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Identification of a haem domain in human soluble adenylate cyclase

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Synopsis

The second messengers cAMP and cGMP mediate a multitude of physiological processes. In mammals, these cyclic nucleotides are formed by related Class III nucleotidyl cyclases, and both ACs (adenylate cyclases) and GCs (guanylate cyclases) comprise transmembrane receptors as well as soluble isoforms. Whereas sGC (soluble GC) has a well-characterized regulatory HD (haem domain) that acts as a receptor for the activator NO (nitric oxide), very little is known about the regulatory domains of the ubiquitous signalling enzyme sAC (soluble AC). In the present study, we identify a unique type of HD as a regulatory domain in sAC. The sAC-HD (sAC haem domain) forms a larger oligomer and binds, non-covalently, one haem cofactor per monomer. Spectral analyses and mutagenesis reveal a 6-fold co-ordinated haem iron atom, probably with non-typical axial ligands, which can bind both NO and CO (carbon monoxide). Splice variants of sAC comprising this domain are expressed in testis and skeletal muscle, and the HD displays an activating effect on the sAC catalytic core. Our results reveal a novel mechanism for regulation of cAMP signalling and suggest a need for reanalysis of previous studies on mechanisms of haem ligand effects on cyclic nucleotide signalling, particularly in testis and skeletal muscle.

Key words: cAMP, carbon monoxide, haem domain, nitric oxide, soluble adenylate cyclase

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INTRODUCTION

The ubiquitous second messenger cAMP mediates a multitude of physiological processes, including glucagon signalling, learning and memory and transcriptional regulation [1]. In mammals, cAMP is generated by a family of ACs (adenylate cyclases), and it mediates its effects via three main targets: PKA (protein kinase A), EPAC (exchange proteins directly activated by cAMP) and CNG (cyclic nucleotide-gated) ion channels [2–4]. All the known eukaryotic ACs, as well as the closely related GCs (guanylate cyclases), belong to the Class III family of cyclases defined by homology within the catalytic domains [2,5]. Two forms of Class III ACs exist in mammals [2]: tmACs (transmembrane ACs) and sAC (soluble adenylate cyclase). The well-characterized tmACs, made up of nine isoforms termed I–IX, are responsive to heterotrimeric G-proteins, which are regulated through binding of extracellular ligands to GPCRs (G-protein-coupled receptors) [1,3]. Thus these ACs predominantly mediate intercellular communication [1]. In contrast, the single known sAC of mammalian cells is an intracellular signalling enzyme uniquely activated by bicarbonate and calcium [2,6–8]. sAC regulates processes such as sperm activation [9], pH homoeostasis in epididymis and kidney [10,11], CO_2-dependent control of ciliary beat in airway [12] and mitochondrial respiration in liver and brain [13,14].

Class III nucleotidyl cyclases have two identical or structurally related catalytic domains: C_1 and C_2, which form dimeric catalytic cores with shared active sites at the dimerization interface [15–18]. In mammalian tmACs and sAC, C_1 and C_2 reside on the same polypeptide chain rendering the core a pseudo-heterodimer. Besides the catalytic domains, sAC and tmACs are completely unrelated. tmACs possess two clusters of six transmembrane helices responsible for proper localization and regulatory interactions [19]. For sAC, there are two known splice variants; the 187-kDa sAC_{fl} (full-length sAC) protein and an approx. 50 kDa alternatively spliced form called sAC_t (truncated sAC) [2]. In sAC_{fl}, the two bicarbonate-regulated catalytic domains comprise the N-terminal approx. 50 kDa (Figure 1A)

Abbreviations used: AC, adenylate cyclase; CO, carbon monoxide; DEANO, diethylamine NO; GC, guanylate cyclase; GST, glutathione transferase; HD, haem domain; NO, nitric oxide; PAS, Per-Ant-Sim; sAC, soluble AC; sAC_{fl}, full-length sAC; sAC-HD, sAC haem domain; sAC_t truncated sAC; sGC, soluble GC; tmAC, transmembrane AC; TPR, tetratricopeptide repeat.

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and are followed by an autoinhibitory 11 residue stretch of unknown physiological function [20]. The remaining C-terminal approx. 1100 residues of sAC are thought to mediate additional regulatory mechanisms, protein–protein interactions and sAC localization. In the Class III enzyme, mammalian sGC (soluble guanylate cyclase), for example, the regulatory region harbours an HD (haem domain), which binds NO (nitric oxide) leading to a approx. 130-fold activation of the GC activity [21,22]. The cAMP-dependent processes, such as sperm capacitation, are also influenced by NO [23], but sAC lacks similarity to the HDs of sGC or other haem proteins, so that the molecular link between cAMP and NO remains to be established. Other Class III cyclases have regulatory domains serving, for example, as pH sensors, membrane anchors and/or signal transducers [24–26]. However, sAC lacks sequence similarity to any of these known cyclase regulatory domains. In fact, no significant sequence similarity of the sAC C-terminal region to any other protein family has been found thus far, limiting our understanding of the spatiotemporal regulation of sAC-mediated cAMP signalling.

In the present paper, we report the identification and characterization of a previously unappreciated regulatory sAC domain. We show that this sAC region comprises an HD that lacks significant sequence similarity to known HDs. One haem cofactor binds non-covalently to each monomer of the oligomeric domain, and the b-type haem with its 6-fold co-ordinated iron atom can bind the ligands NO and CO (carbon monoxide). The HD has an activating effect on the sAC catalytic core and sAC splice variants comprising this domain are expressed in testis and skeletal muscle, revealing a new mechanism for the regulation of cAMP-dependent processes in these tissues.

EXPERIMENTAL

Chemicals

All chemicals were from Sigma if not stated otherwise.

Cloning of sAC fragments and site-directed mutagenesis

All sAC fragments were PCR amplified from a human sAC template [7] and cloned in pGEX4t3 (GE Healthcare), resulting in N-terminally GST (glutathione transferase)-tagged sAC fragments with a thrombin cleavage site in the linker. Mutagenesis was done using the QuikChange® site-directed mutagenesis kit (Stratagene). All steps were performed according to the manufacturer's protocol, except that PCR products for mutagenesis were digested with DpnI for 1.5–4 h at 37 °C before transformation.

PCR analysis of mouse tissue cDNAs

The sAC gene region coding for sAC-HD (soluble adenylate cyclase haem domain) was amplified from 5 μl of different mouse tissue cDNA samples (Clontech) using triple master polymerase (Eppendorf). Primers for sAC-HD (forward: 5′-TTGTCTCTGAAGCCCAGTGAAGG-3′, reverse: 5′-AGATTCCAGAGGGATAATGTCTTCGTCA-3′) and the control reactions, the sAC-C_2 domain (forward: 5′-GAAAAACTGTCATTTGATGGGTCAGGT, reverse: 5′-GAGCAAGGCGGACATCATCC-3′) and cytochrome *c* (forward: 5′-GATCACCCCCAGCCTCCCTTATC, reverse: 5′-AAAATAGAGAATTTAAAAGGCCTAAC-3′), were used at 3.7 pM. PCRs were done with 22 cycles and 3 min extension time, and the products were analysed on a 1 % (w/v) agarose gels.

Expression and purification of sAC-HD and preparation of apo and holo protein

GST-tagged sAC fragments were expressed in *Escherichia coli* Rosetta2 cells (Merck). Cells were grown at 37 °C until an D_{600} value of 0.8 was reached, and then incubated on ice for 20 min. Expression was induced by addition of 0.5 mM IPTG (isopropyl β-D-thiogalactoside) followed by cultivation overnight at 20 °C. Cells were harvested, resuspended in buffer A (50 mM Tris/HCl pH 7.8, and 200 mM NaCl), and disrupted in an Emulsiflex. After centrifugation for 45 min at 40 000 ***g*** and 4 °C, the supernatant was incubated with glutathione–Sepharose (GE Healthcare) for 1 h at 4 °C. The resin was transferred into a column, washed with 20 column volumes of buffer A and eluted with elution buffer (buffer A plus 10 mM GSH). Untagged protein was prepared by 16 h incubation with 10 units of thrombin (GE Healthcare) per mg of fusion protein at 4 °C and subsequent size-exclusion chromatography on a Superose-12 column (GE Healthcare) in buffer A.

For the preparation of apo sAC-HD, purified sAC-HD was incubated in buffer A containing 1 M imidazole overnight at 4 °C. The precipitate was removed by centrifugation for 20 min at 18 000 ***g*** at 4 °C, the supernatant applied to an NAP 10 column (GE Healthcare), and sAC-HD eluted with buffer A. Reconstitution of holo protein was done by titration in increments of 5 μM using a 1 mM haemin stock solution. The samples were mixed gently after each addition and base-line-corrected spectra were recorded immediately.

Limited proteolysis

A 14-μg protein aliquot in buffer A was supplemented with 100 ng of elastase or trypsin respectively, using stock solutions of 0.1 mg/ml protease in 20 mM Tris/HCl, pH 7.8. After 40 min incubation at 4 °C and 22 °C, respectively, reactions were stopped by adding SDS/PAGE sample buffer preheated to 80 °C and immediate boiling for 5 min. Samples were analysed by SDS/PAGE and Coomassie Brilliant Blue staining for approximate size determination. For N-terminal microsequencing, samples were electroblotted to PVDF membrane and analysed by TOPLAB. C-termini of fragments were estimated using the determined N-terminus and the size estimated from the SDS/PAGE.

UV–visible and UV/CD spectroscopy

UV–visible spectra were recorded on a Shimadzu UV-2401-PC at room temperature (20 °C). Protein samples were reduced by adding very few grains of sodium dithionite. The NO-bound

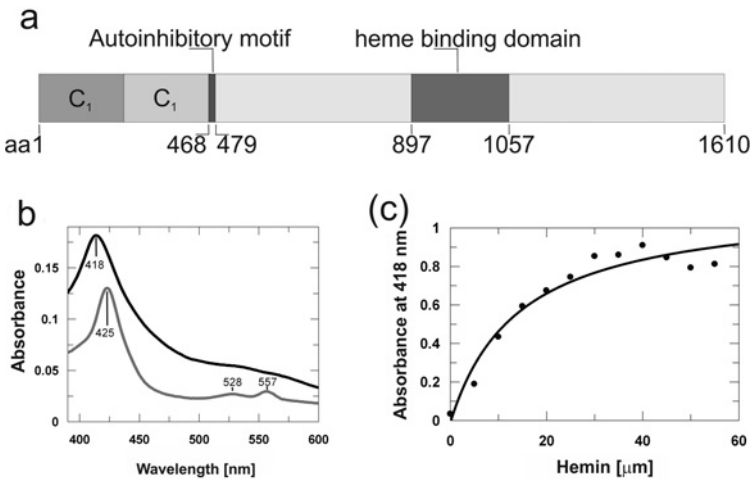

Figure 1 Architecture of human sAC and identification of a haem-binding domain

(**A**) Mammalian sAC consists of two catalytic domains, C_1 and C_2, followed by an autoinhibitory motif (residues 468–479). The regulatory part of sAC is described here to harbour an sAC-HD between residues 897 and 1057. (**B**) UV–visible absorption spectra of the cofactor–binding domain sAC-HD. Spectra of the oxidized (black line) and reduced form (grey line) of the protein show features typical for b-type haem proteins. (**C**) Reconstitution of 45 μM holo sAC-HD by titration of the apo form with haemin. The cofactor was added in increments of 5 μM.

protein was generated by adding 125 μM DEANO (diethylamine NO) to 50 μM sAC-HD followed by 10 min incubation at room temperature. The CO-bound form was generated by bubbling the sample with CO for 20 min and subsequently transferring it into a cuvette flushed with CO. To test for O_2 binding, a protein sample was flushed with argon for 20 min and subsequently transferred to an argon-saturated cuvette. Bicarbonate binding was tested by adding 1 mM sodium bicarbonate, and measurements at different pH were performed in 100 mM sodium citrate, pH 5.5, 100 mM CHAPS, pH 10.0, or 50 mM Tris/HCl, pH 7.8, each supplemented with 200 mM NaCl. All spectra were recorded immediately after sample transferred to the cuvette and baseline-corrected with the respective solution without protein.

CD spectra of 320 μM sAC-HD in buffer C (14 mM NaCl, 2,7 mM KCl, 1 mM Na_2HPO_4 and 180 μM KH_2PO_4, pH 7.3) were recorded with a Jasco J-715 spectropolarimeter and a 0.1 cm pathlength. A total of 60 spectra were recorded at room temperature, averaged, and baseline-corrected. The averaged spectrum was analysed with the Jasco software supplied with the instrument to estimate the secondary structure content.

Preparation of sAC catalytic domain and AC activity assays

Recombinant histidine-tagged sAC_t enzyme was expressed and affinity purified as described before [6], followed by size-exclusion chromatography in 50 mM Tris/HCl pH 7.5, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol and 330 mM NaCl on a Superose-12 column (GE Healthcare). AC activity was tested using 30 min incubation of 0.02 μg sAC in 20 mM Tris/HCl pH 7.8, 5 mM ATP, 5 mM $CaCl_2$ and 10 mM $MgCl_2$ at 37 °C, and subsequent quantification of cAMP formed by using an immunoassay kit (Assay Designs) as recommended by the manufacturer. Assays were done in triplicate, and the data shown are representative of two replications. Error bars shown represent relative percentage differences.

RESULTS

Identification of a haem-binding domain within the C-terminus of sAC

Thus far, the only known regulatory region in sAC is a short autoinhibitory motif of unknown physiological significance next to the catalytic domains [20]; this leaves the majority of the protein with unknown function. To identify sAC domains in the uncharacterized 1131 C-terminal residues, we first analysed the sequence of this region in human sAC for homologies with other proteins and for known protein motifs (Figure 1A). All we detected were a few small and degenerate protein motifs; a P-loop motif (residues 516–523), a putative coiled-coil (residues 900–930) and a potential leucine-zipper region (amino acid 1064–1085). Potential TPR (tetratricopeptide repeat) motifs are located at positions 870–903, 1015–1048 and 1065–1098 (this last putative TPR motif overlaps with the potential leucine zipper). The degenerate nature of these motifs, however, renders their identification in sAC speculative. We further analysed hydrophobicity plots and secondary structure predictions for potential interdomain linkers with low hydrophobicity and no repetitive conformation. Based on all sequence analyses, we dissected the sAC region between amino acids 466 and 1610 into eight fragments covering putative domains (see Supplementary Table S1 at http://www.bioscirep.org/bsr/032/bsr0320491add.htm) for the identification and characterization of functional sAC domains.

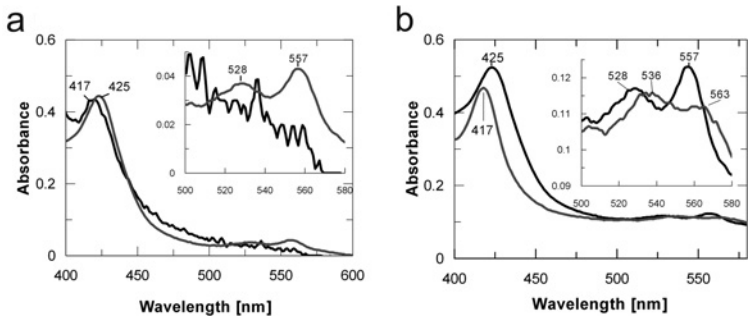

Figure 2 Spectral analysis reveals CO and NO binding to sAC-HD
(**A**) Addition of an NO donor to the reduced protein shifts the maximum of the Soret peak from 425 nm (no NO, grey line) to 417 nm (with NO, black line). (**B**) Spectra of the reduced haem-binding domain in the presence (grey line) or absence (black line) of CO. CO shifts the Soret peak from 425 to 417 nm, the β-peak from 528 to 536 nm and the α-peak from 557 to 563 nm.

We then cloned and tried to purify these putative sAC domains as GST-fusion constructs expressed in *E. coli*.

The affinity purified fragment covering sAC residues 870–1130, which includes the overlapping potential leucine zipper and TPR motifs, showed a strong red colour due to an absorption peak in the visible range. Using limited proteolysis and subcloning, we refined the boundaries of this domain to residues 897–1057 of sAC. The construct $sAC^{897–1057}$ could be purified by affinity chromatography, proteolytic removal of the GST-tag and size exclusion chromatography. In size exclusion chromatography, $sAC^{897–1057}$ eluted at a position expected for a large oligomer, possibly a dodecamer, but later than aggregates (results not shown). To confirm that the purified protein is not an aggregate or aggregation intermediate, the eluted putative dodecamer was re-injected in the size exclusion column several times. After three runs, the construct still eluted at the initial position. We therefore conclude that residues 897–1057 form a stable sAC domain, which might be involved in oligomerization.

To investigate the red coloured cofactor we carried out spectral analyses. Purified $sAC^{897–1057}$ showed an absorption peak at 418 nm. Reduction with sodium dithionite shifted the peak to 425 nm and generated two further peaks, which appeared at 528 and 557 nm (Figure 1B). The spectra thus show typical features for a haem cofactor; a γ-peak (Soret peak) at 418 nm for the oxidized protein, and a Soret peak at 425 nm as well as α- and β-peaks at 557 and 528 nm respectively, for the reduced form. We therefore refer to the region from residues 897–1057 as sAC-HD.

Within sAC-HD, we identified a motif at position 964 (CDHCR), which conforms to the typical CXXCH motif consisting of two cysteines, for covalent haem attachment, followed by a histidine as axial haem ion ligand [27]. We mutated this sequence to SDHCR, CDHSR, SDHSR, SDHSA, CDHCA and CDHCH, but the spectra of each resultant protein showed only insignificant differences compared with wild-type, ruling out this motif as a covalent attachment site. We next tested whether the haem is non-covalently bound. Incubation with imidazole [28] abrogated the sAC-HD haem absorption bands, consistent with the positions of soret and α-peak being more compatible with a non-covalently attached haem (b-type haem) [21]. Finally, the holoprotein could be reconstituted from the obtained apoprotein by titration with free haemin (Figure 1C). During titration, the absorption of the Soret peak increased to higher values than for the original protein sample, indicating that the protein we purified from *E. coli* is not saturated with its haem cofactor. Saturation of the protein with haem was observed at a protein/haemin ratio of approximately 1:1. We thus assume that one haem cofactor binds per sAC-HD monomer, and that the cofactor is a non-covalently bound b-type haem, although the different haem incorporation systems of *E. coli* and mammals render the latter conclusion uncertain (see the Discussion).

The haem cofactor in human sAC binds NO and CO

Haem proteins function as oxygen transporters (i.e. haemoglobin), redox enzymes (i.e. cytochrome P450) or signalling proteins (i.e. sGC). To test whether the sAC-HD might act as a receptor for a gaseous regulator, similar to the activation of sGC through NO binding to its haem [29], we exposed sAC-HD to O_2, CO and NO. Since sAC is directly regulated by bicarbonate [6], we also investigated the influence of bicarbonate on the spectral behaviour of the HD.

We tested NO binding to sAC-HD by adding the NO donor DEANO. DEANO shifted the Soret peak from 425 (indicative of the ligand-free reduced protein) to 417 nm, and it decreased the α- and β-bands to the point where they were no longer clearly visible (Figure 2A). In the oxidized state, the Soret maximum shifted from 418 to 410 nm upon addition of NO, and the β-peak decreased again (results not shown). The spectra thus indicate that sAC-HD can bind NO [30], independent of its oxidation state.

Many haem proteins also bind CO, although physiological functions for this ligand appear to be restricted to a small number of systems. Flushing a solution of the reduced protein with CO gas shifted the Soret peak from 425 to 417 nm. The α- and β-peaks, which are at 557 and 528 nm in the ligand-free form, shifted to 563 and 536 nm, respectively (Figure 2B). In contrast with the results with NO, CO treatment elicited no observable spectral changes upon the oxidized state (results not shown). Thus CO can also bind to sAC-HD, but only to the reduced form.

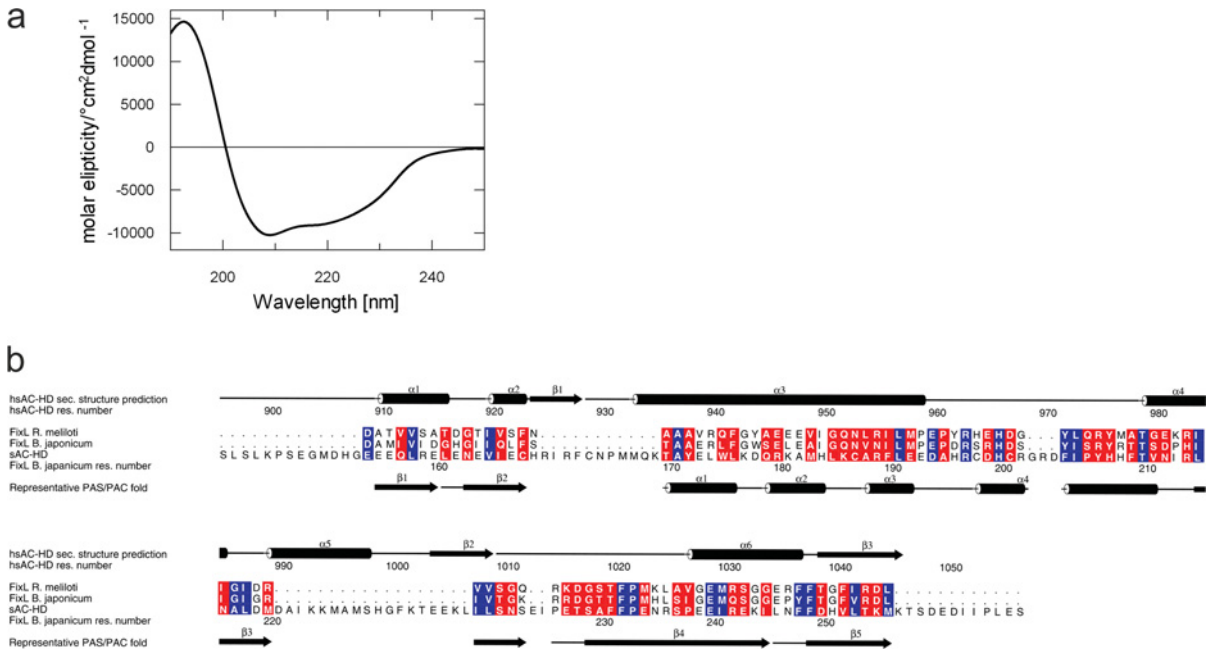

Figure 3 Structural analyses of sAC-HD by UV–CD spectroscopy and structural comparison to haem-containing PAS domains

(**A**) UV–CD spectrum indicating a secondary structure content of 34% α-helix and 30% β-sheet. The molar ellipticities given are relative to the mean amino acid weight. (B) Alignment of sAC-HD and the haem-containing PAS domains and PAC domains of FixL-RHIME (*Rhizobium meliloti*) and FixL-BRAJA (*Bradyrhizobium japonicum*). Blue coloured residues indicate high conservation of physicochemical properties and red colour reflects medium conservation. The secondary structure prediction for sAC-HD on top of the alignment was generated with Jpred [48] and the representative PAS-fold shown below the alignment was deduced from a structural alignment of six PAS structures [33].

Finally, we tested whether sAC-HD can bind oxygen or was affected by bicarbonate addition. The aerobically purified protein was flushed with argon for 20 min in order to displace potentially bound oxygen. The sample was subsequently transferred to a cuvette with argon-saturated atmosphere and subjected to spectral analyses. The spectra exhibited no changes in the peak positions of Soret-, α- or β-band (results not shown) suggesting that O_2 does not bind. Similarly, we observed no spectral changes in the peak positions of Soret-, α- or β-band following the treatment with bicarbonate, indicating that no binding of bicarbonate occur either (results not shown). We thus conclude that sAC-HD is a potential receptor for NO and CO, but apparently not for O_2.

Typical transporters for diatomic gas ligands belong to the globin family, which features an all-α-fold. Secondary structure prediction for sAC-HD suggests a mixed α–β-fold, and UV–CD spectroscopy indeed indicates approx. 34% α-helix and 30% β-sheet content (Figure 3A). While this distribution suggests no similarity to globins, it instead indicates a potential structural resemblance to the mixed-α–β HDs of the H-NOX (haem-NO/oxygen) family, which comprises the sGC HD and the gas sensing PAS (Per-Ant-Sim) domains [30–32]. Both families are highly degenerate on sequence level, but manual alignment of predicted sAC-HD secondary structure elements with two structurally characterized haem-containing PAS domains [33] uncovered weak similarity (Figure 3B). The alignment indicated His^{966} of sAC-HD as a potential axial haem ligand, i.e. one of the possibly two haem iron ligands not provided by the porphyrin system. Positions of soret and α-peak for sAC-HD indeed indicate a six-fold co-ordination [21], which implies the presence of two such axial amino acid ligands. However, when we mutated this histidine residue to alanine, spectra of the sAC-HD-H966A variant showed negligible deviations from wild-type (Table 1). The lack of an additional absorption band above 600 nm also excluded methionine as axial ligand [28], and a Soret shift at acidic pH indicated histidine or lysine and ruled out tyrosine (Figure 4A). Mutating six histidine residues conserved in sAC from different species (Figure 4B) also did not result in significant spectral deviation from wild-type (Table 1). Thus determining the fold and elucidating haem interaction details of sAC-HD will require further structural studies.

sAC-HD is expressed in testis and skeletal muscle and activates sAC

sAC expression has been postulated to be regulated via tissue-specific alternative splicing [34,35]. Expression of sAC_{fl}, which should contain sAC-HD, was demonstrated in testis by immunoprecipitation [36]. We used PCR analysis on a panel of mouse tissue cDNAs with primers for sAC-HD and sAC-C_2 to confirm the presence of sAC-HD in testis and to identify other tissues harbouring HD-containing sAC isoforms (Figure 5A). Amplification of cytochrome *c* cDNA as a control for the PCR reaction yielded

Table 1 Spectral features of sAC-HD variants with exchanges at positions potentially acting as axial haem ligands
ox., oxidized; red., reduced; wt, wild-type.

Protein variant	Soret ox. (nm)	Soret red. (nm)	Soret ox. + NO (nm)	Soret red. + NO (nm)
wt	414	424	409	417
H950A	421	426	398	425
H962A	420, 422	425, 425	394, 401	– 404
H966A	417, 422	425, 425	394, 401	– 404
H976A	412, 413	420, 426	397, 399	425
H977A*	–	–	–	–
H999A	420, 421	425, 426	394, 402	– 402
CDHCA	419	426	399	425
CDHCH	421	426	400	401

*No protein expression was observed.

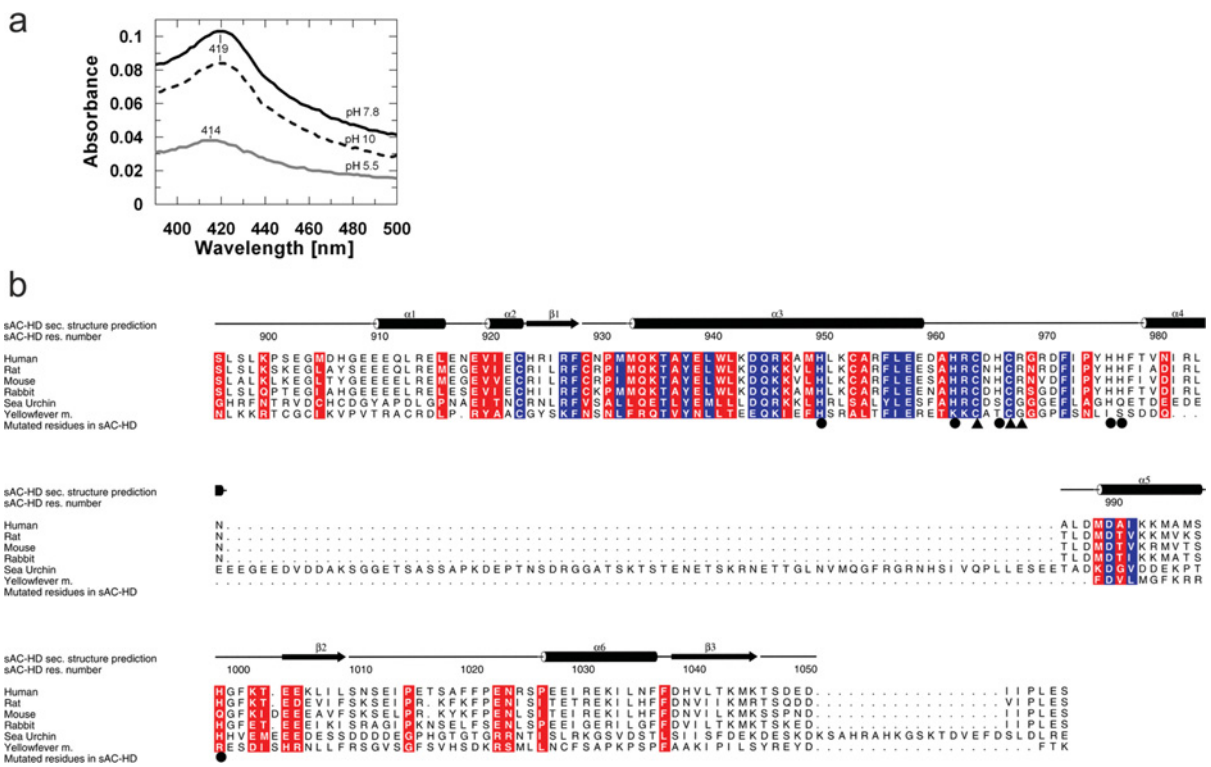

Figure 4 Testing of potential axial haem ligands
(**A**) Spectra of sAC-HD at pH 7.8 (black line), pH 10.0 (dotted line) and pH 5.5 (grey line). Acidic pH causes a shift of the Soret peak from 418 (pH 7.8) to 414 nm (pH 5.5) indicating histidine or lysine as probably axial haem ligands. (**B**) Sequence alignment of sAC-HD and homologous fragments from other sACs reveals six histidine residues (●) with different levels of conservation as putative axial haem ligands. Further, a putative binding motive for covalently bound haem is indicated (▲). The residue numbers and predicted secondary structure elements refer to human sAC-HD. Blue-coloured residues indicate high conservation of physicochemical properties and red colour reflects medium conservation. The secondary structure prediction was generated with Jpred [48].

strong signals for all tissues. The sAC-C_2 domain could also be amplified from cDNAs in all tissues tested, consistent with previous studies demonstrating the presence of active sAC isoforms in a wide variety of tissues [2,6,34,35]. In contrast, PCR analyses of sAC-HD yielded only a strong signal from testis cDNA and a weaker signal from skeletal muscle cDNA. Thus, while confirming that an sAC-HD-containing isoform is present in testis, these data suggest that sAC isoforms harbouring the HD are restricted to select somatic tissues.

Our inability to heterologously express and purify sAC_{fl} hinders our capacity to probe the biochemical function of sAC-HD. Therefore we tested the effect of sAC-HD protein on the

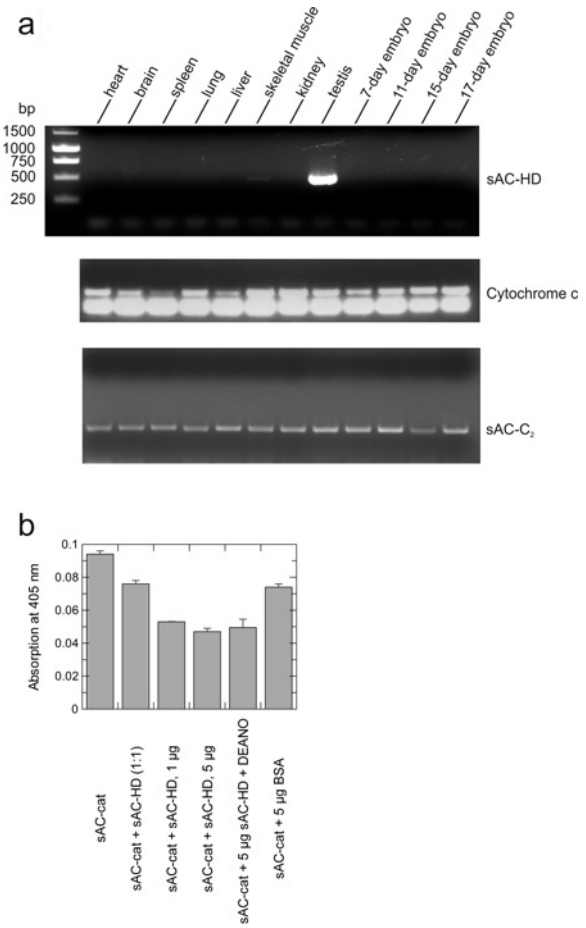

Figure 5 Tissue-specific expression of sAC-HD and effect of sAC-HD on the activity of the sAC catalytic domains

(**A**) The agarose gel shows products of PCR with primer pairs specific for sAC-HD, the sAC-C_2 domain and for cytochrome c as a control, respectively, applied to a panel of mouse tissue cDNA samples as templates. The sAC catalytic domain C_2 could be detected in all tissues, but cDNA coding for sAC-HD was significantly amplified only from skeletal muscle and testis cDNA. (**B**) In a competitive cAMP formation assay (increasing activity leads to decreasing signals), addition of sAC-HD leads to a concentration-dependent increase of the activity of the catalytic domains. BSA addition served as a control. Adding NO together with the sAC-HD did not modulate the activating effect of sAC-HD. Data represent averages of duplicates and are shown as signals relative to control, and error bars represent relative percentage differences.

catalytic activity of isolated sAC-C_1C_2. Adding increasing amounts of isolated sAC-HD increased the AC activity in a dose-dependent manner, whereas a large amount of a control protein (BSA) only slightly influenced the assay (Figure 5B). Thus sAC-HD appears to interact with the catalytic domains to increase their activity. Addition of the NO donor DEANO to the sAC-C1C2/sAC-HD mixture failed to influence the activity further, however, suggesting the mechanism of sAC-HD regulation is probably complex and remains to be fully understood.

DISCUSSION

Numerous biochemical, kinetic and structural studies have revealed the detailed insight into the catalytic domain structure and function of mammalian tmACs and sAC [1,2,18]. In contrast, the knowledge about their regulatory domains remains severely limited. This dearth of information is particularly onerous for sAC, which is involved in various signalling systems in different tissues and cell types, ranging from sperm capacitation to mitochondrial respiration in liver and brain [14,36]. The complexity of sAC functions appears to be reflected by a variety of tissue-specific splice variants [2,34,35], which generate an arsenal of isoforms with catalytic domains fused to suitable regulation and localization modules. We now identified and characterized an HD as the first such module within the regulatory part of sAC.

sAC-HD harbours a non-covalently-attached b-type haem moiety, which is found in the majority of haem proteins [37]. There are different haem incorporation systems in different organisms [37], however, and the non-covalent incorporation observed might be due to the inability of *E. coli* to recognize the haem attachment site in the mammalian sAC-HD protein. Trials to express sAC-HD in mammalian cells have thus far failed, limiting our ability to understand sAC-HD architecture. Although sAC-HD shows no obvious similarity to other proteins, and in particular not to other known haem-based sensors, such as CooA, PAS domains or GCS (globin-coupled sensors) [31], our detailed analysis reveals the possibility that sAC-HD exhibits structural similarity to PAS domains or H-NOX (haem nitric oxide/oxygen) members. Haem-containing PAS domains mostly contain 5-fold-co-ordinated haem moiety serving as oxygen sensor [38], whereas the 6-co-ordinate haem moiety of sAC-HD does not bind oxygen. Mutating the histidine residue derived as a potential axial haem ligand from a secondary structure alignment with PAS domains also did not confirm such a role, suggesting that sAC-HD does not feature a typical PAS-fold. Mutating other histidine residues, the most common haem ligand, also did not result in the spectral changes expected for removal of an axial ligand. Instead, a lysine residue might function as an axial ligand, as observed before in the haem enzyme nitrite reductase [39]. These pronounced differences between sAC-HD and other known haem sensor domains might indicate that sAC-HD represents a novel haem protein class, possibly a novel subclass within the H-NOX family [30]. Definitive answers on the structural relationship to other HDs, however, will have to await an experimentally determined structure for an sAC-HD.

Although we were not yet able to identify the haem ligand that modulates sAC activity through sAC-HD, our results show that NO and CO can bind, making them candidates for this function. While NO is an established second messenger, the role of CO as signalling molecule is still controversial [40], but it has been reported to contribute to regulation of an increasing number of physiological processes, such as smooth muscle contractility and apoptosis [41]. Thus sAC modulation through CO binding to sAC-HD could be a link for this molecule to an established signalling system and should also be considered in future studies.

Furthermore, only the reduced form of sAC-HD binds CO, indicating that, if CO is the ligand, it would simultaneously confer redox-sensitivity to the enzyme. In fact, the physiological function of sAC-HD might be entirely restricted to serve as a redox sensor, i.e. the oxidation state of the haem iron rather than ligands could determine sAC activity. Future studies on physiological systems will have to clarify the regulation mechanism mediated by sAC-HD.

ACs are emerging as attractive therapeutic targets [8,42]. Despite the progress made on the development of specific inhibitors for AC catalytic domains [43–45], novel regulation domains and mechanisms would not only advance our understanding of cAMP signalling but also disclose novel opportunities to specifically modulate this system with drugs. Identification of the sAC-HD is a first such step, but fully clarifying the regulation mechanisms mediated by sAC-HD will require physiological studies in the appropriate biological systems. We therefore identified testis and skeletal muscle as tissues where sAC isoforms harbouring sAC-HD are expressed. It is tempting to speculate that sAC-HD serves as NO or CO sensor in these tissues, providing a direct link between NO/CO and cAMP signalling. Several lines of evidence provoked speculation on such a link for NO, e.g. the NO-dependent, cAMP-mediated response of airway axonemes to alcohol [46] and the stimulatory effect of NO on sperm motility and capacitation [23], a cAMP- and a sAC-dependent process [36]. The HD of mammalian sAC would thus be a functional equivalent to the N-terminal HD in the sAC analogue within the cGMP system sGC. The sGC-HD is the best-established physiological NO receptor [21,22], but several of the many physiological effects of NO are cGMP-independent [47]. It will be interesting to see whether the identified sAC-HD provides a link for NO to an alternative, cAMP-based signalling system. Our characterization of a sAC-HD, its potential ligands, and its tissue distribution has thus identified the framework for further physiological studies on possible roles of this exciting new mechanism in mammalian cAMP signalling.

AUTHOR CONTRIBUTION

Sabine Middelhaufe contributed to the design of the experiments, and data acquisition, analysis and interpretation, and to drafting the paper. Martina Leipelt contributed to the data acquisition and data analysis. Lonny Levin contributed to data analysis and interpretation, and to the intellectual content of the paper. Jochen Buck contributed to the design of the experiments and data analysis, and to the intellectual content of the manuscript. Clemens Steegborn contributed to the design of experiments, data analysis and interpretation, and drafting and revising the paper.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Identification of a haem domain in human soluble adenylate cyclase

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Table S1 Expression constructs for fragments of the human sAC regulatory region fused to GST

Construct (N-terminus→C-terminus)	Expression	Solubility
485–760	−	n.a.
580–760	+	−
870–1091	+	−
897–1081	+	+
897–1130	+	+
965–1130	+	+
1030–1200	+	−
1444–1608	+	−

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