In some cases,

Address correspondence to Dr. Susan S. Taylor, University of California, San Diego, 9500 Gilman Dr. 0654, La Jolla, CA 92093-0654. Tel.: (619) 534-3677. Fax: (619) 534-8193. E-mail: staylor@ucsd.edu

Dr. Durick's current address is Aurora Biosciences, 11010 Torreyana Road, San Diego, CA 92121.

1. *Abbreviations used in this paper:* AKAP, A-kinase-anchoring protein; C, cAMP-dependent protein kinase catalytic subunit; D-AKAP1, dual specificity AKAP1; GFP, green fluorescent protein; PKA, cAMP-dependent protein kinase; R, cAMP-dependent protein kinase regulatory subunit; RI, type I regulatory subunit of PKA; RII, type II regulatory subunit of PKA; SH2, src homology 2.

AKAPs, such as AKAP79, act as scaffolds by recognizing several functionally related enzymes which bind to PKA, PKC, and protein phosphatase 2B (calcineurin; Coghlan et al., 1995; Klauck et al., 1996).

A novel AKAP, D-AKAP1, was discovered that can bind to both type I and type II regulatory subunits (Huang et al., 1997). Despite its single message in most tissues, several D-AKAP1 isoforms or homologues were identified in various species. These isoforms include mouse S-AKAP84 (Chen et al., 1997), AKAP100 (Chen et al., 1997), and AKAP121 (Chen et al., 1997); rat AKAP121 (Feliciello et al., 1998); human S-AKAP84 (Lin, 1995); and AKAP149 (Trendelenburg et al., 1996). Mouse homologues of D-AKAP1, as summarized in Fig. 1 A, share a 525-amino acid core, and differ at their COOH termini, presumably by alternative splicing. This core contains the R-binding domain that is responsible for interacting with the regulatory subunit. Therefore, all isoforms should share the function of anchoring PKA. D-AKAP1a and mouse S-AKAP84 have relatively short COOH termini, whereas D-AKAP1c (AKAP121) and AKAP100 both contain a putative KH domain which was proposed to bind RNA (Siomi et al., 1993; Trendelenburg et al., 1996; Chen et al., 1997; Huang et al., 1997). The COOH-terminal 19 residues of D-AKAP1a are designated as C1, whereas the COOH-terminal 333 residues of D-AKAP1c are designated as C2. In addition to the various COOH-terminal splices, two NH₂-terminal alternative splices, N0 and N1, were identified in the cloning of D-AKAP1 (see Fig. 1 b). The N0 splice is included in the 525-amino acid core, and contains a stretch of hydrophobic residues proposed to be a mitochondrial-targeting sequence (Lin, 1995; Chen et al., 1997). The N1 splice contains 33 additional amino acids at its NH₂ terminus, including a potential myristylation site and a putative PKA phosphorylation site.

Targeting PKA to specific sites in the cell is a major role of the anchoring proteins. In this study, we choose to further investigate this function of D-AKAP1, in particular, to elucidate the functional importance of the two NH₂-terminal splice variants. Using Northern analysis coupled with immunocytochemistry, we demonstrate that the various alternative splice variants are expressed in a tissue-specific manner and dictate distinctly different subcellular localizations. Residues 1–30 of N0 are identified to be necessary and sufficient to target D-AKAP1 to the mitochondria while N1 is expressed primarily in the liver, contains an additional 33 residues and causes D-AKAP1 to localize to the endoplasmic reticulum (ER).

Materials and Methods

Materials

The following materials were purchased as indicated: Mouse Multiple Tissue Northern Blot (Clontech), Ready-to-Go DNA labeling kit (Pharmacia), MitoTracker (Molecular Probes), Triton X-100 and guinea pig IgG (Sigma Chemical Co.), SSC buffer (5'→3' Inc.), enzymes used for DNA manipulations (Life Technologies, Inc.), the DNA sequencing kit (U.S. Biochemical Corp.), monoclonal 12-CA-5 anti-HA tag antibody (BAbCo), biotinylated donkey anti-mouse IgG antibody, FITC donkey anti-mouse IgG antibody, donkey anti-rabbit IgG antibody, and Cy-5 conjugated anti-guinea pig IgG antibody (Jackson ImmunoResearch), Texas red streptavidin (Amersham Corp.), and erythrocyte lysate transcription-and-translation kit (Promega). Antibodies against D-AKAP1

were generated in female rabbits at Cocalico Corp. All oligonucleotides were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego.

Northern Analysis

Blots containing 2 µg of immobilized mRNAs from selected adult mouse tissues were probed with ³²P-radiolabeled cDNA for the various splice variants. Nucleotides 1–90 encoding the first 30 residues of D-AKAP1b were amplified by PCR and used to make the cDNA probe specific for the N1 splice, nucleotides 2071–2366 of D-AKAP1a were used for the C1 splice, and nucleotides 2953–3317 of D-AKAP1c were used for the C2 splice. Fig. 2 a was taken from Huang et al. (1997), in which nucleotides 854–1226 encoding the kinase binding domain in the core region of D-AKAP1 were used for a probe common to all the splice variants. Nitrocellulose filters were prehybridized in 6× SSC (750 mM sodium chloride and 75 mM sodium citrate, pH 7.0), 1% milk, and 0.5 mg/ml salmon sperm DNA for 4 h at 65°C, then hybridized to 1.5 × 10⁶ cpm/ml of denatured radiolabeled cDNA probe in the same buffer. Hybridization was performed at 65°C for 16 h and nonhybridized probe was removed with 0.1× SSC and 0.1% SDS at 65°C. Hybridizing mRNA signals were detected by autoradiography.

Construction of Expression Vectors

To determine the subcellular localization of the two NH₂-terminal splices of D-AKAP1, an epitope tag derived from the influenza hemagglutinin protein (HA) was engineered at either the NH₂ terminus or the COOH terminus for immunofluorescent detection using monoclonal antibodies to the HA tag (Wilson et al., 1984). To make NH₂-terminal HA fusion proteins of N0 and N1, a linker containing the initiation codon of the luciferase gene and the nine-amino acid HA-tag sequence YPYDVPDYA was subcloned into the pcDNA3 vector using the BamHI and EcoRI sites. Another linker was then subcloned into the NotI and XbaI sites downstream from the HA tag to make a one nucleotide frameshift at the NotI site. This new vector was named pcDNA3ML. Finally, cDNAs encoding D-AKAP1a and D-AKAP1b were subcloned into pcDNA3ML using the newly engineered NotI site and XhoI. D-AKAP1a and D-AKAP1b both use the C1 splice but have either N0 or N1, respectively. These two constructs, designated as [HA]-N0 and [HA]-N1 for their different NH₂-terminal splices, correspond to [HA]-D-AKAP1a and [HA]-D-AKAP1b, respectively. To make COOH-terminal HA fusion proteins of D-AKAP1, the cDNA encoding the first 144 residues of D-AKAP1a was first excised from pBS-D-AKAP1a and subcloned into pcDNA3 using EcoRI. A linker containing the HA tag epitope flanked by a blunt end and a XhoI restriction site was then ligated in using EcoRV and XhoI. The final construct was designated N0(1-144)-[HA].

To make [HA]-N0(Δ1-30), which lacks the first 30 amino acids, a NotI site was engineered at residue 30 of [HA]-N0 by site directed mutagenesis (Kunkel et al., 1991), and the cDNA encoding residues 1–30 was excised using NotI. To make deletion mutants of [HA]-N1, a NotI site was engineered at residues Gly13 or Cys24 and cDNAs encoding sequences 1–13 or 1–24 were then excised using NotI. These two constructs lacking the first 13 or 24 residues of [HA]-N1 were designated N1(Δ1-13) and N1(Δ1-24), respectively.

To make (1-30)N0-[GFP], the first 30 residues of N0 were amplified by PCR with EcoRI and BglII engineered at the 5' and 3' termini and subcloned into vector pEGFP-N1 (Clontech) between EcoRI and BamHI sites. To make (1-63)N1-[GFP] and (1-33)N1-[GFP], the first 63 or 33 residues of D-AKAP1b were amplified by PCR with EcoRI and BglII engineered at their 5' and 3' termini, respectively. These two fragments were then subcloned into vector pEGFP-N1 (Clontech) between EcoRI and BamHI sites.

Cell-free Expression of [HA]-N0 and [HA]-N1

Expression vectors for [HA]-N0 and [HA]-N1 were tested using an in vitro transcription-translation kit (TNT T7 Quick coupled system; Promega). In brief, purified DNA was mixed with rabbit reticulocyte extract and 10 µCi [³⁵S]methionine, and incubated at 30°C for 2 h. The assay mixtures were separated by SDS-PAGE and visualized by autoradiography for 48 h. These products were also subjected to immunoblot analysis using antibodies specific for N0 (anti-RPP7) or N1 (anti-N1), and visualized by ECL, which takes <1 min for exposure.

Cell Culture and Transient Transfection

Mouse 10T1/2 fibroblasts were maintained in DME containing 10% FBS, and plated at a density of 1×10^5 cells per 10-cm plate 24 h before transfection. Cells were transiently transfected, using the calcium phosphate method with 10 μ g of DNA per plate (Sambrook et al., 1989). 20 h after transfection, cells were visualized by fluorescent microscopy either directly or after fixing with 4% paraformaldehyde.

Microinjection Analysis

For microinjection, cells were plated on glass coverslips and grown to 70% confluence in DME + 10% FBS. The coverslips were then transferred to DME containing 0.05% calf serum. After 24 h, the cells were injected into their nuclei with solutions of injection buffer (20 mM Tris, pH 7.2, 2 mM $MgCl_2$, 0.1 mM EDTA, and 20 mM NaCl) containing 100 μ g/ml expression plasmid DNA. In some experiments, 6 mg/ml guinea pig IgG (Sigma Chemical Co.) was coinjected as a marker. Experiments were performed using an automatic micromanipulator (Eppendorf) with glass needles pulled on a vertical pipette puller (Kopf). For detection of expressed proteins, cells were fixed in 4% paraformaldehyde 5 h after injection for 10 min, and then washed with 0.1% Tween in PBS. The cells were then incubated successively with appropriate antibodies.

Cell Staining

In general, cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS before staining with antibodies. HA-tagged proteins were stained with primary monoclonal anti-HA tag antibodies (dilution 1:100) for 1 h at 37°C then incubated with rhodamine-conjugated donkey anti-mouse IgG antibodies (dilution 1:200). In some experiments, the secondary antibody is biotinylated donkey anti-mouse IgG, which is in turn stained by Texas red streptavidin. To stain mitochondria, two methods were used. 200 nM of a mitochondrion-specific dye (Mitotracker; Molecular Probes Inc.) was incubated with the microinjected cells for 30 min before fixation. Alternatively, a vector encoding a mitochondrial-specific green fluorescent protein ([GFP]-mito; Llopis et al., 1998) was comicroinjected with the HA-tagged proteins, and mitochon-

dria were visualized directly by GFP. In addition, cells were stained with Cy-5 conjugated anti-guinea pig IgG (dilution 1:100) to discriminate injected from noninjected cells. To stain ER, cells were incubated with rat anti-Bip antibodies (dilution 1:100), washed three times with 0.1% Tween in PBS, and probed with fluorescein-conjugated donkey anti-rat IgG (dilution 1:100). Between incubations with different antibodies, cells were washed three times with 0.1% Tween in PBS for 5 min each time at room temperature to eliminate nonspecific staining.

Laser-scanning Confocal Microscopy

Samples were visualized by confocal microscopy using an MRC-1024 system (Bio Rad Laboratories) attached to an Axiovert 35M (Zeiss AG) and a 40 \times NA objective. Excitation illumination was with 488-, 568-, and 647-nm light from a krypton/argon laser. Individual images (1024 \times 1024 pixels) were converted to PICT format and merged as pseudo-color RGB images using Adobe Photoshop (Adobe Systems). Digital prints were from a Fujix Pictography 3000 printer (Fuji).

Results

Tissue-specific Expression of Splice Variants

To investigate tissue-specific expression of the different splice variants of D-AKAP1, Northern blots were probed with ^{32}P -labeled splice-specific cDNAs. As described previously, probing the blot with cDNA to the core region of D-AKAP1 showed that the overall expression of the 3.8-kb D-AKAP1 message is highest in heart, liver, skeletal muscle, and kidney. In addition, a strong signal at 3.2-kb was detected only in testis (Huang et al., 1997). Probing the same blot with cDNA probes derived from unique regions of the various splice variants allowed detection of splice-specific expression (Fig. 2, b-d). Proteins containing the

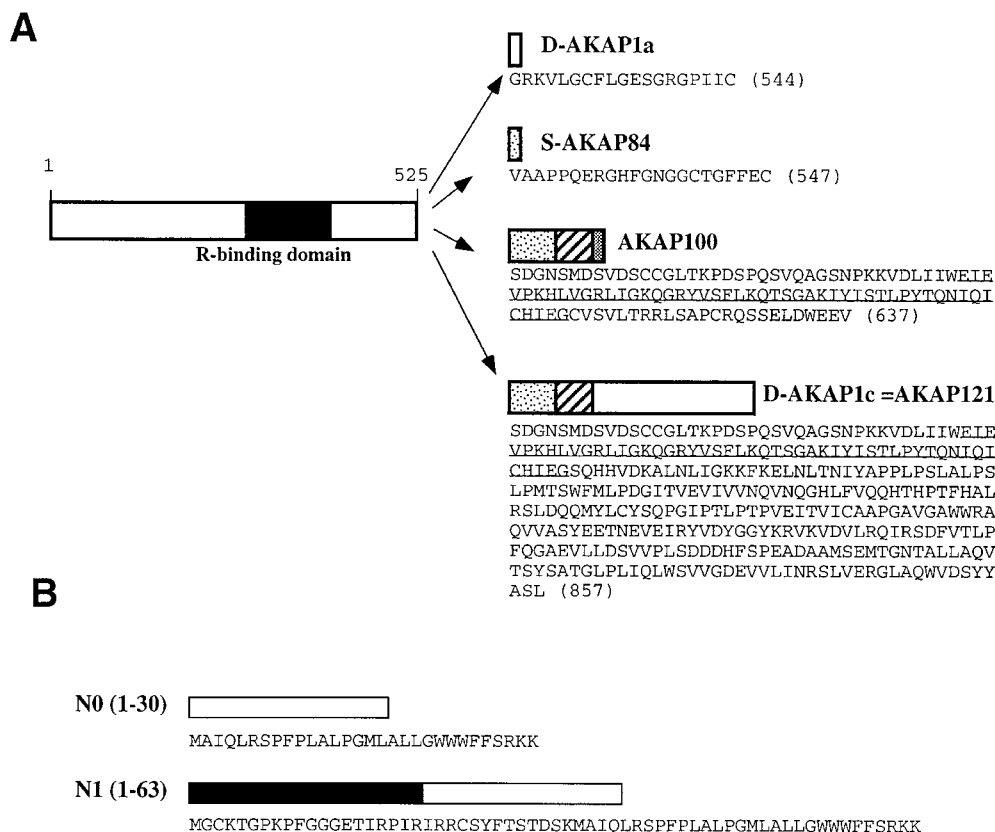


Figure 1. Schematic diagram of the various splices and isoforms of D-AKAP1. (a) Schematic diagram of various mouse homologues of D-AKAP1. All four isoforms share a 525-amino acid core, including the R-binding domain. A putative KH domain was identified in isoforms AKAP100 and D-AKAP1c (AKAP121). This domain is shown as the hatched box and the corresponding sequences are underlined. (b) The NH₂ terminal 30 and 63 residues are shown for N0 and N1, respectively. Residues RRCSY represent the putative PKA phosphorylation site identified in N1.

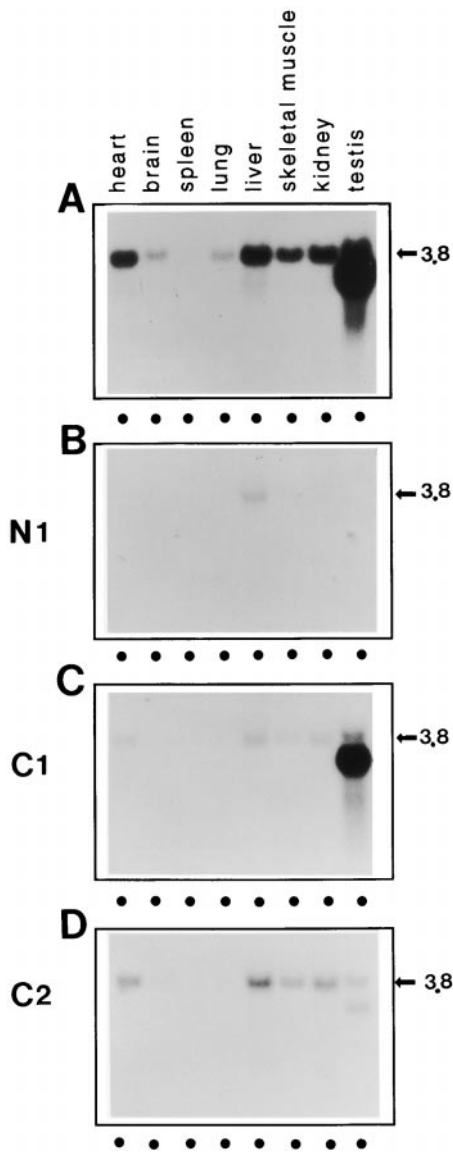


Figure 2. Splice variants of D-AKAP1 are expressed in a tissue-specific manner. The same Northern blot containing immobilized samples of mRNAs of selected adult tissues was probed with ^{32}P -radiolabeled cDNAs specific for the various splice variants: core (A), N1 (B), C1 (C), and C2 (D). The size of the mRNA was indicated on the right side of the blots. Panel a is taken from Huang et al. (1997) with permission.

N1 splice were expressed exclusively in liver, while the C1 splice was expressed predominantly in testis. The C2 splice had a more general expression pattern. Therefore, in addition to the distinct features revealed by amino acid sequences, these splice variants also were expressed in a tissue-specific manner. Because the N0 splice contained sequences shared by the core region, the expression pattern specific for this NH_2 -terminal splice could not be obtained. However, since the N1 splice was only expressed in liver, the D-AKAP1s that are expressed in all the other tissues most likely use the N0 splice.

Expression of [HA]-N0 and [HA]-N1

To determine the location of D-AKAP1 in cells, [HA]-N0

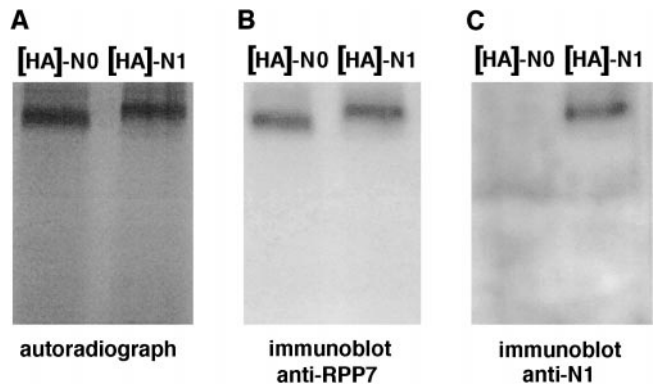


Figure 3. Cell-free expression of [HA]-N0 and [HA]-N1. [^{35}S]Methionine-labeled [HA]-N0 and [HA]-N1 were expressed in an in vitro transcription-translation system and visualized by autoradiography (A). These products were also subjected to immunoblot analysis using anti-RPP7 (B) or anti-N1 (C).

and [HA]-N1 that had an HA-tag at the NH_2 terminus of D-AKAP1a and D-AKAP1b were constructed as described in the Materials and Methods. D-AKAP1a is comprised of the N0 splice and the C1 splice; D-AKAP1b is comprised of the N1 splice and the C1 splice. To determine whether the vectors can express the appropriate proteins, expression was tested first in a cell-free translation system. As seen in Fig. 3, in vitro transcription-translation of [HA]-N0 or [HA]-N1 gave protein products with the apparent molecular mass of 107 and 114 kD, respectively. These are approximately what would be predicted from the sequence. To further verify these expressed products, they were also subjected to immunoblot analysis using antibodies raised against a core region of D-AKAP1 (RPP7) or generated specifically to N1. Only the protein product expressed from [HA]-N1 reacted with antibodies specific for N1 (Fig. 3).

Residues 1–30 of the N0 Splice Variant Target D-AKAP1 to Mitochondria

The NH_2 -terminal 30 amino acids of N0 has a putative mitochondrial-targeting sequence in S-AKAP84 (Lin, 1995; Chen et al., 1997). To further characterize the localization of D-AKAP1 that used this N0 splice, the vector expressing D-AKAP1a with the HA tag at the NH_2 terminus was microinjected into 10T1/2 mouse fibroblasts. Expressed proteins were visualized by immunofluorescence coupled with confocal microscopy. As seen in Fig. 4 A, cells expressing [HA]-N0 had a punctate immunofluorescent staining concentrated around the nucleus. This labeling was mimicked by a fluorescent mitochondrial marker (Mitotracker), indicating its localization to the mitochondria. To test if the HA-tag affected localization, N0-[HA] was constructed. In this vector, the first 144 amino acids of D-AKAP1a were fused to a COOH-terminal HA-tag. The staining pattern was identical for both [HA]-N0 and N0-[HA], and was thus independent of epitope tags attached at either end (Fig. 4 B).

To confirm the mitochondrial localization in living cells as well as in fixed cells, GFP was fused to the targeting se-

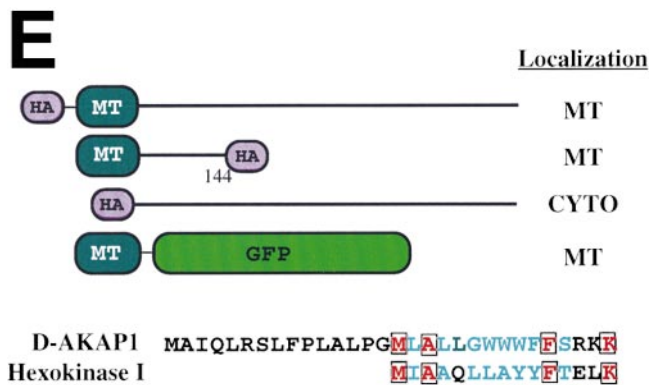
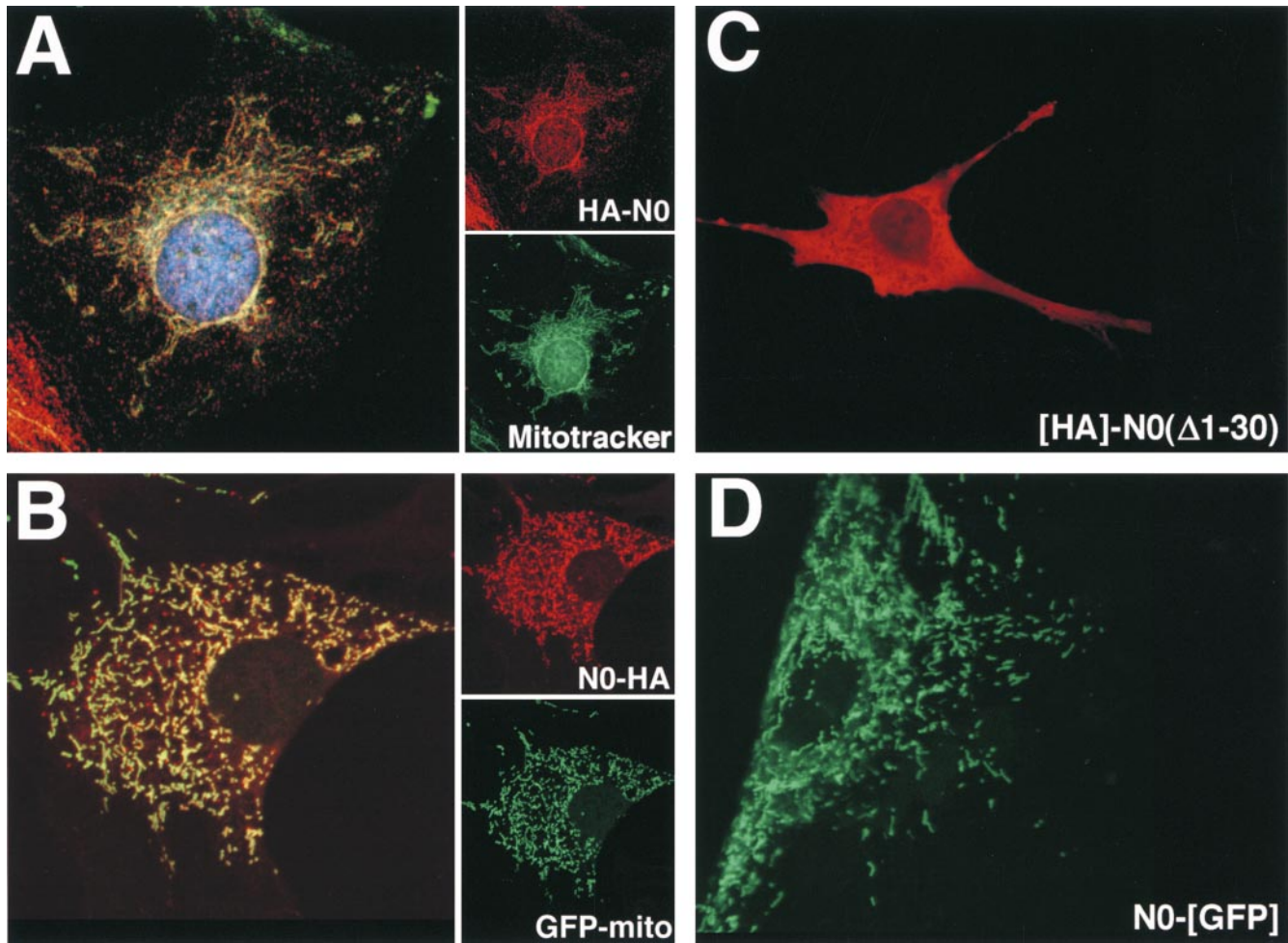


Figure 4. The N0 splice localizes to mitochondria. Confocal images of [HA]-N0 localization using anti-HA antibodies (red) co-stained with a mitochondria fluorescent marker (A) or a mitochondrial targeted GFP marker (B) (both green). Yellow staining indicates overlapped localization. Individual staining patterns were shown in the insets. The blue staining shows immunofluorescent detection of an IgG injection marker (A). [HA]-N0(Δ 1-30) shows a diffused cytoplasmic stain (C), while N0-[GFP] exhibits a mitochondrial pattern (D). These results are summarized schematically in E. The first 30 residues of N0 are aligned with the first 15 residues of Hexokinase I. Identical residues are indicated in black boxes, and homologous residues are indicated in gray boxes.

quence of cytochrome oxidase (Rizzuto et al., 1996). This [GFP]-mitochondrial marker, [GFP]-mito, was used to preclude the possibility of staining artifacts and confirm localization to the mitochondria. Cells microinjected with the expression vector for GFP alone had a diffuse distribution, filling the entire cell as previously reported (data not shown; Pines, 1995). [GFP]-Mito, in contrast, displayed typical mitochondria localization (Fig. 4 B).

To determine whether the first 30 residues of N0 are necessary and/or sufficient to target mitochondria, two additional constructs were engineered. [HA]-N0(Δ 1-30) contained HA-tagged full-length D-AKAP1a lacking the first 30 residues, and (1-30)N0-[GFP] contained only the

first 30 residues of D-AKAP1a fused to GFP. As shown in Fig. 4 C, when cDNA of [HA]-N0(Δ 1-30) was microinjected into the nucleus of 10T1/2 cells, a diffused cytoplasmic staining pattern was observed indicating the loss of mitochondrial targeting. In contrast, (1-30)N0-[GFP] showed a characteristic mitochondrial pattern (Fig. 4 D). Thus, as summarized in Fig. 4 E, the first 30 residues of N0 were not only necessary, but also sufficient for mitochondrial localization.

N1 Targets D-AKAP1b to ER

In addition to the N0 splice, D-AKAP1 has at least one al-

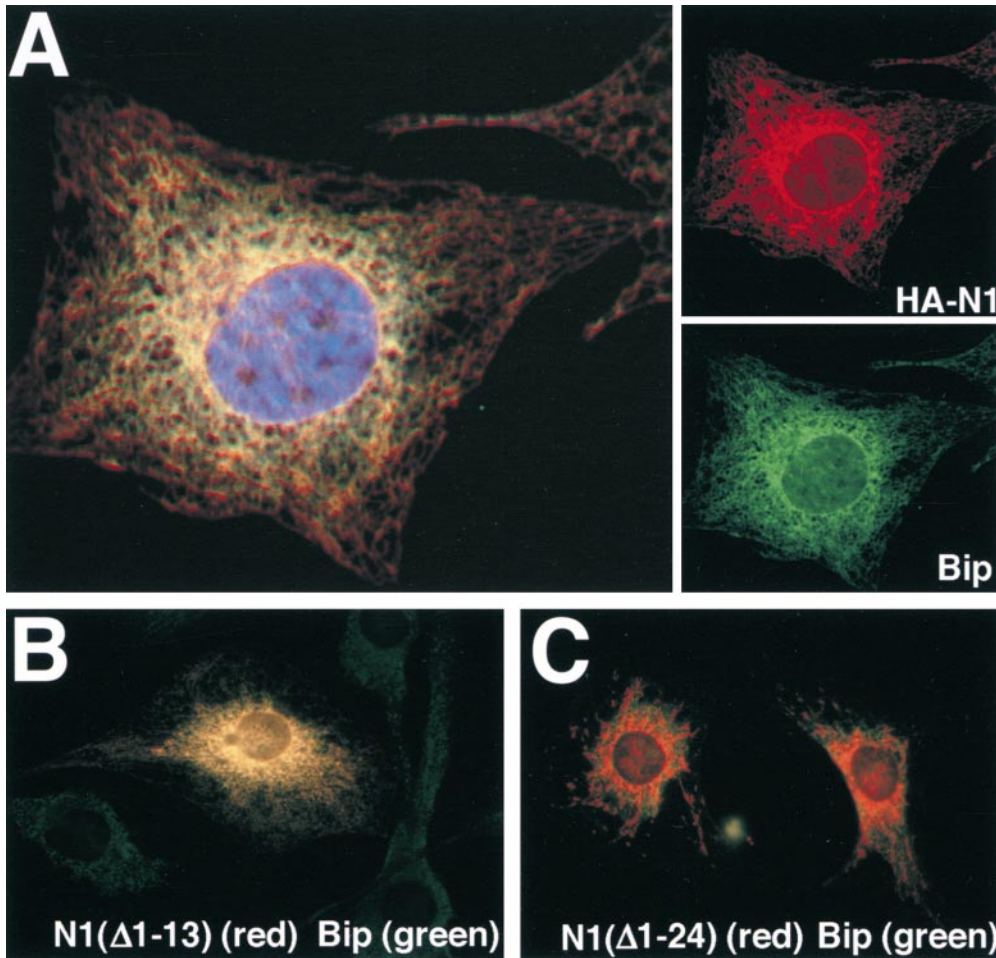
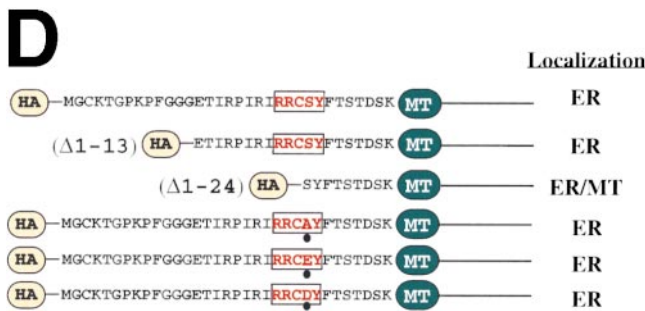


Figure 5. The N1 splice localizes to endoplasmic reticulum. (A) Confocal image of [HA]-N1 localization with anti-HA antibodies (red) costained with anti-Bip antibodies (green). Yellow staining indicates overlapped localization. Individual staining patterns were shown in the insets. The blue staining shows immunofluorescent detection of an IgG injection marker. (B) N1(Δ 1-13) (red) colocalizes with Bip (green). (C) N1(Δ 1-24) (red) does not colocalize with Bip (green). The results in A–C are summarized schematically in D. In addition, mutagenesis studies of the putative PKA phosphorylation site (boxed residues) are also summarized (data not shown). The dotted residues indicate the amino acids used to replace the serine residue.



ternative NH₂-terminal splice variant, N1, that includes 33 extra amino acids NH₂-terminal to the N0 splice. To investigate if these 33 residues affect the localization of D-AKAP1, [HA]-N1 was constructed. [HA]-N1 encodes HA-tagged D-AKAP1b, which consists of the N1 splice in addition to D-AKAP1a. Cells microinjected with an expression construct for [HA]-N1 had a more “lacy” staining pattern. Colocalization of [HA]-N1 with Bip, an hsp70 homologue that resides in the lumen of the ER (Haas, 1994), indicated [HA]-N1 is targeted to the ER (Fig. 5 A). Therefore, the extra 33 residues of the N1 splice not only abolished mitochondrial targeting of D-AKAP1, but also exhibited an ER-targeting function. Cells microinjected with the [HA]-N1 expression vector were also cross-stained

with [GFP]-mito and showed no overlapping staining (data not shown).

To further characterize this ER-targeting function of N1, two deletion mutants, N1(Δ 1-13) and N1(Δ 1-24) were engineered. N1(Δ 1-13) contained the full-length D-AKAP1b with the first 13 residues deleted, while N1(Δ 1-24) contained the full-length D-AKAP1b with the first 24 residues deleted. Both had the HA tag fused to the NH₂-terminus. N1(Δ 1-13), like full-length [HA]-D-AKAP1b, colocalized with Bip, indicating its localization to the ER (Fig. 5 B), whereas N1(Δ 1-24) had a mixed localization pattern between ER and mitochondria. Fig. 5 C shows fluorescence imaging of two cells microinjected with the N1(Δ 1-24) expression construct that exhibited a more prominent mito-

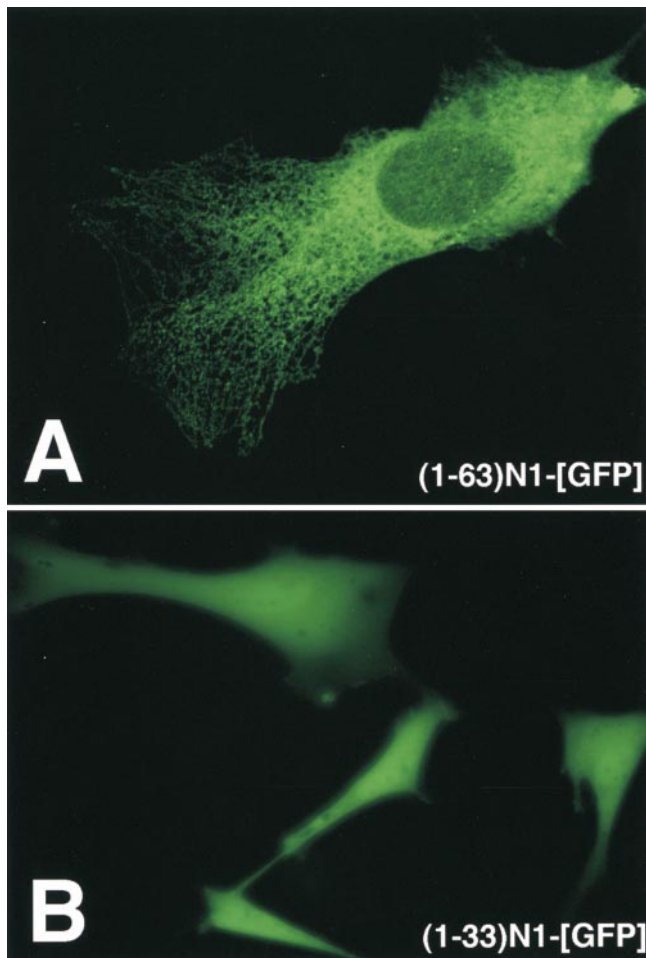


Figure 6. The N1 splice does not contain an ER-targeting signal. Confocal images of 10T1/2 cells transfected with (1-63)N1-[GFP] which displayed ER localization (A), and (1-33)N1-[GFP] with a diffuse pattern (B).

chondrial pattern. Since N1(Δ 1-24) had decreased ability to target D-AKAP1 to ER, residues 14–33 must participate in dictating the ER localization of N1.

Residues 14–33 of N1, as shown in Fig. 5 d, contain a potential PKA phosphorylation site (RRCSY). To test if phosphorylation of this site participated in the ER-targeting function of N1, three mutants with the potentially phosphorylatable Ser changed to Ala, Glu, and Asp were engineered in the construct [HA]-N1. All three mutants exhibited ER localization (original data not shown).

The First 30 Residues of N0 Are Necessary for both Mitochondrial Targeting and ER Targeting

To further localize the ER-targeting signal, two GFP fusion constructs were engineered. (1-63)N1-[GFP] contains the 33 extra amino acids of N1 plus the 30 residues of N0, sufficient for mitochondria targeting, fused to GFP. (1-33)N1-[GFP] contains only the 33 residues of N1 fused to GFP. As shown in Fig. 6 A, cells microinjected with the expression vector encoding (1-63)N1-[GFP] had a lacy staining pattern, characteristic of ER localization, whereas

cells microinjected with the (1-33)N1-[GFP] expression vector showed a diffuse staining pattern, similar to GFP alone (Fig. 6 B). Cells expressing (1-63)N1-[GFP] were also stained with the anti-N1 antibody and ER localization was detected (data not shown). These results indicated that the 33 residues of N1, when fused to the mitochondrial-targeting sequence of N0, were sufficient to switch its localization to the ER. However, these 33 amino acids alone were not able to dictate ER localization. Therefore, the ER-targeting signal is at least partially located within the NH_2 -terminal 30 residues of N0. The 33 additional amino acids at the NH_2 terminus of N1 thus were able to (a) suppress the mitochondrial-targeting function of N0 and (b) generate a new ER-targeting function. Since HA-tagging at the NH_2 terminus of N0 did not alter its mitochondrial localization, it does not appear as though mitochondrial targeting requires the insertion of the NH_2 terminal N0 motif into the membrane.

Discussion

Subcellular localization directed by specific targeting motifs is an emerging theme for regulating signal transduction pathways. These targeting proteins recruit active enzymes into signaling “modules” or place specific enzymes close to their substrates. For example, proteins such as src are brought to the plasma membrane by a myristylation motif at its NH_2 terminus. In addition, src homology 2 (SH2) domains bind to specific phosphotyrosines, thus permitting the assembly of signaling complexes in response to activation of cell surface receptors (Pawson et al., 1993; Waksman, 1994; Pawson and Scott, 1997). Similarly, subcellular compartmentalization of serine/threonine kinases and phosphatases occurs through interactions with targeting subunits or anchoring proteins that localize these enzymes to specific sites (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Klauck et al., 1996). AKAPs play major roles in regulating the compartmentalization of PKA through specific interactions with the NH_2 -terminal dimerization domain of the R subunits (Pawson and Scott, 1997).

D-AKAP1 represents a family of AKAPs with splice variants at both the NH_2 terminus and COOH terminus. We showed here that, based on Northern blot analysis, three of these splice variants are expressed in a tissue-specific manner of the tissues tested. N1 was expressed exclusively in liver, while C1 was expressed predominantly in testis. C2 was expressed in all tissues tested but spleen, consistent with the overall expression pattern of D-AKAP1 core. Furthermore, we demonstrated here that the two NH_2 -terminal splice variants, N0 and N1, act as localization switches to dictate subcellular localization of D-AKAP1 to either mitochondria or endoplasmic reticulum. Fig. 7 summarizes the functional properties of the motifs at the NH_2 terminus of N0 and N1.

The first 30 residues of D-AKAP1 were shown to be necessary for mitochondrial targeting of mouse S-AKAP84 by cellular fractionation (Chen et al., 1997). Using microinjection of the various expression constructs coupled with immunocytochemistry, we directly investigated the localization of D-AKAP1. [HA]-N0 and N0-[HA] both exhibited mitochondrial localization, whereas a construct lacking the first 30 residues of N0 lost the targeting ability.

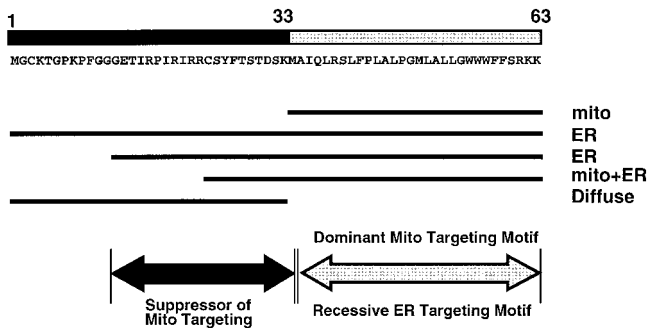


Figure 7. Schematic summary of various NH₂-terminal motifs that target D-AKAP1 to either mitochondria or endoplasmic reticulum.

These results confirmed that for D-AKAP1, the first 30 residues of N0 are necessary for proper targeting to mitochondria. Moreover, we took advantage of GFP-fusion proteins that can be visualized without artificial staining procedures in living cells. (1-30)N0-[GFP], which contains only the first 30 residues of N0 fused to GFP, also exhibited mitochondrial localization. Therefore, the first 30 residues of N0 are not only necessary but also sufficient to target D-AKAP1 to mitochondria.

When the first 30 amino acids of D-AKAP1 were compared with another protein that localizes to the outer mitochondrial membrane, hexokinase I, substantial similarities were identified (Wilson, 1997; Xie and Wilson, 1988). As shown in Fig. 4 E, in addition to the lack of negatively charged residues, hydrophobic residues and consecutive aromatic amino acids were homologous between the two proteins. This conserved stretch of residues (residues 1–15 in hexokinase I) was also identified to be necessary and sufficient to confer mitochondrial binding (Sui and Wilson, 1997). Recently, the crystal structure of the mammalian hexokinase I was determined, revealing that this stretch of mitochondrial-targeting residues forms an amphipathic helix (Mulichak et al., 1998). Whether D-AKAP1 uses the same mechanism for mitochondrial targeting awaits further investigation. However, since the attachment of an epitope tag at the beginning of N0 did not abolish targeting to mitochondria, it is unlikely that the NH₂-terminal motif simply inserts as an amphipathic helix into the membrane bilayer as has been proposed for hexokinase.

Surprisingly, despite the fact that N1 also contains the stretch of hydrophobic residues necessary for mitochondrial targeting, it exhibited a dramatically different localization. [HA]-N1 colocalized with Bip and showed a prominent ER staining, indicating the localization to the ER. Therefore, the extra 33 amino acids in N1, including a potential myristylation site followed by a potential PKA phosphorylation site, not only abolished mitochondrial targeting of N0 but also generated an ER-targeting function. The two distinct localizations for the two NH₂-terminal splice variants of D-AKAP1 defines the first example of an AKAP that shows a localization switch. Since N1 is only expressed in the liver, this switch of mitochondrial to ER localization may have physiological implications. D-AKAP1 does not contain a conventional Lys-Asp-Glu-

Leu (KDEL) sequence that was shown to be essential for ER retention of most soluble proteins (Andres et al., 1990). Therefore, this ER localization of N1 might define a novel mechanism for ER retention. Furthermore, with the NH₂-terminal HA-tagged N1 still localized to ER, myristylation at the NH₂ terminus of N1 is thus not likely to be necessary for its ER targeting.

To further dissect N1, two deletion mutations were constructed and examined. N1(Δ1-13) shows localization to the ER, similar to the full-length [HA]-N1, whereas N1(Δ1-24) has a mixed staining pattern at mitochondrial and ER. Because N1(Δ1-33), i.e., [HA]-N0, localizes exclusively to the mitochondria, residues 14-33 of N1 are especially important for the ER-targeting function. A potential PKA phosphorylation site was identified within this region of N1. Nevertheless, three mutations with the potentially phosphorylatable Ser changed to Ala, Asp, and Glu all localized to ER. The Ser to Ala mutant localized to ER, indicating that the unphosphorylated D-AKAP1 is likely to reside in ER. Since Asp and Glu sometimes do not fully mimic the phosphate moiety, whether phosphorylation affects targeting to ER is still unclear. However, the ER localization is not altered in the presence of exogenous cAMP analogue (data not shown), suggesting that phosphorylation may not be important for targeting.

By investigating the localization of (1-33)N1-[GFP], (1-63)N1-[GFP], and (1-30)N0-[GFP], the localization switch was further characterized. (1-33)N1-[GFP] showed a diffuse pattern in the cytoplasm, similar to GFP, whereas (1-63)N1-[GFP] localizes to ER. (1-30)N0-[GFP], i.e., (34-63)N1-[GFP], exhibited mitochondrial localization. These results not only indicate that the extra 33 residues do not contain an ER-targeting sequence, they also suggest that at least part of the ER-targeting function is embedded within the first 30 residues of N0 that also contain the mitochondrial-targeting signal. Adding N1 to the NH₂ terminus of N0 somehow is able to suppress its mitochondrial-targeting ability and also to generate a new ER-targeting signal. Whether this ER-targeting signal resides exclusively in N0 and is only exposed upon addition of N1, or whether this ER-targeting signal is instead generated by a composite of N0 and a portion of N1 is still under investigation.

The alternative splicing that alters the subcellular localization of D-AKAP1 may represent a novel and important regulatory mechanism to increase regulatory versatility and allow for cell type-specific gene regulation for these anchoring proteins and their signal transduction pathways. Further effort to dissect the molecular mechanism for this ER/mitochondria localization switch is underway.

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