Mutational Analysis Identifies a Short Atypical Membrane Attachment Sequence (KYWFYR) within Caveolin-1†

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ABSTRACT: Caveolae are vesicular invaginations of the plasma membrane. Their formation is strictly dependent on the expression of the caveolin coat proteins. During transit to the plasma membrane, approximately 15 monomers of caveolin-1 assemble into a multivalent homo-oligomer. Caveolae are most likely generated through the subsequent interaction of these caveolin homo-oligomers with one another, with sphingolipids, and with cholesterol. Membrane association of caveolin-1 is critical to this process and is facilitated by an atypical N-terminal membrane attachment domain (residues 82−101), termed N-MAD. To better understand the membrane attachment function of N-MAD, we performed a detailed mutational analysis of the 20 amino acid N-MAD peptide sequence fused to the C-terminus of the soluble reporter green fluorescent protein (GFP). Removal of the distal six residues (KYWFYR) within N-MAD prevents membrane attachment in cells as assessed by hypotonic lysis, detergent solubility, carbonate extraction, and fluorescence microscopy. These six residues (KYWFYR) are sufficient to confer membrane attachment to GFP, an otherwise soluble protein. Both the central aromatic and flanking basic residues in this sequence are required for membrane attachment, as the sequence YWFY does not confer membrane affinity to GFP. Although the KYWFYR sequence within N-MAD facilitates membrane association, we show that the entire N-MAD sequence is required for targeting to lipid rafts/caveolae.

Electron microscopy of endothelial cells and most epithelial tissues reveals 50−100 nm invaginations of the cell membrane termed caveolae. Although most conspicuous when flask-shaped in morphology, caveolae can vary in shape and may even be flat within the membrane. Caveolae are enriched in cholesterol and sphingolipids, rendering them physically distinct from the adjacent phospholipid membrane. The study of caveolae has advanced significantly after identification of the major coat protein caveolin-1 (*1*).

Caveolin-1 is the first of three members of a gene family encoding encoding four different subtypes of caveolin proteins (*2*−*4*). Caveolin-1 (α and *β*) and caveolin-2 have a nearly identical tissue distribution, while caveolin-3 is expressed in muscle (*5*−*9*). Caveolin-1 and caveolin-3 are necessary for the formation of caveolae in their respective tissues of expression (*10*−*12*).

Many experimental approaches have been used to characterize the interaction of caveolins with the caveolar membrane. Collectively, these studies show that caveolin-1 assumes an unusual hairpin-loop membrane topology. Briefly, it is thought that the central portion of caveolin-1 generates a hairpin configuration within the membrane with the N- and C-terminal domains extending into the cytoplasm (*13*−*17*). Detailed molecular characterization of caveolin-1 shows that the regions that flank the central hydrophobic domain, namely, residues 82−101 and and residues 135−150, bind to membranes with high affinity (*1*, *18*−*20*) and serve as targeting signals for retention in different cellular compartments (*1*, *18*).

We recently demonstrated that the caveolin scaffolding domain (CSD, residues 82−101) is sufficient to anchor green fluorescent protein (GFP) to membranes in whole cells (*1*). This region was, thus, given an alternative designation as the N-terminal membrane attachment domain (N-MAD). Given the multifunctional role of the CSD/N-MAD, we were interested in defining a minimal region that facilitates membrane attachment in vivo. In this paper, we identify a short, atypical C-terminal sequence, KYWFYR, within the CSD/N-MAD that is important for membrane association. Although no one residue within this region is necessary to confer membrane attachment, we now show that the KYWFYR sequence alone is sufficient to anchor the soluble reporter protein GFP to membranes. The entire N-MAD sequence, however, is necessary for caveolar membrane targeting.

EXPERIMENTAL PROCEDURES

Materials. Reagents and antibodies were purchased from the following sources: nitrocellulose (0.2 *μ*m pore) (Schleicher & Schuell); Complete Mini tablets and pepstatin (protease inhibitors) and *n*-octyl-glucoside (Roche Molecular

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Biochemicals); rabbit anti-GFP IgG (full-length (FL) raised against the entire GFP molecule) (Santa Cruz Biotechnology); and HRP-conjugated goat anti-rabbit IgG (BD-Transduction Laboratories).

Construction of the GFP−N-MAD Expression Vectors. GFP−N-Cav-1 (82−101) contains the indicated caveolin-1 sequence subcloned as a *Hin*dIII/*Bam*HI fragment within the multiple cloning site of the GFP expression vector pEGFP-C1 (Clontech) (*1*). This construct was used as a template in generating GFP−N-Cav-1 (82−96) through GFP−N-Cav-1 (82−100). A unique *Nhe*I site 5′ to the coding region of EGFP in the pEGFP plasmid was included in the forward primer that was used to amplify GFP−N-Cav-1 (82−101). A series of reverse primers was used to generate the various C-terminal truncations of GFP−N-Cav-1 (82−101). An in-frame stop codon followed by a *Bam*HI site was included in each of the reverse primers. The PCR products were all subcloned as *Nhe*I/*Bam*HI fragments into pEGFP-C1. The alanine mutant constructs were prepared in a similar fashion, using reverse primers coding for an alanine residue at the appropriate position. Other constructs were prepared by standard PCR methods and were subcloned into the same sites within the pEGFP-C1 multiple cloning site.

Cell Culture and Transfection Methods. Human embryonic kidney 293T cells and COS-7 cells were propagated and transfected, as described (*1*). 293T and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. 293T cells were transfected using the calcium phosphate transfection method, and COS-7 cells were transfected using Fugene 6, as detailed by the manufacturer (Roche Molecular Biochemicals).

Protein Assays. Protein concentrations were determined using a commercially available bicinchoninic acid protein assay kit, as described by the manufacturer (Pierce).

Immunoblotting. Samples were subjected to SDS−PAGE under reducing conditions and transferred to nitrocellulose membranes. Protein bands were stained with Ponceau S (Sigma). Membranes were then washed, blocked, incubated with primary antibody, washed again, and incubated with secondary antibody conjugated to horseradish peroxidase. Bound IgG were detected using a chemiluminescent substrate (Pierce).

Hypotonic Lysis. Briefly, cells grown to confluence in a 60 mm diameter dish were scraped into cold PBS, pelleted by centrifugation, and resuspended in 750 μL of hypotonic buffer (5 mM Tris (pH 7.5), 1 mM $MgCl_2$, 1mM EGTA, 0.1 mM EDTA) containing protease inhibitors (*19*). Following a 30 min incubation on ice, the lysate was passed through a 26-gauge needle 10 times and centrifuged at 1000*g* to remove nuclear debris. The postnuclear lysate was transferred to an 11 × 34 mm polycarbonate tube and centrifuged at 56 000 rpm for 30 min in a TLA-100.2 rotor (Beckman). The supernatant was collected, and the pellet was resuspended in an equal volume of 1% SDS. An aliquot of 25 μL from each fraction was subjected to SDS−PAGE and immunoblot analysis.

Alkaline Carbonate Extraction. Cells were grown to confluence in a 60 mm diameter dish and washed twice in ice-cold PBS and once in 150 mM NaCl. After aspiration of the NaCl solution, 1 mL of 200 mM $NaCO_3$ (pH 11.3) containing protease inhibitors was used to scrape the cells off the dish (*19*). The sample was transferred to a tightly fitting 1 mL Dounce homogenizer and homogenized with five strokes. Following a 30 min incubation on ice, the sample was transferred to an 11 × 34 mm polycarbonate tube and centrifuged at 100 000 rpm in a TLA-100.2 rotor. The pellet was resuspended in an equal volume of 1% SDS. Both fractions were sonicated on ice, and 50 μL of each were subjected to SDS−PAGE and immunoblot analysis.

Triton Solubility. Cells were grown to confluence in 35 mm diameter dishes and washed twice with ice-cold PBS. Three hundred microliters of cold MBS (25 mM Mes (pH 6.5), 150 mM NaCl) containing 1% Triton X-100 plus protease inhibitors was then added (*19*). Following a 30 min incubation on ice without agitation, the soluble fraction was collected. An equal volume of 1% SDS was added to the plate to dissolve the remaining Triton X-100-insoluble material. The latter fraction was passed through a 26-gauge needle 10 times in order to lower its viscosity. Twenty microliters of the Triton X-100-soluble and insoluble fractions were each separated by SDS−PAGE and subjected to immunoblot analysis.

Fluorescence Microscopy. COS-7 cells were grown on cover slips, fixed in ice-cold methanol−acetone, viewed with an inverted Olympus microscope, and photographed with a Photonics CCD camera. All microscopy was performed at the Analytical Imaging Facility of the Albert Einstein College of Medicine.

Purification of Caveolae-Enriched Membrane Fractions. Caveolae-enriched membrane fractions were purified, essentially as we described previously (*1*). Cells grown to confluence in two 100-mm diameter plates were washed twice in ice-cold PBS, scraped into 750 μL of MBS containing 1% Triton X-100, passed five times through a tightly fitting Dounce homogenizer, and mixed with an equal volume of 80% sucrose (prepared in MBS lacking Triton X-100). The sample was then transferred to a 4.5 mL ultracentrifuge tube and overlaid with a discontinuous sucrose gradient (1.5 mL of 30% sucrose, 1.5 mL of 5% sucrose; both prepared in MBS, lacking detergent). The samples were then subjected to centrifugation at 200 000*g* (44 000 rpm, Sorval rotor TH-660) for 16 h. A light scattering band was observed at the 5%/30% sucrose interface. Twelve 375-μL fractions were collected, protein concentrations were determined, and equal concentrations of protein from each fraction were subjected to SDS−PAGE and immunoblot analysis.

RESULTS

Deletion Mutagenesis of the N-Terminal Membrane Attachment Domain (N-MAD) of Caveolin-1. We have demonstrated previously that residues 82−101 of caveolin-1 serve as a high-affinity membrane-binding region, termed N-MAD. This domain confers high-affinity membrane attachment to GFP, an otherwise soluble protein, as assessed by hypotonic lysis, alkaline carbonate extraction, detergent solubilization, and fluorescence microscopy (*1*).

As the C-terminal of N-MAD contains a cluster of four aromatic residues flanked by basic amino acids (KYWFYR, residues 96−101), we suspected that this region of N-MAD may mediate its membrane binding activity. Indeed, McLaughlin and colleagues showed that a peptide corresponding to a

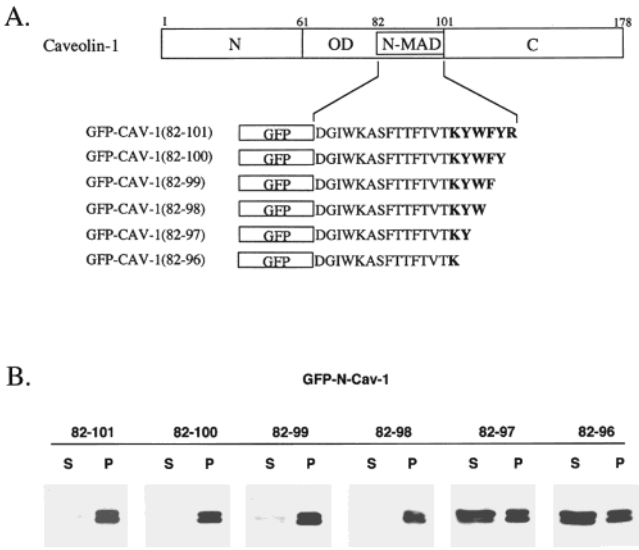

FIGURE 1: Construction and characterization of GFP−N-MAD deletion mutants. (A) Caveolin-1 can be divided into three large domains: the N-terminal domain (N, residues 1−61), the oligomerization domain (OD, residues 61−101), and C-terminal domain (C, residues 102−181). The N-terminal membrane attachment domain ((N-MAD, also known as the Caveolin scaffolding domain (CSD), residues 82−101) exists within the oligomerization domain. GFP−CAV-1 (82−101) represents a fusion of the N-MAD of caveolin-1 (residues 82−101) to GFP; the constructs which follow are successively deleted by one residue at the C-terminus of N-MAD (GFP−CAV-1 (82−100) through GFP−CAV-1 (82−96)). (B) 293T cells were transiently transfected with the indicated GFP−N-MAD deletion constructs. Thirty-six-hours post-transfection cells were subjected to hypotonic lysis, and proteins were fractionated into soluble (S) and particulate (P) portions by high-speed centrifugation. The particulate material was resuspended in a volume equivalent to the soluble fraction, and equal volumes of each were separated by SDS−PAGE. Proteins were transferred to nitrocellulose and probed with anti-GFP antibodies. Deletion of the four C-terminal residues of N-MAD is sufficient to increase the solubility of GFP dramatically (i.e., lessen its affinity for membranes).

similar stretch of aminoacyl residues from the MARCKS protein effector domain binds membranes with high-affinity in vitro (*20*). To test this hypothesis directly, we generated a panel of five N-MAD C-terminal deletion mutants fused to the soluble GFP reporter and assessed their affinity for biological membranes in whole cells (Figure 1A).

To eliminate the possible interaction of these fusion proteins with endogenous caveolins, we chose to express their cDNAs in human embryonic kidney 293T cells. Like many transformed cell lines, 293T cells do not express any known caveolins (*1*). Furthermore, 293T cells do not express other integral caveolae membrane structural proteins (i.e., flotillins), which we demonstrated can hetero-oligomerize with caveolin-1 (*21*). Importantly, we found previously that expression of caveolin-1 in these cells results in proper oligomerization, palmitoylation, and localization of the caveolin-1 protein (*19*). In summary, 293T cells are well-suited for an unbiased analysis of the membrane affinity of GFP when fused to distinct caveolin-1 domains because these cells contain the necessary sorting machinery for assembling and directing caveolin-1 oligomers to caveolae, yet they do not express caveolae structural proteins endogenously.

To assess the membrane association of the N-MAD mutants, we used an established fractionation method (hypotonic lysis) to separate the soluble and particulate components of cellular lysates (Figure 1B). As previously shown, the GFP−N-MAD fusion protein was localized almost exclusively in the particulate fraction. Interestingly, sequential deletion of residues 101 through 99 failed to increase the solubility of GFP. However, the subsequent deletion of residue 98 resulted in GFP being equally distributed in the supernatant and particulate fractions. No further changes in GFP solubility were observed with the loss of residue 97. Thus, GFP−N-MAD shifts from being predominantly membrane-bound to equally dispersed between the soluble and pellet fractions upon the loss of the distal four residues of its C-terminus under the conditions of hypotonic lysis.

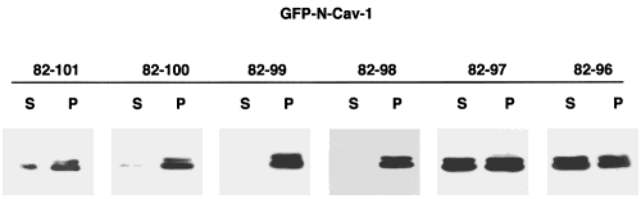

FIGURE 2: GFP is extractable by alkaline sodium carbonate when a C-terminal portion of N-MAD is deleted. 293T cells transiently expressing the indicated GFP−N-MAD deletion constructs were homogenized in 200 mM $NaCO_3$ (pH 11.3). Soluble (S) and particulate (P) fractions were recovered by high-speed centrifugation. Equal volumes of both fractions were subjected to SDS−PAGE and transferred to nitrocellulose. Immunoblot analysis with anti-GFP antibodies demonstrates that deletion of the four C-terminal residues of N-MAD renders GFP−N-MAD soluble in alkaline sodium carbonate.

Carbonate and Detergent Resistance of Caveolin-1 N-MAD Deletion Mutants. A more stringent method for examining the high-affinity association of proteins with membranes is to expose cell membranes to high pH. Those proteins resistant to extraction are considered "integral membrane proteins" (*22*). 293T cells expressing the panel of GFP fusion constructs were exposed to alkaline sodium carbonate (pH 11.3) extraction, and proteins were separated into soluble and membrane-bound fractions (Figure 2). As previously shown, GFP−N-MAD was resistant to alkaline sodium carbonate extraction (*1*). Sequential deletion of residues 101 through 99 of N-MAD failed to restore the solubility of GFP. Only after the deletion of residue 98 did GFP localize to the soluble fraction. Loss of residue 97 had no greater effect on the solubility of GFP.

Caveolae are characterized physically by their insolubility in nonionic detergents, such as Triton X-100 at low temperatures, and buoyancy in a sucrose density gradient (*23*). These properties are due to the relative abundance of cholesterol and sphingolipids within caveolae. In addition to homo-oligomerizing (*16*) and having palmitoylation groups that insert into the membrane (*14*), caveolins also bind sphingolipids (*24*) and cholesterol (*25*). As we showed previously, GFP−N-MAD is localized to the membrane-bound fraction upon Triton X-100 extraction (Figure 3, and ref *1*). The subsequent deletion of C-terminal residues restores GFP's solubility in Triton X-100, with the GFP−Cav-1 (82−96) construct being completely Triton-soluble.

Localization of Caveolin-1 N-MAD Deletion Mutants As Revealed by Fluorescence Microscopy. To examine the subcellular localization of the various GFP-fusion proteins, we performed fluorescence microscopy in transfected COS-7 cells (Figure 4). This cell line, like 293T, shows little or no endogenous caveolin-1 and was selected for these studies

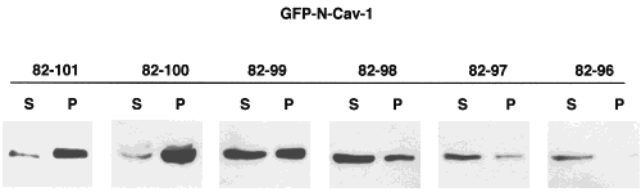

FIGURE 3: GFP is rendered more soluble in Triton X-100 with each subsequent deletion of the C-terminal residues of N-MAD. 293T cells transiently expressing the indicated GFP−N-MAD deletion constructs were extracted with ice-cold Triton X-100, homogenized, and fractionated into soluble (S) and particulate (P) portions. Equal volumes of both fractions were subjected to SDS−PAGE, transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies. Deletion of the arginine at position 101 of the C-terminus of N-MAD failed to alter the solubility of GFP in Triton X-100; however, the subsequent deletion of the C-terminal residues successively increased the solubility of GFP.

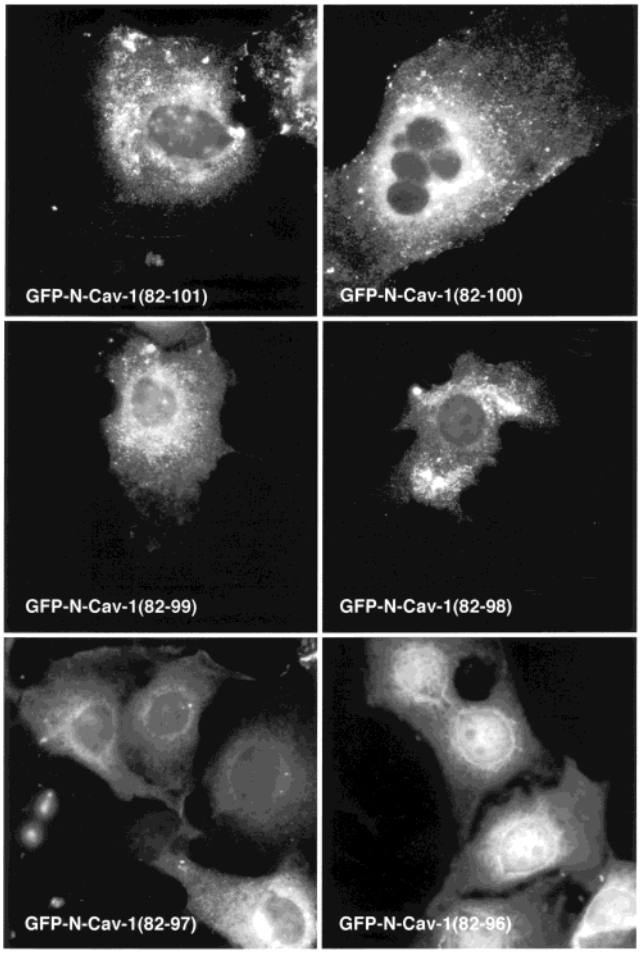

FIGURE 4: Fluorescence microscopy of GFP−N-MAD deletion mutants. Cos-7 cells were transiently transfected with the indicated GFP−N-MAD deletion constructs. Thirty-six-hours post-transfection cells were fixed and viewed using fluorescent microscopy. GFP−CAV-1 (82−101) had a punctate pattern, similar to the pattern seen when full-length caveolin-1 is fused to GFP. Deletion of the distal three C-terminal residues of N-MAD failed to alter this punctate pattern; however, GFP−CAV-1 (82−97) and GFP−CAV-1 (82−96) both had a diffuse cytosolic pattern. These results are consistent with the fractionation data in Figures 1−3.

because it adheres tightly to glass coverslips, whereas 293T cells are less adherent. GFP−Cav-1 (82−101) has a punctate distribution that is similar to the full-length protein, GFP−Cav-1 (1−178), reflecting its membrane localization (*1*). In agreement with the cellular fractionation results, GFP−Cav-

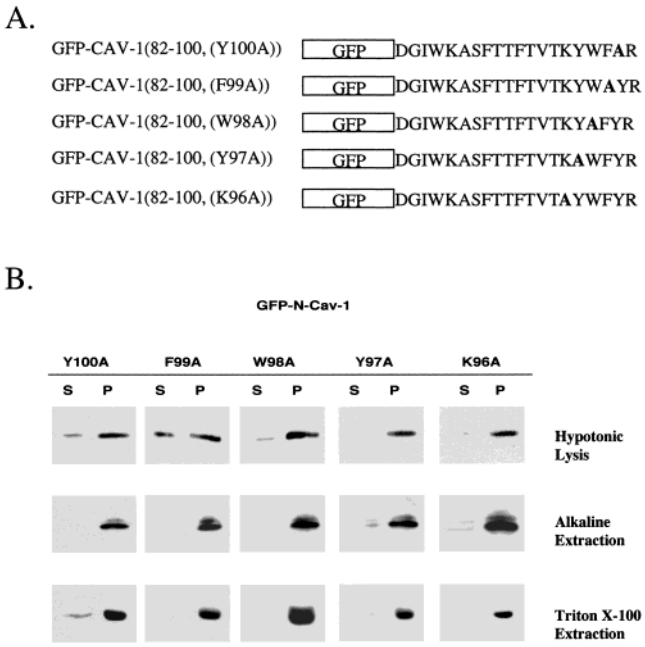

FIGURE 5: Construction and characterization of GFP−N-MAD alanine mutants. (A) The C-terminal residues of GFP−CAV-1 (82−101) were each individually mutated to alanine: GFP−CAV-1 (F100A) through GFP−CAV-1 (K96A). (B) 293T cells were transiently transfected with the indicated GFP−N-MAD alanine mutant constructs. Thirty-six-hours post-transfection cells were subjected to hypotonic lysis, alkaline sodium carbonate extraction, or Triton X-100 extraction, as in Figures 1−3. Immunoblot analysis demonstrated that each fusion protein was found predominantly in the particulate portion after each fractionation procedure.

(82−101) through GFP−Cav-1 (82−98) had a similar punctate fluorescence pattern, while GFP−Cav-1 (82−97) and GFP−Cav-1 (82−96) were diffusely distributed throughout the cytoplasm. These results corroborate the fractionation findings shown in Figures 1−3.

Alanine Scanning Mutagenesis within the N-Terminal Membrane Attachment Domain of Caveolin-1. Having established that the C-terminal residues of N-MAD are important for membrane attachment, we examined the contribution of individual residues by generating and characterizing a panel of GFP−N-MAD alanine-scan mutants (Figure 5A). Figure 5B shows that each of the alanine-scan mutants was located in the pellet fraction upon hypotonic lysis, alkaline carbonate treatment, and Triton X-100 extraction, indicating that all of these mutants are membrane-bound in cells. These results were confirmed by fluorescence microscopy (data not shown). These results indicate that no single residue is responsible for membrane attachment, but rather, as indicated from the deletion mutagenesis, multiple residues within the C-terminus of N-MAD are required for membrane binding.

The KYWFYR Sequence Itself Is Sufficient To Confer Membrane Attachment. The KYWFYR sequence contains a central cluster of four aromatic residues flanked by two basic residues. To determine the relative contributions of these two types of amino acyl residues to membrane attachment, we characterized two additional GFP-fusion proteins: GFP−KYWFYR and GFP−YWFY. Figure 6 illustrates clearly that the YWFY sequence is inadequate to localize GFP to the pellet fraction under all conditions tested. However, the

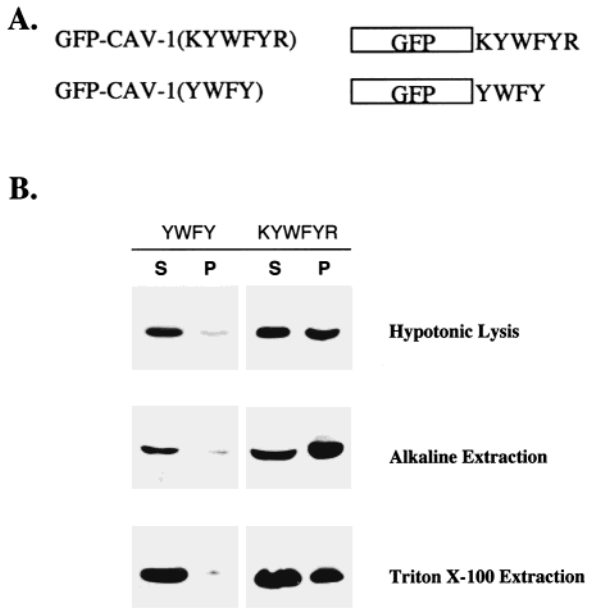

FIGURE 6: Construction and characterization of GFP−N-MAD aromatic/basic residue cluster mutants. (A) The KYWFYR or YWFY sequences were each fused to the C-terminus of GFP. (B) 293T cells were transiently transfected with the indicated fusion protein. Thirty-six-hours post-transfection cells were subjected to hypotonic lysis, alkaline sodium carbonate extraction, or Triton X-100 extraction, as in Figures 1−3. Whereas roughly half of the GFP−KYWFYR protein was in the particulate fraction, nearly all of the GFP−YWFY protein was soluble. Thus, loss of the flanking basic residues resulted in a marked increase in the solubility of the GFP.

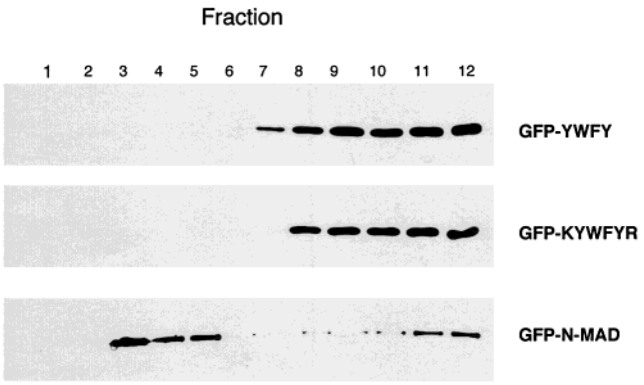

FIGURE 7: Neither the KYWFYR nor the YWFY sequences are caveolar membrane targeting signals. GFP−KYWFYR and GFP−YWFY were each transiently expressed in 293T cells. Thirty-six-hours post-transfection cells were lysed in 1% Triton X-100 in MBS. The lysate was mixed with an equal volume of 80% (w/v) sucrose. The mixture was placed at the bottom of an ultracentrifuge tube and then overlaid with a discontinuous sucrose gradient. After ultracentrifugation, 12 fractions were collected, and protein concentrations were determined. Equal masses of protein from each fraction were subjected to SDS−PAGE. After electrophoresis, proteins were transferred to nitrocellulose. Immunoblot analysis shows, as previously demonstrated (*1*), a proportion of GFP−N-MAD targets to low-density fractions 3−5. However, both GFP−KYWFYR and GFP−YWFY failed to localize to fractions 3−5, arguing that the KYWFYR sequence serves as a membrane attachment domain, but not as a caveolae targeting domain.

KYWFYR sequence was able to render a substantial fraction of GFP membrane bound under these same conditions. These results suggest the need for electrostatic interactions mediated by the flanking basic residues, in addition to the aromatic residues, for N-MAD's membrane association.

The KYWFYR Sequence Itself Is Not Sufficient To Confer Targeting to Lipid Rafts/Caveolae. We used an established equilibrium sucrose density gradient system to separate membranes enriched in lipid rafts/caveolae from other cellular membranes and cytoplasmic proteins (*23*, *26*, *27*). Using this procedure, caveolin-1 is purified ∼2000-fold relative to total cell lysates. Caveolin localizes to the 5%/30% sucrose interface (fractions 3−5), while >99.5% of total cellular proteins are excluded from these fractions. We have previously demonstrated that a significant amount of GFP−N-MAD targets to the low buoyant density fractions (*1*). Figure 7 shows that neither the KYWFYR nor the YWFY sequences were able to target GFP to these fractions. Thus, the KYWFYR sequence within N-MAD is a critical membrane attachment region, but the entire N-MAD sequence is necessary for targeting to lipid rafts/caveolae.

DISCUSSION

Caveolin-1 has a unique hairpin-like organization within the membrane in which its N- and C-terminal domains are localized to the cytoplasm. There is abundant evidence for this membrane topology. First, caveolin-1 does not undergo cell surface biotinylation, indicating that no portion of the molecule is located in the extracellular space (*16*). Second, the caveolin-1 N- and C-terminal domains are each the target of cytoplasmic modifications: tyrosine 14 is a c-Src substrate and cysteines 133, 143, and 156 are palmitoylated in vivo (*14*, *17*, *28*, *29*). Third, caveolin-1 is not sugar-modified when artificial glycosylation sites are placed at the extreme N- or C-termini, arguing that its post-translational modification steps are not those of a typical single-pass integral membrane protein (*15*). Fourth, antibodies directed against the N- and C-terminal domains and epitope tagged caveolin-1 constructs only stain permeabilized cells (*5*, *13*−*15*). Fifth, akin to other integral membrane proteins, caveolin-1 has a central hydrophobic stretch of amino acids, is inextractable from the membrane fraction by alkaline sodium carbonate treatment, can be photoaffinity labeled with a variety of lipids, and binds cholesterol and sphingolipids (*13*, *15*, *23*, *24*, *30*, *31*). Likewise, many studies support the notion that caveolin-1's oligomerization and presence in caveolae provides a mechanism for the sequestration of a variety of caveolar resident proteins (*32*). Much focus has been placed on the caveolin scaffolding domain (CSD, residues 82−101), given its cytoplasmic location and interaction with proteins containing a caveolin binding motif (ΦXΦXXXXΦ or ΦXXXXΦXXΦ, where Φ is an aromatic amino acid) found in many caveolin-interacting proteins.

Because the central hydrophobic region of caveolin-1 most closely resembles the membrane-spanning segment of an integral membrane protein, it was thought that it would play a significant role in membrane attachment. However, we previously demonstrated that this segment (residues 102−134) is not required for membrane attachment in whole cells or in reconstituted lipid vesicles (*19*). Surprisingly, residues 82−101, which flank the central hydrophobic domain, facilitate targeting to low buoyant density membrane fractions and membrane attachment (*1*).

N-MAD is an unconventional membrane attachment domain but has features in common with other membrane binding proteins. It resembles a highly conserved portion of

the homeodomain transcription factor, engrailed, which mediates nonvesicular membrane transport through caveolar membranes, suggesting that the homologous residues may help facilitate caveolar membrane interaction in both cases (*33*, *34*). In addition, there is compelling in vitro data that domains which contain clusters of aromatic and basic residues, including caveolin-1 residues 92−101, have significantly heightened membrane binding affinity due to combined hydrophobic and electrostatic interactions (*19*, *20*). Finally, we note that monotopic membrane association need not involve contiguous hydrophobic and charged residues: juxtaposition of hydrophobic and charged residues from distant portions of clotting factors V and VIII contribute to the membrane affinity of these molecules (*35*, *36*).

In this paper, we have identified a minimal membrane attachment domain within the N-MAD of caveolin-1 in living cells. To do so, we constructed a series of deletion and alanine mutant forms of N-MAD fused to the normally soluble reporter GFP and examined their membrane attachment capacity after transient expression in 293T cells. The results of this analysis indicated that the aromatic/basic residue cluster, KYWFYR (amino acids 96−101), constituting the C-terminal residues of N-MAD, is important for membrane attachment in vivo. We posit that caveolin-1 N-MAD binds membranes through the insertion of the aromatic cluster YWFY into the inner leaflet of membranes and the electrostatic interaction of the flanking charged residues with phospholipid charged headgroups. The present study, in combination with our previous results, contributes to a better understanding of the molecular underpinnings of caveolin-1 membrane attachment. It is our desire to link the molecular characterization of caveolin-1 with functional studies in whole animals in the future.

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