Cross-species Sequence Analysis Reveals Multiple Charged Residue-rich Domains That Regulate Nuclear/Cytoplasmic Partitioning and Membrane Localization of A Kinase Anchoring Protein 12 (SSeCKS/Gravin)*

Received for publication, December 14, 2004, and in revised form, May 10, 2005
Published, JBC Papers in Press, May 27, 2005, DOI 10.1074/jbc.M414017200

Jeffrey W. Streh‡ and Joseph M. Miano§
From the Center for Cardiovascular Research in the Aab Institute of Biomedical Sciences, University of Rochester School of Medicine, Rochester, New York 14642

A kinase anchoring proteins (AKAPs) assemble and compartmentalize multiprotein signaling complexes at discrete subcellular locales and thus confer specificity to transduction cascades using ubiquitous signaling enzymes, such as protein kinase A. Intrinsic targeting domains in each AKAP determine the subcellular localization of these complexes and, along with protein-protein interaction domains, form the core of AKAP function. As a foundational step toward elucidating the relationship between location and function, we have used cross-species sequence analysis and deletion mapping to facilitate the identification of the targeting determinants of AKAP12 (also known as SSeCKS or Gravin). Three charged residue-rich regions were identified that regulate two aspects of AKAP12 localization, nuclear/cytoplasmic partitioning and perinuclear/cell periphery targeting. Using deletion mapping and green fluorescent protein chimeras, we uncovered a heretofore unrecognized nuclear localization potential. Five nuclear localization signals, including a novel class of this type of signal termed X2-NLS, are found in the central region of AKAP12 and are important for nuclear targeting. However, this nuclear localization is suppressed by the negatively charged C terminus that mediates nuclear exclusion. In this condition, the distribution of AKAP12 is regulated by an N-terminal targeting domain that simultaneously directs perinuclear and peripheral AKAP12 localization. Three basic residue-rich regions in the N-terminal targeting region have similarity to the MARCKS proteins and were found to control AKAP12 localization to ganglioside-rich regions at the cell periphery. Our data suggest that AKAP12 localization is regulated by a hierarchy of targeting domains and that the localization of AKAP12-assembled signaling complexes may be dynamically regulated.

Multicellular organisms require extensive signaling networks to coordinate physiological responses to a diverse array of stimuli. As organismal complexity increases, so theoretically does the requirement for an increased complement of signal transduction machinery. Whereas expansion of some kinase families appears to parallel increasing organismal complexity (1), the protein kinase A family has undergone little expansion (2) despite the large number of its physiological substrates distributed throughout the cell. Thus, intrinsic differences between protein kinase A family members cannot fully account for such diversity because, on their own, they do not provide sufficient specificity. Instead, specificity appears to be conferred by an ever expanding pseudo-family of anchoring proteins, the A kinase anchoring proteins (AKAPs),1 that compartmentalize protein kinase A-containing signaling complexes to discrete subcellular locales (3).

The intrinsic targeting and protein-protein interaction domains of each AKAP direct correct spatial and temporal assembly of protein kinase A-containing signaling complexes. Such compartmentalization simultaneously shields neighboring complexes from activated signal cascades and confers specificity to signaling complexes utilizing ubiquitous components (4). Accordingly, these targeting and interaction domains comprise the primary determinants of the role of each AKAP. For example, the ability of AKAP5 (also known as AKAP75, AKAP79, or AKAP150) to amplify cAMP signals to the nucleus is disrupted either by deletion of its membrane-targeting sequence or by mutation of the protein kinase A binding site (5). Moreover, localization of AKAPs can be dynamically regulated through the phosphorylation of critical residues within their targeting motifs, such as the membrane-targeting sequence of AKAP5 (6), to allow for mobilization of AKAP-anchored signaling complexes. Characterization of the domains that direct the localization of each AKAP is thus an essential foundational step toward elucidating AKAP physiological functions.

AKAP12, also known as SSeCKS and Gravin, is an anchoring protein that coordinates the assembly of a multiprotein complex that may include protein kinase A, protein kinase C, protein phosphatase 2B, the β-adrenergic receptor, and calmodulin (7–12). We recently demonstrated that the AKAP12 gene encodes three transcriptionally separate AKAP12 isoforms, α, β, and γ, that have distinct spatio-temporal expression patterns (13). Although all three isoforms share >95% of their coding sequence, an N-terminal myristoylation motif directs AKAP12α to the endoplasmic reticulum. The remaining

---

1 The abbreviations used are: AKAP, A kinase anchoring protein; AR, acidic residue-rich; BR, basic residue-rich; GFP, enhanced green fluorescent protein; GR, ganglioside-rich; ICASM, human coronary artery smooth muscle cell; MARCKS, myristoylated alanine-rich C kinase substrate; NLS, nuclear localization signal.

This paper is available on line at http://www.jbc.org
**Targeting of AKAP12**

isoforms are localized in the cytosol and at discrete locations at the cell periphery, indicating that their targeting information is contained in domains common to all three isoforms. Characterization of the domains controlling AKAP12 targeting will provide a foundation to address how localization of this protein controls its ability to dually regulate nuclear/perinuclear compartmentalization of cyclin D1 (14) and β-adrenergic receptor functions at the cell membrane (10).

In the present study we have characterized the localization of the non-myristoylated AKAP12 isoforms. AKAP12 is a highly charged protein composed of an N terminus of alternating acidic and basic residue-rich regions and a large acidic C-terminal tail. Here we used cross-species sequence analysis and deletion mapping to facilitate the identification of targeting motifs. Seven conserved basic regions in the N terminus were found to be important in determining the localization of AKAP12. The first three basic regions display similarity to the membrane-targeting domain of the MARCKS protein and are important determinants of AKAP12 targeting to ganglioside-rich regions at the cell periphery. Interestingly, the remaining basic regions each contain an SV40-like nuclear localization signal (NLS). Constructs spanning this region are localized to the nucleus. A fifth NLS, revealed through deletion mapping and mutagenesis, represents a novel class of NLS. Nuclear localization, however, is suppressed by the acidic C terminus. The ability of the C terminus to suppress nuclear localization of green fluorescent protein (GFP) chimeras could not be localized to any specific region of the C terminus but instead appears to be related to net charge. Together, our data indicate that the charged residues in the AKAP12 protein are the main targeting determinants and reveal new insight into the interplay between charged residue-rich targeting domains in regulating subcellular localization. Additionally, our data suggest that localization of AKAP12-anchored signaling complexes may be dynamically regulated by factors or modifications (e.g., phosphorylation) that alter the local charge of the AKAP12-targeting domains.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**COS-7 cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transfected with the indicated plasmids using FuGENE 6 (Roche Applied Science) per the manufacturer’s directions. Primary human coronary artery smooth muscle cells (hCASMCs) were obtained from Cascade Biologics and cultured per the manufacturers’ directions. For differentiation, cells were fixed with 4% freshly prepared paraformaldehyde followed by permeabilization with 0.1% Triton X-100. AKAP12 was detected using rabbit anti-Ser/Thr kinase (kindly provided by Dr. Irwin Gelman, Roswell Park Cancer Institute) and goat anti-rabbit fluorescein (Pierce). Smooth muscle actin, a marker of differentiated smooth muscle cells, was detected with mouse anti-smooth muscle actin (Sigma) and goat anti-mouse rhodamine (Pierce). Nuclei were stained by labeling with 4,6-diamidino-2-phenylindole (Molecular Probes). Cells were visualized as described previously (13). For differentiations, cells on glass chamber slides (Nalgene) and culturing as indicated. Cells were fixed with 4% freshly prepared paraformaldehyde followed by permeabilization with 0.1% Triton X-100. AKAP12 was detected using rabbit anti-Ser/Thr kinase (kindly provided by Dr. Irwin Gelman, Roswell Park Cancer Institute) and goat anti-rabbit fluorescein (Pierce). Smooth muscle actin, a marker of differentiated smooth muscle cells, was detected with mouse anti-smooth muscle actin (Sigma) and goat anti-mouse rhodamine (Pierce). Nuclei were stained by labeling with 4,6-diamidino-2-phenylindole (Molecular Probes). Cells were visualized as described previously (13).

**Computer Analysis—**The charge plot profile for AKAP12 was generated using the charge program of EMBOSS via web interface (bioweb.pasteur.fr/seqanal/interfaces/charge.html) (16). The rat AKAP12 β sequence (AY695057) was used. Because of window size constraints, the charge over the first and last 10 amino acids is not included in analysis. The evolutionary conservation profile plot was created using eSHADOW (17). Briefly, sequences for rat, mouse (fusion of open reading frames of BY002721 and NM_031185 to create mouse AKAP12β), and human (NM_144497) AKAP12 were submitted via Internet interface (eshadow.dcdoe.org/). The returned ClustalW alignment was then checked and manually edited as necessary to ensure proper alignment prior to final submission for visualization of conserved regions.

**RESULTS**

**Charge and Conservation of AKAP12—**We recently characterized the AKAP12 gene locus and identified three separate AKAP12 transcripts, α, β, and γ (13). These transcripts encode distinct but largely similar proteins. Although an N-terminal myristoylation motif targets the AKAP12α protein to the endoplasmic reticulum, mutants lacking this motif localize in a similar manner as the β and γ isoforms. This finding indicates that the common AKAP12 coding sequence contains targeting information that directs localization of the AKAP12 isoforms in the absence of myristoylation. Because the subcellular distributions of endogenous AKAP12β and AKAP12γ as well as those of the AKAP12α myristoylation mutants are highly similar, we have used AKAP12β (hereafter referred to as AKAP12) here to identify the targeting domains regulating non-myristoylated AKAP12 localization.

AKAP12 is a large, highly charged protein that is enriched in acidic and basic amino acid residues (nearly 1 of every 3) and has a predicted net charge of -196 at physiological pH. These charged residues are not equally distributed across the protein but rather are organized into alternating acidic and basic residue-rich (AR and BR, respectively) regions (Fig. 1A). The N-terminal half of AKAP12 is composed of seven alternating AR and BR regions. The C-terminal half, in contrast, is more uniformly negatively charged because of the wide distribution of acidic residues across this region. Comparison of the distribution of these regions with the profile of evolutionary conserved regions reveals elevated conservation of the BR regions, as compared with neutral and AR regions (Fig. 1B). Given that targeting motifs regulate a key property of AKAP function and are therefore likely to be well conserved across species, we hypothesized that these basic regions may be important determinants of AKAP12 localization.

**Three Regions Regulate AKAP12 Localization—**To determine whether the targeting regions of AKAP12 are localized to the BR regions, we performed a first pass deletion study to identify general regions important in AKAP12 targeting (Fig. 1C). Whereas deletion of the C-terminal most 404 amino acids does not affect localization, further truncation of the C terminus (1791–1607) results in redistribution of AKAP12-GFP chimeras to the nucleus (Fig. 1D). Nuclear targeting is abolished and normal targeting is restored when we further deleted the region from amino acids 486 to 790 (data not shown). Finally, specific targeting is eliminated by deleting all but the first 78 amino acids.

Our deletion analysis suggests the AKAP12 targeting domains are subdivided into three regions. Interestingly, these
three regions span the first two BR regions, the fourth through seventh BR region, and the AR region C terminus, respectively. The first region spans amino acids 79–286 and directs the cytosolic distribution of AKAP12 as well as targeting at the cell periphery. The second region, spanning amino acids 486–790, directs localization to the nucleus. A third region at the C terminus appears to be involved in suppressing nuclear localization, because a construct containing only the first 78 and last 404 (1–78, 1203–1607) amino acids is excluded from the nucleus (Fig. 1D). However, nuclear localization of AKAP12 appears to be suppressed by an additional C-terminal sequence, as constructs containing the region spanning amino acids 791–1202 were similarly excluded from the nucleus. Thus, based on our deletion analysis, the role of the three AKAP12 targeting regions appears to be subdivided into two functions: 1) nuclear/cytoplasmic partitioning; and 2) cytosolic and peripheral targeting.

**Nuclear Localization Potential of AKAP12**—The nuclear localization potential of AKAP12 is intriguing because a recent study has demonstrated the presence of AKAP12 in nuclear fractions, a finding that likely relates to its ability to control cell cycle progression through regulation of cyclin D1 compartmentalization (14). In addition, we have observed differential compartmentalization of AKAP12 in some primary cell lines. In growing hCASMCs, for example, AKAP12 is predominantly localized in the nucleus with some expression in the cytosol (Fig. 2). Upon differentiation, however, AKAP12 expression diminishes greatly in the nucleus and appears to be redirected to the perinuclear region of the cytosol (Fig. 2). The reduction of AKAP12 nuclear expression is not due to reduced protein expression as detected by Western blotting (data not shown). AKAP12 also appears at times to be localized to the nucleus in vivo. When we examined tissues with high levels of AKAP12β expression, we noted nuclear AKAP12 in a subpopulation of mesenchymal cells of the bladder and lung (data not shown).

Because our deletion study suggested the determinants for nuclear targeting are contained in the region spanning amino acids 486–790, we assessed whether this region was sufficient for nuclear targeting. A fusion construct comprising GFP and a sub-fragment of this region (amino acids 501–767) localizes predominantly to the nucleus, confirming that the nuclear tar-
Targeting of AKAP12

The central targeting region contains four NLSs. A, location and sequence of the four AKAP12 SV40-type NLSs. B, schematic of central targeting-GFP chimera, AKAP12 (501–767)-GFP. The thick black lines and numbers below the schematic indicate the positions of the four NLSs. C, nuclear localization of AKAP12-(501–767)-GFP. Size bars, 10 μm. D, schematics of central targeting region deletion constructs. Multiple deletions were performed to identify regions important for nuclear targeting. The positions of the four SV40-type NLSs are indicated by the thick black lines. All deletion constructs were fused to the N terminus of the GFP. The ability of each GFP chimera to localize to the nucleus is indicated at the right. A.A., amino acid. The nuclear targeting region spans the fourth to seventh BR regions. Using PSORT (psort.org), we scanned this region and the rest of AKAP12 to identify potential NLSs (18). Several bipartite and SV40-type NLSs were found. The bipartite NLSs were found outside of the nuclear targeting region and do not appear to contribute to nuclear localization (data not shown).

However, four SV40-type NLSs were identified in the nuclear targeting region, with one identified in each BR region (Fig. 3A). Because the region encompassing only these four NLSs is sufficient for nuclear localization (Fig. 3, B and C), we constructed further deletions to determine whether the NLSs are necessary for nuclear targeting. Deletion of a single NLS does not affect targeting, nor is the targeting of constructs containing only the two N-terminal NLSs or the two central NLSs altered (Fig. 3D). In contrast, a construct containing only the two C-terminal most NLSs is not specifically targeted to the nucleus, suggesting that the NLSs are not equal in their ability to target to the nucleus. This notion is supported by further deletions, as only the region spanning the second NLS is sufficient on its own for nuclear targeting. Taken with the other deletions, this last finding indicates that nuclear localization is only observed when the second NLS is present. Although NLS2 appears to be the dominant NLS, the result was somewhat surprising because NLS2 differs little in sequence with the other three NLSs (Fig. 3A).

Identification of a Novel Class of NLS—Using mutagenesis, we assessed whether the second SV40 NLS is required for nuclear targeting (Fig. 4A). Mutagenesis of NLS2 in a construct spanning amino acids 559–612 does not affect nuclear localization (Fig. 4B). As this region lacks the other three NLSs, this finding indicates that the targeting of this region is not conferred by NLS2 but instead by another motif contained in this region. To identify the nuclear targeting sequence within amino acids 559–612 we constructed a series of GFP chimeras tiled across this region. Consistent with our mutagenesis data, a sub-region containing NLS2 was not sufficient to target to the nucleus; however, two C-terminal sub-regions displayed specific nuclear localization (Fig. 4C). This region contains several basic residues but does not represent either an SV40-type or a bipartite NLS, the two main NLS classes. Interestingly, this region is similar to the nuclear localization signal of the transcription factor, serum response factor (SRF) (19, 20) (Fig. 4D), and thus represents a novel third class of NLS. This class tentatively has a consensus of GXX/K/R/R/K/R/XX/K/R/K/R/XX-SXX(D/E), although identification of further NLSs of this class will be necessary to define a precise consensus. Based on the spacing of the consensus amino acids, we propose that this new class of NLS be termed X2-NLS to reflect the di-amino acid spacing between the pairs of basic residues.

Both pairs of basic residues are necessary for the X2-NLS to target to the nucleus. Substitution of either pair with alanines abolishes specific nuclear localization (Fig. 4B). In contrast, substitution of the serines with alanines does not affect localization, indicating that the pairs of basic residues are the primary targeting determinants of this class of NLS (data not shown). It should be noted that the other two classes of NLS, the SV40-type and the bipartite, are also composed of two pairs of basic residues, though some substitution is allowed (e.g. proline at position 1 of the SV40-type NLS). In these cases, the spacing between pairs is 9 and 10 amino acids, respectively. In addition to differing in spacing, at least two of the NLS types are substantially different in their ability to target to the nucleus. Whereas no specific nuclear localization is observed with any of the single SV40-type NLS chimaeras, the single X2-NLS is sufficient for targeting to the nucleus (Figs. 3D and 4C). Together, these data indicate that the nuclear targeting region of AKAP12 (amino acids 501–767) contains five NLSs split between two NLS classes that differ in their ability to confer nuclear targeting.
The Five NLSs Contribute to Nucleic Localization Potential—To clarify which class of NLS confers targeting to the AKAP12 nuclear localization region, we mutated either the four SV40-type NLSs or the X2-NLS and then examined localization. Mutation of all four SV40-type NLSs abolishes specific nuclear localization, indicating that these NLSs are important for localization and that the X2-NLS is not sufficient on its own for nuclear targeting in the context of the AKAP12 nuclear targeting region (Fig. 5). Mutation of the X2-NLS alone reduces the extent of nuclear localization, indicating it also contributes to nuclear targeting. However, in support of the X2-NLS being stronger than the SV40-type NLS, mutation of any single SV40-type NLS had no effect on localization (data not shown).

Taken together, these data demonstrate that all five NLSs contribute to the overall nuclear localizing potential of the region between amino acids 486 and 790 of AKAP12. In support of the combinatorial nature of the NLS motifs of AKAP12, inspection of the conservation of these sites revealed that the X2-NLS and at least three of the four SV40-type NLSs are highly conserved in all species examined (human, mouse, rat, dog, chicken, Xenopus, zebrafish, and Tetraodon; data not shown).

The C Terminus of AKAP12 Prevents Nuclear Localization—The presence of five functional NLSs suggests that AKAP12 should be localized to the nucleus. However, nuclear localization of AKAP12 is not always observed, indicating that AKAP12 is being excluded from the nucleus. Our initial deletion mapping experiments indicate that this exclusion is mediated by the AKAP12 C-terminal region spanning amino acids 787–1607 (Fig. 1). Moreover, fusion of this region to GFP is sufficient to exclude GFP from the nucleus (Fig. 6). To determine whether nuclear export mediates AKAP12 exclusion, we scanned this region for nuclear export sequences and examined the effect of the nuclear export inhibitor, leptomycin B, on targeting. Presently known nuclear export mechanisms are unlikely to be the mechanisms by which the C terminus of AKAP12 mediates nuclear exclusion, as this region lacks any clear nuclear export signals, and its targeting is not affected by leptomycin B (data not shown).

To explore other potential targeting mechanisms, we searched the C terminus for conserved regions with similarity to other targeting or protein interaction domains. The only candidate domain we identified was a potential 14-3-3 binding site (data not shown). Because 14-3-3 proteins have been shown to regulate the nuclear localization of other proteins (21), we used deletion mapping to assess if this site was important for targeting. Deletion of the region from amino acid 787 to amino acid 883, which contains the putative 14-3-3 site, does not affect targeting, therefore excluding this site as a mediator (Fig. 6).

Because we were unable to identify any known nuclear exclusion motifs by similarity or pattern searches, we instead used deletion mapping to identify sub-regions in the C terminus that mediate nuclear exclusion. In support of our initial deletion study (Fig. 1), division of the C terminus into two approximate halves does not affect nuclear exclusion, suggesting that at least two domains are capable of this function (Fig. 6). Further division of these sub-regions, however, failed to identify a specific nuclear localization domain, as none of these deletion chimeras is capable of preventing nuclear localization. Thus, the nuclear exclusion property of this region may be conferred by a pair of large complex domains or by the physical composition of the C terminus. For instance, the ability of the C-terminal sub-regions to prevent nuclear localization appears to be correlated with their net charge (Fig. 6), suggesting that the predominance of acidic residues across the C terminus is related to the targeting role of this region.

The C Terminus of AKAP12 Antagonizes NLSs—As the GFP reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the GFP-reporter we used to assess nuclear exclusion is not specifically targeted and lacks a NLS, we sought to determine whether the C terminus can specifically suppress nuclear targeting conferred by a NLS. To generate a specifically targeted GFP reporter, we tandemly fused three copies of the SV40 NLS to the
Targeting of AKAP12

For instance, these chimeras display both the typical perinuclear enrichment and targeting to discrete regions at the cell periphery.

The N-terminal targeting region contains two BR regions that are similar to those found in the membrane-targeting domains of the MARCKS family of proteins (Fig. 8A) (22). A third basic region immediately antecedent of this region also displayed similarity and was included in further analysis. These three N-terminal BR regions alternate with AR regions as described above (Fig. 8B). Using deletion mapping, we assessed the role of the N-terminal BR and AR regions in determining AKAP12 cytosolic and peripheral localization. Interestingly, normal cytosolic targeting (e.g. perinuclear enrichment) is not seen with any of the deletion constructs. Instead, the deletion constructs either localize to the cell periphery or are nonspecifically distributed throughout the cell (data not shown). This finding indicates that correct cytosolic distribution of AKAP12 is determined by the N terminus as a whole.

In contrast to cytosolic targeting, peripheral targeting could be specifically assigned to the BR regions, as each BR region can direct peripheral targeting on its own (Fig. 8C). However, the inclusion of flanking AR regions results in nonspecific distribution throughout the cell, indicating that these regions antagonize the targeting ability of the BR regions. To achieve peripheral localization in the context of these acidic regions, multiple BR regions are required. For instance, GFP chimeras containing the first BR region and first two AR regions are nonspecifically targeted. Extension of this region by the addition of the next 45 amino acids containing the second BR region, however, redirects targeting to the cell periphery.

In the context of the full-length AKAP12 protein, the additive effect of the three BR regions on peripheral targeting is apparent. Internal deletion of these BR regions alone, in pairs, or in total reduces the ability of the full-length AKAP12 to target to the cell periphery in a correlative manner (Fig. 8A). Specifically, deletion of a single BR region only modestly affects peripheral targeting. Compound deletion of any pair of BR regions has a more pronounced effect. The most pronounced reduction in peripheral targeting is observed when all three BR regions are deleted, as would be expected. These data confirm that the BR regions regulate peripheral targeting and demonstrate that maximal targeting requires the contribution of all three BR regions.

Local Charge Controls the Targeting Ability of the N-terminal BR Regions—Each of the N-terminal BR regions contains two sets of sequences similar to that identified as being important for the MARCKS electrostatic switch mechanism of membrane interaction. This motif, which we identify here as a SFKK motif, is also present in a number of other membrane-associated proteins such as Src (data not shown). In this motif, the basic residues interact with the negatively charged phospholipids, the aromatic residue integrates into the membrane to stabilize the interaction, and phosphorylation of the serine residues destabilizes association with the membrane by introducing a negative charge within the local region (23). To address whether charge plays a role in determining the localization of AKAP12 to the cell periphery, we replaced the serines and threonines flanking the MARCKS-like domains with aspartic acid, a substitution that introduces a negative charge (Fig. 8B). This substitution may also mimic phosphorylation of AKAP12 at these residues. Whereas replacing the serines and threonines with a non-charged amino acid, alanine, did not affect targeting (data not shown), substitution with aspartic acid greatly attenuated the localization of AKAP12 to peripheral regions of the cell (Fig. 8C). These data indicate that the charged residues contained in the N terminus, specifically the three BR regions, are the determinants of AKAP12 localization to the cell periphery.

AKAP12 Localizes to Ganglioside-rich Regions at the Cell Periphery—As mentioned above, the first three AKAP12 BR regions have similarity to the membrane-targeting domain of MARCKS. Unlike MARCKS, however, AKAP12 does not appear to be broadly distributed across the plasma membrane but appears to be localized to discrete regions reminiscent of membrane microdomains. Interestingly, the distribution of AKAP12 appeared to be similar to the staining of GR regions that are often used to identify lipid rafts. Indeed, staining of GR regions with the B subunit of the cholera toxin overlapped the AKAP12 peripheral localization (Fig. 9D). This finding indicates that the three AKAP12 N-terminal BR regions target AKAP12 to GR regions at the cell periphery. To our knowledge, this is the first demonstration of such localization for an AKAP.
Targeting of AKAP12

Through our study of AKAP12 targeting we have detailed several targeting domains that regulate AKAP12 subcellular distribution. These targeting domains are segregated into three regions that roughly correspond to the N terminus, the central region, and the C terminus. These regions harbor the domains that regulate peripheral targeting, nuclear localization, and nuclear exclusion, respectively. For instance, we demonstrated that conserved BR regions in the N-terminal and central targeting regions are critical to the targeting function of these regions because they harbor membrane-targeting and nuclear localization signals, respectively. The latter targeting function is suppressed by the C terminus, which functions as a nuclear exclusion domain. On the whole, our study provides foundational insight into the control of AKAP12 targeting and suggests dynamic regulation of this signaling scaffold's localization may be achieved through modulation of it targeting potential.

AKAP12 is a multivalent scaffold that directs assembly of multiprotein signaling complexes at discrete subcellular locales (24). Although some studies have demonstrated a predominant membrane association for AKAP12 (25), most studies have shown that AKAP12 is localized to the perinuclear region of the cytoplasm in addition to the cell surface (11). One possible explanation for the dual localization of AKAP12 is the presence of multiple AKAP12 isoforms that differ in their subcellular distribution. Indeed, we recently demonstrated that the AKAP12 locus encodes for three distinct isoforms that differ only at their N terminus (13). However, although a myristoylation motif directs AKAP12 to the endoplasmic reticulum, our present study indicates that, by virtue of their high degree of similarity, all AKAP12 isoforms, including AKAP12α, retain the targeting information to localize to these subcellular sites.

Control of AKAP12 targeting to these two distinct locales appears to be regulated by the same domain, which is found at the AKAP12 N terminus. However, localization to these compartments is dependent on different properties of this targeting domain. For instance, whereas sub-fragments of the N terminus retained their ability to target to the cell periphery, the entire N-terminal targeting domain was required for correct cytosolic distribution. One potential explanation for this discrepancy is that correct cytosolic targeting is controlled by interplay between the N-terminal AR and BR regions that is disrupted by any amount of truncation. In contrast, the BR regions alone are sufficient for targeting to GR regions at the membrane.

The three BR regions of the N-terminal targeting domain are similar to the membrane-targeting sequence found in MARCKS. Like the MARCKS-membrane interaction, we found that the charge across these regions underlies their targeting ability. For instance, introduction of acidic residues into the N-terminal BR regions disrupts peripheral targeting. Furthermore, AR regions that flank the N-terminal BR regions antagonize the latter's function. Interestingly, several serines within these BR regions have been shown to be phosphorylated (26). Taken with previous reports that show translocation of AKAP12 from the membrane to the perinuclear region upon phosphorylation (11), our data suggest that AKAP12 targeting to GR regions is dynamically regulated by introduction of negative charges into the BR regions through phosphorylation. Specifically, because our mutagenesis overlaps known AKAP12 phosphorylation sites, we propose that phosphorylation of these residues modulates the ability of AKAP12 to target to the membrane. Future studies are warranted to fully characterize the nature of AKAP12 localization to GR regions and agonist-stimulated AKAP12 redistribution.

AKAP12 is most often observed to localize to the cell periphery and the perinuclear region, although recently expression has been documented in nuclear protein fractions (14). Our data support a heretofore unrealized nuclear localization potential for AKAP12 and suggest that under certain conditions AKAP12 can be targeted to the nucleus. Although its function in the nucleus is not clear at this time, AKAP12 may continue to act as a multivalent scaffold inside the nucleus. Nuclear localization of AKAP12 may also be related to its control of cell-cycle progression. For example, AKAP12 has been shown to interact with and redistribute cyclin D1 to the cytosol (14). Although AKAP12 could be detected in nuclear extracts, how and where AKAP12 initially associates with the nuclear localized cyclin D1 is unclear. Taken with our findings, it is tempt-
ing to speculate that at some time during the cell cycle AKAP12 localization is altered and redistributed to the nucleus. In support of this notion, in actively growing primary derived hCASMCs a substantial pool of AKAP12 is localized in the nucleus. Upon differentiation of these cells, however, AKAP12 appears to be redirected from the nucleus to the cytosol. Investigation of the mechanism regulating AKAP12 nuclear/cytosolic partitioning will not only reveal insight into how the relationship between the central NLSs and the C-terminal exclusion domain regulates AKAP12 localization, it will help elucidate the function of nuclear localized AKAP12.

As a whole, our data suggest that a hierarchy of targeting domains determines AKAP12 localization. In this hierarchy, the C-terminal nuclear exclusion domain is the most dominant. However, because this domain lacks the ability to direct specific targeting, subordinate domains must regulate the specific distribution of AKAP12. The myristoylation domain follows in this hierarchy, superseding the central nuclear targeting domain. Interestingly, at the bottom of the AKAP12 targeting hierarchy is the N-terminal targeting domain, which is responsible for the majority of AKAP12 distribution.

The presence of such a targeting hierarchy suggests a means to dynamically regulate AKAP12 localization. For instance, protein modifications such as phosphorylation may functionally inactivate one or more dominant domains, resulting in targeting being directed by alternate domains. Indeed, this notion is supported by our demonstration that alterations in charge, such as those caused by phosphorylation, can affect membrane-targeting ability. A thorough understanding of how the AKAP12 targeting domain hierarchy is maintained and modified will be necessary to elucidate the role of this mobile signaling scaffold at its multiple subcellular locales.

Acknowledgments—We thank the University of Rochester Medical Center’s Functional Genomics Center and the State of New York-funded Academic Medicine Development Company (AMDeC) Microarray Resource Center for performing the sequencing of the constructs used in this study. We thank Mary Georger for immunohistochemical analysis of AKAP12 expression in mouse tissues. We also thank Dr. Keigi Fujiwara for generously providing the microscope used for this study.

REFERENCES

1. Gu, J., and Gu, X. (2003) Gene 317, 49–57
2. Canaves, J. M., and Taylor, S. S. (2002) J. Mol. Biol. 386, 17–29
3. Michel, J. J., and Scott, J. D. (2002) Annu. Rev. Pharmacol. 42, 235–257
4. Collode, M., and Scott, J. D. (1999) Trends Cell Biol. 9, 216–221
5. Indolfi, C., Stabile, E., Coppola, C., Gallo, A., Perrino, C., Alevato, G., Cavuto, L., Torella, D., Di Lorenzo, E., Troncone, G., Feliciello, A., Avvedimento, E. V., and Chiariello, M. (2001) Circ. Res. 88, 319–324
6. Dell’Acqua, M. L., Faux, M. C., Thorburn, J., Thorburn, A., and Scott, J. D. (1996) EMBO J. 17, 2246–2250
7. Nauert, J. B., Klauck, T. M., Langeber, L. K., and Scott, J. D. (1997) Curr. Biol. 7, 52–62
8. Chapline, C., Mousseau, B., Ramsay, K., Duddy, S., Li, Y., Kiley, S. C., and Jaken, S. (1996) J. Biol. Chem. 271, 6417–6422
9. Shih, M., Lin, F., Scott, J. D., Wang, H., and Malbon, C. C. (1999) J. Biol. Chem. 274, 1588–1595
10. Tao, J., Wang, H. Y., and Malbon, C. C. (2003) EMBO J. 22, 6419–6429
11. Lin, X., Tombler, E., Nelson, P. J., and Gelman, I. H. (1996) J. Biol. Chem. 271, 28430–28438
12. Lin, X., and Gelman, I. H. (2002) Biochem. Biophys. Res. Commun. 290, 1368–1375
13. Streb, J. W., Kitchen, C. M., Gelman, I. H., and Miano, J. M. (2004) J. Biol. Chem. 279, 13681–13686
14. Lin, X., Nelson, P. J., and Gelman, I. H. (2000) Mol. Cell. Biol. 20, 7259–7272
15. Kenworthy, A. K., Petranova, N., and Eddin, M. (2000) Mol. Biol. Cell 11, 1645–1655
16. Rice, P., Longden, I., and Bleasby, A. (2000) Trends Genet. 16, 276–277
17. Orchorencourt, I., Belfi, D., and Leots, G. G. (2004) Genome Res. 14, 1191–1198
18. Nakai, K., and Horton, P. (1999) Trends Biochem. Sci. 24, 34–36
19. Rech, J., Bari, L., Veyrune, J. L., Vie, A., and Blanchard, J. M. (1994) J. Cell Biol. 110, 3029–3036
20. Gauthier-Rouviere, C., Vandromme, M., Lautredou, N., Cai, Q. Q., Girard, F., Fernandez, A., and Lamb, N. (1995) Mol. Cell. Biol. 15, 433–444
21. grozinger, C. M., and Schreiber, S. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7835–7840
22. Swierczynski, S. L., and Blackshear, P. J. (1996) J. Biol. Chem. 271, 23424–23430
23. McLaughlin, S., and Adren, A. (1995) Trends Biochem. Sci. 20, 272–276
24. Gelman, I. H. (2002) Front. Biosci. 7, d1782–d1797
25. Grove, B. D., and Bruchey, A. K. (2001) J. Vasc. Res. 38, 163–175
26. Chapline, C., Cotton, J., Tobin, H., Hulmes, J., Crabb, J., and Jaken, S. (1998) J. Biol. Chem. 273, 19482–19489

2 J. W. Streb and J. M. Miano, unpublished data.