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# Analysis of A-Kinase Anchoring Protein (AKAP) Interaction with Protein Kinase A (PKA) Regulatory Subunits: PKA Isoform Specificity in AKAP Binding

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Compartmentalization of cAMP-dependent protein kinase (PKA) is in part mediated by specialized protein motifs in the dimerization domain of the regulatory (R)-subunits of PKA that participate in protein-protein interactions with an amphipathic helix region in A-kinase anchoring proteins (AKAPs). In order to develop a molecular understanding of the subcellular distribution and specific functions of PKA isozymes mediated by association with AKAPs, it is of importance to determine the apparent binding constants of the R-subunit-AKAP interactions. Here, we present a novel approach using surface plasmon resonance (SPR) to examine directly the association and dissociation of AKAPs with all four R-subunit isoforms immobilized on a modified cAMP surface with a high level of accuracy. We show that both AKAP79 and S-AKAP84/D-AKAP1 bind RIIα very well (apparent $K_D$ values of 0.5 and 2 nM, respectively). Both proteins also bind RIIβ quite well, but with three- to fourfold lower affinities than those observed *versus* RIIα. However, only S-AKAP84/D-AKAP1 interacts with RIα at a nanomolar affinity (apparent $K_D$ of 185 nM). In comparison, AKAP95 binds RIIα (apparent $K_D$ of 5.9 nM) with a tenfold higher affinity than RIIβ and has no detectable binding to RIα. Surface competition assays with increasing concentrations of a competitor peptide covering amino acid residues 493 to 515 of the thyroid anchoring protein Ht31, demonstrated that Ht31, but not a proline-substituted peptide, Ht31-P, competed binding of RIIα and RIIβ to all the AKAPs examined ($EC_{50}$-values from 6 to 360 nM). Furthermore, RIα interaction with S-AKAP84/D-AKAP1 was competed ($EC_{50}$ 355 nM) with the same peptide. Here we report for the first time an approach to determine apparent rate- and equilibria binding constants for the interaction of all PKA isoforms with any AKAP as well as a novel approach for characterizing peptide competitors that disrupt PKA-AKAP anchoring.



*Keywords:* A-kinase anchoring protein; cAMP-dependent protein kinase; cAMP; surface plasmon resonance; SPR; protein-protein interaction

## Introduction

Cyclic AMP regulates a number of discrete physiological responses through cAMP-dependent protein kinase (PKA). Although PKA has broad substrate specificity, one intriguing aspect of its action is the ability to phosphorylate selectively individual substrates in response to distinct hormonal stimuli. Selectivity in PKA action may be mediated by particular pools of kinase compartmentalized at different subcellular loci through interaction with A-kinase anchoring proteins (AKAPs, reviewed in (Colledge & Scott, 1999;

Abbreviations used: AKAP, A-kinase anchoring protein; ATP, adenosine triphosphate; ADP, adenosine diphosphate; C, catalytic subunit of PKA, cAMP, adenosine 3′,5′-cyclic monophosphate; PKA, cAMP-dependent protein kinase; CM, carboxymethyl; DTT, dithioerythreitol; EDTA, *N*,*N*,*N*′,*N*′-ethylenediamine tetracetic acid; GST, glutathione-*S*-transferase; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; Mops, 4(*N*-morpholino) propanesulfonic acid; MW, molecular weight; Pipes, 1,4 piperazinebis(ethansulfonic acid); R, regulatory subunit of PKA, SDS, sodium dodecyl sulfate.

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Rubin, 1994)) which target PKA towards specific substrates. The PKA holoenzyme complex forms a tetramer consisting of two regulatory (R) and two catalytic (C) subunits. Four different regulatory subunits (RIα, RIβ, RIIα and RIIβ) of PKA have been identified and serve to regulate catalytic activity by binding and inactivating the C-subunit. The C-subunit is released and activated upon the binding of four molecules of cAMP to the R-subunit dimer (for review and references, see (Francis & Corbin, 1994; Scott, 1991; Taylor *et al*., 1992)).

Targeting of PKA to various subcellular loci is mediated by interaction of the R-subunits with different AKAPs (Colledge & Scott, 1999; Rubin, 1994). Whereas PKA type I (containing RIα or RIβ) is known to be mainly soluble, it has also been demonstrated to localize in proximity to membrane receptors such as antigen receptors on lymphoid cells and nicotinic acetylcholine receptors in neuromuscular junctions (Skålhegg *et al*., 1994; Imaizumi-Scherrer *et al*., 1996). In contrast, PKA type II (containing RIIα or RIIβ) is primarily particulate and associated with cytoskeletal elements and a number of organelles. While the subcellular targeting of RI *via* mono or dual-specific AKAPs is emerging (Angelo & Rubin, 1998; Huang *et al*., 1997a,b; Miki & Eddy, 1998, 1999), it is well known that RII can be specifically bound to AKAPs that bind exclusively RII or both RII and RI. AKAPs that bind RII have been localized to microtubules (MAP2 and AKAP150), postsynaptic densities and cortical actin (AKAP79/75), actin (AKAP78/ezrin, AKAP-KL), nuclear matrix (AKAP95 and nuclear AKAP150), sarcoplasmic reticulum and nuclear envelope (mAKAP), endoplasmic reticulum (D-AKAP1), peroxisomes (AKAP220), Golgi apparatus (AKAP85), mitochondria (S-AKAP84/149/D-AKAP1), centrosomes (AKAP450), filopodia (gravin/AKAP250), sperm flagella (AKAP110, FSC1/AKAP82) and shown to target to membrane receptors as β2-adrenoreceptors (gravin/AKAP250), and various ion channels (AKAP15/18, AKAP79, yotiao/AKAP450, ezrin) (for review and references, see (Colledge & Scott, 1999; Fraser & Scott, 1999)).

The interaction of PKA RII subunits with various AKAPs is shown to involve an amphipathic helix motif with a conserved structure (X{L,I,V}X3{A,S}X2{L,I,V}{L,I,V}X2{L,I,V}{L,I,V}X2{A,S}{L,I,V}) in the AKAP where the hydrophobic face binds the RII dimer (Carr *et al*., 1991; Vijayaraghavan *et al*., 1999) Furthermore, the recent solution of the structure of the dimerization and AKAP binding domains of RIIα have shown that high-affinity AKAP binding involves an X-type four-helix bundle dimerization motif with an extended hydrophobic face in residues 1-44 (Newlon *et al*., 1999). In contrast to the interaction of RIIα with AKAPs, the RIα N terminus has a stable, α-helical dimerization and docking motif that involves residues 12 to 61 and includes two stable disulfide bonds (Leon *et al*., 1997). In addition to the distinct subcellular distribution of PKA type I and II, isozymes with RIIα and RIIβ have also been demonstrated to localize differently in the Golgi-centrosomal area (Keryer *et al*., 1999). A recent study addressed the specificity of the interaction of the dimerization/AKAP binding domain of RIIα and RIIβ and showed clearly that, whereas some anchoring proteins bind both RIIα and RIIβ, although with different affinity, other anchoring molecules such as AKAP95 almost exclusively bind RIIα (Hausken *et al*., 1996). The biochemical basis for the distinct distribution of PKA isozymes is based on the specificity and affinity in PKA interaction with available AKAPs. In order to develop a molecular understanding of the subcellular distribution and specific functions of PKA isozymes, we found it of importance to develop methods to assess the apparent binding constants of the R-AKAP interaction. We present a novel approach to examine directly binding of AKAPs to all R-subunit isoforms with a high degree of accuracy where the R-subunit is immobilized on a modified cAMP surface, and the association and dissociation of AKAP proteins is examined by surface plasmon resonance. We examine the PKA-interaction of AKAP79, reported to bind both RIIα and RIIβ, with the four R-subunits (RIα, RIβ, RIIα and RIIβ) and compare it with that of AKAP95, reported to bind more selectively RIIα, and with that of S-AKAP84/D-AKAP1 (AKAP121, AKAP149), which is reported as a dual-specific AKAP binding both RI and RII (Huang *et al*., 1997b) and show selectivity in AKAP binding for the different R-subunits. Apparent binding constants and $EC_{50}$ values for competitor peptides are determined.

## Results

The number of A-kinase anchoring proteins (AKAPs) is growing fast and several of these highly specific PKA-binding proteins are in the same cellular compartment, sharing the four available R-subunits, RIα, RIβ, RIIα and RIIβ. Therefore accurate numbers for AKAP/R-subunit interactions are needed to understand the basis for differential subcellular distribution of PKA R-subunits. We expressed fragments of S-AKAP84/D-AKAP1, AKAP79 and AKAP95 containing the respective RII binding domains as GST-fusion proteins or non-fusion proteins. Two different approaches were used to determine AKAP R-subunit interaction. As a first approach, we incubated GST-AKAP proteins with radiolabeled R-subunits with both partners at a concentration of 100 nM, and precipitated GST-proteins by glutathione agarose beads. Figure 1 shows the R-subunits, photoaffinity-labeled with 8-azido-[$^{32}$P]cAMP, co-precipitated with the indicated GST-AKAP. As seen in the upper panel, the dual-specific S-AKAP84/D-AKAP1 interacted with RIα (lane 1) and both with RIIα and RIIβ (lanes 3 and 4). AKAP79 precipitated RIα at low levels (middle panel, lane 1) and RIIα and RIIβ approximately equally well (lanes 3 and 4). In contrast, GST-

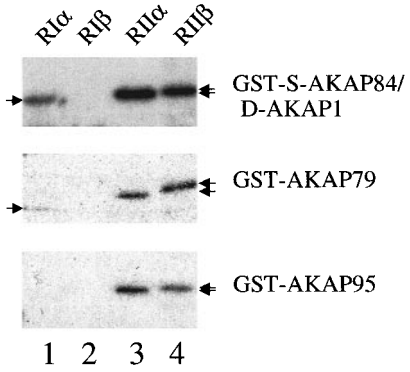


**Figure 1.** Co-precipitation of regulatory subunits (RIα, RIβ, RIIα, RIIβ) with GST-AKAPs. Regulatory subunits were photoaffinity-labeled with 8-azido-[$^{32}$P]cAMP, incubated with fusion proteins (GST-S-AKAP84/D-AKAP1, GST-AKAP79, GST-AKAP95), containing the respective R-binding domain in the various AKAPs (100 nM of both R and AKAP). GST-proteins were subsequently precipitated with glutathione-agarose beads. Precipitates were analyzed for the presence of labeled R-subunits by SDS-PAGE and autoradiography. Note: whereas covalent labeling of RII by 8-azido-[$^{32}$P]cAMP is at 1:1 stoichiometry, labeling of RI occurs at a 2:1 molar ratio, and RI will thus appear twice as strong as RII.

AKAP95 appeared not to interact with RIα (lower panel, lane 1) and interact better with RIIα (lane 3) than with RIIβ. In the case of RIα, the stoichiometry of labeling by 8-azido-[$^{32}$P]cAMP is two *versus* one for RII, thus the signal for RIα appears comparably stronger than for an equimolar amount of RII. No interaction was detected with RIβ for any of the AKAPs. Furthermore, N-terminally truncated RIα (lacking amino acid residues 1 to 45) and RIIα (lacking amino acid residues 1 to 57) did not interact with the AKAPs (data not shown). However, this kind of assay was limited to an equilibrium binding constant of about 50 nM and affinities below that could not be measured. Therefore surface plasmon resonance (SPR) was employed as a highly accurate method to determine protein-protein interaction. With this technique it is possible to measure the association and dissociation rate constants separately and thereby give an accurate measure how these proteins interact.

We employed a novel capturing chemistry to immobilize the R-subunits using an analog of cAMP, 8-AHA-cAMP, covalently coupled *via* a linker at the 8′ position of the adenine ring to a CM5 chip by amine coupling. Control experiments were performed using a surface with unmodified cAMP to ensure that under conditions used for coupling, the amine group in the 6′ position of the adenine ring does not react with the activated chip. No specific binding to this cAMP-surface was detected (data not shown). These cAMP chips bound very specifically and tightly all four types of cAMP-free (urea-stripped) R-subunit. After binding of the R-subunit, the association and dissociation of AKAP proteins could be monitored (Figure 2). A crucial basis for the determination of accurate rate constants is the highly reproducible immobilization of ligand, i.e. R-subunit on the sensor surface. Since the binding of the R-subunit to the cAMP-surface is purely a mass transfer-controlled and the dissociation rate constant is very slow (F.W.H., unpublished data), the level of immobilization can be accurately controlled by the time of injection.

To date there has been no physiological agent known to dissociate the AKAP from the R-subunit once the interaction has taken place. To overcome this problem, the entire R-subunit/AKAP complex was removed from the cAMP-surface using SDS (Figure 2). Control experiments were performed to ensure that the surface activity is unchanged after using SDS (data not shown). This efficient regeneration method allows the reuse of a single surface and the R-subunit subsequently could be captured again on the cAMP surface to determine a different AKAP interaction. The regeneration could also be performed physiologically by adding the catalytic subunit of PKA in the presence of cAMP in the dissociation buffer (data not shown).

Steric hindrance in protein-protein interactions has been shown to be a problem when using biomolecular interaction analysis, since interacting surfaces may be blocked due to the site of immobilization or because of fusion parts. In control experiments where AKAP95 was used as an immobilized ligand and the R-subunits were used

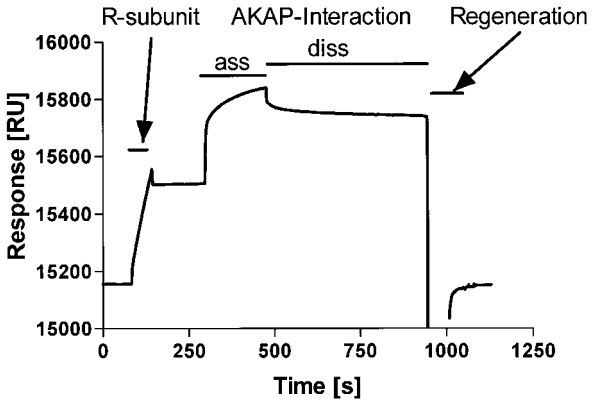


**Figure 2.** Principle of AKAP-interaction studies using surface plasmon resonance on a Biacore 2000 instrument. Binding given in response units (RU) as a function of time during the capture of cAMP-free (urea-stripped) RIIα subunit at a concentration of 50 nM on an 8-AHA-cAMP chip and the subsequent binding of AKAP95. After the interaction with the AKAP protein, the entire surface was regenerated with 0.05% SDS as indicated on the figure and R-subunit could be captured again at the same level (not shown). Control experiments using another analog of cAMP for the capture of the R-subunits yielded similar results (data not shown).

as analyte, binding stoichiometries were poor (data not shown). In the second set of control experiments, the RIα, RIβ and RIIα-subunits were used with the GST-fusion part. No binding to the AKAP proteins was observed either with the GST-R-subunit or the AKAP as immobilized ligands indicating steric problems. Once the GST-fusion part of the R-subunits was cleaved, however, binding of the RIα and RIIα subunits, but not with the RIβ subunit was restored. In contrast, GST-fusion parts on the AKAP-proteins had no influence on the binding (data not shown).

In order to screen quickly for the relative binding affinity of any AKAP to the four isoforms of the R-subunits, a single sensor chip was used with the same amount of R-subunit captured on each surface. Figure 3 shows the interactions of all R-subunits, with S-AKAP84/D-AKAP1 (a), AKAP79 (b) and AKAP95 (c). The interaction with each AKAP was then monitored simultaneously for all R-subunits together. The data clearly characterized S-AKAP84/D-AKAP1 as an AKAP with an apparent dual specificity, binding both to the RII-subunits as well as to the RIα subunit (Figure 3(a)). However, the binding to the RIα subunit was somewhat lower. AKAP79 also bound very well to the RIIα- and β form but weakly to the RIα (Figure 3(b)). No binding to the RI-subunits could be observed for AKAP95 which only interacted with RII-subunits (Figure 3(c)). None of the AKAPs tested bound to RIβ at concentrations up to 20 μM.

To obtain accurate numbers for association and dissociation rate constants, a series of different concentrations (see Figure 4 as an example) of each AKAP protein was injected over a fixed amount of R-subunit (about 250 Response Units (RUs). From those numbers, rate constants ($k_{ass}$ and $k_{diss}$) as well as equilibrium binding constants ($K_D$) were calculated (Table 1). The association and dissociation rate constants were determined simultaneously using a global fit analysis as described in Material and Methods. A Langmuir 1:1 binding stoichiometry was assumed.

As seen from the prescreen approach using only one high concentration of analyte and ligand (Figure 3), AKAP84/D-AKAP1 qualifies as a dual-specificity AKAP displaying a high level of affinity binding of $K_D = 0.5$ nM and 2.1 nM for the RIIα and RIIβ subunits, respectively, and an almost three orders of magnitude higher binding constant of 185 nM for the RIα-subunit (Figure 4 Table 1). This difference was mainly due to a 100-fold accelerated dissociation rate constant for the RIα-subunit. AKAP79 bound to RIα was even weaker, not allowing for an accurate determination of either rate or equilibrium binding constants. However, a binding was clearly detectable, as shown in Figure 3. Smaller differences were detected when comparing the binding of all AKAP proteins to RIIα and RIIβ (Figure 4, Table 1). The binding of AKAP84/D-AKAP1 and AKAP79 to RIIβ was three to fourfold weaker than binding to RIIα, which was almost exclusively due to changes in the dissociation rate constant. A much clearer difference was seen for AKAP95 which bound RIIα an order of magnitude better than RIIβ. Here the association rate constant was twofold slower, and the dissociation rate constant fivefold faster.

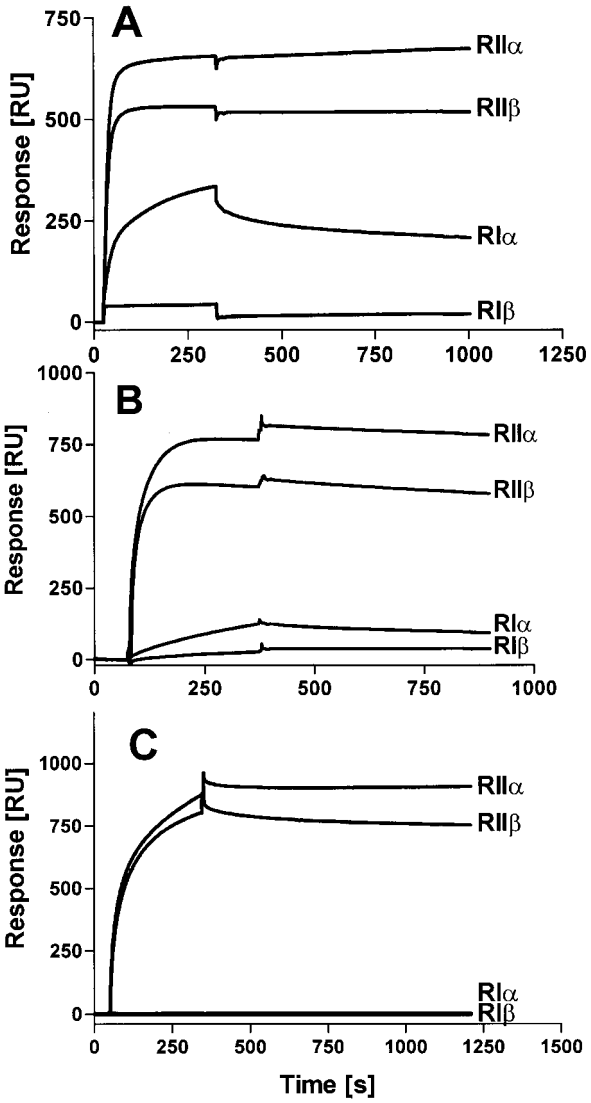


**Figure 3.** Interaction of S-AKAP84/D-AKAP1, AKAP79 and AKAP95 with immobilized RIα, RIβ, RIIα and RIIβ subunits. A sensor chip with 60 RUs of 8-AHA-cAMP immobilized on each surface was used to capture 1500 RU of each R-subunit to a separate flow cell. Each AKAP (0.5 μM) was run simultaneously over the immobilized RIα, RIβ, RIIα and RIIβ subunits for 300 seconds and the association phases of S-AKAP84/D-AKAP1 (a), AKAP79 (b), and AKAP95 (c) were monitored in 20 mM Mops, pH 7.0, 150 mM KCl, 1 mM DTT and 0.005% surfactant P 20. The dissociation phase was monitored for another 700 seconds after omitting the AKAP in the running buffer. The immobilization of the R-subunits and the regeneration are not shown.

Competition experiments with a synthetic peptide derived from Ht31 ($Ht31_{493\text{-}515}$) were performed to make sure that the interactions moni-

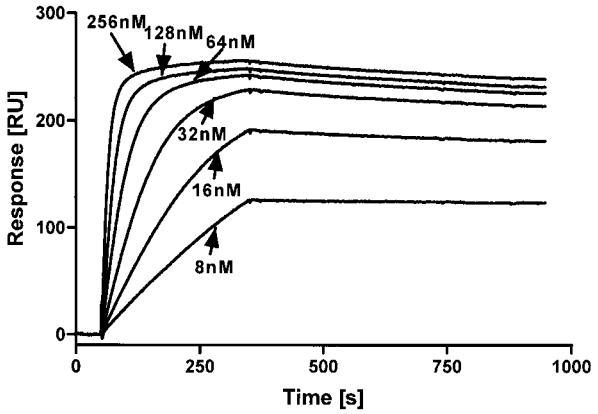


**Figure 4.** Determination of association and dissociation rate constants for the binding of S-AKAP84/D-AKAP1 to the RIIα subunit. Interaction studies were performed as described in Material and Methods with 250 RU of immobilized RIIα subunit. S-AKAP84/D-AKAP1 at concentrations indicated on the plot were injected for 300 seconds and after the end of the injection the dissociation phase without any AKAP present was monitored for 300 seconds. The interaction was monitored in 20 mM Mops, 150 mM KCl, 1 mM DTT and 0.005% surfactant P 20 at a flowrate of 30 µl/minute. After subtracting a blank surface, a Langmuir 1:1 model for the interaction was assumed using a global fit analysis algorithm provided in Biaevaluation 3.0. The plot represents a representative experiment for each AKAP-R-interaction. Each experiment was repeated at least as triplicate using different enzyme preparations.

tored with the immobilized R-subunit and the AKAP proteins as analyte were not affected by steric hindrance. From these experiments, additional detailed information about the relative binding affinity in comparison to Ht31 could be derived. In a first approach, a surface competition experiment was performed. R-subunit was immobilized to a surface concentration of 1500, 1000 and 500 RUs on three different flow cells of a chip. The designated AKAP protein was then injected in the presence or absence of 10 µM $Ht31_{493\text{-}515}$ or Ht31-P as a control. Ht31-P is the corresponding peptide to $Ht31_{493\text{-}515}$ with two isoleucine residues substituted by proline residues which disrupt the amphipathic helix structure necessary for R-subunit binding. Figure 5 is a bar diagram representing the binding of the AKAPs to the immobilized R-subunits at equilibrium in the presence or absence of the competitor peptides during the injection. Each R-subunit-AKAP interaction could be competed with 10 µM $Ht31_{493\text{-}515}$ (+Ht31) but not with Ht31-P (+Ht31-P) at the same concentration. This clearly indicates the specificity of the interaction of the respective R-subunit and the AKAP protein. Since $Ht31_{493\text{-}515}$ should bind to the immobilized R-subunits, a small increase in mass detected as an increase in resonance signal was expected. Therefore, control experiments were performed using 10 µM of $Ht31_{493\text{-}515}$ (Ht31) and Ht31p (Ht31P) without AKAP present. A significant binding could be observed for $Ht31_{493\text{-}515}$, but not for Ht31P (Figure 5).

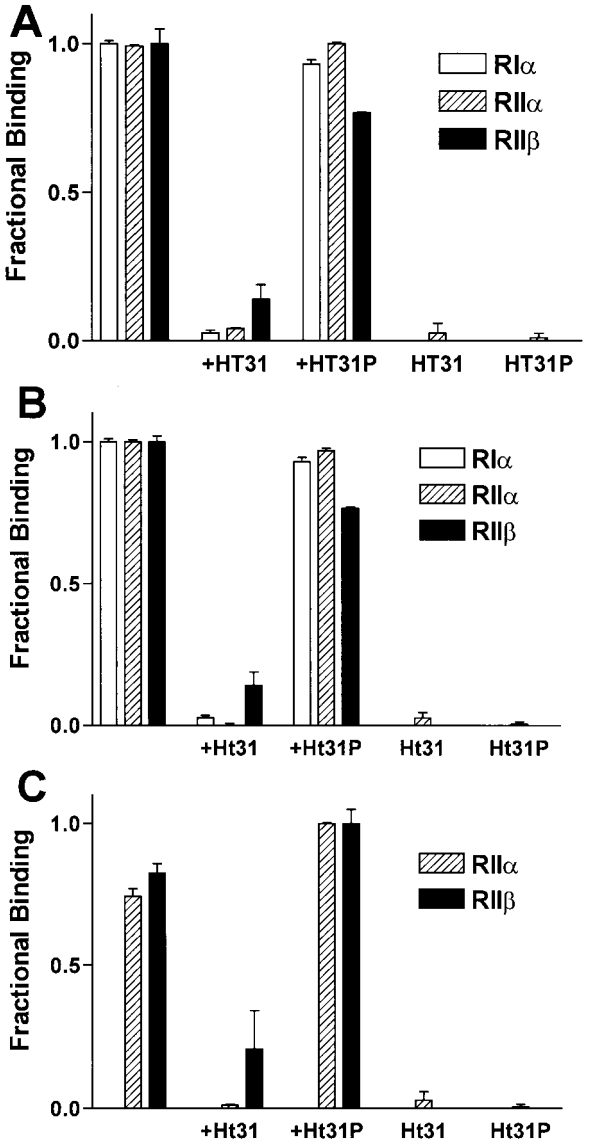


**Figure 5.** Qualitative surface competition experiments with $Ht31_{493\text{-}515}$. RIα, RIIα and RIIβ were immobilized to an 8-AHA-cAMP surface at resonance levels of 500, 1000 and 1500 RUs. S-AKAP84/D-AKAP1 (a), AKAP79 (b), and AKAP95 (c) in a concentration of 500 nM each were injected in the absence or presence of $Ht31_{493\text{-}515}$ (10 µM; +Ht31) and the residual binding to the R-subunits was determined. As a control, 10 µM of a peptide containing a proline residue in the AKAP interaction site (+Ht31P) was injected with each AKAP protein. Since $Ht31_{493\text{-}515}$ binds to the immobilized R-subunits, a small increase in mass was expected. Therefore control experiments were performed for all interactions using 10 µM of $Ht31_{493\text{-}515}$ (Ht31) and Ht31p (Ht31P) without AKAP present. A significant binding could be observed for $Ht31_{493\text{-}515}$, but not for Ht31P. Data were normalized and standard errors are given as SD from three different experiments.

**Table 1.** Apparent association and dissociation rate constants for AKAP interaction with RIα, RIβ, RIIα and RIIβ immobilized to a sensor surface

| | $k_{ass}$ ($M^{-1} \times s^{-1}$) | $k_{diss}$ ($s^{-1}$) | $K_D$ |
|---|---|---|---|
| A. *S-AKAP84/D-AKAP1* | | | |
| RIα | $6.5 \times 10^4$ | $1.2 \times 10^{-2}$ | 185 nM |
| RIβ | - | - | - |
| RIIα | $2.3 \times 10^5$ | $1.3 \times 10^{-4}$ | 0.5 nM |
| RIIβ | $2.4 \times 10^5$ | $5.0 \times 10^{-4}$ | 2.1 nM |
| B. *AKAP79* | | | |
| RIα | | | >> 1μM |
| RIβ | - | - | - |
| RIIα | $1.1 \times 10^5$ | $1.5 \times 10^{-4}$ | 1.5 nM |
| RIIβ | $9.3 \times 10^4$ | $4.2 \times 10^{-4}$ | 4.5 nM |
| C. *AKAP 95* | | | |
| RIα | - | - | - |
| RIβ | - | - | - |
| RIIα | $4.4 \times 10^4$ | $2.6 \times 10^{-4}$ | 5.9 nM |
| RIIβ | $2.7 \times 10^4$ | $1.4 \times 10^{-4}$ | 52 nM |

300 RU of the RIIα and β subunits and 600 RU of RIα were immobilized to a 8-AHA-cAMP surface. Varying concentrations of S-AKAP84/D-AKAP1, AKAP79 and AKAP95 were injected with a flow rate of 30 μl/minute as shown in Figure 4 and the complete association phase, except the first five seconds, were used for evaluation. For the dissociation phase after the initial first five seconds, 60 seconds were evaluated. Apparent association ($k_{ass}$) and dissociation ($k_{diss}$) rate constants were calculated from three different independent experiments using global fit analysis as described in Material and Methods. No kinetic data could be obtained for the RIα/AKAP79 interaction.

These qualitative surface competition experiments were extended to a more detailed study. In a first step, the binding of the R-subunits to the $Ht31_{493\text{-}515}$ peptide was determined using a biotinylated $Ht31_{493\text{-}515}$-peptide immobilized to a streptavidin chip (data not shown). Association and dissociation rate constants for the RIIα-subunit of $1.5 \times 10^5$ $M^{-1}$ $s^{-1}$ and $3.5 \times 10^3$ $s^{-1}$ were obtained yielding a $K_D$-value of 24 nM. Accordingly, values for RIIβ were determined to $1.1 \times 10^5$ $M^{-1}$ $s^{-1}$ ($k_{ass}$) and $1.6 \times 10^3$ $s^{-1}$ ($k_{diss}$), yielding a $K_D$ of 15 nM, indicating similar association behaviors of both type II R-subunits, but different dissociation patterns displaying a higher affinity for RIIβ to biotinylated $Ht31_{493\text{-}515}$ than for RIIα. Only weak binding could be observed to the RIα-subunit, not permitting a kinetic analysis.

In a second step, the concentrations of $Ht31_{493\text{-}515}$ for each injection were varied to obtain $EC_{50}$ values (see Figure legend for experimental details). Figure 6 shows a panel of data for the competition of AKAP-binding to RIα, RIIα and RIIβ. Data were obtained from competition curves taken at various R-subunit concentrations to control for concentration effects by the immobilized ligand. Table 2 summarizes the apparent $EC_{50}$-values giving a measure of how efficiently PKA interaction with each AKAP could be competed away by the peptide derived from another AKAP, Ht31. Characterization of AKAP95's interaction with PKA revealed that anchoring *via* this AKAP was competed most efficiently by $Ht31_{493\text{-}515}$. In particular, the RIIβ interaction was competed with an $EC_{50}$ of 6 nM, indicating a strongly similar binding pattern of Ht31 and AKAP95 (and the other AKAPs tested in this study). This correlates with the higher affinity of biotinylated $Ht31_{493\text{-}515}$ to RIIβ. AKAP79 was competed from RIIα only poorly by $Ht31_{493\text{-}515}$ with an $EC_{50}$ of 360 nM, however the RIIβ was competed away almost an order of magnitude more efficiently. Surprisingly, this peptide also competed anchoring of the RIα subunit with a submicromolar $EC_{50}$ value as shown for competition of S-AKAP84/D-AKAP1 binding to RIα, although only weak binding of RIα to biotinylated $Ht31_{493\text{-}515}$ was observed.

## Discussion

Compartmentalization of signal transduction enzymes into signaling complexes is an important mechanism to ensure the specificity of intracellular events. The formation of these complexes is mediated by specialized protein motifs that participate in protein-protein interactions. The N-terminal regions of both types of R-subunit, the type I and II, bind to amphipathic α-helical motifs in different AKAP proteins. However, distinct distributions of PKA isozymes are observed based on differences in both the specificity and the affinity of the R-subunit's interaction with AKAPs. Simple analyses of protein-protein interactions as GST-precipitation assays does not allow any real quantification of the interaction between RI and RII and AKAPs, which underlines the need of a more quantitative assay. Therefore, it is important to develop accurate methods to assess the apparent binding constants of the R-subunit - AKAP interaction.

Several approaches have been described recently using either the AKAP-interaction partner as a ligand (biotinylated calmodulin interacting with AKAP79 (Faux & Scott, 1997)) or using immobilized $Ht31_{493\text{-}515}$ as a ligand (Burton *et al.*, 1997; Newlon *et al.*, 1999) and utilizing a cuvette system

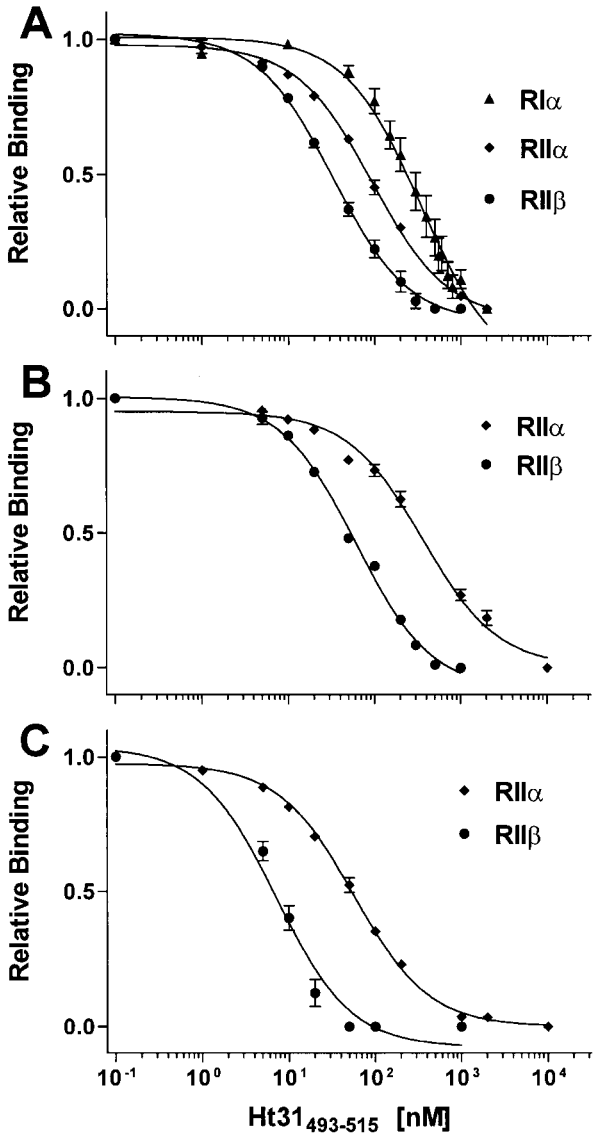


**Figure 6.** Quantitative surface competition experiments with $Ht31_{493\text{-}515}$. Each R-subunit was immobilized to a surface concentration of 500, 300 and 100 RU and AKAP proteins (200 nM, S-AKAP84 in (a) AKAP79 in (b) and AKAP95 in (c) were injected in the presence of $Ht31_{493\text{-}515}$ at 0, 5, 10, 20, 50, 100, 200, 300, 500, and 1000 nM. The residual binding of the respective AKAP to the R-subunit was plotted after an injection time of 180 seconds shown in (a)-(c). The data represent the mean of three different competition experiments with three different R-subunit concentrations to ensure that the concentration of R-subunit on the surface does not influence the competition.

(Iasys, Affinity sensors). For the interaction of biotinylated $Ht31_{493\text{-}515}$ with RIIα or a fragment of RIIα, dissociation constants ($K_D$) of 11 nM (Burton *et al.*, 1997) or 16 nM (Newlon *et al.*, 1999) was described, which is in good agreement with data presented here. For the $Ht31_{493\text{-}515}$/RIα-subunit a dissociation constant of 2.1 μM was described (Burton *et al.*, 1997). However, no data have been published so far using larger constructs or entire AKAP-proteins as interaction partners. Furthermore, to date association and dissociation rate constants of the R-subunit-AKAP interaction have not been published. Competition assays to assess the effect of Ht31 on the relative competition/disruption of various PKA-AKAP interactions have not been performed previously; however, competition assays between RI and RII with $Ht31_{493\text{-}515}$ were completed (Burton *et al.*, 1997). Here, we present a novel approach to examine directly the binding of AKAPs to all R-subunit isoforms with a high degree of accuracy and surface competition experiments with $Ht31_{493\text{-}515,}$ both of which are based on surface plasmon resonance (SPR).

SPR is an excellent technique to determine protein-protein interactions by monitoring the binding of a component in the buffer phase flowing over an immobilized interaction partner. However, besides technical limitations such as mass transfer-limited reactions and unspecific binding effects (for details, see (Herberg & Zimmermann, 1999)) several problems must be considered. Steric hindrance might limit interaction due to immobilization or to fusion parts, and indeed this problem occurred when GST-fusions proteins of the R-subunit were used. Several studies using site-directed mutagenesis or structural studies employing NMR clearly show that the very N-terminal regions of RI (Huang *et al.*, 1997b, 1999) and RII (Hausken *et al.*, 1996; Newlon *et al.*, 1997, 1999) are important for AKAP interaction. Therefore, it is very likely, that the N-terminal fusion parts interfere with the binding to the AKAP. Another problem derives from non-directed coupling of a ligand molecule (for example the R-subunit) *via* primary amine groups, possibly inducing structural or functional changes that impair specific interaction. Again, initial experiments using amine coupling with either the R-subunit or the AKAP protein were unsatisfying. Thus, it was important to develop a method for the directed immobilization of the R-subunit without changing its functional patterns. In the method presented in this study, analogs of cAMP attached to the surface were used in SPR to bind tightly the R-subunits *via* their cAMP-binding sites. This stable interaction is mainly due to rebinding and yielded an R-subunit surface with a high degree of stability. The cAMP surfaces could be reused several hundred times.

For these investigations, the R-subunit was stripped of cAMP and immobilized on a cAMP analog-surface where both the association and dissociation rates of AKAP proteins to R-subunits were determined, and the effect of a specific peptide, derived from the AKAP Ht31 ($Ht31_{493\text{-}515}$), on the R-subunit - AKAP interaction was quantified. For our experiments, we characterized the interaction of all R-subunits (RIα, RIβ, RIIα and RIIβ) with recombinant fragments of S-AKAP84/D-AKAP1, AKAP 79 and AKAP95. Interactions of the AKAP constructs as used here may influence the binding properties, since limitations due to size or method of expression might occur, however, *in vivo*

**Table 2.** $EC_{50}$ values for the competition of immobilized R-subunits with AKAP protein by $Ht31_{493-515}$ - peptide

| | S-AKAP84/D-AKAP1 (nM) | AKAP79 (nM) | AKAP95 (nM) |
|---|---|---|---|
| RIα | 355 ± 70 | N.D.[a] | - |
| RIβ | - | - | - |
| RIIα | 94 ± 3 | 360 ± 122 | 56 ± 9 |
| RIIβ | 31 ± 2 | 60 ± 3 | 6 ± 2 |

Each AKAP (200 nM) was preincubated with $Ht31_{493-515}$ in varying concentrations as described in Material and Methods and indicated in Figure 6. The residual binding to three different concentrations of the immobilized R-subunits (500, 300 and 100 RUs, respectively) was determined and plotted according to Figure 6. The normalized binding curves were averaged and a dose-response curve with a fixed slope was calculated with the software GraphPad Prism. Apparent $EC_{50}$-values are given with the SEM of three different experiments using different concentrations of immobilized R-subunit, to exclude concentration effects affecting the competition with $Ht31_{493-515}$.

[a] N.D., Not determined.

affinity of different AKAPs for R-subunits may be altered as a consequence of association of the AKAP targeting domain with its cognate subcellular docking site and by other structures in the vicinity. Thus, the *in vitro* examination of a correctly folded PKA-interaction domain of the AKAP in solution may be a better approximation of the *in vivo* situation than the examination of the full-length protein in solution. Indeed, AKAP79/75 has been precipitated or purified from cells in association with both RIIα and RIIβ, whereas there are no reports of *in vivo* association of S-AKAP84/D-AKAP1 with RIα or on *in vivo* association of AKAP95 with RIIβ. The approach used here provides a tool to characterize AKAP-R-subunit isoform interactions which are not detected in an *in vivo* assay. To date, no AKAP have been shown to interact with the RIβ subunit of PKA, and we were not able to detect interaction with any AKAP used here. However, as RIβ has been implicated in the presynaptic regulation of hippocampal LTP/LTD and *in vivo* synaptic plasticity in the visual cortex (Brandon *et al*., 1995; Hensch *et al*., 1998), one may speculate that specific RIβ anchoring proteins exist in neurons.

During the cell cycle, redistribution of PKA associated with centrosomal anchoring proteins (Keryer *et al*., 1998) and with AKAP95 (Eide *et al*., 1998) has been observed. Whereas RIIα dissociates from centrosomes at mitosis, the interaction between RIIα and AKAP95 occurs only after breakdown of the nuclear envelope at mitosis. How RIIα dissociates from centrosomal AKAPs at mitosis, and from AKAP95 in anaphase/telophase to then be excluded from the nucleus upon reformation of the nuclear envelope is unknown, but these observations indicate that there are physiological mechanisms that regulate the interaction of PKA with AKAPs; such mechanisms will be interesting to investigate in the future, both *in vitro* using the method presented here, and inside cells.

## Materials and Methods

### Reagents

8-AHA-cAMP (8-aminohexyl amino adenosine 3′-,5′-cyclic monophosphate) was purchased from Biolog, Bremen, Germany, the peptide substrate, LRRASLG, from Bachem Biochemicals, NHS, EDC and Surfactant P20, Sensor chips CM 5 research grade from Biacore AB, Sweden. Other reagents were purchased as follows: ATP and cAMP (Sigma), PMSF (Boehringer Mannheim), media supplies (Difco). All other reagents were obtained in the purest grade available.

### Expression and purification of A-kinase anchoring proteins

Human cDNA clones encoding AKAP79 and S-AKAP84/D-AKAP1 (K. T., unpublished results) were isolated by RII-overlay screening of a Jurkat T cell expression library (Stratagene, λZAP Express/EcoRI) using [$^{32}$P]-labeled human RIIα as described (Bregman *et al*., 1989). A *Hin*cII-*Eco*RI fragment corresponding to nucleotides 1830 to 2612 (encoding amino acid residues 178 to 427) of the published human AKAP79 sequence (Carr *et al*., 1992) was subcloned into the *Sma*I/*Eco*RI sites of the vector pGEX-KG (Guan & Dixon, 1991); kindly provided by Dr J. E. Dixon, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI). Nucleotides 979 to 1285 (encoding amino acid residues 285 to 387) of the published human S-AKAP84/D-AKAP1 (AKAP149) sequence (Trendelenburg *et al*., 1996) was amplified by PCR using standard conditions and primers that introduced a *Bam*HI restriction site at the 5′-end and an in-frame stop codon and an *Xba*I site at the 3′-end and subcloned into the *Bam*HI/*Xba*I sites of the vector pGEX-KG. The resulting constructs were sequenced by the dideoxy chain termination method in order to verify correct insertion of the fragments. Protein fragments AKAP79(178-427), and S-AKAP84/D-AKAP1(285-387), containing the respective RII binding domains were expressed as GST-fusion proteins in the *Escherichia coli* strain BL21/DE3 (Studier & Moffatt, 1986) and purified from bacterial lysates by absorption to glutathione-agarose beads as described (Guan & Dixon, 1991; Smith & Johnson, 1988; Tasken *et al*., 1993). A GST-AKAP95 fragment covering amino acid residues 387 to 692 with the RII binding domain of AKAP95 was expressed as described (Eide *et al*., 1998). To remove the GST-tag, purified fusion proteins were digested with thrombin (Sigma) and GST was absorbed on glutathione-agarose beads in order to yield soluble AKAP fragments.

### Expression and preparation of PKA regulatory subunits

For GST-pulldown assays, human regulatory subunits (RIα, RIβ, RIIα, RIIβ) were expressed as fusion proteins

with glutathione *S*-transferase (GST), purified and proteolytically cleaved to yield full-length R-subunits with an N-terminal extension of 15 (RIα, RIβ) and two (RIIα, RIIβ) amino acid residues from the glycine linker segment of pGEX-KG for RI and RII respectively (Solberg *et al.*, 1994; Tasken *et al.*, 1993; and K. T., unpublished results).

For surface plasmon resonance studies, the recombinant R-subunits (type Iα, Iβ, IIα and IIβ) were overexpressed in *E. coli* E222 (Saraswat *et al.*, 1986) and all except the RIβ were purified by ion-exchange chromatography on DEAE-cellulose (Buechler & Taylor, 1991) or over a 8-AHA-cAMP resin (Biolog, Bremen). RIβ was purified *via* GST-agarose chromatography and the GST-part was cleaved using thrombin (as described above). To obtain cAMP-free R-subunits, the R-subunits were unfolded with 8 M urea (RIα) or 6 M urea (all other R-subunits) as described and refolded in buffer A (150 mM KCl, 20 mM Mops, pH 7.0, 0.005 % surfactant P20 (Biacore AB)) (Buechler *et al.*, 1993). All R-subunits were additionally purified using gel filtration on a Superdex 200 column (Pharmacia) in buffer A. The proteins were then subjected to SDS-gelelectrophoresis under reducing and non-reducing conditions to check if the proteins are (1) intact, and (2) still formed dimers as described by (Solberg *et al.*, 1994). All R-subunits were checked for their ability to inhibit the C-subunit stoichiometrically using a spectrophotometric assay as described by (Cook *et al.*, 1982) (data not shown).

### GST-pull down assays

Human R-subunits were photoaffinity-labeled with 8-azido-[$^{32}$P]cAMP as described (Tasken *et al.*, 1993). Labeled R-subunits were then diluted to 100 nM and mixed separately with 100 nM of different GST-AKAP fragments in a buffer containing 20 mM Tris HCl (pH 7.4) 300 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1 % Triton X-100 and incubated at room temperature for 30 minutes. Subsequently, 20 µl of glutathione agarose beads were added and incubation continued for two hours at 4 °C with shaking, after which beads were pelleted by centrifugation at 3000 ***g*** for five minutes and washed three times in the same buffer. Precipitates were then eluted by boiling in SDS-sample buffer and subjected to SDS-PAGE and autoradiography.

### Surface plasmon resonance (SPR)

Studies on the interaction between the R-subunits of PKA and AKAP proteins were performed by SPR spectroscopy using a Biacore 2000 instrument (Biacore AB, Sweden). In this method binding partner, referred to as the ligand, is immobilized on a sensor chip and the interaction with an interactant in free solution, the analyte, is detected. Changes in the mass concentration are proportional to changes in the refractive index on the sensor surface, resulting in changes in the SPR signal (for review of the technique see (Szabo *et al.*, 1995)). To summarize the phenomenon, light reflected at an interface between media of two refractive indices separated by a thin film of conducting material will resonate (Surface Plasmon Resonance) at a specific angle and result in a reduction in intensity of the reflected light at that angle. The angle is very sensitive to refractive index changes in the less dense medium on the opposite side of the interface from the incident and reflected light. In general, the refractive index change for a given change of mass concentration at the surface layer, is practically the same for all proteins and peptides (Stenberg *et al.*, 1991). The change in the resonance angle is expressed in Response Units (RU). A response (i.e. a change in the resonance signal) of 1000 RU corresponds to a change in surface concentration on the sensor chip of about 1 ng protein/mm$^2$ (Stenberg *et al.*, 1991).

### Immobilization of 8-AHA-cAMP

CM-5 chips (research grade, Biacore AB) were activated for seven minutes with NHS/EDC according to the manufacturers instructions (amine coupling kit, Biacore AB). 8-AHA-cAMP was dissolved in 100 mM Hepes-KOH, pH 8.0 at a concentration of 3 mM by heating slightly and injected for seven minutes at a flowrate of 5 µl/minute. Each surface of a sensor chip was activated and coupled separately. Finally, all surfaces were deactivated with ethanolamine/NaCl (amine coupling kit, Biacore AB).

### Capturing of the R-subunits

All R-subunits were stripped of cAMP using urea denaturation (see above) and injected over the 8-AHA-cAMP surface at a flowrate of 5 µl/minute. Since the interaction of the R-subunit with the cAMP surface is purely mass transfer-controlled and the dissociation rate constant is extremely slow (Herberg & Zimmermann, 1999), the level of R-subunit on the cAMP-surface could be adjusted very accurately. For qualitative experiments, the surface concentration was adjusted to 300, 800 and 1200 RU of R-subunit, respectively. The association and dissociation rates of the AKAPs were determined at a flow rate of 30 µl/minute in buffer A at 20 °C using various concentrations as indicated. Regeneration was performed with a single injection of 0.2 % or 0.05 % SDS for the RI-subunits and the RII-subunits, respectively, followed by an injection of water to remove residual SDS. Each surface could be used several hundred times. To determine unspecific binding, blank runs were performed with identical samples and solutions using a 8-AHA-cAMP surface without immobilized R-subunit and these values were subtracted.

To determine the affinity between $Ht31_{493\text{-}515}$ and the R-subunits, a different experimental setup was used. $Ht31_{493\text{-}515}$ was biotinylated at the N terminus and 500 RU were immobilized on a streptavidin surface (SA-chip, Biacore AB) in buffer A. R-subunits at varying concentration between 15 to 250 nM were injected over the peptide surface and the association and dissociation phase were monitored. After each interaction cycle the entire surface was regenerated using 100 mM NaOH restoring the binding capacity of the SA-chip.

Surface activity was calculated using the equation: $S = MW_L R_A / MW_A R_L$ where S is the stoichiometry, subscript L defines ligand (immobilized protein), subscript A defines analyte (injected protein), R represents response in RUs and MW is the molecular weight of ligand or analyte.

Constants for the rates of association and dissociation of free analyte to the bound ligand were calculated from the changes in response by non-linear regression using the Biaevaluation 3.0 software. Control experiments were performed to detect mass-transfer limited interactions affecting the kinetics (Karlsson & Falt, 1997). Therefore firstly, sensor chips were produced with varied ligand density on the surface and secondly, the flow

rate was changed to overcome mass transfer limitations. For kinetic studies the immobilization level of the R-subunits was reduced to 250-300 RUs and the flowrate was set to 30 µl/minute.

Kinetic constants were calculated by non-linear regression of data using the Biaevaluation software version 2.1 or 3.0 (Biacore). The association rate constant was calculated according to:

$$R = k_a C R_{max}/(k_a C + k_d) \times (1 - e^{-(k_a C + k_d)t})$$

where $R$ is the SPR-signal in response units, $k_a$ the association rate constant, $k_d$ the dissociation rate constant, and $C$ the concentration of the injected analyte at any time $t$ during association. This equation describes the response at any time during association and can be used for non-linear regression analysis of single curves. The dissociation rate constant was calculated according to:

$$R_t = R_0 e^{-k_d(t-t_0)}$$

where $R_t$ is the response at time $t$ and $R_0$ the time at an arbitrary starting point $t_0$. With the rate constants determined and known analyte concentrations, the equilibrium binding constants were calculated according to:

$$K_D = k_d/k_a$$

A 1:1 binding model assuming Langmuir conditions was applied to the data.

Global fit analysis was used in the kinetic studies between the R-subunits and the AKAP proteins.

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