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Cloning and characterization of a *Drosophila* adenylyl cyclase homologous to mammalian type IX

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Abstract A novel *Drosophila* adenylyl cyclase (AC) was identified by PCR using degenerate primers specific for the known metazoan ACs. The full-length cDNA predicts a protein displaying significant sequence homology with mammalian Type IX AC (AC9). The abundance and size of the message for the *Drosophila* AC9 homolog (DAC9) changes through development. Biochemical analysis of DAC9 confirms it encodes a functional enzyme which can be activated by forskolin or G protein. Together with the *Drosophila* Type I AC homolog encoded by the learning and memory gene, *rutabaga*, the molecular identification of DAC9 demonstrates there is a family of *Drosophila* AC isoforms reflecting at least part of the diversity of mammalian AC isoforms.

Key words: *Drosophila*; Adenylyl cyclase; cAMP; Forskolin; G protein; Signal transduction

1. Introduction

In mammals, adenylyl cyclase (AC), the enzyme responsible for generating the second messenger cAMP, is encoded by a family of at least nine genes. These mammalian AC isoforms possess a conserved structure comprised of two sets of six transmembrane spans with each set followed by a distinct but homologous catalytic domain [1]. The two catalytic domains, termed C_{1a} and C_{2a}, are both necessary and sufficient for enzymatic activity [2]. Differences in tissue distribution and modes of regulation imply that each isoform plays a distinct role in cAMP production. For certain isoforms, specific functions have been predicted from their individual biochemical properties [1], restricted localization [3,4], or expression patterns in different physiological states [5–7] or following pharmacological treatments [8–10]. However, only Type I AC (AC1) has been definitively assigned a specific physiological role. Molecular genetic analysis in *Drosophila melanogaster* revealed the AC1 ortholog is encoded by the learning and memory *rutabaga* gene [11,12]. Functional conservation of mammalian AC1 was later confirmed by knockout experiments in mouse [13].

A wide array of powerful genetic tools and an extensive list of molecularly uncharacterized mutations make *Drosophila* an excellent system to investigate gene functions. Specifically for the study of ACs, the fruit fly appears to be a valid indicator of mammalian physiology; the only characterized *Drosophila* AC is structurally, biochemically and functionally conserved with its mammalian ortholog AC1. However, for *Drosophila* to be a relevant model system for characterizing mammalian ACs, it must display the diversity of AC isoforms present in mammals. We now describe the cloning of a second *Drosophila* AC displaying significant homology to a specific mammalian isoform. In addition to indicating that cAMP production in *Drosophila* is accomplished by multiple AC isozymes, the evolutionary conservation of primary structure of at least two AC isoforms in *Drosophila*, the one described here and the previously characterized Rutabaga AC, suggest there is conservation of specific functions between fly and mammalian AC isozymes.

2. Materials and methods

2.1. Amplification of a novel Drosophila AC

A cDNA fragment was amplified by Polymerase Chain Reaction (PCR) using degenerate oligonucleotide primers [14] designed to recognize the conserved regions within the second intracellular catalytic domain of mammalian ACs (LRL87 for the amino acid sequence KIKT(I/V)G and LRL88 for the amino acid sequence WG(N/K/D/E)TVN). Briefly, first-strand cDNA was prepared from 1 μg of *D. melanogaster* adult poly(A)$^+$ RNA (Clontech) using SuperScript Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Approximately 2% of the resultant first-strand cDNA was subjected to PCR amplification in a total volume of 25 μl under standard conditions (Perkin Elmer). After denaturation at 94°C for 5 min, reactions were cycled 30 times for 1 min at 94°C, 30 s at 60°C and 30 s at 72°C followed by final extension at 72°C for 10 min. Products were separated on 1% agarose or 2% TreviGel (Trevigen) agarose gels, and a band of the appropriate size (270 base pairs) was excised and TA cloned into pCR-II (Invitrogen). The subcloned fragment was sequenced by chain termination using Sequenase-2 (Amersham). Clone AR7.1 corresponded to a novel *Drosophila* AC-like sequence.

2.2. cDNA library screening

Random-prime labeled AR7.1 was used to screen approximately 10^6 clones of a *Drosophila* adult cDNA library constructed in our laboratory using the ZAP Express cDNA synthesis kit (Stratagene). Five positive clones were recovered. The longest clone (AR7.1 #3) was 3.1 kb and encoded only a portion of the putative *Drosophila* AC. Its predicted open reading frame was homologous to AC sequences extending N-terminally from the second set of transmembrane domains through its presumptive C-terminus. The 5′-most sequences from the longest clone were used to screen a randomly primed *Drosophila* head cDNA library cloned into λ-gt11 kindly provided by Dr. Mike Forte [15]. The longest clones from this screen extended the putative open reading frame further, predicting a potential AC with 12 membrane-spanning domains, but it did not appear to correspond to the full-length cDNA. The final 5′-end sequence was obtained after an additional round of screening the random-primed *Drosophila* head library (Fig. 1). The final, full-length cDNA was reconstructed in pBluescript KS (Stratagene).

The full-length cDNA was sequenced on both strands by Rockefeller University or Cornell University Sequencing Cores using fluorescent dye terminator thermal cycle sequencing (PE-Applied Biosystems).

2.3. Northern blotting

Commercially obtained (Clontech) *Drosophila* Embryonic, Larval and Adult poly(A)$^+$ RNA (2.5 μg each) were separated on a 1%

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denaturing agarose gel and transferred to nylon membrane [16]. The 4.2-kb *Xho*I-*Xmn*I fragment of AR7.1 (Fig. 1) was labeled by random priming (Amersham). The filter was hybridized with probe overnight at 60°C in 50% formamide, 5× Denhardt's, 5×SSPE, 5% SDS, 200 μg/ml ssDNA, washed in 2×SSC, 0.1% SDS+0.1×SSC, 0.1% SDS at 65°C and exposed to X-ray film (Kodak) with intensifying screen for the indicated times.

2.4. Expression and assay of Drosophila ACs

The AR7.1 putative AC was heterologously expressed in mammalian cells using the cytomegalovirus promoter/enhancer of pRK5. Human embryonic kidney cells (HEK293) were transiently transfected using Lipofectamine (GIBCO BRL) with *Drosophila* AR7.1 cDNA or pRK5 vector with or without human luteinizing hormone receptor (LHR) subcloned into pKNH expression vector [17]. For cAMP accumulation assays, two days after transfection, cells were labeled for an additional 24 h with [^{3}H]adenine (2 μCi/ml) and assayed in the absence or presence of 1 μg/ml human β-chorionic gonadotropin (hCG) (Sigma). The level of intracellular cAMP was measured and expressed as the ratio of [^{3}H]cAMP to total adenine nucleotides as described previously [18]. AC activity in crude lysates was determined 72 h after transfection as described previously [12]. Data points represent triplicate assays and are reported as means ± S.D.

2.5. Isolation of P1 clones

A *Drosophila* P1 genomic library (Genome Systems) densely plated on filters was screened by hybridization with the 4.2-kb *Xho*I-*Xmn*I fragment corresponding to the coding sequence of AR7.1 cDNA (Fig. 1). Hybridization to positive clones was confirmed by Southern hybridization. Map positions of positive P1 clones were determined by Berkeley *Drosophila* Genome Project and were obtained via internet access (http://fruitfly.berkeley.edu/).

3. Results

3.1. Cloning a new Drosophila adenylyl cyclase

A novel AC-like sequence, AR7.1, was identified by PCR on *Drosophila* adult cDNA using degenerate primers designed to recognize C_{2a} domain sequences conserved in all previously described mammalian ACs and *Drosophila* Rutabaga AC. Using this AR7.1 PCR product as probe, five independent clones falling into two overlapping classes were isolated from a *Drosophila* adult oligo dT-primed cDNA library. In two of the five clones (AR 7.1 #2 and AR 7.1 #3 in Fig. 1), the site of poly-A addition was 600 nucleotides upstream compared to the other 3 clones. All five clones contained typical polyadenylation signal sequences (AATAAA) 25 nucleotides upstream from their respective poly-A$^+$ addition sites. These 2 classes of cDNAs appear to reflect differential polyadenylation usage in vivo (see below and Fig. 3). Two successive screenings of a randomly primed *Drosophila* head cDNA library resulted in the isolation of an additional 3 overlapping clones which were used to reconstruct the full-length AR7.1 cDNA (Fig. 1). This cDNA contained a single large open reading frame consisting of 1708 amino acids. The hydrophobicity profile of the presumptive protein, calculated using the Kyte and Doolittle algorithm in the Protean sequence analysis program (DNASTAR), predicts the postulated structure for all cloned metazoan ACs (Fig. 2A).

According to BLAST sequence analysis, the predicted protein sequence of *Drosophila* AR7.1 was most closely related to mouse AC9. Database searches revealed two other potential AC9 orthologs. A recently described *Xenopus laevis* oocyte AC [19] and a putative gene product in *Caenorhabditis elegans* detected by the Sanger Centre Genome Project (http://www.sanger.ac.uk/Projects/C_elegans/). Comparison of *Drosophila* AR7.1 with these two newly described ACs, the nine known mammalian ACs and *Drosophila* Rutabaga AC using the Clustal multiple sequence alignment algorithm within Megalign program (DNASTAR) suggests that AR7.1, the *X. laevis* AC (xlAC), the *C. elegans* AC (ceAC), and mouse AC9 define an interspecies subfamily comprised of apparent AC9 orthologs (Fig. 2B). Based on these sequence similarities, we refer to the *Drosophila* AR7.1 clone as DAC9.

Genomic Southern hybridization data demonstrated that DAC9 is present in the *D. melanogaster* Canton S genome as single-copy gene (data not shown), and isolation of a *Drosophila* P1 library clone indicates that the DAC9 locus maps to position 35C7–9.

3.2. Expression of DAC9

DAC9 mRNA expression, determined by Northern Blot analysis using a coding sequence-specific probe, varied in abundance and size through the *Drosophila* life cycle (Fig. 3). An 8.9-kb DAC9 mRNA was expressed throughout development and was the predominant species in embryos and adult flies. Adults also expressed an approximately equally abundant 7.5-kb message. The presence of two DAC9 mRNA species in adult flies correlates with the diversity of

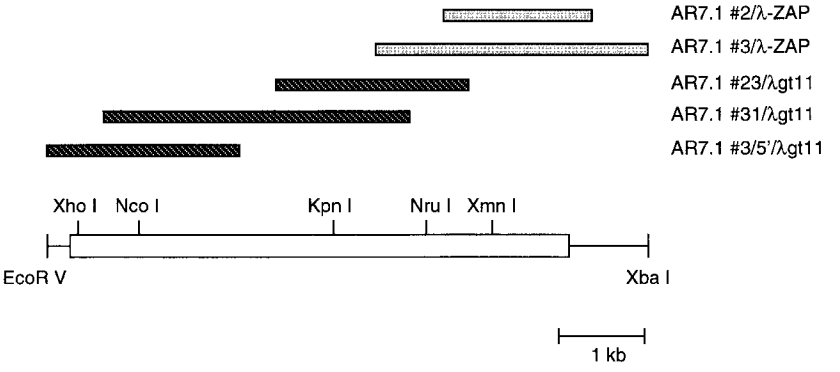

Fig. 1. cDNA clones corresponding to DAC9 *D. melanogaster* adenylyl cyclase. Overlapping clones corresponding to AR7.1 are shown schematically. Light gray bars represent the 2 groups of clones isolated from the *Drosophila* adult cDNA library (clones AR7.1 #2 and #3) differing in their 3′ ends, and dark gray bars represent clones isolated from the randomly primed *Drosophila* head cDNA library (clones AR7.1 #23, #31 and #3/5′) [17]. The full-length open reading frame is represented as an open bar with restriction sites used in reconstruction indicated (*Nco*I, *Kpn*I and *Nru*I). The *Xho*I-*Xmn*I 4.2-kbp fragment was used as probe in Northern blot analysis and P1 library screen.

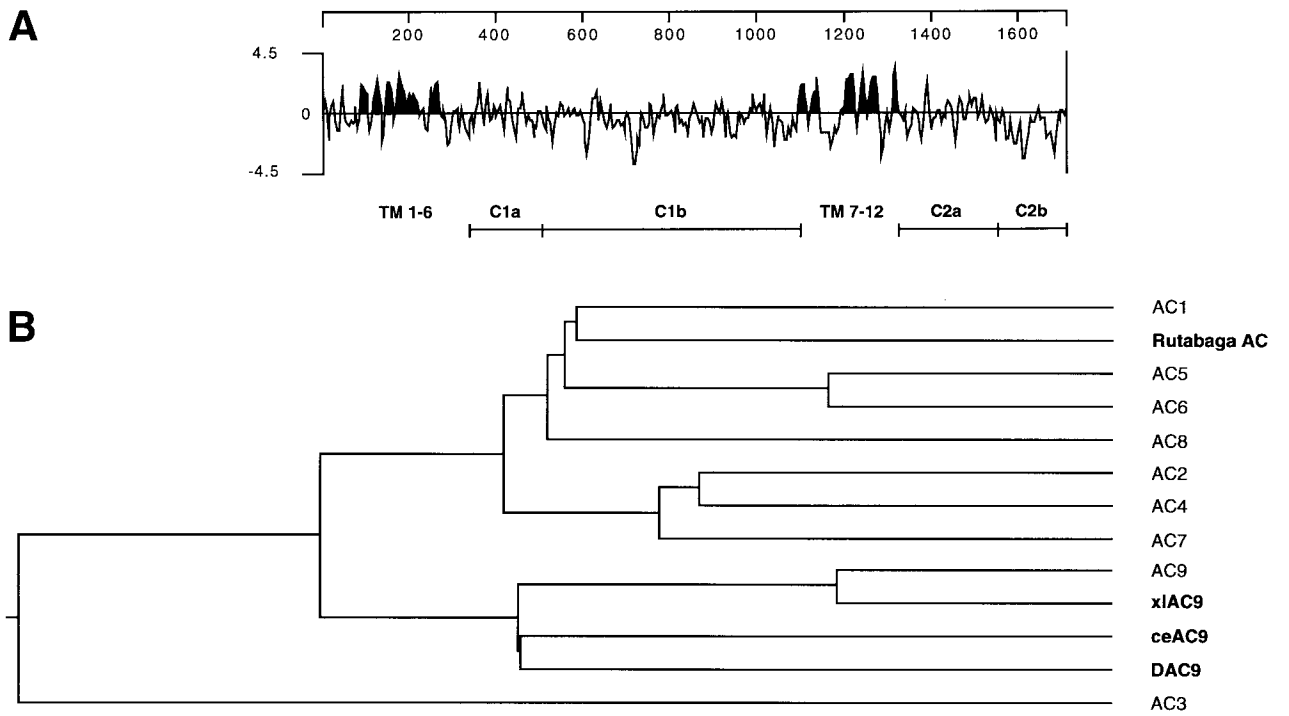

Fig. 2. Deduced amino acid sequence of DAC9. (A) Calculated hydrophobicity profile of DAC9. Twelve predicted transmembrane spans are filled in and labeled TM 1–6 and TM 7–12. Regions corresponding to the conserved catalytic domains are labeled C_{1a} and C_{2a} and the variable domains are labeled C_{1b} and C_{2b}. The approximate size, in amino acids, is indicated above. (B) Alignment of predicted amino acid sequences of known *Drosophila* ACs with the 9 known mammalian ACs. *Drosophila*, *Xenopus* and *C. elegans* ACs are shown in bold. Previously published AC sequences were as follows; AC1-bovine AC1 [25]; AC2-rat AC2 [26]; AC3-rat AC3 [3]; AC4-rat AC4 [27]; AC5-rat AC5 [28]; AC6-rat AC6 [28]; AC7-mouse AC7 [29]; AC8-rat AC8 [30]; AC9-mouse AC9 [22]; Rutabaga AC [12], *Xenopus* laevis oocytes AC-xlAC9 [19], *C. elegans* AC-ceAC9 (http://www.sanger.ac.uk/Projects/C_elegans/).

3′ ends among cDNA clones detected in the adult cDNA library; as described above, this very likely reflects alternative polyadenylation site utilization. Interestingly, the predominant mRNA in larvae was 7.2 kb. This larval-specific 7.2-kb DAC9 mRNA has not been further investigated but might imply a specific function for DAC9 during larval development.

3.3. Enzymatic activity of DAC9

We first tested whether DAC9 protein had enzymatic activity in response to activated G_s protein in vivo. HEK293 cells were cotransfected with DAC9 and the G_s-coupled LH receptor [17]. Treatment of cotransfected cells with hCG, a specific LH receptor agonist, induced cAMP production in DAC9 expressing cells 4-fold over vector transfected cells (Fig. 4). These results confirm that this novel *Drosophila* cDNA encodes a functional AC and is capable of coupling to the endogenous human G_s protein.

The in vitro biochemical properties of DAC9 were then compared with those of its mammalian counterpart. Basal activities in crude lysates of HEK293 cells transfected with DAC9 exhibited less basal activity than murine AC9 lysates; however, the DAC9 responses to activated G protein and to forskolin were much more pronounced than those of its mammalian counterpart (Fig. 5A). Dose-dependent stimulation of DAC9 activity was consistently greater than vector control at GTPγS concentrations as low as 0.5 μM (Fig. 5B) and forskolin concentrations as low as 5 μM (Fig. 5D). In contrast to mammalian AC9, DAC9 is potently stimulated by forskolin.

4. Discussion

The use of *Drosophila* as a genetic model for studying mammalian ACs is dependent upon it possessing a gene family of AC isoforms of similar extent and complexity as that found in

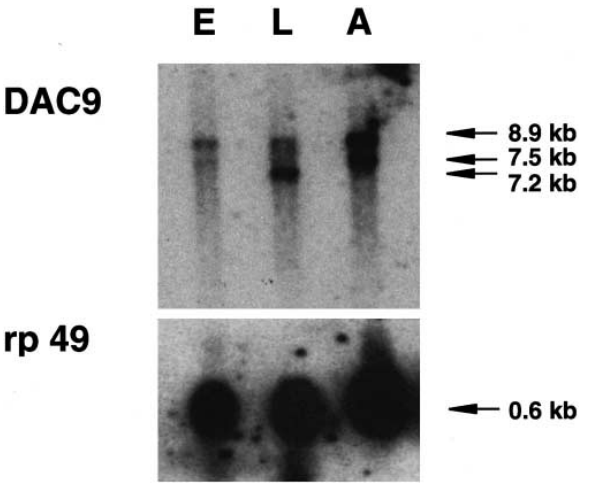

Fig. 3. Expression of DAC9 mRNA through development. 2.5 μg of *Drosophila* embryonic (E), larval (L) and adult (A) poly(A)+ RNAs were hybridized to DAC9 (*top*) coding region-specific probe. As load control, the blot was probed with the ribosomal protein 49 (rp 49) specific probe (*bottom*). Film exposure times were as follow 30 h for DAC9 and 1 h for rp 49. The approximate sizes of each message were calculated in relation to a commercially available RNA ladders (Life Technologies).

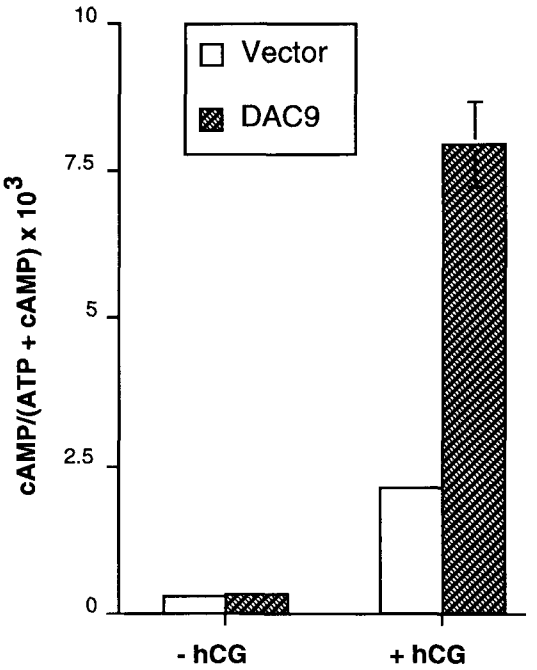

Fig. 4. In vivo cAMP accumulation of DAC9. cAMP accumulation in HEK293 cells coexpressing DAC9 with LH receptor cDNA determined in the absence (−hCG) or presence (+hCG) of human chorionic gonadotropin. Values correspond to the ratio of cAMP to total adenine nucleotides and represent means of triplicate assays with error bars indicating standard deviation.

mammals. Residual cyclase activity in flies deficient for the only previously known *Drosophila* AC, Rutabaga AC [11,20], and distinct AC-like sequences detected in *Drosophila* genomic DNA [12,21], suggested the existence of additional AC isoforms in flies. However, there has so far been no direct demonstration of multiple AC isoforms in *Drosophila* nor an indication of their molecular nature. The additional *Drosophila* AC gene described here confirms that cAMP synthesis in flies is accomplished by a family of AC isoforms showing significant structural homology to mammalian AC isozymes.

DAC9 is highly homologous to mouse AC9 as well as to newly described isoforms from *Xenopus* and *C. elegans*. There is 55% amino acid identity between the catalytic domains of DAC9 and those of mouse AC9. The *C. elegans* isoform is similarly conserved, exhibiting 54% identity within its catalytic domains compared to mammalian AC9. This significant structural conservation of type 9 AC across evolution suggests it performs an important physiological function. Additionally, AC9 isoforms appear to be nearly ubiquitously expressed, also implying a fundamental biological role. Mammalian AC9 is widely expressed in adult mice [22], DAC9 is expressed throughout fly development, and *Xenopus* AC9 mRNA is abundant in oocytes but was not detected in early embryos [19]. Expression patterns of *C. elegans* AC9 or of *Xenopus* AC9 later in development are not yet known.

DAC9 is the longest of the AC9 isoforms primarily due to its large C_{1b} domain (Fig. 2A). This region does not appear to be required for G_s or forskolin responsiveness [2]; W.-J. Tang, personal communication) and might be involved in subcellular localization or have as yet undiscovered regulatory interactions.

Consistent with previous reports [22], heterologously ex-

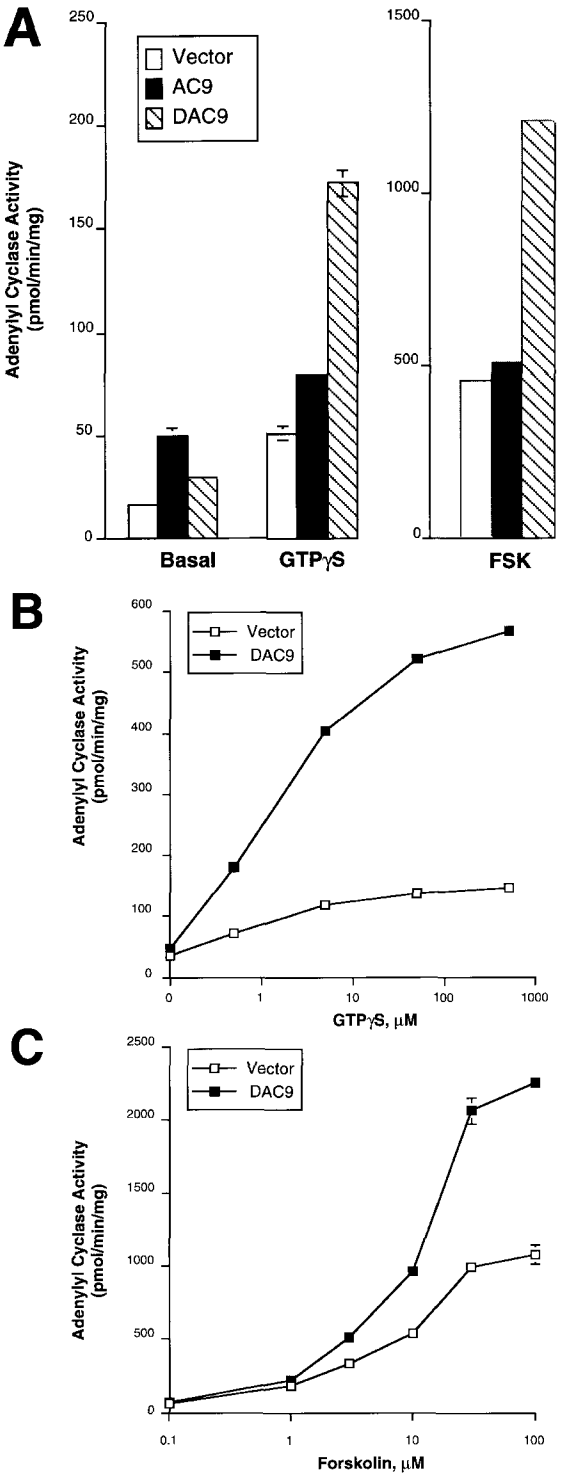

Fig. 5. In vitro AC activity of DAC9 compared to mammalian AC9. (A) AC activity was measured in crude lysates prepared from HEK293 cells expressing vector alone (open bars) DAC9 (striped bars), or mouse AC9 (black bars) without any addition (basal), with 100 μM GTPγS or with 100 μM forskolin (FSK). AC activity in crude lysates of vector-transfected (open symbol) or DAC9-transfected (filled symbol) HEK293 cells determined in the presence of increasing concentrations of (B) GTPγS or (C) forskolin. Values correspond to pmoles cAMP formed per min per mg protein and represent means of triplicate assays with error bars indicating standard deviations.

pressed murine AC9 exhibited high basal activity which was essentially insensitive to forskolin. In contrast, DAC9 basal activity was undetectable in transiently transfected HEK293 cells but was significantly stimulated by forskolin. The physiological significance of activation by the diterpene forskolin is not known, but this difference in responsiveness would be predicted from the amino acid sequences of murine AC9 and DAC9. Two residues in AC9 C_{2a} which differ from every other known member of the AC superfamily (residue 1082 in murine AC9 is tyrosine instead of the conserved leucine and residue 1112 is alanine instead of the conserved serine) appear to be involved in forskolin responsiveness. In particular, the Tyr^{1082} substitution is sufficient to abrogate forskolin responsiveness in AC9 (S.-Z. Yan, Z.-H. Huang, and W.-J. Tang, personal communication). Interestingly, the analogous residues in DAC9, Leu^{1407} and Ser^{1437}, are conserved with the AC superfamily and correctly predict DAC9 should be forskolin responsive.

There are many uncharacterized mutations corresponding to the genetic map position of DAC9, one in particular is a potentially interesting candidate. A female sterile mutation, *midway*, maps near 35C [23], and it has long been known that there is an important role for an AC in fly fertility [24]. We are currently pursuing an extensive genetic analysis of this and other genetic defects mapping to 35C to determine whether they are caused by mutation of DAC9.

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References

[1] Sunahara, R.K., Dessauer, C.W. and Gilman, A.G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 461–480.
[2] Tang, W.J. and Gilman, A.G. (1995) Science 268, 1769–1772.
[3] Bakalyar, H.A. and Reed, R.R. (1990) Science 250, 1403–1406.
[4] Glatt, C.E. and Snyder, S.H. (1993) Nature 361, 536–538.
[5] Tzavara, E.T., Pouille, Y., Defer, N. and Hanoune, J. (1996) Proc. Natl. Acad. Sci. USA 93, 11208–11212.
[6] Torgan, C.E. and Kraus, W.E. (1996) Am. J. Physiol. 271, E253–260.
[7] Espinasse, I., Iourgenko, V., Defer, N., Samson, F., Hanoune, J. and Mercadier, J.J. (1995) J. Mol. Cell. Cardiol. 27, 1789–1795.
[8] Granneman, J.G. (1995) Endocrinology 136, 2007–2012.
[9] Avidor-Reiss, T., Nevo, I., Levy, R., Pfeuffer, T. and Vogel, Z. (1996) J. Biol. Chem. 271, 21309–21315.
[10] Matsuoka, I., Maldonado, R., Defer, N., Noel, F., Hanoune, J. and Roques, B.P. (1994) Eur. J. Pharmacol. 268, 215–221.
[11] Livingstone, M.S., Sziber, P.P. and Quinn, W.G. (1984) Cell 37, 205–215.
[12] Levin, L.R., Han, P.L., Hwang, P.M., Feinstein, P.G., Davis, R.L. and Reed, R.R. (1992) Cell 68, 479–489.
[13] Wu, Z.L., Thomas, S.A., Villacres, E.C., Xia, Z., Simmons, M.L., Chavkin, C., Palmiter, R.D. and Storm, D.R. (1995) Proc. Natl. Acad. Sci. USA 92, 220–224.
[14] Premont, R.T. (1994) Methods Enzymol. 238, 116–127.
[15] Demchyshyn, L.L., Pristupa, Z.B., Sugamori, K.S., Barker, E.L., Blakely, R.D., Wolfgang, W.J., Forte, M.A. and Niznik, H.B. (1994) Proc. Natl. Acad. Sci. USA 91, 5158–5162.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] Rosenthal, W. et al. (1993) Adv Second Messenger Phosphoprotein Res. 28, 47–55.
[18] Levin, L.R. and Reed, R.R. (1995) J. Biol. Chem. 270, 7573–7579.
[19] Torrejon, M., Echeverria, V., Retamales, G., Herrera, L., Hinrichs, M.V. and Olate, J. (1997) FEBS Lett. 404, 91–94.
[20] Han, P.L., Levin, L.R., Reed, R.R. and Davis, R.L. (1992) Neuron 9, 619–627.
[21] Cann, M.J. and Levin, L.R. (1997) in: Advances in Second Messenger and Phosphoprotein Research (Cooper, D.M.F., Ed.), Lippincott-Raven, New York (in press).
[22] Premont, R.T., Matsuoka, I., Mattei, M.G., Pouille, Y., Defer, N. and Hanoune, J. (1996) J. Biol. Chem. 271, 13900–13907.
[23] Schupbach, T. and Wieschaus, E. (1991) Genetics 129, 1119–1136.
[24] Bellen, H.J., Gregory, B.K., Olsson, C.L. and Kiger Jr., J.A. (1987) Dev. Biol. 121, 432–444.
[25] Krupinski, J. et al. (1989) Science 244, 1558–1564.
[26] Feinstein, P.G., Schrader, K.A., Bakalyar, H.A., Tang, W.J., Krupinski, J., Gilman, A.G. and Reed, R.R. (1991) Proc. Natl. Acad. Sci. USA 88, 10173–10177.
[27] Gao, B.N. and Gilman, A.G. (1991) Proc. Natl. Acad. Sci. USA 88, 10178–101782.
[28] Premont, R.T., Chen, J., Ma, H.W., Ponnapalli, M. and Iyengar, R. (1992) Proc. Natl. Acad. Sci. USA 89, 9809–9813.
[29] Watson, P.A., Krupinski, J., Kempinski, A.M. and Frankenfield, C.D. (1994) J. Biol. Chem. 269, 28893–28898.
[30] Cali, J.J., Zwaagstra, J.C., Mons, N., Cooper, D.M.F. and Krupinski, J. (1994) J. Biol. Chem. 269, 12190–12195.