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SHORT COMMUNICATION

The identification of novel cyclic AMP-dependent protein kinase anchoring proteins using bioinformatic filters and peptide arrays

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A-kinase anchoring proteins (AKAPs) localize cyclic AMP-dependent protein kinase (PKA) to specific regions in the cell and place PKA in proximity to its phosphorylation targets. A computational model was created to identify AKAPs that bind to the docking/dimerization domain of the RII alpha isoform of the regulatory subunit of PKA. The model was used to search the entire human proteome, and the top candidates were tested for an interaction using peptide array experiments. Verified interactions include sphingosine kinase interacting protein and retinoic acid-induced protein 16. These interactions highlight new signaling pathways mediated by PKA.
Keywords: A-kinase anchoring proteins/cAMP signaling/computational prediction/peptide array/protein–protein interactions

Introduction

Protein kinase A (PKA) plays an integral role in many cellular signaling pathways (Colledge and Scott, 1999; Skalhegg and Tasken, 2000; Hundsrucker *et al*., 2006b). The PKA holoenzyme is composed of four polypeptide chains, two regulatory subunits and two catalytic subunits. The regulatory subunits bind to a catalytic subunits and maintains them in an inactive state (Kim *et al*., 2005). When the regulatory subunits become bound by cyclic AMP (cAMP) upon a rise of cAMP within the cell, the active forms of the catalytic subunits are released from the regulatory subunits. An additional role of the regulatory subunit is to mediate localization of PKA within the cell. Two regulatory subunits dimerize to form a domain referred to as the docking/dimerization (D/D) domain, and the D/D domain provides an interaction surface for A-kinase anchoring proteins (AKAPs). AKAPs localize PKA to proper cellular compartments where PKA can encounter its targets while in the active form.

The primary sequences of AKAPs are diverse and the region that binds to PKA is approximately 27 amino acids long (Burns-Hamuro *et al*., 2003). There are four isoforms of PKA's regulatory subunits RI alpha, RI beta, RII alpha and RII beta. Each can be utilized for interactions with AKAPs. The isoforms can bind to different subsets of AKAPs, and the subsets can overlap. Binding to both RI and RII isoforms means that an interaction is dual specific (Burns-Hamuro *et al*., 2003). Sequences within AKAPs that bind to the RII alpha isoform have been characterized with a position-specific scoring matrix (PSSM), as derived from an alignment of five sequences that have high affinity (Alto *et al*., 2003). The sequence motif was used to identify potential binding sites (Carnegie and Scott, 2003). The binding site motif with AKAPs for the RII interactions was also generated using more, i.e. 13, of the known binding sequences (Scholten *et al*., 2006). That motif identified a putative AKAP, the sphingosine kinase interacting protein (SKIP), within a set of proteins found by cAMP pull-down experiments of whole-cell lysates.

Interactions between PKA for AKAPs have been characterized three-dimensionally using NMR spectroscopy and X-ray crystallography (Banky *et al*., 2003; Kinderman *et al*., 2006). These structural studies confirmed the prediction that AKAP binding sequences are in an amphipathic alpha-helical conformation for the interaction with the D/D domain of the regulatory subunit of PKA (Carr *et al*., 1991). Analysis of the amphipathic helices within the AKAPs has demonstrated that the hydrophobic side is composed of small branched chain hydrophobic residues, such as valine and isoleucine, for the interactions with the RII isoform.

In the current study, an alignment of 22 known interaction sequences was used to generate a hidden Markov model (HMM) (Durbin, 1998) of sites that interact with the RII alpha isoform. The HMM was applied to the entire human proteome and potential binding sites were ranked according to their similarity to the HMM. Putative false positives were removed based on a low likelihood of adopting an alpha-helical conformation and/or were derived from a region with low evolutionary conservation. Alpha-helical propensity was assessed by a prediction from primary sequence with the P.S.HMM program (Won *et al*., 2007). Binding site conservation was tested as done previously (McLaughlin *et al*., 2006), based on the observation that protein interaction sites tend to be evolutionarily conserved (Lichtarge and Sowa, 2002).

The highest ranking peptide interaction candidates were tested by peptide array experiments. In a comparable study,

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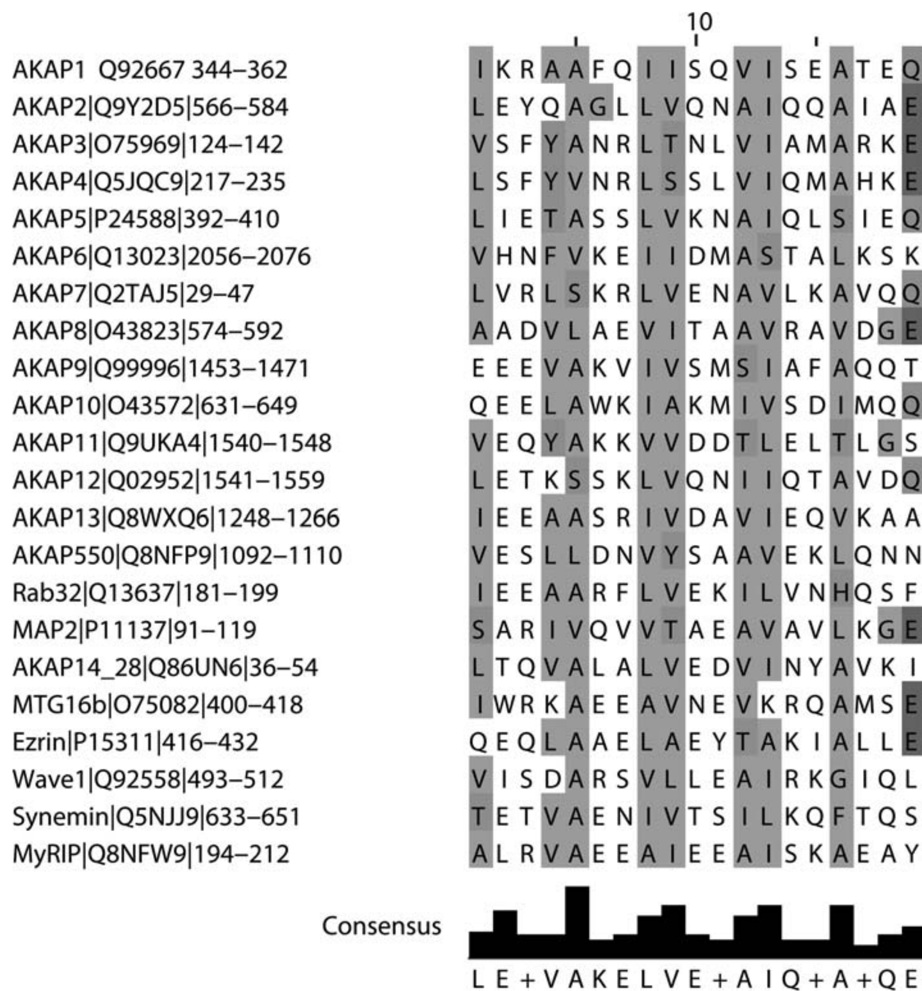

Fig. 1. Alignment of 22 sites documented to interact with the docking/dimerization (D/D) domain of the RII alpha regulatory subunit of PKA that were used to create a hidden Markov model of the binding site. SWISS-PROT accession codes and the residue ranges are given for each peptide sequence.

binding specificity measurements were derived computationally and verified experimentally with peptide array for SH3 domains (Hou *et al.*, 2009). Here we use a computational technique to predict new interactions of PKA, and putative AKAPs were validated with peptide array experiments. The signaling pathways implicated for PKA are discussed.

Materials and methods

Generation of the AKAP binding site HMM

Lists of binding sequences of RII isoform of PKA within AKAPs and their alignments were compiled by Neal Alto (personal communication of his dissertation work) and by Hundsrucker *et al*. (2006b). Fourteen of these peptide interactions were documented to interact in the context of the native proteins (Rubino *et al.*, 1989; Carr *et al.*, 1992; Carrera *et al.*, 1994; Lin *et al.*, 1995; Nauert *et al.*, 1997; Dong *et al.*, 1998; Eide *et al.*, 1998; Fraser *et al.*, 1998; Kapiloff *et al.*, 1999; Vijayaraghavan *et al.*, 1999; Witczak *et al.*, 1999; Reinton *et al.*, 2000; Wang *et al.*, 2000, 2001; Klussmann *et al.*, 2001; Alto *et al.*, 2002). Human sequences were retrieved and the list was expanded to include six more interaction sites upon review of the literature (Dransfield *et al.*, 1997; Kultgen *et al.*, 2002; Schillace *et al.*, 2002; Rawe *et al.*, 2004; Russell *et al.*, 2006; Goehring *et al.*, 2007). The 22 sequences found are presented in Fig. 1 and in the same order in the References section. For cases where the exact position of the binding site was not apparent from the documentation, an alignment with that sequence with the other binding sequences was done in the following way. Different registers of the sequence were aligned and an HMM was created based on each alignment (Durbin, 1998). The alignment that produced an HMM that retrieved all the known binding sequences at highest ranks upon the sequence database search was kept as the proper alignment. The region that directly interacts with PKA is approximately 19 amino acid long based on three-dimensional structural analysis (Gold *et al.*, 2006; Kinderman *et al.,* 2006), and the final alignment was made with that 19 amino acid stretch.

Searches and filters used to identify new AKAP sequences

The human proteomic sequences, as represented in the UniProt database (Apweiler *et al.*, 2004), timestamp 3 July 2007, were searched using the sequence HMM created from an alignment of the 22 known binding sequences of AKAPs. The secondary structural contents of the proteins retrieved by the search were predicted with the program P.S.HMM (Won *et al.*, 2007). The P.S.HMM program can predict the secondary structure based one protein sequence (Won *et al.*, 2007),

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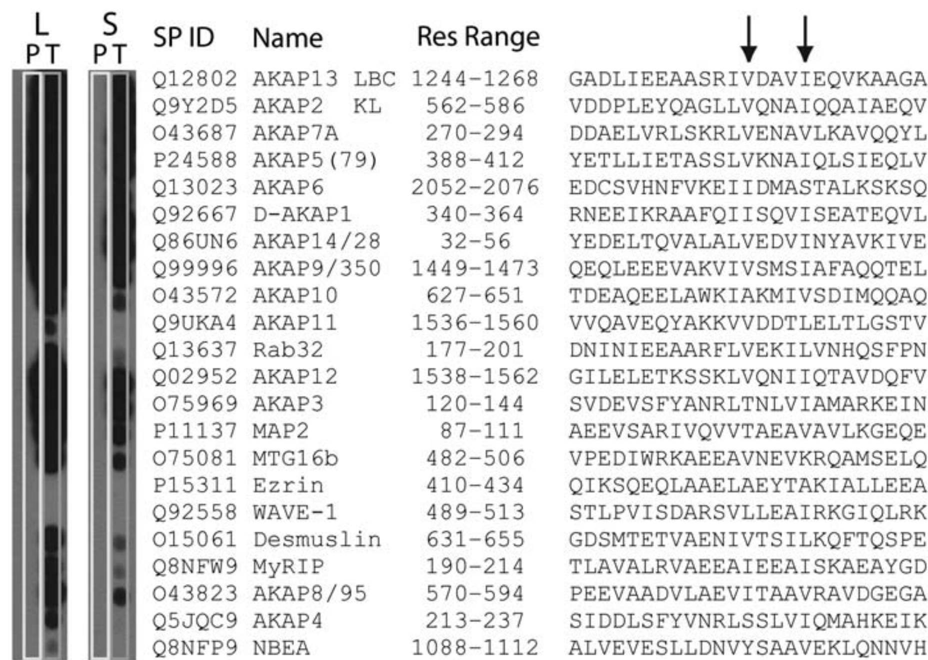

Fig. 2. Peptide array results for the 22 known binding sequences and controls with double-proline mutations. Arrows indicate the positions of the proline mutations. Peptide blots were exposed to long (L) and short (S) periods to the chemiluminescent signal. Sequences with the double-proline mutations (P lanes) were not found to bind. Of the 22 known binding sequences listed in the test lanes (T lanes), 17 indicated to bind after a short exposure. Twenty indicated binding at the longer exposure to the chemiluminescent signal.

which makes it easily applied to the each protein found by the search. If <12 residues with >80% probability of adopting an alpha-helical conformation within the entire protein found by the search, the peptide was removed as false positive. Evolutionary conservation of the binding site was tested by determining whether the corresponding peptide sequence in a mouse homolog contained the binding site motif (McLaughlin *et al.*, 2006). Homologous sequences in mouse were found as the highest scoring protein upon a BLAST search of the human proteome. Figure S1 diagrams the use of the evolutionary conservation method. Top-ranking peptides found by the sequence HMM that passed the secondary structure and evolutionary conservation filters were prepared for the analysis by experimental peptide array (Frank, 1992). Leave-one-out cross-validation was used to estimate the accuracy of predicted interactions.

Peptide array experiments

Peptide arrays were synthesized on nitrocellulose membranes using the SPOT technology as previously described (Frank, 1992; Alto *et al.*, 2003; Burns-Hamuro *et al.*, 2003; Hundsrucker *et al.*, 2006a). The peptides were 25 residues in length, as there were four residues N-terminal and two residues C-terminal to the 19mer region used to generate the HMM. The flanking residues were added to include more of the native structural context of the peptides. Peptide arrays were immersed in ethanol for 1 min and preincubated with T-TBS blocking buffer (TBS pH 7.4, 0.05% Tween 20, 5% skim milk) for 2 h. Murine RII alpha protein in blocking buffer was incubated at a concentration of 1 μg/ml with the array for 2 h followed by four 5 min washes with T-TBS. Polyclonal rabbit primary antibody against the mouse RII alpha regulatory subunit of PKA was incubated on the array in blocking buffer for 1.5 h. The array was washed four times for 5 min with T-TBS. The arrays were incubated for 1.5 h with the secondary antibody followed by four 5 min washes in T-TBS buffer. Detection was either by chemiluminescence as facilitated by a secondary antibody fused to horseradish peroxidase or by fluorescence with an antibody labeled with IRDye 800CW from Licor Biosciences. Fluorescence was detected with the Odyssey Infared Imaging System. All interactions were tested using the chemiluminescent technique. Results for the alanine scan of the SKIP and the D-AKAP1 peptides were also tested using the fluorescent technique.

Results and discussion

Computational prediction of PKA binding sequences

The alignment of the known interaction sequences of the RII alpha isoform of the regulatory subunit, shown in Fig. 1, was used to generate an HMM of the binding site motif (Durbin, 1998). The model was used to search 69 525 human proteins available in the UniProt database (Apweiler *et al.*, 2004). The top 500 ranked non-identical peptides that were most similar to the model were subsequently filtered based on evolutionary sequence conservation and secondary structure content filter described above. The remaining 126 candidates were tested by experimental peptide array. The sensitivity and specificity was estimated with a leave-one-out cross-validation. Based on the 22 known binding AKAP protein sequences and the other 70 046 protein sequences from human, sensitivity was estimated to be 0.1904 and specificity to be 0.9993.

Tests with experimental peptide array

Known binding sequences were first verified as controls. Shown in Fig. 2 are the results for 22 known binding

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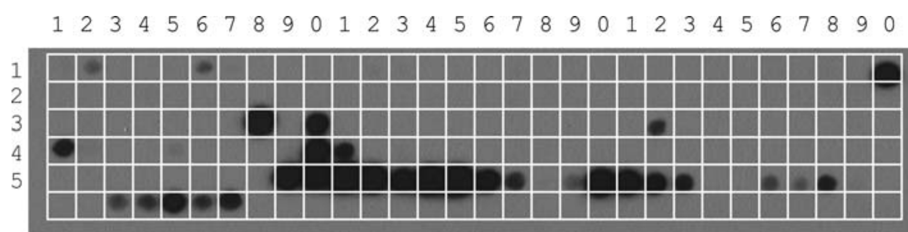

Fig. 3. Results of the peptide array experiments. The first 126 grid entries, starting from the top left are the positions of candidate peptide sequences found by the HMM sequence search. There were 10 candidates found after short exposure to the chemiluminescent signal. The following is the row and column positions: 1,2; 1,6; 1,30; 2,8; 3,10; 3,22; 4,1; 4,5; 4,10; and 4,11. These peptides were subsequently tested with alanine substitution scans.

Table I. List of the candidates found by the computational search and found to bind to the RII alpha isoform of the regulatory subunit of protein kinase A based on the peptide array overlay experiment

SWISS-PROT accession	Name	Residue range	Sequence
Q06455	MTG8B	441–465	VPEEIWKKAEEAVNEVKRQAMTELQ
Q2M3C7	SKIP protein	926–944	DIYCITDFAEELADTVVSMATEIAA
Q9Y4A5	Transformation/transcription domain-associated protein	2896–2920	PEEQQLSFIERLVEMASSLAIREWR
Q86V87	Retinoic acid-induced protein 16	348–372	YCDHLITEAHTVVADALAKAVAENF
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	1777–1801	SHRHLSDHLSELVEQTLSDLEQSKC
Q5BJF6	Cenexin	707–731	ECGTLARQLESAIEDARRQVEQTKE
O43175	D-3-phosphoglycerate dehydrogenase	48–72	CEGLIVRSATKVTADVINAAEKLQV
Q6UXC1	Apical endosomal glycoprotein precursor	930–954	SMDVQAERAWRVVFEAVAAGVAHSY
Q9HCK8	Chromodomain-helicase-DNA-binding protein 8	174–198	EHQKKQEKANRIVAEAIARARARGE
O14513	Nck-associated protein 5	206–230	QREQYERCLDEVANQVVQALLTQKD

The 10 candidate sequences with the highest intensity of the chemiluminescent signal are listed.

sequences. Proline mutations abolish binding of the peptides due to the perturbation of the alpha-helical conformation (Dell'Acqua and Scott, 1997; Hundsrucker *et al.*, 2006a), and were also used as controls. Twenty known binding peptides showed an interaction, while none of the proline mutants had an interaction. Two of the known binding sequences did not bind, possibly because the required structural contexts, as found their native proteins, were absent. In addition, for the AKAP11 protein, there may be multiple binding sequences within the protein (Reinton *et al.*, 2000; Hundsrucker *et al.*, 2006a), and the one tested may be of lower affinity.

For the 126 candidate peptides identified by the computational search, 31 showed an interaction with PKA. Of these, 10 sites were detected after short exposure to the chemiluminescent signal. These interactions are shown in Fig. 3 and listed in Table I. Site-specific alanine mutations were done to further characterize these interactions. Literature reviews were conducted to identify previous documentation for an interaction with PKA and/or an implied role in a PKA signaling.

The first of the 10 peptides was derived from a protein homologous to MTG8 called MTG16b (SWISS-PROT: O75082). MTG8 and MTG16b are known AKAPs within the same protein sequence family (Fukuyama *et al.*, 2001; Schillace *et al.*, 2002). MTG8 was chosen as the representative for list of known binding sequences. The second peptide found was from sphingosine kinase interacting protein or SKIP (SWISS-PROT:Q2M3C7). SKIP was previously been identified as an AKAP through an elution by a cAMP column pull-down experiment with PKA and AKAP11. A potential interaction site with PKA was found through a bioinformatic analysis (Scholten *et al.*, 2006). The same site within SKIP was independently identified here as a putative PKA binding site by the computational search.

As further validation of the interaction, an alanine scan of the putative binding site with the SKIP protein was performed (Figs 4 and 5). The scan demonstrated that the corresponding peptide had the specificity features of an AKAP. Positions 7 and 11 are predicted to face the hydrophobic surface of D/D domain of PKA, and the presence of branched chain hydrophobic amino acids is associated with high affinity (Gold *et al.*, 2006; Kinderman *et al.*, 2006; Chang *et al.*, 2008). Mutations to alanine are known to decrease binding (Burns-Hamuro *et al.*, 2003), as was found here. An alanine scan of a known AKAP (D-AKAP1) is provided in Fig. 4 for comparison. A recent report demonstrated that SKIP binds specifically to the RI isoform of PKA in the heart and spleen tissue (Kovanich *et al.*, 2010). The results presented here indicate that it also binds to the RII isoform. That is, it is dual specific.

Experimental evidence that SKIP is an AKAP, together with the observation that it interacts with sphingosine kinase (SK), prompted a review of its function role. An implied role of SKIP is that it integrates signaling pathways mediated by second messenger molecules sphingosine/sphingosine-1-phosphate and cAMP, and it can act as a converging scaffold protein for these signaling pathways (Lacana *et al.*, 2002; Scholten *et al.*, 2006). Changes in sphingosine activity can lead to changes in PKA activity or vice versa. Here we extend the discussion of the potential cross-talk between the cAMP and sphingosine signaling pathways (Scholten *et al.*, 2006). We describe evidence that via the cross-talk, a rise in cAMP can lead to an inhibition of apoptosis.

One study demonstrated that an increase in cAMP stimulates SK and inhibits apoptosis (Machwate *et al.*, 1998). Also, sphingosine-1-phosphate, the product of the phosphorylation of sphingosine by SK, inhibits apoptosis (Spiegel

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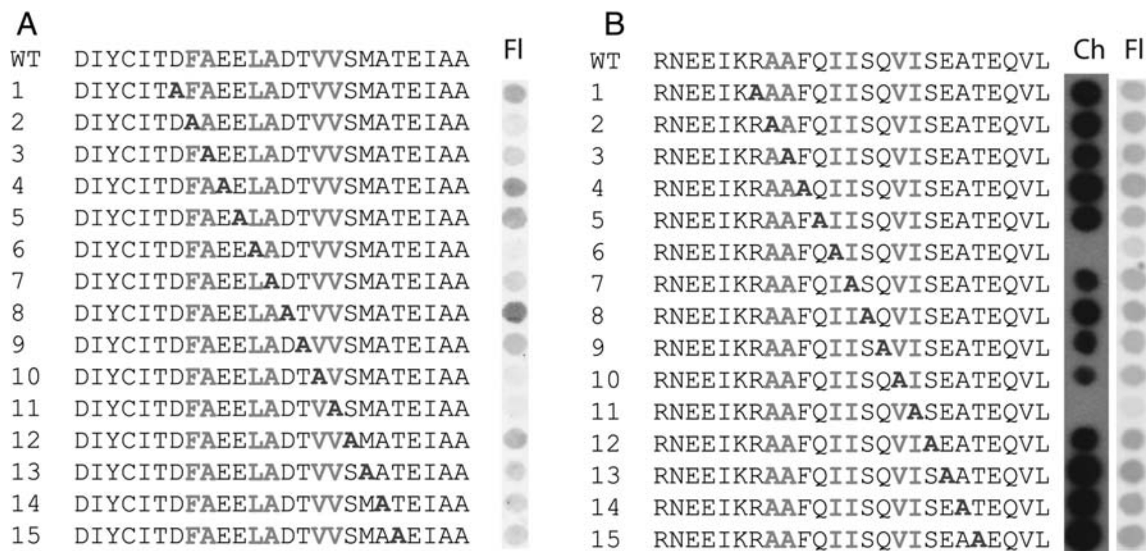

Fig. 4. An alanine substitution scan of the PKA-binding sequence within the SKIP protein is shown in (**A**). Substitutions of the branched chain amino acid residues valine, isoleucine, or leucine with alanine at the hydrophobic binding interface showed a decrease in binding affinity. That is consistent with the binding characteristics of an AKAP. A comparable alanine substitution scan of a known AKAP, D-AKAP1, in shown in (**B**). Note that alanine substitutions at positions 3, 7 and 14 correspond to the wild-type sequence. Abbreviations are Ch, detection with chemiluminescence, and Fl, detection with fluorescence.

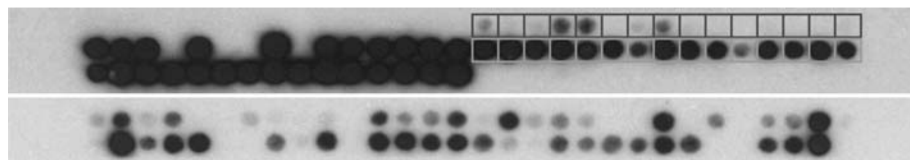

Fig. 5. Alanine substitution scans of the 10 candidates found to have relatively high affinity. The peptides are ordered left to right, top to bottom. The results for SKIP are the second group of 15 and are highlighted highlighted in white boxes. The results for retinoic acid-induced protein 16 are the fourth group of 15 and are highlighted in the white boxes. These two peptides have the binding characteristics of an AKAP.

and Milstien, 2000). The mechanism by which cAMP may activate SK may involve the colocalization of PKA with SK through dual anchoring/binding functions of SKIP (Scholten *et al.*, 2006). Close proximity of PKA with SK may place PKA substrates within reach to facilitate the cross-talk.

Evidence that colocalization of PKA and SK is required for a cross-talk is that staurosporine, a broad-spectrum protein kinase inhibitor which inhibits PKA, reduces the apoptotic-sparing effects that can be observed through an increase in SK levels (Olivera *et al.*, 1999). An increase in the inactive *unphosphorylated* SK does not lead to an increase in these apoptotic-sparing effects, which indicates that phosphorylation of SK is required. The role that PKA has on the phosphorylation of SK may be direct or through secondary kinase, possibly through an ERK1/2 kinase or a close relative (Lindquist and Rehnmark, 1998; Pitson *et al.*, 2003). In Fig. 6, we present a model for the integration of cAMP/sphingosine-1-phosphate and the suppression of apoptosis, given a rise in cAMP within the cell.

The SKIP example highlights the utility of our approach to model a novel interaction and signaling pathway of PKA. A second protein found to have the characteristics of an AKAP based on the alanine scan of the binding site is retinoic acid-induced protein 16 (SWISS-PROT: Q86V87) (Fig. 5). Further evidence that retinoic acid-induced protein 16 is an AKAP is that it has been demonstrated that PKA II activity increases upon retinoic acid-induced growth and differentiation (Kim *et al.*, 2000; Kvissel *et al.*, 2004). Also, the type I regulatory subunit of PKA is down-regulated retinoic acid stimulation; and novel forms of the PKA with its regulatory subunits are found under the stimulation (Kvissel *et al.*, 2004). The predicted AKAP may highlight a role for PKA and cAMP in signaling through previously indentified pathways that functions only under the altered expression state initiated by retinoic acid stimulation.

Interactions of PKA with AKAPs contribute the localization of PKA to specific cellular compartments (Wong and Scott, 2004), and the level of second messenger molecule cAMP is similarly controlled (Dodge-Kafka *et al.*, 2006; Zaccolo, 2009). AKAPs bind directly to phosphodiesterases to further localize cAMP concentrations (Tasken and Aandahl, 2004; Fischmeister *et al.*, 2006; Carnegie *et al.*, 2009, Houslay, 2009), and AKAPs bind to phosphatases to maintain local control of signal transduction and termination (Zaccolo, 2009). Our ability to manipulate the control points in a spatiotemporal manner may allow for advances the treatment of various diseases (Houslay, 2009; Zaccolo, 2009).

Conclusion

An integrated set of computational tools was used to predict new AKAPs that interact with the RII alpha regulatory subunit of PKA. Peptide array experiments verified some of the predictions. Novel interactions of PKA include SKIP, which is expressed cardiac muscle cells, and retinoic acid induced protein 16, which is expressed under cellular

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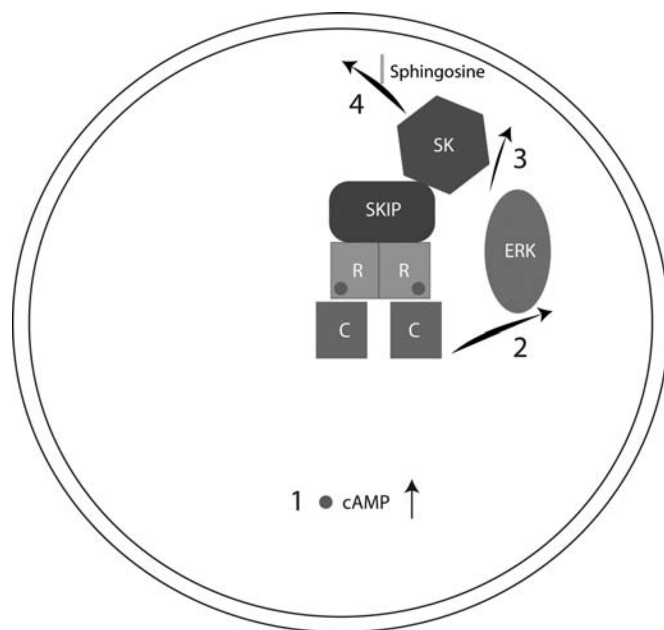

Fig. 6. A model for integration of cAMP signaling and sphingosine second messenger signaling to control apoptosis. A rise in cAMP can activates PKA that can phosphorylate ERK kinase. ERK kinase phosphorylates and activates sphingosine kinase (SK). Active SK phosphorylates sphingosine to generate sphingosine-1-phosphate. The rise in sphingosine-1-phosphate suppresses apoptosis.

activation by retinoic acid. The interactions provide starting points for further experiments to characterize the signaling pathways of PKA. As a general approach, the combination of the computational and experimental techniques described here provides a systematic way to identify more of the full complement of AKAPs. The approach can overcome challenges of the identification of AKAPs that are present in different cell types and/or present in different expression/activation states of a cell. Such interactions can remain refractory to identification through pull-down assays with PKA, as the large variety of conditions cannot be simultaneously tested. The computational technique places *bona fide* interaction sequences within reach of number of peptides that can be feasibly tested by peptide array.

Supplementary data

Supplementary data are available at *PEDS* online.

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