Published in final edited form as:

Mol Reprod Dev. 2006 March ; 73(3): 361–368. doi:10.1002/mrd.20409.

Autoinhibitory Regulation of Soluble Adenylyl Cyclase

James A. Chaloupka, Stewart A. Bullock, Vadim Iourgenko, Lonny R. Levin*, and Jochen Buck

Department of Pharmacology, Weill Medical College of Cornell University, New York, New York

Abstract

Soluble adenylyl cyclase is an evolutionarily conserved bicarbonate sensor that plays a crucial role in cAMP dependent processes that occur during mammalian fertilization. sAC protein is expressed at the highest levels in male germ cells, and is found to occur as one of two known isoforms: a truncated protein (sAC_t) that consists almost exclusively of the two conserved catalytic domains (C1 and C2), and a full-length form (sAC_{fl}) that contains an additional noncatalytic C-terminal region. Several studies suggested sAC_t was more active than sAC_{fl}. We now demonstrate that the specific activity of sAC_t is at least 10-fold higher than the specific activity of sAC_{fl}. Using deletion analysis and a novel genetic screen to identify activating mutations, we uncovered an autoinhibitory region just C-terminal to the C2 domain. Kinetic analysis of purified recombinant sAC revealed this autoinhibitory domain functions to lower the enzyme's V_{max} without altering its affinity for substrate or regulation by any of the known modulators of sAC activity. Our results identify an additional regulatory mechanism specific to the sAC_{fl} isoform.

Keywords

cAMP; bicarbonate; sperm; alternative splicing; capacitation; fertility

INTRODUCTION

Adenylyl cyclases (AC) generate the ubiquitous signaling molecule cAMP, which mediates cellular responses to extracellular stimuli and nutritional conditions in prokaryotes and eukaryotes. Until recently, the production of cAMP in higher eukaryotes was solely attributed to a family of G-protein regulated transmembrane adenylyl cyclases (tmACs). We now know that mammalian cells express an additional, distinct source of cAMP, the soluble adenylyl cyclase (sAC). Soluble adenylyl cyclase is structurally, molecularly, and biochemically distinct from tmACs. The sAC protein does not appear to have any membrane spanning domains, and its catalytic domains are more closely related to those of cyanobacterial ACs than other eukaryotic ACs (Buck et al., 1999). Soluble adenylyl cyclase activity was originally identified using Mn^{2+} -ATP as substrate (Braun and Dods, 1975), and sAC in vitro activity is highest in the presence of Mn^{2+} -ATP (Litvin et al., 2003). sAC is insensitive to the prototypical regulators of heterotrimeric G proteins and forskolin (tmACs), that is, heterotrimeric G proteins and forskolin, and its physiologically relevant activity

© 2005 WILEY-LISS, INC.

*Correspondence to: Lonny R. Levin, Department of Pharmacology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. llevin@med.cornell.edu.

Vadim Iourgenko's present address is Department of Functional, Genomics, Novartis Institute for Biomedical Research, 100 Technology, Square, Cambridge, Massachusetts

The authors declare that they have no competing financial interests.

(measured using Mg^{2+} -ATP as substrate) is directly stimulated by bicarbonate (Chen et al., 2000) and by calcium ions (Jaiswal and Conti, 2003; Litvin et al., 2003).

In mammals, cAMP is integral to a number of processes essential for male fertility. During spermatogenesis, cAMP modulation of gene expression plays an essential role (Blendy et al., 1996; Nantel et al., 1996; Hess et al., 2005). Following spermatogenesis, mature sperm are stored in the epididymis, but they remain unable to fertilize an egg. Upon ejaculation, the sperm mix with seminal and prostatic fluids, and they become motile and acquire the competence to fertilize an egg by undergoing a series of cAMP-dependent changes termed capacitation. cAMP is essential for motility and multiple maturational capacitative processes essential for fertilization (Fraser, 1981; Tash and Means, 1988; White and Aitken, 1989; Visconti et al., 1995; Lefievre et al., 2002; Hess et al., 2005). sAC mRNA is most abundantly expressed in testis (Buck et al., 1999), specifically in the developing germ cells (Sinclair et al., 2000), and sAC protein accumulates throughout spermatogenesis reaching high levels in mature spermatozoa (Xie and Conti, 2004). In fact, sAC seems to be the prevalent source of cAMP in testis and sperm (Hess et al., 2005), yet there appears to be functional roles for both families of cyclases in male fertility. We have identified defects in mature sperm from mice with their *sAC* gene deleted (sAC-KO) suggestive of a spermatogenic role for sAC. Although testis in sAC-KO mice appear morphologically normal (Esposito et al., 2004), we have found sAC-KO sperm to develop a kink at their midpiece rendering them recalcitrant to fertilizing an egg even in the presence of membrane permeable cAMP (Hess et al., 2005).

Two of the essential signals in seminal and prostatic fluids for elevating sperm cAMP and inducing motility and capacitation are bicarbonate and calcium (Visconti et al., 2002), both of which can directly stimulate sAC activity (Chen et al., 2000; Jaiswal and Conti, 2003; Litvin et al., 2003). Both genetic knockout (Esposito et al., 2004; Hess et al., 2005) and pharmacological inhibition (Hess et al., 2005) demonstrate that sAC is required for sperm motility and for a subset of maturational changes termed capacitation. The best-characterized molecular marker for capacitation is a prototypical pattern of tyrosine phosphorylated proteins, and sAC activity is essential for induction of this pattern (Hess et al., 2005). The final cAMP-dependent process in sperm essential for fertilization, the exocytosis of its acrosomal compartment, did not appear to be affected in sperm treated with sAC inhibitor or in sperm from sAC-KO animals (Hess et al., 2005). In contrast, the reduced fertility in knockouts of the Type III tmAC might be due to a specific defect in acrosomal exocytosis (Livera et al., 2005), suggesting there are roles for cAMP generated by distinct sources.

There are two isoforms of sAC expressed in testis (Buck et al., 1999) generated by alternative splicing of a single gene (Jaiswal and Conti, 2001) and both are deleted in sAC-KO mice (Hess et al., 2005). To clone the *sAC* gene, we purified soluble adenylyl cyclase activity from rat testes and identified a protein of approximately 48 kDa (Buck et al., 1999; Buck et al., 2002). Upon screening a testis cDNA library, we found two cDNAs, one encompassing 34 exons and predicting a protein of 187 kDa and a second cDNA skipping the 12th exon, altering the reading frame and introducing a stop codon predicting a 53 kDa protein (Buck et al., 1999). The two cDNAs were termed sAC_{fl} encoding full-length protein, and sAC_t encoding the truncated protein (Fig. 1A). Subsequently, reverse transcription-polymerase chain reaction (RT-PCR) and RNase protection experiments confirmed the sAC_t mRNAs to be a bonafide splice variant conserved in mouse and rat testis (Jaiswal and Conti, 2001).

Both splice variants are bicarbonate regulated (Chen et al., 2000; Jaiswal and Conti, 2003) but heterologous expression of the sAC_t cDNA, which consists almost exclusively of the

two cyclase catalytic domains, resulted in a 10–20-fold higher cyclase activity relative to the full-length protein (Buck et al., 1999; Jaiswal and Conti, 2001, 2003). We now demonstrate that this difference represents an intrinsic difference in their specific activities due to the presence of an inhibitory domain within the noncatalytic regions of the sAC_{fl} splice variant. A series of C-terminal deletions of sAC_{fl}, as well as a screen for mutations disinhibiting the longer forms identified an inhibitory function for the region immediately C-terminal to the conserved C2 catalytic core.

MATERIALS AND METHODS

Chemicals

ATP and the ANTI-FLAG monoclonal antibody were purchased from Sigma; all tissue culture reagents were from Invitrogen and [$\alpha^{32}\text{P}$] ATP and [^3H] cAMP were from PerkinElmer Life Sciences (Boston, MA). MacConkey agar used in the complementation assays was purchased from Difco.

E. coli Strains and Plasmids

The *E. coli* TP2000 strain, a *cya* deletion strain (kind gift of Wei-Jen Tang, University of Chicago) was used to screen for activating mutations in the complementation assay. Deletion of the *cya* gene abrogates all endogenous cyclase activity, resulting in a pleiotropic defect in carbohydrate metabolism (Perlman and Pastan, 1968, 1969). Specifically, the TP2000 strain is impaired in its ability to utilize maltose (Mal[−]) as a carbon source, producing white colonies on MacConkey Maltose agar. Complementation of the *cya* mutation was detected on MacConkey Maltose agar supplemented with ampicillin (100 μl /ml) and IPTG (100 μM) by the production of red colonies following transformation with rat sAC expression constructs. Positive colonies were re-streaked to confirm complementation, and plasmids were isolated with the Qiagen plasmid purification kit and sequenced.

Cell Growth and Transfections

HEK 293 cells were grown in DMEM supplemented with 10% FBS. Where indicated, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Briefly, cells were seeded in 60-mm dishes and grown until they were 90% confluent. Eight micrograms of plasmid (pBK-CMV or pcDNA3.2 based constructs) were allowed to form complexes with Lipofectamine 2000 and placed on HEK293 cells for 4 hr in OPTI-MEM before switching to normal growth media. Cells were harvested 24 hr after transfection with lysis buffer (50 mM Tris-HCl, pH = 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM PMSF) and lysed by sonication. Cell lysates were centrifuged at 14,000g 10 min at 4°C to obtain a crude soluble fraction.

Molecular Cloning of Rat sAC Expression Vectors

Rat sAC_i and sAC70 were subcloned using the Gateway Technology expression system (Invitrogen) into a baculovirus expression vector to generate an N-terminal GST fusion protein. Recombinant GST-rat sAC_i and sAC70 expressing baculovirus was generated as previously described (Litvin et al., 2003).

Expression and Purification of GST-sAC Fusion Protein

SF9 cells, grown to a density of $\sim 1 \times 10^6$ cells/ml, were infected with GST-rat sAC_i or sAC70 baculovirus. After 48 hr, cells were pelleted by centrifugation, resuspended in lysis buffer (phosphate buffered saline, pH = 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF), and lysed by sonication. The cell lysate was

cleared by centrifugation at 100,000g 60 min at 4°C, and the soluble fraction was applied to Glutathione-Sepharose 4B column (Amersham Biosciences, Piscataway, NJ). The GST-rat sAC recombinant fusion proteins were eluted from the column with glutathione elution buffer (50 mM Tris-HCl, pH = 8.0, 10 mM reduced glutathione, 10 µg/ml aprotinin, 10 µg/ml leupeptin) and stored as 50% glycerol stocks at -20°C.

Construction of sAC70 Random Mutant Libraries

Libraries containing random mutations to the C-terminal domain of sAC70 were constructed by error-prone PCR using the Genemorph kit (Stratagene, La Jolla, CA). The 0.5 kb fragment generated by PCR was digested with *Bam* HI and *Kpn* I and was subcloned into pProExHT-sAC70 plasmid in order to replace the wild-type C-terminus that was removed by digestion with the same enzymes as above. Following ligation, XL-Blue cells were transformed according to standard protocol, and allowed to grow on LB ampicillin plates for 24 hr. All colonies were collected in a pool of LB medium (containing ampicillin) grown overnight, and a pool of mutagenized plasmids was prepared. Site-directed mutagenesis was performed with the QuikChange II XL kit (Stratagene) according to the recommendation of the manufacturer.

Cyclase Assay

Cyclase assays were performed in a final volume of 100 µl, using ~100 ng of purified recombinant sAC protein in the presence of 50 mM Tris-HCl, pH = 7.5, substrate [α -³²P]ATP, and either MnCl₂, MgCl₂, and/or CaCl₂ and NaHCO₃ as indicated. Reactions were incubated for 30°C for 30 min and were stopped by the addition of 200 µl of 2% SDS. The [α -³²P]cAMP generated in the reactions was recovered using the two-column method that has been previously described (Levin et al., 1992; Levin and Reed, 1995). In vitro cyclase assays for HEK293 cell lysates were also performed in a total volume of 100 µl using ~25 µg of the crude soluble fraction in the presence of 50 mM Tris-HCl, pH = 7.5, 20 mM Creatine Phosphate, 100 U/ml CPK, 1 mM DTT, 5.0 mM ATP, and either 10 mM MnCl₂, MgCl₂, or CaCl₂ in the presence or absence of 40 mM NaHCO₃, unless otherwise as indicated. Reactions were incubated at 30°C for 30 min and were stopped by the addition of 100 µl of 0.2 N HCl. cAMP formed was detected using a Correlate-EIA Direct cAMP Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI), and data analyses were performed using the program Prism, Version 4.0a (GraphPad Software, Inc.).

Western Analysis

Proteins were separated under reducing conditions using 7.5% SDS-PAGE, transferred to PVDF membrane, and blocked in 5% milk solution. The blots were probed with antibodies against the FLAG epitope (M2 monoclonal, 1:4,000; Sigma-Aldrich, St. Louis, MO) overnight. Following several washes, the blots were incubated with HRP-conjugated secondary antibodies, and bands were visualized using ECL reagent.

RESULTS

C-Terminal Truncations Define an Autoinhibitory Regulatory Domain in sAC_{fl}

To explore the difference in activity between sAC_t and sAC_{fl}, we used PCR to generate a series of C-terminal deletions of rat sAC_{fl} protein. We expressed the two naturally occurring sAC isoforms and each of the generated truncations in HEK293 cells, and because we remain unable to adequately purify sAC_{fl} protein, we relied upon Western blotting to quantitate the relative expression of the different sAC proteins for determining their specific activities (Fig. 1). To rule out any differences due to sensitivity to physiological regulators, we compared maximal activities of the sAC constructs by using Mn²⁺-ATP as substrate.

Sequential removal of most of the noncatalytic region of the sAC full-length isoform did not result in a concomitant increase in cyclase activity. In fact, truncating the full-length isoform to a protein approximately 70 kDa in size did not lead to an appreciable increase in cyclase activity. All C-terminal truncations we generated resembled sAC_{fl} in specific activities and were significantly less active than sAC_t (Fig. 1). Consistent with the previous results from various heterologous expression systems (Buck et al., 1999; Chen et al., 2000; Jaiswal and Conti, 2003), the specific activity of the sAC_t isoform was at least 10-fold higher than the specific activity of sAC_{fl}, and this difference was present even in the smallest of the artificially generated truncations, the approximately 70 kDa (sAC70) construct. Thus, we concluded that there were sequences responsible for diminishing the activity of sAC_{fl} contained within the 17 kDa immediately C-terminal to the end of the second catalytic domain.

Kinetic Analysis Reveals Catalytic Rate Is Diminished by Autoinhibition

Unlike sAC_{fl}, we were successful at purifying the artificially generated sAC70 truncation as a GST fusion protein (Fig. 2 Upper). Our previous experience with sAC_t revealed that presence of the GST fusion was of no consequence to its activity (Litvin et al., 2003). Direct comparison of the specific activities of purified sAC_t and sAC70 revealed a 10-fold difference in their intrinsic specific activities (Fig. 2 Lower) excluding the possibility that their activity difference was due to differential interaction with regulatory factors in the various heterologous expression systems.

Historically, soluble adenylyl cyclase activity from testis had been defined and measured using Mn²⁺-ATP as substrate (Braun and Dods, 1975; Braun, 1990). Since its molecular isolation, we (Chen et al., 2000; Litvin et al., 2003) and others (Jaiswal and Conti, 2003; Geng et al., 2005) have defined conditions to measure sAC activity using its more physiologically relevant substrate Mg²⁺-ATP. The activities of sAC_t and sAC70 in the presence of Mg²⁺-ATP alone were approximately nine and sevenfold lower than their Mn²⁺-ATP dependent activities, respectively (Fig. 2B); thus, the physiologically relevant basal activity of sAC70 was also inhibited relative to sAC_t. Consistent with our previously published data (Chen et al., 2000; Litvin et al., 2003), the activity of sAC_t was stimulated approximately twofold by the addition of bicarbonate and synergistically activated by calcium and bicarbonate approximately sixfold (Fig. 2 Lower). We found very similar regulation of sAC70 activities; bicarbonate alone stimulated activity approximately twofold and calcium and bicarbonate stimulated activity greater than fourfold.

Kinetic analysis of sAC70 in the presence of its substrates Mn²⁺-ATP or Mg²⁺-ATP revealed that its catalytic activity behaves similar to sAC_t in all aspects but with a diminished V_{max}. The rat sAC70 enzyme displayed a K_m for Mn²⁺-ATP of 2.9 mM (Fig. 3, inset), which is relatively close to our previously determined K_m for human sAC_t (0.8 mM Mn²⁺-ATP) and in agreement with the previously reported values for purified soluble adenylyl cyclase activity from rat testis (between 1 and 2 mM Mn²⁺-ATP) (Stengel and Hanoune, 1984; Braun, 1991). The basal activity of rat sAC70, that is, in the presence of Mg²⁺-ATP, also behaved very similarly to the basal activity of human sAC_t (Litvin et al., 2003). As previously reported, the basal activities of sAC70 and sAC_t do not display a true K_m since their activities do not plateau within the limits of our assay conditions, but the apparent K_m of sAC70 was measured to be above 13 mM for Mg²⁺-ATP (Fig. 3) which is indistinguishable from the apparent K_m we found for sAC_t (>10 mM Mg²⁺-ATP) and very similar to the values reported for soluble adenylyl cyclase activity purified from rat testis (12–16 mM) (Stengel and Hanoune, 1984).

We previously showed that bicarbonate stimulates sAC_t activity by elevating its V_{max} with little effect on its K_m for substrate ATP. In contrast, calcium stimulates sAC_t activity by

lowering its K_m for ATP with little effect on its V_{max} (Litvin et al., 2003). Structural analysis of a bacterial sAC-like cyclase with conserved regulation provided a mechanistic explanation for these observations; bicarbonate induces an allosteric change facilitating catalysis while calcium helps to coordinate the substrate Mg^{2+} -ATP in the catalytic center (Steebhorn et al., 2005). Because bicarbonate stimulates sAC_t activity exclusively by stimulating its V_{max} , we determined sAC70's dose dependency for bicarbonate. The median effective concentration (EC₅₀) for bicarbonate activation of sAC70 was 28.7 ± 6.8 mM bicarbonate (Fig. 4), which is nearly indistinguishable from the value we measured for rat sAC_t (EC₅₀ of 26.7 ± 4.6 mM) and the value previously reported for rat sAC_t purified as a His-tagged protein (EC₅₀ = $25.4 \text{ mM} \pm 7.5 \text{ mM}$) (Chen et al., 2000). As with sAC_t, calcium lowers sAC70's affinity for Mg^{2+} -ATP to 3.7 mM (from 14.3 mM) without significantly affecting the enzyme's maximal velocity (Fig. 3). Thus, kinetic analysis of sAC70 reveals that differences in the intrinsic specific activities of rat sAC_t and recombinant sAC70 are not attributable to differences in substrate affinities, but rather, are due to differences in the two enzymes' maximal velocities. sAC70 responds to all identified modulators of sAC_t but with diminished V_{max} . These data suggest that the additional sequences in sAC70 act by decreasing the catalytic rate of the enzyme via an autoinhibitory mechanism.

Definition of the Minimal Autoinhibitory Domain

We adopted two parallel strategies to delineate the molecular requirements of this autoinhibitory domain; we determined the extent of the autoinhibitory domain by generating additional C-terminal truncations, and we identified amino acid residues essential for autoinhibition by selecting activating mutations in sAC70. The highly active sAC_t isoform is approximately 53 kDa, and we introduced stop codons in sAC by site directed mutagenesis to generate C-terminal truncations of approximately 60, 55, and 54 kDa. Heterologous expression of these constructs in HEK293 cells revealed that they were autoinhibited similar to the sAC70 protein (data not shown). Therefore, addition of the immediate nine amino acids following the sAC_t (53 kDa) isoform proved sufficient for autoinhibition suggesting that the sequences responsible for regulating sAC activity are immediately downstream or perhaps contiguous with the second catalytic domain.

We also employed a genetic screen of randomly mutagenized sAC70 constructs to identify substitutions that relieve autoinhibition. We took advantage of an *E. coli* strain (TP2000) deficient in adenylyl cyclase activity (*cya*⁻) that exhibits a pleiotropic deficiency in carbohydrate utilization (Wang and Morse, 1968; Perlman and Pastan, 1969; Botsford, 1981). In the absence of a carbon source such as glucose, *E. coli* require cAMP to induce the enzymes needed to metabolize replacement carbon sources, such as maltose; mutants deficient in adenylyl cyclase are therefore unable to utilize these alternate carbon sources and fail to grow on minimal media. A simple colorimetric screen, the ability to turn MacConkey agar red, reveals which bacteria possess sufficient adenylyl cyclase activity to induce the enzymes enabling them to ferment maltose. We first determined that the catabolic defect in the TP2000 mutant strain could be complemented by transformation with a sAC_t expression construct, but not with a construct expressing either sAC_{fl} or sAC70. TP2000 bacteria expressing sAC_t grow on minimal medium and as red colonies on MacConkey agar (Fig. 5). In contrast, bacteria expressing the sAC_{fl} or sAC70 (Fig. 5) proteins grow as white colonies on MacConkey agar, revealing that these two autoinhibited sAC proteins do not generate sufficient levels of cAMP to utilize maltose as a carbon source. We took advantage of this phenotypic difference to screen a randomly mutagenized pool of sAC70 constructs to identify amino acid substitutions that disinhibited sAC70.

Screening several thousand colonies from three independent randomly mutagenized pools of sAC70 plasmids, we identified several disinhibited mutants harboring a single amino acid substitution. A preponderance of mutations map to the area between codons 453 and 469 of

the sAC70 protein. Interestingly, these residues are retained within the highly active, that is, naturally disinhibited, sAC_t isoform. In particular, point mutations affecting three residues (C462, L465, and E467) occurred with the greatest frequencies.

To validate the mutations identified by the genetic screen, we introduced one of the genetically identified substitutions into a naive sAC70 construct by site-directed mutagenesis and expressed the mutant protein in HEK 293 cells. As predicted from its ability to complement the *cya*⁻ *E. coli* strain, introduction of the E467K mutation into the sAC70 protein relieves autoinhibition. Although not as active as the native sAC_t isoform, expression of sAC70 E467K resulted in significantly greater normalized activity than sAC70. The E467K substitution stimulated activities approximately sevenfold when using Mn²⁺-ATP as substrate and approximately three or fourfold when measuring basal (i.e., using Mg²⁺-ATP as substrate) or physiologically stimulated (i.e., in the presence of bicarbonate and calcium) activities, respectively (Fig. 6). Similar results were obtained with other site-directed mutants generated to confirm the results from the genetic screen.

DISCUSSION

sAC is the predominant source of cAMP in sperm (Hess et al., 2005), and it plays a pivotal role in male fertility (Esposito et al., 2004; Hess et al., 2005). In testis and sperm, sAC is present as at least two protein isoforms (Hess et al., 2005) that are generated by alternative splicing of a single gene (Jaiswal and Conti, 2001). One isoform is 187 kDa in size; it is encoded by all known sAC exons and is therefore considered the full-length protein (sAC_{fl}). The second isoform (sAC_t) is truncated relative to sAC_{fl}; it is approximately 53 kDa and is essentially comprised of just the catalytic portions of sAC (Buck et al., 1999; Jaiswal and Conti, 2001). Thus far, in vitro studies of sAC activity have focused on modulators affecting the catalytic center of the enzyme. Detailed biochemical analysis was facilitated by the ability to purify heterologously expressed sAC_t protein (Chen et al., 2000; Litvin et al., 2003). Characterization of sAC_{fl} activity has been restricted to study of extracts from HEK293 cells transfected with the sAC_{fl} cDNA (Chen et al., 2000; Jaiswal and Conti, 2003), or immunoprecipitations of native sAC isoforms from testis (Jaiswal and Conti, 2001). Such studies suggested regulatory properties were conserved in sAC_{fl}; however, it remained unclear whether either of these methods measured exclusively sAC_{fl} activity in the absence of any contaminating sAC_t or other, as yet unidentified, sAC isoforms. Additionally, each of these methods revealed that sAC_{fl} was subject to additional regulation, but it remained unclear what this regulation was.

To identify putative regulatory elements present in the noncatalytic portion of sAC_{fl}, we generated several deletions of sAC_{fl} and compared their activities to the full-length protein and to sAC_t. We found sAC_{fl} to have diminished specific activity relative to sAC_t, and this inhibitory function was retained by a very small (nine amino acids) region C-terminal to the second catalytic domain. Kinetic analysis of one particular artificial truncation, sAC70, revealed that isoforms longer than sAC_t are regulated via autoinhibition of their catalytic rate. Autoinhibition does not appear to be an artifact of these isoforms having an artificial C terminus; sAC70 (and each of the heterologously expressed deletion mutants) displayed similar affinity for ATP (either Mn²⁺-ATP or Mg²⁺-ATP) as sAC_t and retained responsiveness to both bicarbonate and calcium. Thus, the catalytic centers of longer isoforms are properly folded, and autoinhibition appears to represent a regulatory property specific to sAC_{fl} (and any as yet unidentified naturally occurring isoforms longer than sAC_t).

In general, autoinhibition of enzymatic activity can occur either by blockade of substrate from the active site or by allosteric alteration of the active site, causing the enzyme to

function sub-optimally. In the case of sAC_{fl} (and sAC70), the latter appears to be the more likely mechanism. The enzyme is still active, with unaltered affinity for substrate in the presence or absence of calcium, implying substrate access to the active site is not occluded. Furthermore, our limited attempts to supply the autoinhibitory domain in *trans* to modulate sAC_i activity proved unsuccessful (data not shown), suggesting the autoinhibitory domain must be linked which is consistent with an allosteric inhibitory mechanism. On the other hand, the autoinhibited forms are still regulated by bicarbonate, revealing they are still susceptible to the bicarbonate-induced allosteric shift to the conformation favoring catalysis (Steebhorn et al., 2005).

The autoinhibitory domain does not contain any recognizable modulatory motifs. As it was finally defined, the minimal region necessary for autoinhibition did not contain any discernible binding or regulatory motifs, nor did it possess any homology to other mammalian cyclases (i.e., G protein regulated tmACs or guanylyl cyclases) precluding identification of important regulatory amino acids based upon conservation with other ACs (Linder et al., 2002). Our initial deletion series identified the sAC70 protein as autoinhibited. This fragment retains a canonical P-loop or Walker A motif, which is used in nucleotide binding. But site directed mutagenesis of a conserved lysine residue within the motif (data not shown) as well as subsequent deletion analyses revealing autoinhibition of sAC proteins smaller than sAC70, ruled out the P-loop sequence as being essential for the diminished specific activity of sAC_{fl}. This finding does not rule out the P-loop sequence in regulation of sAC_{fl}; this motif may prove essential for activation, or disinhibition, of sAC_{fl}. Our attempts to disinhibit the activity of purified sAC70 using a nonsubstrate nucleotide (i.e., GTP or nonhydrolyzable ATP) failed (data not shown), but nucleotide binding to the P-loop sequence may still represent the activation mechanism of sAC_{fl}. sAC70 may not have the entire nucleotide interaction domain, and/or we may not have tried the correct nucleotide. It also remains possible the P-loop sequence will be involved in sAC_{fl} activation by a mechanism not involving nucleotide binding.

Autoinhibition does not appear to depend upon interacting factors. We observed diminished catalytic activity in sAC_{fl}, and all deletion constructs expressed in HEK293 cells and in rat sAC70 purified from an insect cell expression system. If autoinhibition were dependent upon an interacting factor, it would have to be a widely expressed protein (or molecule) that is extremely well conserved from insects to mammals. In contrast, the existence of this autoinhibitory domain suggests that interaction with a regulatory protein or factor represents a possible mechanism for regulating sAC_{fl} activity. Our future efforts will be to identify such putative activating factors or binding proteins, as well as to test the possibility that post-translational modification (such as phosphorylation) of the autoinhibitory region provides the mechanism for disinhibition, and therefore activation.

Finally, our use of cyclase deficient bacteria to select activating mutations in sAC70 defines a novel method for genetic analysis of adenylyl cyclase mutations. Complementation of *E. coli* was previously used for identification of active forms of solubilized tmACs (Tang and Gilman, 1995), and genetic selections for activating mutations have been performed in *Dictyostelium*, on G proteins involved in adenylyl cyclase activation (Jin et al., 1998) and in yeast, on heterologously expressed mammalian tmACs (Zimmermann et al., 1998, 1999; Haney et al., 2001). Our use of complementation of *cya*⁻ bacteria permits quantitative assessment of adenylyl cyclase activity; we screened for activating mutations in sAC70, but it would be equally possible to use *cya*⁻ bacteria to select inactivating mutations. Additionally, our screen proved to be quite sensitive. While sAC_i turns *cya*⁻ *E. coli* red because it has approximately 20-fold higher specific activity than sAC_{fl} or sAC70, we identified a mutation in sAC70, E467K, which turned *cya*⁻ *E. coli* red with only approximately six to seven fold higher specific activity.

The autoinhibitory domain in sAC_{fl} provides an additional level of regulatory complexity, which may be essential under certain conditions or in different subcellular compartments within spermatozoa. Both identified sAC isoforms are lost in sAC-KO mice and both are inhibited by the sAC-specific inhibitor KH7; therefore, it is thus far unclear what individual contributions to sperm function are played by each isoform. Because both isoforms are regulated in a similar manner by the known chemical modulators of sAC (bicarbonate and calcium), distinct functional roles of the two isoforms may depend upon regulatory properties, which are unique to one or the other isoform. The existence of an autoinhibitory domain in the full-length isoform defines such a unique modulatory mechanism in which its activity could be temporally and spatially regulated in a tightly controlled manner.

Acknowledgments

We thank Wei-Jen Tang for the original isolates of TP2000 and control plasmids, Fritz Muhlschlegel for the BW26356 cya-strain, and Jason B. Kiviat and Sara Wieselthier for assistance with the genetic selection. We also thank the LevBuck Laboratory for critical reading of the manuscript. This work was supported by NIH 5T32 CA062948 (J.A.C.), NIH HD38722 and the Hirschl Weil-Caulier Trust (L.R.L.), NIH HD42060, GM62328 and the Ellison Medical Foundation (J.B.).

REFERENCES

- Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E, Schutz G. Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature*. 1996; 380:162–165. [PubMed: 8600391]
- Botsford JL. Cyclic nucleotides in procaryotes. *Microbiol Rev*. 1981; 45:620–642. [PubMed: 6276705]
- Braun T. Inhibition of the soluble form of testis adenylate cyclase by catechol estrogens and other catechols. *Proc Soc Exp Biol Med*. 1990; 194:58–63. [PubMed: 1970182]
- Braun T. Purification of soluble form of adenylyl cyclase from testes. *Methods Enzymol*. 1991; 195:130–136. [PubMed: 1851925]
- Braun T, Dods RF. Development of a Mn-2+-sensitive, “soluble” adenylate cyclase in rat testis. *Proc Natl Acad Sci USA*. 1975; 72:1097–1101. [PubMed: 1055368]
- Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Natl Acad Sci USA*. 1999; 96:79–84. [PubMed: 9874775]
- Buck J, Sinclair ML, Levin LR. Purification of soluble adenylyl cyclase. *Methods Enzymol*. 2002; 345:95–105. [PubMed: 11665644]
- Chen Y, Cann MJ, Litvin TN, Lourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionary conserved bicarbonate sensor. *Science*. 2000; 289:625–628. [PubMed: 10915626]
- Esposito G, Jaiswal BS, Xie F, Krajnc-Franken MA, Robben TJ, Strik AM, Kuil C, Philipsen RL, Van Duin M, Conti M, Gossen JA. Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proc Natl Acad Sci USA*. 2004; 101:2993–2998. [PubMed: 14976244]
- Fraser LR. Dibutyryl cyclic AMP decreases capacitation time in vitro in mouse spermatozoa. *J Reprod Fertil*. 1981; 62:63–72. [PubMed: 6262510]
- Geng W, Wang Z, Zhang J, Reed BY, Pak CY, Moe OW. Cloning and characterization of the human soluble adenylyl cyclase (hsAC). *Am J Physiol Cell Physiol*. 2005
- Haney SA, Xu J, Lee SY, Ma CL, Duzic E, Broach JR, Manfredi JP. Genetic selection in *Saccharomyces* of mutant mammalian adenylyl cyclases with elevated basal activities. *Mol Genet Genomics*. 2001; 265:1120–1128. [PubMed: 11523785]
- Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS, Levin LR, Williams CJ, Buck J, Moss SB. The “soluble” adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev Cell*. 2005; 9:249–259. [PubMed: 16054031]

- Jaiswal BS, Conti M. Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. *J. Biol Chem.* 2001; 21:21.
- Jaiswal BS, Conti M. Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. *Proc Natl Acad Sci USA.* 2003; 100:10676–10681. [PubMed: 12958208]
- Jin T, Soede RD, Liu J, Kimmel AR, Devreotes PN, Schaap P. Temperature-sensitive Gbeta mutants discriminate between G protein-dependent and-independent signaling mediated by serpentine receptors. *Embo J.* 1998; 17:5076–5084. [PubMed: 9724643]
- Lefievre L, Jha KN, de Lamirande E, Visconti PE, Gagnon C. Activation of protein kinase A during human sperm capacitation and acrosome reaction. *J Androl.* 2002; 23:709–716. [PubMed: 12185106]
- Levin LR, Reed RR. Identification of functional domains of adenylyl cyclase using *in vivo* chimeras. *J Biol Chem.* 1995; 270:7573–7579. [PubMed: 7706305]
- Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, Reed RR. The *Drosophila* learning and memory gene rutabaga encodes a Ca^{2+} /Calmodulin-responsive adenylyl cyclase. *Cell.* 1992; 68:479–489. [PubMed: 1739965]
- Linder JU, Schultz A, Schultz JE. Adenylyl cyclase Rv1264 from *Mycobacterium tuberculosis* has an autoinhibitory N-terminal domain. *J Biol Chem.* 2002; 277:15271–15276. [PubMed: 11839758]
- Litvin TN, Kamenetsky M, Zarifyan A, Buck J, Levin LR. Kinetic properties of “soluble” adenylyl cyclase. Synergism between calcium and bicarbonate. *J Biol Chem.* 2003; 278:15922–15926. [PubMed: 12609998]
- Livera G, Xie F, Garcia MA, Jaiswal B, Chen J, Law E, Storm DR, Conti M. Inactivation of the mouse adenylyl cyclase 3 gene disrupts male fertility and spermatozoon function. *Mol Endocrinol.* 2005; 19:1277–1290. [PubMed: 15705663]
- Nantel F, Monaco L, Foulkes NS, Masquillier D, LeMeur M, Henriksen K, Dierich A, Parvinen M, Sassone-Corsi P. Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature.* 1996; 380:159–162. [PubMed: 8600390]
- Perlman RL, Pastan I. Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of *Escherichia coli*. *Biochem Biophys Res Commun.* 1969; 37:151–157. [PubMed: 4310317]
- Sinclair ML, Wang XY, Mattia M, Conti M, Buck J, Wolgemuth DJ, Levin LR. Specific expression of soluble adenylyl cyclase in male germ cells. *Mol Reprod Dev.* 2000; 56:6–11. [PubMed: 10737962]
- Steegborn C, Litvin TN, Levin LR, Buck J, Wu H. Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. *Nat Struct Mol Biol.* 2005; 12:32–37. [PubMed: 15619637]
- Stengel D, Hanoune J. The sperm adenylyl cyclase. *Ann NY Acad Sci.* 1984; 438:18–28. [PubMed: 6398658]
- Tang WJ, Gilman AG. Construction of a soluble adenylyl cyclase activated by Gs alpha and forskolin. *Science.* 1995; 268:1769–1772. [PubMed: 7792604]
- Tash JS, Means AR. cAMP-dependent regulatory processes in the acquisition and control of sperm flagellar movement. *Prog Clin Biol Res.* 1988; 267:335–355. [PubMed: 2853368]
- Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development.* 1995; 121:1139–1150. [PubMed: 7538069]
- Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol.* 2002; 53:133–150. [PubMed: 11730911]
- Wang RJ, Morse ML. Carbohydrate accumulation and metabolism in *Escherichia coli*. I. Description of pleiotropic mutants. *J Mol Biol.* 1968; 32:59–66. [PubMed: 4868120]
- White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. *Gamete Res.* 1989; 22:163–177. [PubMed: 2540081]

- Xie F, Conti M. Expression of the soluble adenylyl cyclase during rat spermatogenesis: Evidence for cytoplasmic sites of cAMP production in germ cells. *Dev Biol.* 2004; 265:196–206. [PubMed: 14697363]
- Zimmermann G, Zhou D, Taussig R. Genetic selection of mammalian adenylyl cyclases insensitive to stimulation by G α . *J Biol Chem.* 1998; 273:6968–6975. [PubMed: 9507003]
- Zimmermann G, Zhou D, Taussig R. Activating mutation of adenylyl cyclase reverses its inhibition by G proteins. *Mol Pharmacol.* 1999; 56:895–901. [PubMed: 10531392]

Fig. 1.

Adenylyl cyclase activity of heterologously expressed rat sAC_t, sAC_{fl}, and sAC_{fl} C-terminal deletions. HEK293 cells were transfected with empty vector, or the indicated sAC expression constructs (Diagrammed at **left**, with gray boxes denoting catalytic domains and C-terminal Flag tag). After 24 hr, cytosolic extracts were prepared and assayed for adenylyl cyclase activity (**center**) and sAC construct expression (**right**). Adenylyl cyclase activity was measured in the presence of 5.0 mM ATP and 10 mM MnCl₂. Data are expressed as nmoles of cAMP formed per minute per milligram of cytosolic protein, and values represent averages of duplicate determinations with error bars indicating SD from the mean. Shown is a representative of at least three independent experiments. Cytosolic extracts were separated by electrophoresis on SDS/7.5 % PAGE and probed with a FLAG M2 monoclonal antibody.

Fig. 2. Adenylyl cyclase activity of purified rat sAC_t and sAC70 proteins. **Upper:** sAC proteins, purified as recombinant GST fusion proteins, were separated by electrophoresis on SDS/7.5% PAGE and probed with a FLAG M2 monoclonal antibody. Different volumes were loaded (indicated on the top of each lane) to determine equivalent amounts of protein for in vitro cyclase assays. **Lower:** sAC_t and sAC70 were assayed in the presence of 5 mM ATP and either 10 mM MnCl₂ (hatched bars), 10 mM MgCl₂ (gray bars), or 10 mM MgCl₂ plus 40 mM NaHCO₃ and 10 mM CaCl₂ (dark bars). Values represent the average of triplicate determinations with error bars indicating SD from the mean. Shown is a representative of at least three independent experiments.

Fig. 3.

Calcium activates sAC70 by lowering its K_m for ATP-Mg²⁺. sAC activity was measured as a function of substrate [ATP-Mg²⁺] in the presence (dashed curve) or absence (solid curve) of 10 mM CaCl₂ with excess NaHCO₃ (80 mM) and MgCl₂ (80 mM). Values represent the average of triplicate determinations with error bars indicating SD from the means. Shown is a representative of at least two independent experiments. (Inset), For comparison, corresponding sAC70 Mn²⁺-dependent activity was measured as a function of substrate [ATP-Mn²⁺] in the presence of excess MnCl₂ (10 mM).

Fig. 4. Bicarbonate activation of sAC70 activity. Purified sAC70 was assayed in the presence of the indicated concentrations of NaHCO₃ with 10 mM ATP and 40 mM MgCl₂. Values represent the average of duplicate determinations, with error bars indicating SD from the mean. Shown is a representative of at least three independent experiments.

Fig. 5.

Phenotypic complementation assay revealing activity of sAC constructs in *cyd⁻* bacteria. **A:** TP2000 bacteria were transformed with the indicated sAC expression plasmids (pProExH6 backbone) and plated on MacConkey-maltose plates supplemented with ampicillin and 100 μ M of IPTG. Plates were incubated at 30°C for 36–48 hr. **B:** Sequence alignment of rat, mouse, rabbit, and human sAC (differences highlighted with gray box) indicating substitutions identified in the screen (denoted by black boxes and arrows above the alignment). The sequence shown is the minimal autoinhibitory domain; the first amino acid aligned is immediately C-terminal of C2 and the final amino acid shown is the C-terminal amino acid of sAC54, the smallest autoinhibited sAC construct. Arrow below sequences denotes the end of the naturally occurring sAC_t isoform (Buck et al., 1999; Jaiswal and Conti, 2001). [See color version online at www.interscience.wiley.com].

Fig. 6.

The E467K mutation of sAC70 relieves autoinhibition. The E467K mutation, identified by the complementation assay, was introduced into a sAC70 pBK-CMV expression construct by site-directed mutagenesis. HEK293 cells were then transfected with the indicated sAC expression constructs as previously indicated. After 24 hr, cytosolic extracts were prepared, and adenylyl cyclase activity was measured in the presence of 2.5 mM ATP and either 10 mM MnCl₂ (hatched bars), 10 mM MgCl₂ (gray bars), or 10 mM MgCl₂ plus 40 mM NaHCO₃ and 10 mM CaCl₂ (dark bars). Data are expressed as pmoles of cAMP formed per minute per milligram of sAC protein normalized via quantitation of Western blotted protein (not shown), and values represent averages of duplicate determinations with error bars indicating SD from the mean. Shown is a representative of at least four independent experiments.