A Molecular Switch for Targeting between Endoplasmic Reticulum (ER) and Mitochondria

CONVERSION OF A MITOCHONDRIA-TARGETING ELEMENT INTO AN ER-TARGETING SIGNAL IN \( \text{dAKAP}^1 \)B

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\( \text{dAKAP}^1 \) (AKAP121, S-AKAP84), a dual specificity PKA scaffold protein, exists in several forms designated as a, b, c, and d. Whether \( \text{dAKAP}^1 \) targets to endoplasmic reticulum (ER) or mitochondria depends on the presence of the N-terminal 33 amino acids (N1), and these N-terminal variants are generated by either alternative splicing and/or differential initiation of translation. The mitochondrial targeting motif, which is localized between residues 49 and 63, is comprised of a hydrophobic segment, however, does not contain an abundance of hydrophobic residues and therefore does not increase the hydrophobicity of the mitochondrial targeting signal. The specificity of ER or mitochondria targeting is determined and switched by the availability of the negatively charged residue, Asp31.

cAMP-dependent protein kinase (PKA)\(^2\) is an important regulator in cells, and it is involved in many cellular functions (1–4). Increasing evidence indicates that PKA fulfills its specific cellular regulation in part by subcellular localization in addition to selective recognition of its substrates (5). Subcellular localization of PKA is typically achieved through the binding of the regulatory subunits to scaffold proteins termed AKAPs (a kinase-anchoring proteins) where different AKAPs are expressed in different cell types. Depending on the targeting domain or motif an AKAP carries, it can bring PKA to different subcellular locations such as the nucleus, plasma membrane, endoplasmic reticulum (ER), Golgi, mitochondria, microtubules, etc. (6–8).

\( \text{dAKAP}^1 \) (also known as AKAP1, S-AKAP84, and AKAP121) is one of the few dual specificity AKAPs that were identified through interaction with R1α for binding to both RI and RII (9). A unique feature of this protein is that it can bind both PKA-I and PKA-II. In addition to binding to PKA, \( \text{dAKAP}^1 \) binds PP1, tubulin, a Myc-binding protein (AMY-1), PPD1, and mRNA (10–15). Several isoforms of \( \text{dAKAP}^1 \) have been identified in mouse, rat, and human varying at both the N terminus and the C terminus, with the PKA-binding domain located within the common core in the center of the molecule (9, 16, 17). The ER/mitochondrial targeting domain of \( \text{dAKAP}^1 \) is located at the N terminus (17, 18). This targeting domain also contains an overlapping mitotic spindle-targeting site (11). Variation in the N terminus can alter the distribution of PKA in the cell (18, 19). The alternative anchoring of PKA on either mitochondria or ER implies that PKA may be involved in important cellular metabolism and regulations related to these two organelles, such as energy production, protein synthesis, Ca\(^{2+}\) signaling, and/or apoptosis (8, 14).

\( \text{dAKAP}^1 \) bearing a shorter N terminus (\( \text{dAKAP}^1 \)a and 1c) targets exclusively to mitochondria, whereas the molecule bearing 33 additional residues (N1) at the N terminus (\( \text{dAKAP}^1 \)b and 1d) targets to ER (18). Similar to many known mitochondria-targeting signals, the mitochondria-targeting motif in \( \text{dAKAP}^1 \) contains a hydrophobic region and positively charged residues to the immediate C terminus (19–25). The N1 segment, however, does not contain an abundance of hydrophobic residues and therefore does not increase the hydrophobicity of the targeting motif, as found in other ER-targeting motifs in the molecules targeting to both ER and mitochondria (24–27). However, how the extra 33 residues convert the mitochondrial targeting motif into an ER-targeting signal is not clear. To understand the role of PKA anchoring to ER and mitochondria and how this anchoring is regulated, we explored the molecular mechanism for \( \text{dAKAP}^1 \) binding to both mitochondria and ER. Here we describe a novel mechanism that \( \text{dAKAP}^1 \) employs to switch the anchoring of PKA between ER and mitochondria.

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2 The abbreviations used are: PKA, cAMP-dependent protein kinase; ER, endoplasmic reticulum; GFP, green fluorescent protein; CytC, cytochrome c.
AKAP, Mitochondria, ER, cAMP-dependent Protein Kinase

EXPERIMENTAL PROCEDURES

Reagents—Antisera raised in the lab against PKA-Cat and S-DAKAP1 were used to detect PKA-Cat and S-DAKAP1. Other antibodies are from commercial sources: anti-mouse RIα from Santa Cruz Biotechnology, anti-CytC from BD Biosciences, anti-calnexin (against the cytosol-exposed C-terminal tail), and anti-calreticulin both from Abcam.

DNA Constructs and Sequence Analysis—Murine S-DAKAP1 isoforms 1a and 1b were subcloned into the pCMV-Sport1 vector (Invitrogen) containing a C-terminal FLAG tag (19). The N-terminal regions of S-DAKAP1a (amino acids 34–175) or S-DAKAP1b (amino acids 1–175) were subcloned into pCDNA3 (Invitrogen) using EcoRI and Xhol. The proteins were in vitro translated with the quick-coupled T7 Tnt system (Promega) as previously described (19). The N-terminal sequences of S-DAKAP1a and 1b (amino acids 1–63) were inserted into pEGFP-N1 (Clontech) with PCR. To avoid the interference of charged residues encoded by the polylinker, the sequence between KpnI and AgeI sites in the vector were replaced with a new linker (GGATCCGTGGCTCAGCTCACCGGT) that has a single BlpI recognition site. The sequences of anchoring domain were therefore inserted in between EcoRI and BlpI sites. Most of the mutations in the anchoring domain of S-DAKAP1 were made using Kunkel methods (28). A few difficult mutants were made by a modified quick change method (29). The mitochondria marker, mtGFP (30), was a gift from Dr. R. Y. Tsien (University of California, San Diego). The ER targeting marker, ER-GFP was a gift from Dr. D. G. Pestov (University of Illinois) (31). The genomic organization of S-DAKAP1 was generated with online-genomic Blast analysis.

Transfection Assay—10T 1/2 cells were cultured with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in 6-cm dishes containing 1.2-mm round coverslips (Fisher). The cells at 60–70% confluence were then transferred to a 24-well dish and transfected. Transfection was performed using either CytoFectine (Bio-Rad) or LipofectAmin-plus (Invitrogen) according to the manufacturer’s protocols. When cells were co-transfected, different DNA constructs were incubated at an equal molar ratio, adjusted to the same total weight with blank vector. The cells were incubated for 6–7 h post-transfection before fixings and indirect immunofluorescence.

Immunofluorescence and Photo-microscopy—Immunofluorescent labeling was performed as previously described (19, 32). The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min followed by permeabilization with 0.3% Triton X-100 in phosphate-buffered saline for 15 min at room temperature. S-DAKAP1a was detected using a monoclonal αFLAG antibody (clone m2; Sigma) and a rhodamine-conjugated donkey anti-mouse secondary antibody (Jackson Laboratory, Bar Harbor, ME). To detect wild type and mutants of S-DAKAP1b, a polyclonal antibody against N1 (18) was used n conjunction with a rhodamine-conjugated donkey anti-rabbit antibody (Jackson Laboratory). The final concentration of the antibodies was 10 μg/ml. All of the antibodies were diluted in a solution containing 0.5% Nonidet P-40, 5 mg/ml bovine serum albumin in phosphate-buffered saline, pH 7.2, and incubated with cells at 37 °C. The coverslips were mounted on glass slides with Slowfadem medium (Molecular Probes) and analyzed on a Leica microscopy equipped with a Mitsubishi digital camera.

Fractionation of Mitochondria and ER—Mitochondria and ER were isolated and purified from mouse liver according to Pagliarini et al. (33) and Sharma et al. (34). Mouse liver was homogenized with a motor-driven 30-ml Potter-Elvehjem tissue grinder five times in MSHE (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, and 1 mM EGTA). Nuclear material and unbroken cells were removed by centrifugation for 10 min at 2300 rpm (600 × gmax) in a Sorval SS34 rotor. Crude mitochondrial fractions were collected by centrifugation for 10 min at 11,200 rpm (15,000 × gmax). This fraction was then further purified by centrifugation on a Percoll-Histodenz gradient. The material between 17.5 and 35% Histodenz was collected as purified mitochondria (31). The post-mitochondria supernatants were fractionated by centrifugation at 100,000 × g for 1 h in a Ti70 rotor to collect crude ER as pellets (microsome) and supernatants (S100) (31). Crude ER was further fractionated on a discontinuous sucrose gradient by centrifugation at 29,000 rpm (100,000 × gmax) in a sw55 rotor for 16 h (34). Smooth ER was collected between 0.25 and 1.3 M sucrose, and rough ER was collected between 1.3 and 2.5 M. Protein (20 μg) from each fraction was analyzed by Western blotting. When fractions were protease treated, 5 μg of trypsin was incubated with the samples (350 μg of protein) for 0–10 min, followed by the addition of soybean trypsin inhibitor 100 μg and Complete (Roche Applied Science) protease inhibitors. When the fractions were washed with high salt and/or alkaline solutions, the samples (350 μg of protein) were incubated with 200 or 500 mM KCl in 5 mM HEPES, pH 7.4, or 0.1 M sodium carbonate, pH 11.5 for 30 min on ice, according to previously published reports (35, 36).

RESULTS

The 33-residue N1 Fragment Is Generated by Alternative Splicing at the N Terminus of S-DAKAP1—S-DAKAP1 was cloned from a mouse embryonic library with multiple isoforms bearing several N and C termini (9). Different clones from sperm, S-DAKAP84 and AKAP121, have similar C-terminal variations but lack the N-terminal variant (17). In Fig. 1 we show the exon organization of S-DAKAP1. Mouse genomic BLAST analysis of these cDNA clones and the newly deposited S-DAKAP1 alleles AK076216, AK132749, and BC065135 revealed that these various termini match different exons, indicating that these isoforms are generated by alternative splicing (Fig. 1A). For simplicity, only one allele is shown to represent the three. S-DAKAP1a is the shortest protein. It is encoded by exon 4 and then directly linked exon 5. S-DAKAP1b is identical to 1a at the 3’ end. Other isoforms have two additional 3’ compositions beyond exon 4: S-DAKAP1c, 1d, AKAP121, AK076216, AK132749, and BC065135 have exons 4 and 5 plus exons 7–15 at the 3’ end that encode two putative RNA-binding domains, a K homology (KH) domain, and a Tudor domain (Fig. 1, A and B); S-DAKAP84 is similar to the above variants except that it uses exon 6 instead of exon 5. S-DAKAP84 is similar to S-DAKAP1a in size, because exon 6 contains an in-frame stop codon that encodes 24 residues, whereas S-DAKAP1a has 20 residues.
encoded by exon 5 in the same place. At the 5' end, however, 
\( \text{AKAP1c} \) is identical to \( \text{AKAP1a} \).

There are three longer forms that are derived from differences at the 5' end: one has exon 1 proceeding exon 4 (in \( \text{AK076216} \) and \( \text{BC065135} \)), the second has exon 2 proceeding exon 4 (in \( \text{AKAP1b} \) and 1d), and the third has exon 3 in front of exon 4 (in S-\( \text{AKAP84} \), \( \text{AK132749} \) and \( \text{AKAP121} \)). Exon 3 in \( \text{AK132749} \) is 12 nucleotides shorter than \( \text{AKAP121} \), probably because of strain variation. Because neither exon 1 or 3 have an in-frame initiation codon, translation of \( \text{AKAP121} \), S-\( \text{AKAP84} \), \( \text{AK076216} \), \( \text{AK132749} \), and \( \text{BC065135} \) would start from exon 4, which corresponds to \( \text{AKAP1a} \) and 1c, with the first initiation codon 32 nucleotides within the 5' boundary of the exon 4.

d\( \text{AKAP1b} \) and 1d, on the other hand, carry an initiation codon in exon 2 that is 68 nucleotides to the 3' boundary and is in-frame with the protein encoded by exon 4. Therefore, in \( \text{dAKAP1b} \) and 1d, the merger of 68 nucleotides in exon 2 and the first 31 nucleotides at the beginning of exon 4 generates an extra fragment of 99 nucleotides encoding for 33 residues (Fig. 1C).

The production of two isoforms at the N terminus by alternative splicing could therefore provide the primary mechanism to generate two anchoring proteins that target to different organelles in different tissues.

**Targeting to Different Organelles Can Be Generated by Alternative Initiation of Translation from a Single mRNA**—Previously, we identified two N-terminal splice variants of \( \text{dAKAP1} \) (18). The first splice variant is found in isoforms 1a and 1c, initiates transcription at exon 4, and encodes a protein that is targeted to the mitochondria. The second splice variant is found in isoforms 1b and 1d, initiates transcription at exon 2, and encodes a protein that is targeted to the ER. In addition to alternative splicing, initiation of translation from different codons provides an alternative mechanism for generating two proteins from a single gene (37).

In our initial studies, we wanted to examine whether different initiation codons exist within a single mRNA from \( \text{dAKAP1} \). As diagramed in Fig. 2A, in \( \text{dAKAP1} \), codons 1, 34, and 49 are all located in a consensus with optimal initiation of translation (38) and can, therefore, potentially encode several \( \text{dAKAP1} \) variants by a leaky scanning mechanism (37). *In vitro* translation was used to determine whether multiple start sites in \( \text{dAKAP1a} \) and \( \text{dAKAP1b} \) exist. Fig. 2C shows that full-length \( \text{dAKAP1b} \) clearly has two distinct bands, one that co-migrates with \( \text{dAKAP1a} \) and a second slightly larger band, reflecting initiation from at least two positions (Fig. 2C). It was unclear from these experiments whether multiple start sites for \( \text{dAKAP1a} \) exist. To show the different initiation products more clearly, we analyzed the *in vitro* translation products with shorter constructs from the first 175 residues of \( \text{dAKAP1a} \) and 1b (Fig. 2D). *In vitro* translation of \( \text{dAKAP1b} \) generated two products from codons 1 and 34 (Fig. 2C and D), whereas a mutant at Met34 and Met49 only produced the longer
not wash away RIIα. From this information, we conclude that  

\( \text{dAKAP1} \) is inserted in the outer membrane of mitochondria.

A similar set of experiments was undertaken for the rough ER (Fig. 3C) and smooth ER (Fig. 3D) to determine the localization of  \( \text{dAKAP1} \). Similar to results obtained for mitochondria,  \( \text{dAKAP1} \) was sensitive to trypsin and digested. The cytosol-exposed C-terminal tail of calnexin was also digested, but calreticulin, a lumen-associated protein, was not. Therefore,  \( \text{dAKAP1} \) appears to be located on the cytosolic surface of both smooth and rough ER. Most type II PKA on rough ER was digested away similar to  \( \text{dAKAP1} \), suggesting that most of the type II PKA on mitochondria co-exist with  \( \text{dAKAP1} \) (Fig. 3B). We next washed the mitochondria with high concentrations of KCl (0.2 M and 0.5 M) and Na₂CO₃ (0.1 M, pH 11.5) to determine whether the association of  \( \text{dAKAP1} \) to mitochondria is through specific insertion in the membrane (Fig. 3B) (35, 36).  \( \text{dAKAP1} \) was resistant to all of these washes. Alkaline treatment breaks the outer membrane and released CytC, but it does not wash away  \( \text{dAKAP1} \). Alkaline treatment removed most of the PKA-Cat but did not wash away RIIα. From this information, we conclude that  

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\( \text{dAKAP1} \) is inserted in the outer membrane of mitochondria.
Therefore, we conclude that the association of ΔAKAP1 with rough ER or smooth ER is mediated through specific insertion of the protein into the membrane. The localization of ΔAKAP1 to the cytosolic surface of both ER and mitochondria suggest that similarities exist in targeting ΔAKAP1 to these organelles.

A Negatively Charged Residue Is Required for ER Targeting—As previously established, ΔAKAP1b and ΔAKAP1d are alternatively spliced with exon 2 from the ΔAKAP1 gene and have 33 amino acids added to the N terminus (18, 19). These splice variants are localized to the ER rather than the mitochondria. Thus the extra 33 amino acids contain an element that modifies and converts the mitochondrial targeting signal in ΔAKAP1a into an ER-targeting signal. Our previous results indicate that deletion of the first 13 amino acids does not affect ER targeting in ΔAKAP1b (18). Therefore, the modifier element for ER targeting must be within residues 15–33. To determine the key element required for the conversion from mitochondrial to ER targeting, we conducted a series of substitution mutations within this region (Fig. 4). Of one double- and seven triple-amino acid scanning mutations tested, only one mutation region failed to target to ER (amino acids 31–33; Sub31A). As shown in Fig. 4B (panels g–i), this mutant targeted to mitochondria like ΔAKAP1 (Fig. 4B, panels a–c). To further identify the key element, residues 31–33 were mutated individually to Ala. Only Asp31 was found to be required for ER targeting; mutation of D31A targeted to mitochondria rather than ER (Fig. 4B, j–l). Other mutations at position 31 were tested. Mitochondrial localization was observed when aspartic acid 31 was replaced with asparagine, and tyrosine but not glutamic acid. Only the mutation to glutamic acid maintained ER targeting (Fig. 4B, panel m–o); the other mutations abolished ER targeting and targeted to mitochondria (Fig. 4A). The single acidic residue at position 31 is thus required in ΔAKAP1b to suppress the fused mitochondrial signal and convert it into an ER-targeting signal.

The Negatively Charged Residue Asp31 Is a Separate Element From the Mitochondria-targeting Motif—It has previously been shown that the hydrophobic region and the positive charged residues are integral functional elements for mitochondria targeting (21). Because the negatively charged Asp31 is only two residues away from the first residue of ΔAKAP1a, we wanted to determine whether Asp31 integrates with the existing mitochondrial targeting signal to retarget ΔAKAP1 to the ER. To detect the integrity of the ER-targeting signal, two or four residues were inserted at the N-terminal junction between ΔAKAP1a and ΔAKAP1b (Fig. 4A). These insertions push the negative charge further away from the mitochondrial targeting sequence and introduce a half or one potential helix turn if an α-helix is formed near the junction. As summarized in Fig. 4A, neither insertion affected ER targeting, suggesting that the distance between the negative charge and the mitochondrial targeting signal is not critical and that there is no well defined structural element in this region (data not shown). Furthermore, these results indicate that no α helix is required to correctly position the negative charge, and the negatively charged residues are not integrated with the remainder of the targeting domain.

A putative PKA phosphorylation site exists at Ser25, six residues N-terminal to Asp31. To determine whether a negative charge in this position can support ER targeting, we generated acidic amino acid substitutions, S25D and S25E, in ΔAKAP1b to mimic the phosphorylated state individually. Fig. 4B shows that neither S25D nor S25E had any noticeable effect on ER targeting. However, when the negative charge was removed in a double mutation (D31A/S25D or D31A/S25E), either Asp or Glu at residue 25 was able to suppress the mitochondrial targeting of D31A and restore ER targeting (Fig. 4, C and B, panels p–r). The newly introduced negative charge compensated for the missing negative charge in D31A and rescued the ER signal. These data agree with the insertion experiments, indicating that the positioning of the negative charges is not critical for ER targeting. It is likely that the element in N1 for ER targeting is not part of an integrated structure with the mitochondria targeting
domain. The single negative charge in the N1 segment could therefore be a separate switch motif that serves as a suppressor to the mitochondrial-targeting signal.

The C-terminal Positively Charged Residues of the Targeting Domain Are Critical for Both ER and Mitochondria Targeting—

It has been reported that the balance of hydrophobic and positively charged residues in the targeting domain is important for both ER and mitochondria localization. More hydrophobic residues promote ER targeting, whereas more positive charges prefer mitochondria targeting (21, 26, 39). One role of the positive charged element is to suppress binding of the hydrophobic segments to ER and promote mitochondria targeting (22–24, 26, 35). We wanted to determine the role of the positively charged motif in ER targeting and whether Asp31 promotes ER targeting through muting this suppressive positive charged element in the C terminus of the mitochondria-targeting motif (residues Arg61-Lys62-Lys63 and Arg65).

To test this, we made double mutations at Asp31 and at the positively charged residues in ΔAKAP1b (Fig. 5). We first generated the mutations in the full-length ΔAKAP1b. In these constructs, the run of five charged residues RKKDR(61–65) was replaced with three alanines (Ala4). All these mutations target to ER, independent of whether Asp31 was present or not (Fig. 5A). D31A mutation is unable to cause mitochondrial targeting without the positively charged element. However, if we only changed residues 61–63 to Alanine (SubA), we obtained a mitochondrial-targeting signal. It appears that this positively charged element is required for mitochondrial targeting and acts as a suppressor of an ER-targeting signal, although this ER-targeting signal could be nonspecific (23, 24, 26, 39–41). This element has to be on the C-terminal side of the targeting domain. When we removed the charged residues at position 61–65 and placed two positively charged residues (RK) at the N terminus of ΔAKAP1a, or in front of the 15AA minimal targeting sequence to generate Ins34 and Ins49, respectively, mitochondria targeting were not restored (Fig. 5A).

The positively charged residues also have some different effects on ER and mitochondria signals. Deletion of basic amino acids 61–63 (RKR) retained ER targeting; however, longer deletions (amino acids 58–63 or 34–63) completely abolished ER targeting (Fig. 5B). Reducing the net positive charges by substituting residues 61–63 with three Glu (Sub(E)) does not affect targeting in ΔAKAP1a, whereas this same acidic mutation in ΔAKAP1b abolished all targeting (Ref. 19 and Fig. 5B).

To study how Asp31 generates an ER-targeting signal with the positively charged element in the targeting domain, we put the double mutations into constructs that contained the N-terminal targeting domain fused to GFP. Asp31 was replaced with
Ala and RKK (61–63) was replaced with three alanines (Ala₃). We also removed all charged residues from the linker region of the vector, pEGFP, to avoid possible interference. An anti-N1 antibody was also used along with GFP to detect the translation products starting from Met1. As expected, the wild type targeting domain fusion protein targets to ER and the mutant 1–63 (D31A) targets to mitochondria (Fig. 5C). Ala₃, however, destroyed all targeting capability, regardless of whether Asp₃¹ was present. Thus the positive charges appear to be necessary for both ER targeting and for mitochondrial targeting as shown previously (19). The presence of Asp₃¹ muted the mitochondria-targeting signal and formed a new ER-targeting signal. Both positive and negative charged elements are required for ER targeting. nAKAP1 will target to mitochondria if a negative charged element is missing from the N1 segment.

DISCUSSION

nAKAP1 has several isoforms that can be generated by alternative splicing. All have a PKA-binding element in the center, with variations at the N and C termini. At the N terminus, two isoforms (nAKAP1b and 1d) have exon 2 and generate a 33-residue modifier segment that is essential for ER targeting. DAKAP1a and 1c lack exon 2 and target to mitochondria. At the C terminus, DAKAP1 has three forms. The longer form includes two RNA-binding domains, a K homology and a Tudor domain, whereas the shorter form does not have these RNA-binding elements. A putative caspase site exists at the junction between the shorter and longer forms (Asp₅₆⁹ in DAKAP1d) and may lead to the conversion of an RNA-binding form into a non-binding form post-transcriptionally. In the previous study, we have found that a helix (residues 49–57) is essential for targeting to both mitochondria and ER (19). For mitochondria targeting an amphipathic helix is sufficient,
AKAP, Mitochondria, ER, cAMP-dependent Protein Kinase

![Diagram of targeting domains]

FIGURE 6. Schematic diagram of the targeting domain of dAKAP1. Asp31 suppresses the mitochondrial targeting signal and converts it into an ER-targeting signal in dAKAP1b. The sequences required for mitochondrial targeting are the C-terminal positively charged residues and a few hydrophobic residues on the opposite side of an α-helix. It requires more hydrophobic residues for ER localization. In addition to the C-terminal positively charged residues, the targeting signal requires a run of hydrophobic residues plus a negatively charged residue to the N-terminal side. The essential elements are highlighted with lines and boxes, and the essential residues identified are marked with dots and small boxes.

whereas a fully hydrophobic helix is required for ER targeting. In dAKAP1a and 1c, the targeting domain is composed of this hydrophobic core with a positively charged patch at the immediate C terminus. The addition of 33 additional residues in 1b and 1d suppresses the mitochondrial targeting signal and converts it into an ER-targeting signal. In this study, we found that dAKAP1 is located on the cytosolic surface of the mitochondria outer membrane and ER membrane. We also took a mutagenic approach to study the N-terminal bi-functional targeting motif of dAKAP1 for its role in mitochondria and ER targeting (summarized in Fig. 6). We demonstrate here that a single amino acid, Asp31, is critical for this mitochondria-ER switch. With this unique targeting signal, the suppression by Asp31 provides an organelle-specific mechanism for a subcellular switch and therefore provides an easy way to regulate the switch of the localization of PKA.

This negative charged switching element is in a separate motif from the mitochondria-targeting signal. It can be moved further apart and be substituted by a different negatively charged residue at another location. The existence of a negatively charged residue helped convert a mitochondria signal into an ER signal. It seems that the ER signal uses a novel mechanism to preferentially associate with ER in cells that does not allow the molecule to access mitochondria. Whether there are any interacting proteins that may help ER targeting for dAKAP1 needs to be confirmed.

Several molecules that have overlapping targeting domains are known to target to both ER and mitochondria including Bclx, Cytb5, TOM 20, TOM 70, VAMP1, and cytochrome P-450, etc. (22, 25, 26, 35, 41, 42). Binding to signal-recognition particle (SRP) on ER is mediated by the hydrophobic regions co-translationally (22, 35, 42). These molecules can also change their preferences from ER to mitochondria and are transported to mitochondria post-translationally but with distinct mechanisms (22, 35). Several isoforms of cytochrome P-450 have an N-terminal "chimeric signal" domain for ER and mitochondria targeting, which is similar to dAKAP1 (27, 42, 43). Switching from ER to mitochondria is achieved by proteolysis in P4501A1 to remove an N-terminal segment and generate a shorter mitochondria signal (27, 43). However, P4502A1 and P4502E1 use cAMP-dependent phosphorylation on Ser128 or Ser129, respectively, to increase the affinity to mitochondria (42, 44). The phosphorylation can activate a "cryptic" mitochondrial targeting signal embedded within the targeting domain and increased the affinity of the molecules to chaperones, and this promotes mitochondria binding (42, 44). Although Ser25 in dAKAP1b is a potential PKA phosphorylation site, the effect of the phosphorylation on Ser25 is unknown. Treatment of the cells with forskolin has no effect on the targeting of dAKAP1b (data not shown). Treatment of the in vitro translation products of dAKAP1b or mutant D31A with PKA-Cat also has no effect in an in vitro mitochondria binding assay (data not shown). How dAKAP1 attaches to mitochondria and ER and whether phosphorylation by PKA has any effect on the affinity to the organelles needs to be determined.

It has been shown that the positively charged element can suppress nonspecific signal-recognition particle binding mediated by hydrophobic motifs and promote mitochondria targeting (22, 35). Such a nonspecific ER targeting element seems to exist C-terminal to the positively charged motif in certain dAKAP1 mutants (such as Ala4) when the suppressive positive motif is removed. However, this downstream ER element may not contribute to ER targeting in wild type dAKAP1, because the targeting signal is fully contained within the N-terminal 63 residues (Fig. 5). There are no residue requirements that are C-terminal to residue 63 for ER targeting. In addition, the positively charged motif is required for ER targeting and is probably a part of the ER signal. Kaufmann et al. (24) proposed that the positive element could also prevent the molecule from leaking out during transport to mitochondria and in this way enhance mitochondria targeting. We postulate that Asp31 does not negate this effect to reduce mitochondria targeting and enhance ER targeting, because we do not see much change of mitochondria targeting in vitro. Our data suggest that Asp31 altered the mitochondria-targeting signal and transformed it into an ER signal.

In summary, the production of two isoforms at the N terminus, by alternative splicing, provides a mechanism to generate two PKA-anchoring proteins that target to different organelles. Generation of several dAKAP1 molecules with different initiation sites for translation could be an alternative way to produce multiple AKAPs that target to ER, mitochondria, and microtu-
bules. The mechanism of switching from mitochondria to ER targeting by adding a single negatively charged residue is an economic way to modulate different targeting locations, and this can be further augmented by phosphorylation. This switch mechanism alters the targeting domain but does not need to encode an entirely new protein.

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