Structural insight into the mechanism of synergistic autoinhibition of SAD kinases

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The SAD/BRSK kinases participate in various important life processes, including neural development, cell cycle and energy metabolism. Like other members of the AMPK family, SAD contains an N-terminal kinase domain followed by the characteristic UBA and KA1 domains. Here we identify a unique autoinhibitory sequence (AIS) in SAD kinases, which exerts autoregulation in cooperation with UBA. Structural studies of mouse SAD-A revealed that UBA binds to the kinase domain in a distinct mode and, more importantly, AIS nestles specifically into the KD-UBA junction. The cooperative action of AIS and UBA results in an 'αC-out' inactive kinase, which is conserved across species and essential for presynaptic vesicle clustering in C. elegans. In addition, the AIS, along with the KA1 domain, is indispensable for phospholipid binding. Taken together, these data suggest a model for synergistic autoinhibition and membrane activation of SAD kinases.

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The SAD (synapses of amphids defective) kinases, conserved across a wide range of species, play important roles in multiple phases of axonal development and function. The founder member Caenorhabditis elegans SAD-1 was identified from a screen for mutations affecting presynaptic vesicle clustering at active zones, and SAD-1 was demonstrated to regulate axon termination and neuronal polarity. Later, mammalian SAD-A and SAD-B (also referred to as BRSK2 and BRSK1, respectively) have emerged as essential regulators of early axon–dendrite polarization and axonal arborization. The SAD kinases have also been reported to localize to synaptic sites and promote the maturation of nerve terminals or modulate neurotransmitter release in adults. In addition to the significant functions in neurons, mammalian SAD-B and the yeast orthologs Cdr2 and Hsl1 are involved in cell cycle by regulating mitotic entry and centrosome biogenesis. Recently, evidence had unravelled striking roles of the SAD kinases in diverse biogical processes renders them attractive drug targets for treating neurological diseases, cancer and metabolic disorders. Like other kinases, functions of the SAD kinases depend on their catalytic activities. The activated SAD kinases can phosphorylate various downstream effector proteins including Tau, RIM1, Wee1, Cdc25, γ-tubulin and PAK1, and thereby regulate diverse biological processes. However, little is known about the regulatory mechanisms of SAD activity.

In human genome, BRSK1/SAD-B and BRSK2/SAD-A are closely related to AMPK-a1/2 subunits and 10 other kinases (MARK1/2/3/4, SIK1/2/3, NUAK1/2 and MELK), and these kinases constitute a unique branch of the human kinome tree, the AMPK family. The principal member AMPK, a heterotrimer consisting of the catalytic α- and regulatory β-, γ-subunits, is a key regulator of cellular and whole-body energy homeostasis. Recently, AMPK activation under metabolic stress have been reported to suppress axon initiation and neuronal polarization, and Aβ-induced activation of AMPK can trigger dendritic spine loss. The MARK/Par-1 subfamily regulates neuronal differentiation and migration, as well as various biological processes including cell polarity, cell cycle control and glucose metabolism. Despite the diverse functions, the AMPK family members display similar structural organization, with an N-terminal kinase domain followed by a non-catalytic region containing an ubiquitin associated (UBA) domain and, in some cases, a kinase associated (KA1) domain. Their catalytic activities are generally coupled to phosphorylation of a conserved threonine in the kinase domain by upstream kinases, in addition, AMPK was recently reported to be synergistically activated by A-769662 and AMP, bypassing the need for threonine phosphorylation. The UBA domain in AMPK (also referred to as AID) binds to the kinase domain and plays important roles in autoinhibition and allosteric activation of AMPK holoenzyme. The KA1 domain of MARKs has been identified as membrane association module and may also regulate their kinase activities. The C-terminal non-catalytic region of SAD kinases includes two short conserved regions, SCR1 and SCR2, which respectively contain the characteristic UBA and KA1 domains. Yet, whether and how UBA and KA1 regulate SAD activity remains elusive.

In this study, structural and biochemical analyses of mouse SAD-A revealed a synergistic model for the autoinhibition of SAD activity by two separable elements, a newly identified autoinhibitory sequence (AIS) and the UBA domain. The assembly of a fully autoinhibited SAD kinase depends on the distinct intramolecular interactions among the kinase domain, the UBA domain and the AIS sequence, which provides the basis for developing specific inhibitors of SAD kinases, particularly targeting the unique pocket at kinase domain (KD)-UBA junction. We further applied the genetic approaches in C. elegans neurons and demonstrated that the cooperative autoinhibition by AIS and UBA in C. elegans SAD-1 is essential for presynaptic vesicle clustering. In addition, the AIS-KA1 fragment, but not the KA1 domain alone, can bind acidic phospholipids, which may localize SAD kinases to the membrane. We thus propose a model for the synergistic autoinhibition and membrane-binding triggered activation of SAD kinases.

**Results**

**Two fragments of SAD-A autoregulate its kinase activity.** To dissect the precise function of the non-catalytic region of SAD kinases, we examined the effects of step-wise C-terminal truncations of mouse SAD-A on enzyme activity (Fig. 1a and Supplementary Fig. 1). Bacterially expressed SAD-A proteins were unphosphorylated, and the conserved Thr175 in the activation segment could be readily phosphorylated by LKB1 (Fig. 1b). To quantitatively determine the phosphorylation stoichiometry of the SAD-A kinase domain, we employed a continuous spectrophotometric assay to monitor the dephosphorylation of SAD-A by PP2Cz. The reaction was near 100% complete in 2 min, and the phosphorylation stoichiometry was determined to be close to 1 mol of phosphate per mol of full-length SAD-A (Fig. 1c). Thus, LKB1 only phosphorylates the conserved Thr175 in the activation segment in vitro.

The unphosphorylated SAD-A proteins had no detectable enzymatic activity, while the LKB1-activated (Thr175 phosphorylated) SAD-A proteins can phosphorylate Ser216 of Cdc25C (ref. 12; Fig. 1d). Notably, the full-length SAD-A displayed a relatively low activity for Cdc25C phosphorylation (Fig. 1e). Removal of the sequence C terminal to the UBA domain by truncation at residues 519, 436 or 342 increased the initial velocity approximately sixfold, while further truncation to residue 286 resulted in an additional threefold increase. Thus, all C-terminal truncation mutants were more active than the full-length protein, and the kinase domain alone displayed the highest activity, ~18-fold higher than that of the full-length SAD-A.

We then determined the steady-state kinetic parameters of various SAD-A mutants, each showing hyperbolic concentration dependence on the Cdc25C peptide (Fig. 1f). Consistent to the increased initial velocities, the C-terminal truncations resulted in striking increase in the values when compared with the full-length enzyme. However, little change was observed for the values, indicating that the C-terminal regulatory region barely affects the binding of Cdc25C peptide to the substrate recognition site of SAD-A kinase domain. The values for these SAD-A proteins were determined to be 0.22 ± 0.04 μM −1 s−1 and represent the largest values measured for protein kinases, indicating that Cdc25C is an efficient substrate of SAD-A. These kinetic data provide clear evidence for the presence of two autoinhibitory elements within the non-catalytic region of SAD-A, the UBA region (residues 286–342) and the C-terminal fragment (519–653), both of which regulate the rate of phosphoryl transfer.

SAD-UBA binds to kinase domain in a distinct mode. To understand the autoinhibitory mechanisms, we first determined the crystal structure of mouse SAD-A fragment containing both kinase and UBA domains (Fig. 2a and Table 1). This KD-UBA structure comprises a head-to-tail dimer per asymmetric unit, which is probably a crystallization artifact owing to its monomeric state in solution (Supplementary Fig. 2a,b). The two
represent the best fitting results to the Michaelis–Menten equation with face of the catalytic cleft28,34,35. However, the relative KD-UBA domains bind to their respective kinase domains on the opposite (Supplementary Fig. 2e,f). In all KD-UBA structures, the UBA domain to the UBA domain of three-helix bundle.

The AMPK kinase family is characterized by possessing the non-canonical UBA domain, and the UBA domains are structurally conserved in spite of the low sequence identity (Supplementary Fig. 2e,f). In all KD-UBA structures, the UBA domains bind to their respective kinase domains on the opposite face of the catalytic cleft28,34,35. However, the relative KD-UBA orientations are different (Fig. 2b). The UBA/AID domain of AMPK binds to both N- and C-lobes of the kinase domain, while those of MARKs and MELK interact exclusively with the N-lobes. Distinctly, the SAD-A kinase adopts a third binding mode, mainly with the N-lobe of the kinase domain and weakly with the C-lobe.

The KD–UBA interaction in SAD-A primarily involves two helices, α3 from the UBA domain and αC from the kinase domain, which buries ~1,500 Å² exposed surface area. Six conserved residues from the UBA domain pack against a hydrophobic patch at the C terminus of the prominent αC helix (Fig. 2c). The imidazole ring of His136 from the kinase αE helix also nests into a hydrophobic pocket lined by residues from kinase αC, UBA α3 and the linker. In addition, several polar or charged side chains from the UBA domain and the linker form hydrogen bonds with residues from both kinase N- and C-lobes (Fig. 2d). For instance, Gln330 on UBA α3 tethers the two lobes by interacting with Glu67, Arg66 from helix αC and Ser137 C terminal to helix αE; in turn, Arg66 and Ser137 interact with two glutamates from UBA α3. Residues at SAD-A KD-UBA interface are conserved among SAD kinases; however, due to the different KD-UBA binding modes the interacting residues vary from one AMPK family member to another (Fig. 2e,f and Supplementary Fig. 1). In particular, GLu331 on helix α3 of the SAD-A UBA domain is charged and involved in KD–UBA interactions, while the corresponding residues in all other AMPK-RKs are hydrophobic and essential for integral of the UBA domains (Fig. 2d and Supplementary Fig. 2f). Therefore, the UBA α3 helix similarly predominates the KD–UBA interaction in AMPK family members, yet the binding modes and interacting residues are distinct.

**Figure 1 | Two elements within the non-catalytic region autoinhibit SAD-A activity.** (a) Schematic diagram of mouse SAD-A. The structural elements are coloured as follows: the kinase domain (N-lobe, pink; C-lobe, yellow; activation segment, red), UBA (slate blue), AIS (dark green) and KA1 (green). The sequence of AIS is provided, with the key residues highlighted in red. (b) Phosphorylation of SAD-A Thr175 by LKB1 analyzed using an anti-AMPK-pT172 antibody. (c) Representative time course of PP2Cz-catalyzed dephosphorylation of activated SAD. The reaction mixture contains 1μM SAD-A full-length protein and 250 nM PP2Cz. (d) Time courses of SAD-catalyzed phosphorylation of Cdc25C. Reactions were initiated by adding 5nM indicated SAD-A fragments towards 7.5 μM Cdc25C peptide. (e) Comparison of the initial rates of 5nM various SAD-A fragments towards 7.5 μM Cdc25C peptide (mean ± s.e.m., n = 3). (f) Plots of initial rate of SAD-catalyzed Cdc25C phosphorylation versus the Cdc25C concentration. The solid lines represent the best fitting results to the Michaelis–Menten equation with kcat and Km values listed at the top.

**Binding of UBA orients helix αC in an inactive conformation.** The UBA domains from different AMPK family members regulate the kinase activities through distinct mechanisms. The AMPK-AID directly inhibits the kinase domain by constraining the mobility of helix αC (ref. 28). By contrast, the UBA domains...
of MARKs and MELK indirectly regulate the catalytic activities by enhancing LKB1-activation or promoting proper folding\(^{35,36}\).

Consistent with the structural observation that SAD-A largely resembles AMPK, we found the SAD-UBA directly and specifically inhibits the activity of SAD-A kinase domain (Supplementary Fig. 3a). Structural comparison reveals that the KD–UBA interaction in SAD-A brings helix $\alpha$C into an orientation that is rotated outwards approximate 90° from that in the active kinases including AMPK ortholog Snf1 and the prototype PKA (Fig. 3a). The N-terminal portion of the partially disordered activation segment folds back into the active site, thereby inhibiting the catalytic activity, particularly the $\alpha$C helix.

To assess the UBA inhibition, we generated a series of point mutations on the KD-UBA fragment of SAD-A and evaluated their effect on kinase activity (Fig. 3b and Supplementary Table 1). When the interacting residues on or near the UBA $\alpha$3 helix were individually substituted, the catalytic efficiencies of all mutants, but R341A, were increased two to threefold, comparable to that of the kinase domain alone; in contrast, substitution of the amino acids on the linker and $\alpha$1-$\alpha$2 loop had little, if any, effect on basal activity. Similar to the truncation results shown in Fig. 1f, the effective UBA mutations led to increase in the $k_{cat}$ values but little change in the $K_m$ values (Supplementary Table 1). These results corroborate that the UBA $\alpha$3 helix plays a predominant role in displacing the orientation of helix $\alpha$C and thereby inhibiting the catalytic activity, particularly the phosphoryl transfer step, of SAD-A kinase domain.

The AIS binds to KD-UBA junction. In addition to the UBA domain, the C-terminal fragment (residues 519–653) of SAD-A can remarkably reduce the SAD activity (Fig. 1). The C-terminal regions of the yeast SAD kinase Hsl1 and certain AMPK family members were suggested to exert regulatory function as well\(^{32,33,37–39}\). This additional inhibitory fragment of SAD-A contains a KA1 domain and two flanking loops, and we carried out trans-inhibition assays to determine the precise inhibitory segment (Fig. 4a and Supplementary Fig. 4a). Unexpectedly, neither the KA1 domain alone (530–640) nor the fragment including the C-terminal loop (530–653) exhibited inhibitory effect on the catalytic activity of KD-UBA. By contrast, the SAD-A fragment or the chimeric protein containing the sequence N terminal to KA1 (506–653) effectively trans-inhibited the
Table 1 | Data collection and refinement statistics for crystals.

|                      | KD-UBA                  | KD-UBA + AIS-KA1             |
|----------------------|-------------------------|-----------------------------|
| **Data collection**   |                         |                             |
| Space group          | PT                      | CI21                        |
| Cell dimensions      |                         |                             |
| a, b, c (Å)          | 43.3, 60.7, 73.9        | 98.1, 87.3, 80.3            |
| α, β, γ (°)          | 103.7, 106.5, 107.4     | 90, 92.3, 90                |
| Resolution (Å)       | 30.00–2.00 (2.03–2.00)† | 50.00–2.49 (2.58–2.49)†    |
| Rmerge (%)           | 8.7 (46.0)              | 8.7 (71.9)                  |
| Rfree (%)            | 7.6 (36.8)              | 3.4 (27.8)                  |
| I/m (%*              | 82.2 (1.3)              | 26.9 (4.8)                  |
| CC1/2 (%)            | 0.618                   | 0.934                       |
| Completeness (%)     | 96.2 (85.7)             | 100.0 (100.0)               |
| Redundancy (%)       | 2.3 (1.9)               | 7.5 (7.5)                   |
| **Refinement**       |                         |                             |
| Resolution (Å)       | 29.68–2.00 (2.05–2.00)  | 34.58–2.49 (2.59–2.49)      |
| No. reflections      | 41,937                  | 23,731                      |
| Rwork/Rfree (%)      | 19.0/22.9               | 19.7/23.6                   |
| No. atoms            | 5,153                   | 3,440                       |
| Protein              | 0                       | 8                           |
| Ligand/ion           | 163                     | 67                          |
| Water                | 48.6                    | 54.5                        |
| B-factors            | 48.6                    | 63.7                        |
| Water                | 48.1                    | 43.3                        |
| R.m.s. deviations    | Bond lengths (Å)        | 0.008                       |
|                      | Bond angles (°)         | 1.110                       |
|                      |                         | 0.008                       |
|                      |                         | 1.085                       |

AIS, autoinhibitory sequence; KA1, kinase associated; KD, kinase domain; r.m.s., root mean squared; UBA, ubiquitin associated.

*Each data set was collected from a single crystal.
†Values in the parentheses are for highest resolution shell.
*Values for highest resolution shell.

Kinase activity. These two proteins incompletely inhibited the KD-UBA activity, plateauing at ~15%, which is consistent with the approximately sixfold inhibition in the truncation experiments (Fig. 1). Similarly, the shorter fragment containing residues 519–653, albeit with low solubility, suppressed the KD-UBA activity with comparable efficiency (Supplementary experiments (Fig. 1)). To confirm the significance of the segment N terminal to KD-UBA activity, plateauxing at 519KKSWFGNFINLE530 exerts an inhibitory effect on activity (Fig. 4a). These data clearly demonstrated that the trans- and 519–530, and both peptides the KA1 domain, we synthesized two peptide of residues 506–530 the approximately sixfold inhibition in the truncation fragment sits on the top of KD-UBA (Fig. 4b). Comparison of two KD-UBA conformations, in isolation and in complex with AIS-KA1, revealed that the AIS-KA1 binding does not induce a large rearrangement of the kinase domain, and that the major inactivating effect is likely due to the influence of AIS on the αC conformation (Fig. 4c).

Consistent with the trans-inhibition results, the non-inhibitory KA1 domain makes few contacts with KD-UBA (Supplementary Fig. 4b). Contrarily, the AIS sequence, adopting an extended conformation, makes many interactions with both the kinase and UBA domains (Fig. 4d,e and Supplementary Fig. 4c). Two consecutive aromatic residues from the AIS, Trp522 and Phe523, penetrate into a hydrophobic crevice between the β-sheet and αC helix of kinase domain. The following Phe526 and Ile527 stick into an adjacent hydrophobic pocket formed by residues from three helices, the kinase αC and the UBA α1, α3. The conformation of AIS is further stabilized by multiple hydrogen bonds, directly or water mediated; in particular, Trp522 and Ser521 from the AIS interact with two acidic residues Glu87 and Asp84 on the kinase β4 strand. The extensive interactions ensure the stable complex between KD-UBA and AIS-KA1 and reinforce the inactive conformation of the essential αC helix. More importantly, the AIS sequence pries away the N-lobe β-sheet from the αC helix, resulting in further separation of the pivotal Lys49 and Glu67 (Supplementary Fig. 4d). These structural observations and the incomplete trans-inhibition provide evidence for a non-competitive manner of AIS inhibition.

UBA and AIS synergistically autoinhibit SAD activity. To confirm the AIS autoinhibition, we first generated various AIS and KA1 mutations on the AIS-KA1 fragment and examined their effects on the trans-inhibition on wild-type KD-UBA (Fig. 5a). Individually mutating four central hydrophobic residues of AIS to charged side chains largely abolished the AIS inhibition on the KD-UBA activity; however, substitutions of three interface residues on the KA1 domain had no effects. These data corroborated that it is the AIS, but not the KA1 domain, that exhibits the inhibitory effect.

Unexpectedly, the AIS-KA1 fragment did not inhibit the activity of kinase domain alone, indicating that the AIS autoinhibition on SAD activity requires the UBA domain (Supplementary Fig. 5). To examine the importance of UBA, we then carried out trans-inhibition assay with various KD-UBA.
proteins bearing UBA mutations (Fig. 5b). Indeed, mutations of several essential KD-UBA interface residues (E331K, I334D and L337D) completely or greatly abolished the AIS inhibition. However, the AIS retained evident inhibition on activities of some KD-UBA mutants (E328A, Q330A and M333D) in spite of their dramatic effects on UBA autoinhibition. One potential explanation is that the AIS makes interactions with both the kinase and UBA domains, which partially compensates the effect of these mutations. Notably, substitution of Leu310 and Arg341 also restored both synaptic organization at the distal end of DA9’s dendritic puncta; by contrast, 30% of the single mutant animals display the ectopic phenotype with less than three ectopic vesicle clusters in the dendrite (Fig. 6d,e), while the double mutants (SI W637D/F638D and SI L641D/A642D) caused more severe polarity defects (Fig. 6f,g). The ectopic puncta of synaptic vesicles were restricted to the axon and no puncta were detected at the dendritic region41 (Fig. 6b). Overexpression of sad-1 led to increased SAD-1 activity and thereby stimulated the ectopic accumulation of synaptic vesicles in the DA9 dendrite, similar to that observed in ASI neuron1 (Supplementary Fig. 6b). To examine the importance of synergistic autoinhibition, we mutated the conserved interacting residues on UBA α3 helix or in the AIS sequence, and introduced a single-copy insertion of sad-1 into C. elegans. Animals bearing the single-copy insertion of wild-type sad-1 (SI wt) displayed similar phenotype as wildtype (Fig. 6c). Imaging analysis of sad-1 point mutations (SI M361D and SI L641D) revealed ectopic vesicle clusters in the dendrite (Fig. 6d,e), while the double mutants (SI W637D/F638D and SI L641D/A642D) caused severe polarity defects (Fig. 6f,g). Barely 30% of the single mutant animals expressing the double mutants have more than four ectopic puncta (Fig. 6h). In addition, we determined whether these mutations could rescue the DA9’s phenotype in the sad-1(ky289) protein-null animals (Supplementary Fig. 6c). As expected, the wild-type sad-1 restored both synaptic organization at the distal end of DA9’s axon and neuronal polarity in the dendrite region42–44. The UBA and AIS mutants, by contrast, rescued synaptic organization defect but not neuronal polarity defect. The ectopic accumulations of synaptic vesicles in the mutant rescue experiments were likely due to the increased activities of these sad-1 mutants. Together, the in vitro and in vivo experiments demonstrated that the SAD activity was cooperatively regulated by AIS and UBA, and this synergistic autoinhibition is of great importance for axonal–dendritic polarity.

To date, there are 5,911 reported single-nucleotide polymorphisms (SNPs) for human BRSK2/SAD-A (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?genid=9024) and 2,262 for BRSK1/SAD-B (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?genid=84446). In particular, seven UBA and one AIS residues of hBRSK2 and seven UBA and five AIS residues of hBRSK1 are altered (Supplementary Fig. 6d). Most of the SNPs would have little effect on the UBA and AIS autoinhibition, including the conserved substitution of the important AIS residues, human BRSK2 F525L and BRSK1 L600V (corresponding substituted, the catalytic efficiencies of the single or double mutants were evidently enhanced, as that observed in the trans-assay shown in Fig. 5a. Particularly, the double mutation of Trp522 and Phe523 (WF/DD) exhibited the same activity as the KD-UBA fragment, completely abolishing the AIS inhibition. We also generated triple mutations to release both the UBA and AIS inhibitions, and these mutants indeed yielded dramatic increase in the SAD activity, comparable to that of the kinase domain alone. By contrast, the KA1 mutations had no effect. Taken together, the structural and biochemical data demonstrated that the AIS and UBA synergistically inhibit the SAD activity by orienting helix αC into an inactive conformation.

Synergistic autoinhibition is essential for SAD function. Considering the highly conserved sequence and the identical domain organization, we proposed that the synergistic autoinhibition observed in mouse SAD-A would most likely be conserved in C. elegans SAD-1 (Fig. 6a and Supplementary Fig. 1). Indeed, the AIS sequence from SAD-1 can efficiently inhibit the KD-UBA activity in vitro (Supplementary Fig. 6a). SAD-1 was known to regulate neuronal polarity and synaptic organization in C. elegans, and the kinase activity was required for its functions1,40. We then analyzed presynaptic vesicle clustering in DA9 motor neuron using GFP::RAB-3 as marker. In wild-type worm, the synaptic vesicles were restricted to the axon and no puncta were detected at the dendritic region11 (Fig. 6b).
to mouse SAD-A Val303, Phe313 and Met307, would disrupt the intra-UBA hydrophobic interactions and thus destabilize the UBA structure. Notably, the V319I mutation of human BRSK1 was reported in the large cell carcinoma of the lung. Therefore, some SNPs might affect the UBA and AIS function, and more studies are needed to explore their physiological significances.

Both AIS and KA1 are indispensable for phospholipid binding. The KA1 domain of SAD-A folds into two helices and a five-stranded β-sheet, which, despite the low sequence homology, is structurally conserved to the KA1 domain of MARKs and the C-terminal domain of AMPK α-subunits 46,47 (Fig. 7a,b and Supplementary Fig. 7a,b). Since the KA1 domain of MARKs can mediate membrane association, we performed in vitro protein-lipid overlay assays with various mouse SAD-A C-terminal fragments (Fig. 7c). The C-terminal fragments of SAD-A, including both the AIS sequence and the KA1 domain, were able to bind to acidic phospholipids, such as phosphatidylserine, phosphatidylinositol-4,5-bisphosphate and phosphatidic acid. Unexpectedly, the KA1 domain alone failed to bind to phospholipids, which indicated that the AIS sequence is required for lipid binding of SAD-A.

Certain surface-exposed basic residues from the KA1 domains are required for membrane association of MARK and Cdr2 kinases 31,48; however, the distribution of positively charged patches on SAD-A KA1 domain is different (Fig. 7b and Supplementary Fig. 7b). There are two spatially adjacent clusters of basic residues at one end of the KA1 domain, one in the β3'-β4' hairpin and the other at the N terminus of helix α2'. In addition, the AIS sequence contains two consecutive basic residues (Fig. 7b). We then validated the importance of these basic clusters for lipid binding (Supplementary Fig. 7c). Replacement of the positively charged side chains in each cluster by uncharged Ser led to complete lipid detachment of the SAD-A AIS-KA1 fragment; by contrast, substitution of three discrete basic residues on the other end of SAD-KA1 had no effect on lipid interaction. These results suggest that the phospholipid binding and likely membrane association of the SAD kinases require three basic clusters within the AIS-KA1 regulatory element of SAD autoinhibition and lipid binding.

Discussion

Protein kinases are important regulators of intracellular signal transduction, and the precise regulation of kinase activity is
AMP family members (Supplementary Figs 1 and 7a). Thus, we propose that the synergistic autoinhibition of AIS and UBA is unique to the SAD kinases. However, it is an open question whether the variant linkers between the UBA domain and the AIS sequence and the divergent C-terminal tails following the KA1 domain play certain role(s) in SAD regulation.

Several molecular mechanisms have been described for kinase regulation, such as restricting the orientation of helix αC, hindering the binding site for ATP or substrate and modulating the conformation of activation segment11–13. In SAD-A, AIS and UBA synergistically act on helix αC, immobilizing the kinase domain in an ‘αC-out’ inactive conformation or slowing down the transition rate from inactive to active form. Noteworthily, this extensive interface involves three out of four spatially conserved pockets (i, ii and iv) on helix αC, presenting in different kinase branches of the kinome tree25 (Fig. 7d). The aromatic rings of two highly conserved AIS residues Trp522 and Phe523, respectively, penetrate into pockets i and iv, and several hydrophobic residues from SAD-UBA insert into pocket ii. More importantly, the other two essential residues of AIS, Phe526 and Ile527, nestle into a specific binding pocket between the kinase and UBA domains (Fig. 7d). This long, narrow AIS-binding site, including the conserved αC pockets i, iv and the specific KD-UBA interface pocket, is present only in SAD kinases. The UBA domains from AMPK, MARK and MELK would sterically clash with the bound AIS of SAD-A, especially residues Phe526 and Ile527 (Supplementary Fig. 7d). Consistently, the AIS sequence of SAD-A cannot inhibit the kinase activities of the KD-UBA fragments from AMPK and other AMPK-RKs (Supplementary Fig. 7e). This unique autoinhibition by AIS provides the basis for developing specific inhibitors of SAD kinases, particularly targeting the specific pocket at the junction of kinase and UBA domains.

Release of autoinhibition can occur by several mechanisms, including post-translational modification, interaction with regulatory molecules and lipid/membrane association19,56–58. The membrane association of MARK via its KA1 domain has been suggested to coincide with the increase of kinase activity30,31. SIK2 can co-localize with the p97/VCP ATPase to ER membrane and regulate ER-associated protein degradation59. Recently, evidence suggested that AMPK locates on the endosomal surface where it can be activated by LKB1 at lower AMP threshold concentrations on energy stress60. Thus, membrane localization might be a common mechanism for the regulation and function of AMPK family members. The SCR2 region of human BRSK1/SAD-B has been found associated with synaptic vesicles, and the C-terminal fragment of Schizosaccharomyces pombe SAD kinase Cdr2 was required for its anchoring to the cortex7,48. Since the AIS-KA1 fragment of SAD-A can interact with acidic phospholipids (Fig. 7c), we believe that AIS-KA1 might target SAD-A to particular membrane locations. However, all three basic clusters required for lipid binding are sterically buried or hindered in the autoinhibited SAD, which suggests that the membrane association of AIS-KA1 would drag the AIS away from the KD-UBA junction and thereby activate, at least partially, the SAD kinase (Fig. 7e). To verify this hypothesis, we then carried out the protein-lipid overlay assay with full-length SAD-A. Unexpectedly, the full-length protein did not interact with phospholipids (Supplementary Fig. 7f). Moreover, when the AIS-KA1 fragment was preincubated with KD-UBA, the complex of AIS-KA1 and KD-UBA did not bind phospholipids either. These data suggest that the presence of the KD-UBA fragment can block the lipid binding of AIS-KA1, or the AIS-KA1 fragment in the intact SAD kinase prefers to interact with KD-UBA, rather than phospholipids or membrane.
Then, how would SAD kinases be activated and located to membrane? The C-terminal domain of AMPK α-subunit mediates assembly of AMPK holoenzyme by interacting with the β- and γ-subunits (Supplementary Fig. 7b). The scaffold protein GAB1 and death-associated protein kinase can bind the non-catalytic region of MARKs and regulate their kinase activities. Moreover, the synaptic protein neurexin can bind to the C-terminal non-catalytic region of C. elegans SAD-1 in vivo and in vitro, and this physical interaction plays a role in regulating neuronal polarity. The cytoskeletal septin filaments had also been proved to directly associate with the C-terminal region of Saccharomyces cerevisiae SAD ortholog Hsl1, which localizes Hsl1 to the bud neck and relieves its autoinhibition. We thus speculate that the certain regulatory proteins might bind to the non-catalytic region of SAD kinases, alter the intramolecular interactions between AIS, UBA, and kinase domain, and lead to relief of the AIS (and UBA) inhibition. Then, three exposed basic clusters in the free AIS-KA1 fragment can cooperatively bind to acidic phospholipids, resulting in a membrane-bound, activated SAD kinase (Fig. 7e). Indeed, the SAD-A W522D/F523D mutant alone was capable of binding to acidic phospholipids in the protein-lipid overlay assay, confirming that the AIS-KA1 fragment in the AIS-released/free full-length SAD-A plays a bona fide phospholipid binding role (Supplementary Fig. 7f). Based on the results presented in this study and knowledge of AMPK family, we propose this synergistic autoinhibition and lipid/membrane activation model for the SAD-A/BRSK2 kinases. We are keenly interested in the remaining questions, such as which regulatory protein(s) could release the synergistic autoinhibition by UBA and AIS and trigger membrane association of SAD kinases, and whether drugs could activated SAD-A independently of the Thr175 phosphorylation, similar to that reported for AMPK (ref. 27).

**Methods**

**Protein preparation.** The complementary DNAs of mouse SAD-A and C. elegans SAD-1 were kindly provided by Dr Joshua R. Sanes (Harvard University) and Dr Mei Zhen (University of Toronto), respectively. The full-length SAD-A and various fragments were amplified by standard PCR and cloned into pGEX-6P-1 and/or pET21b vectors with N-terminal GST-tag or C-terminal His6-tag. The short segments containing the AIS sequences of mouse SAD-A (residues 506–530) and C. elegans SAD-1 (621–645) were fused to a C-terminal thioredoxin-tag to generate high-quality peptides. All site-specific mutations were generated by overlap PCR procedure and verified by DNA sequencing. The plasmids of human LKB1, STRADα and MO25α were kindly provided by Dr Gail Fraser and Dr Maria Deak (University of Dundee), respectively. The active LKB1-STRAD-MO25 complex was similarly expressed in S9 cells and purified. The human pET28a-PP2Cx expression plasmid was a generous gift from Dr Mark Solomon (Yale University).

 alors, overexpressed in Escherichia coli BL21 (DE3) cells at 18 °C, were purified over Ni-NTA (Qiagen) and MO25x were kindly provided by Dr Gail Fraser and Dr Maria Deak (University of Dundee), respectively. The active LKB1-STRAD-MO25 complex was similarly expressed in S9 cells and purified. The human pET28a-PP2Cx expression plasmid was a generous gift from Dr Mark Solomon (Yale University).

All proteins, overexpressed in E. coli BL21 (DE3) cells at 18 °C, were purified over Ni-NTA (Qiagen) or GST (GE Healthcare) columns, followed by ion exchange and gel filtration chromatography (Source-15Q/1S and Superdex-200/75, GE Healthcare). The purified proteins in a buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl and 2 mM DTT were stored at −80 °C and subjected to crystallization trials. Protein stocks used for enzymatic assay were supplemented with glycerol at a final concentration of 20% (v/v).

**Phosphorylation of SAD-A by LKB1.** The SAD-A proteins (5 μM) were incubated with the LKB1 complex (10 nM) in a phosphorylation buffer containing 50 mM MOPS, pH 7.0, 2 mM DTT, 100 mM NaCl, 10 mM MgCl2 and 1 mM ATP at 25 °C. The reactions are initiated by adding LKB1. At indicated time intervals, aliquots were removed from the reaction mixture and phosphorylation was terminated by adding EDTA to a final concentration of 50 mM. The samples were then subjected to western blot analysis to examine the phosphorylation state of the conserved Thr175 in the kinase activation loop of SAD-A using an antibody against the Thr175 phosphorylation.
phospho-Thr172 of AMPK (1:5,000; Cell Signaling Technologies, #2535). The uncropped blots were shown in Supplementary Fig. 8.

The maximum phosphorylation was observed after 60 min. To generate fully phosphorylated proteins, various SAD-A fragments or mutants (5 μM) were incubated with 50 nM LKB1 and 1 mM ATP for at least 60 min. The samples were then subjected to a coupled kinase assay for SAD-A by using a Cdc25C peptide as substrate.

**Figure 7 | Model for the autoinhibition and activation of SAD-A.**

(a) Sequence alignment for the AIS-KA1 regions of SAD and MARK kinases. The positively charged residues required for phospholipid binding are highlighted in blue. h, human; m, mouse; Sc, Schizosaccharomyces pombe; sc, Saccharomyces cerevisiae. (b) Three basic clusters in the AIS-KA1 fragment. The basic residues are shown as blue sticks, and the surface representation is coloured according to electrostatic potential (positive, blue; negative, red). (c) Lipid-binding assays for two representative C-terminal fragments. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Chl, cholesterol; SPM, sphingomyelin; PS, phosphatidylserine; PIP2, phosphatidylinositol-4,5-bisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine. (d) Conserved (i–iv) and specific (SAD) hydrophobic pockets surrounding helix αc. Three AIS-binding pockets are indicated by green circles, and that for UBA binding is coloured in blue. For clarity, only helices α1 and α2 of UBA are displayed.

(e) Regulation model for SAD-A. The extensive intramolecular interactions keep SAD-A in an autoinhibited conformation, where the kinase activity is synergistically inhibited by UBA and AIS. Membrane association of the AIS-KA1 fragment, probably triggered by regulatory protein(s), might release the AIS (and UBA) inhibition.

**Kinetic analysis of SAD kinases.** The kinase activity of SAD-A was determined using the synthetic Cdc25C peptide (201DQEAKVSRSGLYRSPSPMENLNRPRLKQVE230, SciLight Biotechnology) as substrate. The standard assay was performed with 5 mM SAD-A protein (full-length, fragments or mutants) and indicated amounts of the Cdc25C peptide at 25 °C, in 1.8-ml reaction mixture containing 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, 1 mM ATP, 200 μM NADH, 1 mM phosphoenolpyruvate, 20 U ml⁻¹ lactate dehydrogenase, and 15 U ml⁻¹ pyruvate kinase. This spectrophotometric kinase assay couples the production of ADP with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. Progress of the reaction was monitored continuously by following the decrease of NADH at 340 nm on the PerkinElmer Lambda 45 spectrophotometer, and the initial rates were determined from the linear slopes of the progress curves. The kinetic parameters (kcat and Km) were obtained by fitting the experimental data to the Michaelis–Menten equation using a nonlinear regression analysis programme. The concentrations of ADP generated in the SAD-catalyzed reaction were calculated with an extinction coefficient for NADH of 6,220 M⁻¹ cm⁻¹ at 340 nm, and concentration of the Cdc25C peptide was determined by turnover with the activated SAD-A under condition of limiting Cdc25C peptide.

The trans-inhibition studies were performed at 25 °C in the 1.8-ml kinase reaction mixture with indicated inhibitory fragments containing the UBA domain or the AIS sequence. The AIS peptide (519KKSWFGNFINLE530) and the AIS-containing peptide (506MSNLTPESSPELAKSWFGNFINLE526) were synthesized...
was fitted to the non-competitive inhibition equation

\[
m_100 \frac{[S]}{[S] + K_a}
\]

Standard refinement was performed using Phenix66 and Coot67. The data with MARK1 KD-UBA structure (PDB code: 2HAK) as search model. The reaction was initialized by adding the substrate Cdc25C peptide (20

\[
\text{AMPK KD-UBA/AID (100 nM, towards 10}
\]

The 3 kb promoter sequence of sad-1 (Psd-1) was amplified from C. elegans genome and cloned into pCF151 through Spel/Xhol sites. The wild-type or mutant sad-1 sequence was inserted into pCF151-Psd-1 through Xhol site. All constructs were verified by DNA sequencing.

The nematode strains (N2, EG4322 and wlsy85) were maintained, cultured and crossed using standard techniques69. The single-copy insertion of transgenes was verified by DNA sequencing. The data processing and refinement statistics were summarized in Table 1. The structure validated was cca1.25, but using Molprobity all protein residues in the favoured and allowed regions of the Ramachandran plot, and none are in disallowed regions. The clash score were 6.7 (94th percentile) for the KD-UBA structure and 5.16 (99th percentile) for the KD-UBA-AIS-KA1 structure, while the overall MolProbity score was 1.97 (74th percentile) and 2.02 (95th percentile), respectively. All structural representations in this paper were prepared with PyMOL (http://www.pymol.org).

Observation of presynaptic puncta in C. elegans DA9 neuron.

Lipid overlay assay. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, sphingomyelin and cholesterol were purchased from Avanti Polar Lipids, phosphatidylinositol-4,5-bisphosphate (PIP2) was purchased from Echelon Biosciences, and phosphatidic acid was purchased from Larodan Fine Chemicals. The phospholipids were dissolved in methanol/ chloroform/water (2:1:0.8, v/v/v), and 0.5 nmol of each phospholipid was spotted and air-dried on immobilon-NC membrane (Millipore). The membrane was blocked with 4% BSA for 1 h at room temperature, and then incubated with GST-tagged SAD-A proteins for 4 h at 4°C. After extensive washing with PBS buffer, the bound proteins were detected by immunoblotting analysis with an anti-GST antibody (1:5,000; Sigma-Aldrich, G1160).

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