Mechanism for Targeting the A-kinase Anchoring Protein AKAP18δ to the Membrane*§

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A-kinase anchoring proteins (AKAPs) are a family of scaffolding proteins that target PKA and other signaling molecules to cellular compartments and thereby spatiotemporally define cellular signaling events. The AKAP18 family comprises AKAP18α, AKAP18β, AKAP18γ, and AKAP18δ. The δ isoform targets PKA and phosphodiesterase PDE4δ to AQP2 (aquaporin-2)-bearing vesicles to orchestrate the acute regulation of body water balance. Therefore, AKAP18δ must adopt a membrane localization that seems at odds with (i) its lack of palmitoylation or myristoylation sites that tailor its isoforms AKAP18α and AKAP18β to membrane compartments and (ii) the high sequence identity to the preferentially cytoplasmic AKAP18γ. Here, we show that the electrostatic attraction of the positively charged amino acids of AKAP18δ to negatively charged lipids explains its membrane targeting. As revealed by fluorescence correlation spectroscopy, the binding constant of purified AKAP18δ fragments to large unilamellar vesicles correlates (i) with the fraction of net negatively charged lipids in the bilayer and (ii) with the total amount of basic residues in the protein. Although distantly located on the sequence, these positively charged residues concentrate in the tertiary structure and form a clear binding surface. Thus, specific recruitment of the AKAP18δ-based signaling module to membranes such as those of AQP2-bearing vesicles must be achieved by additional mechanisms, most likely compartment-specific protein-protein interactions. AKAP18δ is involved in maintaining body water homeostasis. The rapid increase in water permeability of renal collecting ducts in response to antidiuretic hormone (arginine-vasopressin) is achieved through the fast increase of AQP2 (aquaporin-2) abundance in the plasma membrane, which results from the exocytic insertion of AQP2-containing intracellular vesicles (1). The underlying signal cascade starts with the binding of arginine-vasopressin to the vasopressin V2 receptor of renal principal cells. Stimulation of the V2 receptor leads to a rise in cAMP (2). The subsequent activation of PKA results in AQP2 phosphorylation, which, in turn, initiates an AQP2 redistribution from vesicles distributed throughout the cell to the apical plasma membrane (2).

PKA consists of a dimer of regulatory (R)2 subunits (R1α, R1β, R11α, and R11β) and two catalytic subunits, each of which binds to an R subunit. Thereby, the R subunits maintain the catalytic subunits in an inactive state. Upon binding of two molecules of cAMP to each R subunit, the catalytic subunits are released and phosphorylate substrates in close proximity. A-kinase anchoring proteins (AKAPs) direct PKA to defined cellular sites to limit the kinase’s access to a subset of substrates (3). This compartmentalization of PKA by AKAPs is crucial for various biological processes, including the arginine-vasopressin-induced redistribution of AQP2 (3–5). All canonical AKAPs possess an amphipathic helix, which directly binds PKA. The helix interacts with the dimerization and docking (D/D) domain formed by the dimerized R subunits. AKAP18δ (353 amino acids) is the largest member of the AKAP18 family, which comprises AKAP18α, AKAP18β, AKAP18γ, and AKAP18δ (6). AKAP18δ tethers PKA to AQP2-bearing vesicles as shown by analysis of immunosolated vesicles from principal cells of the inner medullary collecting duct (6, 7). In addition, AKAP18δ directly interacts with the phosphodiesterase PDE4δ (7). This signaling module, consisting of AKAP18δ, PKA, and PDE4δ, controls the local cAMP level and thus PKA activity in the vicinity of AQP2. Thereby, it participates in the control of the localization of AQP2 and thus in the control of arginine-vasopressin-mediated water reabsorption (7). In cardiac myocytes, AKAP18δ directly interacts with phospholamban. This interaction is involved in Ca2+ reuptake into the sarcoplasmic reticulum and thereby participates in the regulation of cardiac myocyte contractility (8).

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2 The abbreviations used are: R, regulatory; AKAP, A-kinase anchoring protein; D/D, dimerization and docking; LUV, large unilamellar vesicle; DPH/PC, diphytanoylphosphatidylcholine; DPH/PG, diphytanoylphosphatidylglycerol; PLE, polar lipid extract; tris-NTA, tris-nitrotriacetic acid; FCS, fluorescence correlation spectroscopy.
Membrane Localization of AKAP18δ

The shorter isoforms of the AKAP18 family, AKAP18α (81 amino acids) and AKAP18β (104 amino acids), are targeted to plasma membranes by (i) myristoylation and palmitoylation and (ii) binding to ion channels such as Na⁺ channels (9, 10). In contrast, there is no evidence for specific binding of AKAP18δ to membrane channels (e.g. AQP2). This agrees with the observation made for another AKAP, gravin. Its myristoylation sites are not required for membrane localization or function (11). Gravin possesses short positively charged domains (the so-called MARCKS (myristoylated alanine-rich C-kinase substrate) effector domain-like regions), which serve to electrostatically link the protein with the membrane. However, similar domains are not present in the AKAP18δ primary structure (supplemental Fig. 1). The only domain with basic amino acids is the so-called conserved nuclear localization sequence between positions 78 and 86. All of the other positively charged residues are (i) scattered throughout the protein and (ii) well balanced by acidic residues (supplemental Fig. 1). The nuclear localization sequence is unlikely to be responsible for the membrane localization of AKAP18δ. It is also part of AKAP18γ, another splice variant (324 amino acids) that is localized mainly in the cytoplasm (12). AKAP18γ is 26 amino acids shorter and ~75% identical to AKAP18δ (6). The additional N-terminal sequence (amino acids 1–26) of AKAP18δ has a net negative charge of ~4, and its electrostatic interactions with the membrane are thus unlikely to be responsible for the difference in cellular location between AKAP18δ and AKAP18γ.

Although not localized in well defined domains, the tertiary structure of AKAP18δ(87–292) (13) reveals numerous positively charged amino acids that are concentrated into one plane (Fig. 1). We speculate that this arrangement may represent the binding plane, i.e. that the number of positive charges in that plane is large enough to attract the protein to negatively charged membranes. This would explain how AKAP18δ adopts its membrane localization. Membrane anchoring is mandatory because AKAP18δ would otherwise be unable to orchestrate the regulation of water balance in renal principal cells and Ca²⁺ reuptake into the sarcoplasmic reticulum of cardiac myocytes.

To test whether electrostatic interactions of AKAP18δ with lipids may be sufficient for membrane recruitment, we purified AKAP18δ fragments that contained different numbers of charged residues and monitored their interaction with lipid bilayers. We found that the binding energy increased with the total length of the fragments, even though no specific targeting domains were identified. The mere increase in the number of positively charged residues was sufficient.

EXPERIMENTAL PROCEDURES

Lipid Vesicle Preparation—Large unilamellar vesicles (LUVs) were prepared from a lipid mixture in chloroform. The lipids were dried on a rotary evaporator, hydrated in a solution containing 300 mM NaCl and 20 mM HEPES buffered at pH 7.3, and put through 21 extrusion cycles stacked with two polycarbonate filters with 400- and 100-nm pore sizes using a mini-extruder from Avanti Polar Lipids. The final stock solution subsequently contained 35 mg/ml lipids.

To count the number of vesicles in the confocal volume, lipid in chloroform was mixed with DiIC₁₅(5)-DS (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid; Invitrogen) in ethanol to obtain a final concentration of ~3 dyes per lipid vesicle. During our study, we used liposomes containing diphytanoylphosphatidylcholine (DPhPC) and diphytanoylphosphatidylglycerol (DPhPG) at different molar ratios and brain polar lipid extract (PLE) (all from Avanti Polar Lipids).

Protein Expression and Purification—AKAP18δ versions encoding amino acids 87–292, 1–292, and 76–353 were generated as N-terminally His₁₅-tagged fusion proteins using the vector pET-30 EK/LIC and Escherichia coli strain Rosetta(DE3). To improve the solubility of AKAP18δ(76–353), the vector was coexpressed with a plasmid (pET-46) encoding the AKAP-binding domain of the RIIα subunits of PKA (D/D domain, amino acids 1–44) or full-length RIIα, both lacking the His₁₅ tag. Precultures were grown overnight in 50 ml of LB medium (25–37 °C) and centrifuged, and cell pellets were resuspended and used to inoculate up to four 500-ml cultures in Overnight Express™ Instant TB medium (autoinduction medium; Novagen). Cultures were grown for at least 24 h at 25–37 °C and 110 rpm. Cells were harvested by centrifugation, washed with PBS, and stored at ~80 °C. Cells were thawed on ice, resuspended in extraction buffer (40 mM phosphate buffer (pH 7.5), 300 mM NaCl, 5–10 mM imidazole, 5 mM β-mercaptoethanol, 0.5 mMEnable extraction of the text from the image.
PMSF, one protease inhibitor tablet (Roche Applied Science), and 5 μl/100 ml Benzonase, and lysed using a French press. Cell debris was removed by centrifugation (21,000 rpm, 4 °C, 30 min) and filtration through a 0.45-μm filter. The cleared supernatant was applied to a Talon cobalt affinity column (Clontech) pre-equilibrated with 40 mM phosphate buffer (pH 7.5), 300 mM NaCl, and 5–10 mM imidazole in an AKTA system. The Talon column was washed with equilibration buffer, and protein was eluted with 40 mM phosphate buffer (pH 7.5), 300 mM NaCl, and an imidazole gradient up to 300 mM. Fractions containing AKAP18 truncations were pooled (supplemental Fig. 2). If necessary, the pool was dialyzed against 20 mM NaCl in 20 mM Tris-HEPES and 300 mM NaCl (pH 7.0). Pure fractions were pooled, supplemented with 20% glycerol, and stored at −80 °C.

**Protein Labeling**—We used the high affinity of tris-nitrotriacetic acid (tris-NTA)(Atto565) for the His6 tag (14) to label our AKAP18 constructs. For this, the tris-NTA(Atto565) solution was saturated with NiCl2 and mixed with AKAP18 versions for 30 min. Depending on the final tris-NTA(Atto565) concentration (~10 mM), the fraction of free tris-NTA(Atto565) varied between 40 and 70%. The fraction of labeled AKAP18 amounted to ~10%. In the presence of 4.5 μM NiCl2, tris-NTA(Atto565) does not readily dissociate from His6 tags (15).

**Fluorescence Correlation Spectroscopy (FCS)**—FCS is an accurate and valuable means for quantifying the interaction of proteins with lipid bilayers (16). We first monitored bulk diffusion of AKAP18 versions (Confocor 3 attached to an LSM 510 META laser scanning microscope, both from Carl Zeiss, Jena, Germany) (17). tris-NTA(Atto565) was excited at 561 nm. We calibrated the confocal volume by measuring the residence time (τ_e) of rhodamine 6G in solution. On the basis of a diffusion coefficient of 426 μm² s⁻¹ (18), we arrived at confocal plane radii (r_o) of 0.22 and 0.25 μm for the two wavelengths 561 and 633 nm.

We obtained the number of particles (N) of each diffusing species from the autocorrelation function (G(τ)) of the fluorescence temporal signal from DiIC18(5)-DS-labeled lipid vesicles or tris-NTA(Atto565)-labeled proteins (Equation 1),

\[
G(\tau) = 1 + \frac{1}{\sum_{j=1}^{J} N_j \left( \frac{1}{1 + \frac{\tau}{\tau_1}} \right)} \cdot \frac{1}{1 + \left( \frac{\tau}{\tau_2} \right)^2} \cdot \frac{\tau}{\tau_2}
\]

(Eq. 1)

where \( j \) and \( z_o \) are the number of different species and the depth of the confocal volume (in the direction of the laser beam), respectively. Recordings (5 × 30 s/measurement) were made in a small self-made measurement chamber (<100 μl) 100 μm above the glass slide to estimate the fraction of lipid-bound proteins. The measurement chamber consisted of a plastic cylinder that was glued (Norland optical adhesive NOA63) to a glass slide. To exclude unwanted protein and dye adsorption to the chamber walls at nanomolar protein and dye concentrations, we preincubated the chamber with 1 mg/ml BSA (Sigma) overnight and rinsed it four times with measurement buffer before use.

We started the titration experiments with 70 nM AKAP18δ to be well above the detection limit of FCS. We fitted a standard diffusion model for \( j = 3 \) to the FCS autocorrelation curves, where we fixed four out of six parameters. These four parameters, namely the diffusion times of (i) free tris-NTA(Atto565) (45 μs), (ii) labeled AKAP18δ versions (180–220 μs), (iii) labeled AKAP18δ versions bound to lipid vesicles (2.5 ms), and (iv) the fraction of free tris-NTA(Atto565) compared with protein-bound tris-NTA(Atto565), were determined in 12 independent experiments.

**Determination of Binding Constants and Binding Energies from Experimental Measurements**—Assuming Langmuir adsorption, we quantified protein binding to lipid bilayer membranes using the molar partition coefficient (\( K \)) (19). \( K \) is the proportionality factor between the mole fraction of AKAP18δ versions bound to the membrane (\( \chi \)) and the concentration of AKAP18δ versions free in bulk solution ([A]) (Equation 2),

\[
\chi = \frac{[A]_m}{[A]_m + [A]_{tot}} = k[A]
\]

(Eq. 2)

where \([A]_m\) and \([L]_{acc}\) denote the concentration of membrane-bound AKAP18 versions and the concentration of the accessible lipid, respectively. As AKAP18δ versions cannot reach the inner leaflet, \([L]_{acc}\) amounts to only half of the total lipid concentration. For all of our experiments, \([L]_{acc} \gg [A]_m\). Equation 2 can be written as shown in Equation 3.

\[
[A]_m = k[A][L]_{acc}
\]

(Eq. 3)

The total concentration of AKAP18δ versions in the solution is the sum of bound and free AKAP18δ (Equation 4).

\[
[A]_{tot} = [A]_m + [A]
\]

(Eq. 4)

Combining Equations 3 and 4 gives the expression shown in Equation 5.

\[
\frac{[A]_m}{[A]_{tot}} = \frac{k[L]_{acc}}{1 + k[L]_{acc}}
\]

(Eq. 5)

Fitting Equation 5 to a plot of \([L]_{acc}\) as a function of \([A]_m/[A]_{tot}\) allows estimation of \( K \). \( K \) is associated with the Gibbs free energy by Equation 6,

\[
\Delta G = -N_A k_B T \ln(K)
\]

(Eq. 6)

where \( N_A \) is Avogadro’s number, \( k_B \) is the Boltzmann constant, and \( T \) is the temperature. Equation 6 can be used to convert the molar partition coefficients gained by Equation 5 into energy units, the binding free energy (\( \Delta G \)).

**ζ-Potential Measurements**—The surface charge of the extruded (100-nm filter) lipid vesicles was evaluated by measuring their electrophoretic mobility with a Zetasizer (Malvern Instruments) in 50 mM KCl, 2 mM Tris (pH 7.3), and 0.5 mM EDTA (20).
Membrane Localization of AKAP18δ

RESULTS

Diffusion of single fluorescent particles in and out of the focus of a laser beam gives rise to intensity fluctuations of the emitted light. We first recorded these fluctuations from the free tris-NTA(Atto565) dye in an aqueous solution using the FCS extension of a laser scanning microscope. Fluctuation analysis in terms of autocorrelation functions (see Equation 1) allowed determination of the residence time of the dye in the focus to be equal to $\tau_{R1} = 45 \mu s$. Linkage of the small dye to the much larger AKAP18δ(1–292) fragment was expected to increase the residence time. In line with these expectations, the experimentally determined residence time ($\tau_{R2}$) of the labeled protein was equal to 180–220 μs (Fig. 2, black line). The binding of the AKAP18δ(1–292)-tris-NTA(Atto565) complex to the even larger liposomes should result in a further decrease in diffusion, which the residence time of $\tau_{R3} = 2.5 ms$ confirmed (Fig. 2, cyan line). In control experiments, we demonstrated that the free tris-NTA(Atto565) dye did not bind to the vesicles (supplemental Fig. 4).

At intermediate lipid concentrations (Fig. 2, red, green, blue, and pink lines), protein binding was incomplete, i.e. the resulting autocorrelation curves were a superposition of (i) protein diffusing free in solution, (ii) protein bound to vesicles, and (iii) free dye diffusing in solution. Setting $j = 3$ in Equation 1 and fitting it to the autocorrelation curves allowed determination of the number of particles $N_1$, $N_2$, and $N_3$ of tris-NTA(Atto565), AKAP18δ(1–292)-bound tris-NTA(Atto565), and lipid vesicle-bound AKAP18δ(1–292)-tris-NTA(Atto565), respectively. To reduce the error in the assessment of the lipid-bound AKAP18δ fraction, $N_j/(N_1 + N_2)$ = [A]$_{m}$/[A]$_{tot}$, we fixed $\tau_{R1}$, $\tau_{R2,1}$, $\tau_{R2,3}$, and $\tau_{R3,3}$. $N_j/(N_1 + N_2 + N_3)$ is the fraction of the free dye. It was determined at the beginning of each lipid titration experiment (i.e. in the absence of the lipid). The above procedure assumes that the diffusing species do not vastly differ in their brightness. This requirement is fulfilled (i) if all of them are holding just 1 dye molecule, which (ii) changes neither its adsorption spectra nor its quantum yield upon binding to the membrane.

To verify that this must have been the case, we analyze the worst case, i.e. the situation with the highest number of dyes per vesicle. The plot of $[A]_m/[A]_tot$ against $[L]_{ac}$ (Fig. 3) shows that the lowest lipid concentration corresponds to this situation because it indicates the highest protein-to-lipid ratio of $7 \times 10^{-8}/1.5 \times 10^{-4} = 4.7 \times 10^{-4}$. Assuming that one lipid occupies $68 \text{ Å}^2$, we arrive at $4.6 \times 10^4$ accessible lipids per vesicle and at an outer leaflet area of $3.1 \times 10^6 \text{ Å}^2$ for 100-nm lipid vesicles, i.e. we added 21.6 ($4.6 \times 10^4 \times 4.7 \times 10^{-4}$) proteins per vesicle into the aqueous solution. 30% of these AKAPs (compare Fig. 3) were bound to the vesicular membrane, so the average vesicle held seven AKAPs. This transforms into 0.7 dyes per vesicle because only 10% of these proteins were labeled. Thus, it is safe to conclude that the diffusing species, which entered the autocorrelation analysis, all had just one fluorescent label.

Decreasing the fraction of acidic lipids in the vesicular membrane also decreased the fraction of the protein, which was bound to the lipids (Fig. 3). For a quantitative analysis, we fitted Equation 5 to the data shown in Fig. 3. The only fitting parameter was the molar partition coefficient ($K$). For 58, 20, and 0 mol % DPhPG, $K$ was equal to 2520 ± 450, 350 ± 60, and 20 ± 13 $M^{-1}$, respectively (Fig. 4), indicating that binding is driven by electrostatic attraction. Besides electrostatics, other factors such as lipid packing density may affect binding. We tested this assumption by substituting DPhPG for natural brain PLE. The resulting $K = 2160 ± 570 M^{-1}$ was close to the $K$ value for 58 mol % DPhPG (Fig. 4) even though only 23.6% (w/w) 18.5% (w/w) phosphatidylserine and 4.1% (w/w) phosphatidylinositol (manufacturer's information) of the lipids were charged.

We verified the acidic lipid content of extruded brain PLE LUVs by comparing the electrophoretic mobility of these LUVs with that of LUVs from synthetic lipids of known composition. Because particle velocity depends on the electric field strength, the so-called $\zeta$-potential served as the output parameter. $\zeta$ indi-

FIGURE 2. Representative FCS measurements of AKAP18δ(1–292) binding to DPhPC/DPhPG (58 mol % DPhPG) LUVs. Normalized autocorrelation curves show vesicle titration to $7 \times 10^{-8}$ M AKAP18δ(1–292). Lipid concentrations were (top to bottom) 0, $3 \times 10^{-5}$, $9 \times 10^{-5}$, $3 \times 10^{-4}$, $9 \times 10^{-4}$, and $3.7 \times 10^{-3}$ M, corresponding to 0, 20, 35, 50, 70, and 100% of vesicle-bound AKAP18δ(1–292), respectively. tris-NTA(Atto565) was used as a fluorescent label. The solution contained three different freely diffusing fluorescent species: (i) free label ($\tau_{R1}$ = 45 μs), (ii) labeled protein ($\tau_{R2} = 180–220 \mu s$), and (iii) labeled protein bound to lipid vesicles ($\tau_{R3} = 2.5 ms$).

FIGURE 3. FCS measurements of AKAP18δ(1–292) binding to DPhPC/DPhPG. The protein-bound fraction is plotted versus the accessible (acc.) lipid concentration (about half of the actual lipid concentration). The fractions were deduced from data similar to those in Fig. 2. The solution contained 300 mM NaCl, 20 mM HEPES (pH 7.3), $4.5 \times 10^{-6}$ M NiCl$_2$, $7 \times 10^{-8}$ M AKAP18δ(1–292), and DPhPC/DPhPG vesicles composed of 0 (%), 20 (gray circles), and 58 (red) mol % DPhPG. Error bars were calculated as standard variance. Straight lines represent the least-square fits of Equation 3 to the data. The poor fit for DPhPC indicates very weak binding.
Membrane Localization of AKAP186

![Graph](https://via.placeholder.com/150)

**FIGURE 4.** Molar partition coefficient (K) as a function of mole percent acidic lipids for $7 \times 10^{-5}$ m AKAP186(1–292). Measurements of 0 (white bar), 20 (gray bar), and 58 (black bar) mol % DPhPG are compared with brain PLE (cross-hatched bar) as a natural mixture. The acidic lipid content of brain PLE was calculated from the manufacturer's information and cross-checked via $\zeta$-potential control measurements of lipid vesicles.

![Graph](https://via.placeholder.com/150)

**FIGURE 5.** Molar partition coefficient (K) of the indicated AKAP186 variants as a function of positively charged residues per construct at 58 mol % DPhPG. The arginine and lysine residues were counted for positively charged amino acids at pH 7.3. The numbers in parentheses indicate the amino acids of AKAP186, the D/D domain, and RIIa. AKAP186(76–353) was co-purified with these proteins.

cates the electric potential at a distance of 2–4 Å from the vesicle surface depending on whether one or two layers of adsorbed water molecules and ions move along with the vesicle. At $-40.2 \pm 2$ mV, $\zeta$ of brain PLE vesicles was smaller than the $\zeta$ value of $-42 \pm 2$ mV for LUVs containing 30% DPhPG and 70% DPhPC. Extruded DPhPC/DPhPG LUVs with 10 and 60 mol % DPhPG exhibited $\zeta$ values of $-23.5 \pm 2$ and $-52.5 \pm 3$ mV, respectively, in 50 mM KCl.

We also performed binding experiments with two more AKAP186 fragments, namely AKAP186(87–292) and AKAP186(76–353). AKAPs interact with dimers of RI subunits, which are formed through interactions of the N-terminal 45 amino acids of each protomer. The dimerized N termini form the so-called D/D domain of RIIa. Either the D/D domain or full-length RIIa was added to AKAP186(76–353) to exclude the possibility that we mistook basic residues involved in protein-protein interaction for those residues that are responsible for lipid binding. These experiments revealed a linear dependence of ln K on the number of positively charged residues per construct for AKAP186(87–292), AKAP186(1–292), and AKAP186(76–353)/DD (Fig. 5).

This is in agreement with the observation made for oligopeptides that $\Delta G$ (or ln K; see Equation 6) scales with the amount of positively charged residues. The surprising observation is that part of a soluble protein, i.e. the D/D domain, increases membrane affinity. The D/D domain offers a pocket that accommodates the hydrophobic interface of an amphipathic helix of AKAP186(76–353). The outer edges of the binding crevice are acidic and thus could contribute to the electrostatic interaction with the membrane (21, 22). The remainder of RIIa adds little to the binding affinity, as would be expected for a water-soluble protein (Fig. 5).

To further prove the electrostatic nature of the AKAP186-membrane association, we screened the surface charges of both membrane and protein by increasing the salt concentration from 300 to 800 mM NaCl. The decreased surface potentials of membrane and protein resulted in significantly decreased membrane affinity of AKAP186 fragments, thereby confirming the crucial role of electrostatics in the binding process. For instance, the fraction of vesicle-bound AKAP186(76–353)/RIIa was halved (Fig. 6).

Structural investigations of AKAP186 revealed that the central domain can bind 5′-AMP (13). The binding of 5′-AMP to AKAP186 neutralizes a region of positive charge at the base of the binding groove, and both hydroxyl groups of the 5′-AMP become available for interaction. The 5′-AMP-mediated change in the charge and shape of the molecule could potentially be involved in regulating AKAP186-membrane affinity. However, the addition of 5 mM 5′-AMP to AKAP186(76–353)/RIIa did not induce a significant change in K (supplemental Fig. 3). In addition, 5′-AMP apparently did not change the location of full-length AKAP186 overexpressed in HEK293 cells (data not shown).

**DISCUSSION**

We have defined a hitherto unobserved mechanism through which a scaffolding protein can anchor to membranes. The affinity is provided solely by electrostatic attraction of amino acid residues, which, although distantly located on the sequence, concentrate in the tertiary structure to form a binding surface. Neither lipid anchors nor interactions with other proteins are required.

In contrast to other AKAPs (11, 23), the charged amino acids are not bundled in domains. This conclusion stems from the investigation of four different AKAP186 versions (AKAP186(87–292), AKAP186(1–292), AKAP186(76–353)/DD, and AKAP186(76–353)/RIIa). AKAP186(87–292) lacks the highly positively charged sequence between positions 76 and 86 that is present in AKAP186(1–292). To identify a possible role for PKA in modifying binding affinity, we co-purified AKAP186(76–353), which comprises the PKA-binding domain, with the D/D domain of RIIa or full-length RIIa subunits of PKA. Only the acidic residues of the RIIa-binding crevice (21) increased membrane affinity. The remainder of the water-soluble RIIa had little effect on K. K of the other three AKAP versions is an exponential function of their total number (m) of positively charged amino acids (Table 1).
bend away from a negatively charge surface. This very special arrangement of charges suggests that this is the lipid-interacting interface (Fig. 1). The hypothesis is supported by the calculations of $\Delta G$. If $\Delta G$ was derived from the interaction of these positively amino acids with negatively charged lipids, we arrive at a binding free energy per positive charge of about $-0.44$ kcal/mol (Table 1), which is in the order of the interaction energy derived from short polylsine model peptides (19).

The pure electrostatic targeting mechanism does not impose any selectivity to the location of the scaffolding protein. This seems at odds with its task of regulating water homeostasis. One would expect that AKAP18δ binds specifically to AQP2-containing vesicles instead of binding randomly to any negatively charged membrane. Hence, we have to assume that this specificity is achieved through other mechanisms such as protein-protein interactions. AKAP18δ may directly interact with a transmembrane- or membrane-associated protein on AQP2-bearing vesicles in a similar manner as with phospholamban in the membrane of the sarcoplasmic reticulum in cardiac myocytes (8). Alternatively, AKAP18δ may be specifically targeted to AQP2-bearing vesicles through interactions of one of its binding partners, PKA or PDE4D (7), with a protein on AQP2-bearing vesicles. A similar concept most likely applies to many other scaffolding proteins, where myristoylation or palmitoylation of the protein does not accomplish targeting to a specific membrane compartment (25, 26).

In summary, we conclude that AKAP18δ is anchored to membrane lipids by electrostatic interactions. Membrane affinity stems from the special spatial arrangement of positively charged amino acids into a binding plane (Fig. 1).

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