Regulation of Adenylyl Cyclase from *Paramecium* by an Intrinsic Potassium Conductance

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Hyperpolarization of the cell membrane of *Paramecium* stimulates adenosine 3′,5′-monophosphate (cAMP) formation. Manipulations of the K^+ resting conductance of the ciliate by adaptation in different buffers affected excitability of the cAMP generating system. Blockade of K^+ channels inhibited hyperpolarization-stimulated cAMP formation. A mutant of *Paramecium* that is unable to control its K^+ resting conductance had a defect in cAMP formation. Purified adenylyl cyclase, when incorporated into an artificial lipid bilayer membrane, revealed properties of a voltage-independent K^+ channel. This indicates that the adenylyl cyclase of *Paramecium* has a secondary function as carrier of the K^+ resting conductance. A hyperpolarization-activated K^+ efflux appears to directly regulate adenylyl cyclase activity in vivo.

ADENOSINE 3′,5′-MONOPHOSPHATE (cAMP) serves as a second messenger in mammalian cells. Many hormones stimulate or inhibit cAMP synthesis via three functionally coupled membrane-associated proteins: hormone receptors, guanine nucleotide-binding proteins (G proteins), and adenylyl cyclase (*1*). The amino acid sequence of an adenylyl cyclase from bovine brain shows a topographical similarity with ion channels such as Ca^{2+} and K^+ channels (*2*). However, experimental evidence for a channel-related function of adenylyl cyclase has not yet been obtained. The adenylyl cyclase of *Paramecium* is unresponsive to cholera toxin, pertussis toxin, fluoride, guanosine triphosphate, and its nonhydrolyzable analogues 5′-guanylylimidodiphosphate (GMPPNP) or guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) in vitro (*3*). These data exclude regulation by G proteins. In vivo, cAMP formation is triggered not by hormones but by changes in the concentration of extracellular ions that cause hyperpolarization (*4*). It is unknown whether the membrane potential itself or a current resulting from hyperpolarization is coupled to adenylyl cyclase activation. As a freshwater ciliate *Paramecium* must actively regulate its K^+ resting conductance to maintain the membrane potential within narrow limits in changeable environments (*5, 6*). Therefore, the extent of hyperpolarization caused by dilution of external cations depends on the composition of the equilibration buffer, which determines the existing resting conductance (*6*). Because hyperpolarizing stimuli increase cAMP production in *Paramecium* (*4*), we investigated whether the K^+ resting conductance itself may be coupled to cAMP regulation.

Paramecia that were adapted for 4 hours in solutions containing between 1 and 24 mM K^+ were hyperpolarized by proportional dilution of the concentration of potassium, $[K^+]$. The amount of cAMP present was determined 10 s later. In cells adapted to 16 mM K^+, in which the resting conductance is high, dilution of $[K^+]$ to one eighth of its original concentration increased the accumulation of cAMP fourfold (Fig. 1A). In cells adapted to 1 mM K^+, in which the resting conductance is lower (*6*), dilution of $[K^+]$ to 0.125 mM did not enhance cAMP production (Fig. 1A). The stimulation of cAMP formation correlates with the change of resting membrane potentials that

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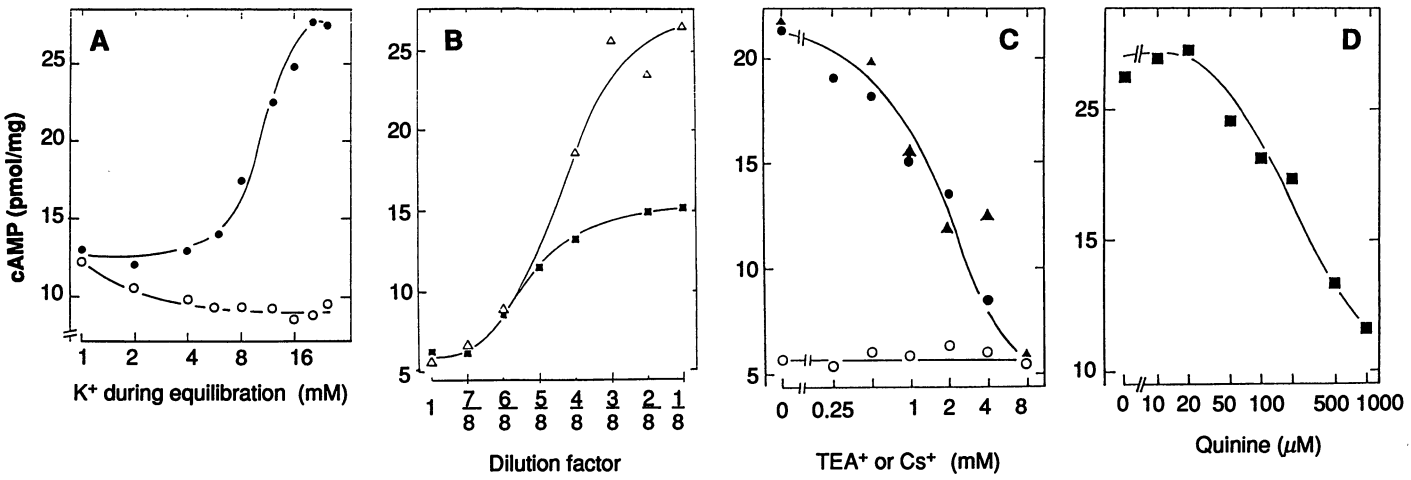

Fig. 1. Formation of cAMP in *Paramecium tetraurelia* wild-type 51s cells stimulated by hyperpolarization after dilution of extracellular $[K^+]$. (**A**) Cells grown at 25°C in an axenic complex medium (*18*) were collected by centrifugation at 250*g*, washed, and equilibrated for 4 hours at 50,000 cells/ml (90,000 cells = 1 mg of protein) in 10 mM 4-morpholine propanesulfonic acid (MOPS)–tris (pH 7.2), containing the $[K^+]$ indicated on the abscissa and 0.5 mM Ca^{2+}. For stimulation, $[K^+]$ was diluted to one eighth of its original concentration by transfer of 50 μl of equilibrated cells with a wide-bore pipette into 350 μl of K^+-free buffer (●). Viability and swimming behavior were checked microscopically before and during the experiments. Incubations were stopped after 5 s by adding perchloric acid (final concentration 1 M). The amount of cAMP present was determined by radioimmunoassay (*19*). Cross-reactivity of the antibody with cGMP (guanosine 3′,5′-monophosphate) was <5%. No cAMP was found when samples were digested with phosphodiesterase before the assay. Amounts of cGMP were measured concomitantly; no changes were found. Basal amounts of cAMP before K^+ dilution are indicated by open circles. (**B**) Relation between hyperpolarization and cAMP formation. Cells adapted for 4 hours at 8 (■) or 16 mM (△) K^+ and 1 mM Ca^{2+} were stimulated by dilution of external K^+ proportionally as indicated on the abscissa and analyzed as indicated above. Representative experiments are shown (n = 2 to 5). (**C** and **D**) Inhibition of cAMP formation by K^+ channel blockers TEA^+, Cs^+, and quinine. Cells were adapted for 4 hours in buffers containing 16 mM K^+, 0.5 mM Ca^+ and the indicated concentrations of TEA^+ (●) or Cs^+ (▲) (C), and quinine (D). For stimulation, external K^+ was diluted to one eighth of its original concentration. Amount of cAMP in unstimulated cells (○). In all instances, stimulations were for 5 s.

is observed after dilution of extracellular [K^+] (7). The apparent co-regulation of the K^+ resting conductance and cAMP formation suggests that the two targets are coupled. The differences in cAMP production were not due to (i) a shift in the time course (peak amounts were obtained between 5 and 10 s), (ii) cAMP leakage from cells, (iii) an osmotic or surface charge effect (balance with choline or tris was without effect), nor (iv) changes in basal adenylyl cyclase or phosphodiesterase activities (*8*).

The relation between hyperpolarization and cAMP production was quantitated by diluting an adaptation solution to different extents. When similarly graded stimuli were applied to paramecia adapted to 8 or 16 mM K^+, hyperpolarization response curves for cAMP formation were obtained (Fig. 1B). The dilutions necessary for half maximal stimulations were comparable in both instances (5/8); however, the maximal cAMP response was reduced in cells adapted to 8 mM K^+ in which the resting conductance is attenuated (*6*). This indicates that at low [K^+] the number of ion pores that participate in maintaining the resting potential is decreased.

The K^+ resting conductance in *Paramecium* is regulated not by K^+ itself but by the membrane potential. Hyperpolarization upon dilution is, therefore, self-limiting in *Paramecium* (Fig. 1A) (7). A further indication that the membrane potential regulates the K^+ resting conductance is that other ions, for example Na^+ and Ca^{2+}, that also affect the membrane potential, regulate this conductance as well (*5*). Hyperpolarization of *Paramecium* by dilution of [Na^+] or by an increase in [Ca^{2+}] also stimulates cAMP formation (*4, 8*).

We used blockers of K^+ channels to investigate the coupling of the K^+ resting conductance and cAMP synthesis. Tetraethylammonium (TEA^+), Cs^+, or quinine, which block K^+ channels in many cells including *Paramecium* (*9, 10*), inhibited cAMP production upon dilution of [K^+] in a dose-dependent manner (Fig. 1, C and D). The IC_{50} (dose that inhibited by 50% the effect of diluting [K^+]) was 1.5 mM for TEA^+ and Cs^+ and 100 μM for quinine. In agreement with electrophysiological data (*10*), the effect of TEA^+ did not require prior incubation, whereas Cs^+ and quinine had to be added 30 min prior to stimulation. Production of cAMP stimulated by dilution of Na^+ or an increase in Ca^{2+} was also blocked by TEA^+. Concentrations of these blockers needed to inhibit cAMP formation were lower than those required in electrophysiological tests (*10*). This is not surprising because the experimental methods and the integrity of the specimens differ substantially. Neither TEA^+, Cs^+, nor quinine affected cell-free adenylyl cyclase activity at concentrations used in these experiments (*8*).

A mutant of *Paramecium*, *restless*, is oversensitive to low external [K^+] (*7, 11*). The mutant lacks control of the K^+ resting conductance; it cannot curb its membrane hyperpolarization upon K^+ dilution and behaves like a passive K^+ electrode (7). When cells were adapted to 4 mM K^+, dilution of external [K^+] to 0.125 mM elicited a large accumulation of cAMP in *restless* cells but not in wild-type cells (Fig. 2). This result agrees with the reported membrane resistances of *restless* (30 megohms) and wild-type cells (60 megohms) at 4 mM external K^+ (7). Blocking the K^+ conductance of *restless* cells with TEA^+ inhibited the cAMP response with an IC_{50} of 1.5 mM. An extra-

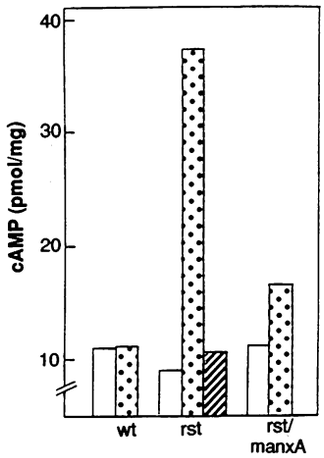

Fig. 2. Hyperpolarization-stimulated formation of cAMP in *Paramecium tetraurelia* wild-type 51s, K^+ channel mutant *restless* (rst), and the double mutant *rst/manxA*. Cells equilibrated in 4 mM K^+ for 4 hours were stimulated for 5 s by an eightfold dilution of K^+ (dotted bars). Open bars represent unstimulated controls. Addition of 8 mM TEA^+ with the dilution buffer inhibited the exaggerated response in *restless* (hatched bar). Because the mutant cells do not grow in axenic media, these experiments were carried out with cells grown in cerophyl medium inoculated with *Enterobacter aerogenes* (*20*). A representative experiment is shown (n = 3 to 6).

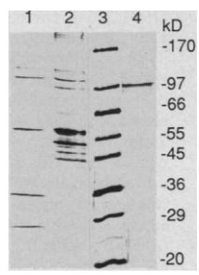

Fig. 3. Analysis of adenylyl cyclase purified from cilia of *Paramecium tetraurelia* wild-type 51s by SDS–polyacrylamide gel electrophoresis (10%). Lane 1, unbound material from second phenyl-Superose; lane 2, active fractions eluted from phenyl-Superose; lane 3, molecular size standards; lane 4, adenylyl cyclase after the final column chromatography. Proteins were detected by silver stain.

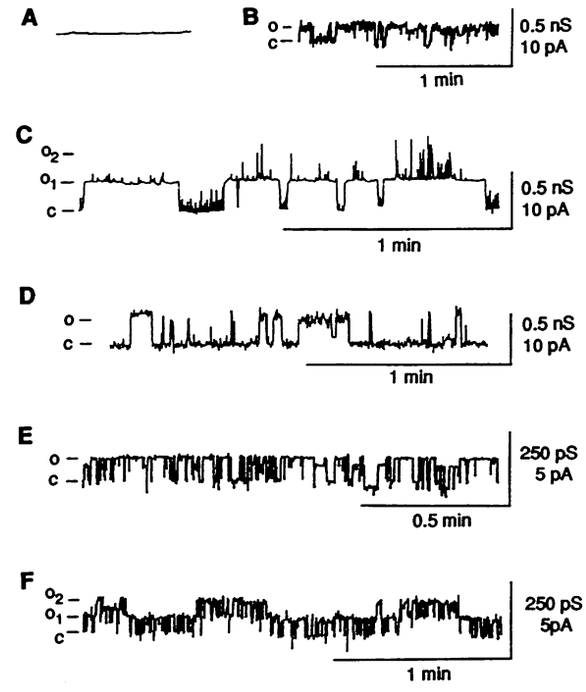

Fig. 4. Single ion channel activity of purified adenylyl cyclase from *Paramecium* reconstituted into artificial lipid-bilayer membranes. (**A**) Recording in solution containing 1 M KCl in the presence of 10 μl of elution buffer per milliliter in the measuring cell. (B to D) Approximately 1 ng of purified adenylyl cyclase (10 μl) was added to each side. Recording at 150 mM KCl (**B**); 1 M KCl (**C**); 1 M NaCl (**D**); 0.5 M $CaCl_2$ (**E**); 0.5 M $MgCl_2$ (**F**). Bilayer membranes of 0.08 mm^2 were formed from a solution of diphytanoyl phosphatidylcholine (1%, w/v) (from Avanti Polar Lipids, Birmingham, Alabama) in n-decane in a 2 × 5 ml teflon chamber as described (*15*). The salt solutions were buffered with 10 mM tris-HCl (pH 7.2). Adenylyl cyclase (approximately 1 ng in 10 μl) was added to each side of the bilayer membrane. The membrane potential was held at 20 mV with a pair of calomel electrodes with salt bridges. The current was amplified with a current to voltage converter (Burr Brown operational amplifier). Data were monitored with a storage oscilloscope (Tektronix 7633) and stored on a tape recorder (Racal DS 20) or on a chart recorder. The records were analyzed by hand. Experiments were carried out at ambient temperature (22° ± 2°C). Purification of adenylyl cyclase and reconstitution into lipid bilayers were carried out six times with identical results. Negative controls with inactive fractions or detergent-containing buffer were included in all reconstitution experiments. Continuous recording from single bilayers was possible for more than 60 min. Representative original graphs from different experiments are shown.

genic suppressor mutation, *manxA*, suppresses the electrophysiological defects of *restless* without affecting other ion currents (*9*). The hyperpolarization-induced overproduction of cAMP caused by the *restless* lesion was also corrected in the double mutant (Fig. 2). Because cell-free adenylyl cyclase activities of the *restless* mutant and the *restless-manxA* double mutant were identical to those of wild-type cells, the mutant data strongly link the K^+ resting conductance to regulation of cAMP formation.

Hyperpolarization by dilution does not require a net ion current per se. The shift of the K^+ equilibrium potential alone is sufficient to hyperpolarize the membrane potential. Nevertheless, hyperpolarization transiently increases those ion fluxes that, under conditions of complete adaptation, are delimited by the K^+ and Ca^{2+} resting conductances (*5, 6*). These K^+ and Ca^{2+} fluxes do not contribute to electrogenesis because they are electrically balanced (*5, 6*). Application of the Nernst equation to the K^+-dilution experiments indicates that only a K^+ efflux is possible. This efflux occurs through the channels that carry the major K^+ resting conductance and appears to serve a non-electrical function in that it directly controls cAMP formation.

Considering the proposed transmembrane topography of adenylyl cyclase (*2*), it is conceivable that in *Paramecium* the adenylyl cyclase itself operates as an ion channel. Therefore, the enzyme was purified. Because cilia constitute only 1% of cellular protein, but contain 50% of adenylyl cyclase activity they were used as starting material (*12*). The enzyme activities in the ciliary membranes (as cAMP, 0.5 nmol mg^{-1} min^{-1}) and of the purified protein (as cAMP, 25 μmol mg^{-1} min^{-1}) were similar to those of preparations from bovine brain (*13*). Analysis by SDS–polyacrylamide gel electrophoresis showed one major band at 96 kD (Fig. 3). About 20 ng of purified adenylyl cyclase were obtained from 170 g of paramecia.

Adenylyl cyclase was tested in artificial lipid-bilayer membranes for pore-forming activity. When partially enriched adenylyl cyclase was used for reconstitution, two transient channels with mean values of ion conductances (±SD) of 0.34 ± 0.13 (380 events) and 1.5 ± 0.3 nS (420 events) were observed with equal frequency in 28 of 30 membranes at 1 M KCl. The mean open times varied from 10 ms to 10 s. When purified enzyme was used for reconstitution, the large conductances were absent and almost all channels (>95%) had a conductance of 320 ± 60 pS (800 events) in 18 of 20 membranes (Fig. 4C). In medium containing 0.15 M KCl, single channel conductance was 150 ± 50 pS (230 events) (Fig. 4B); this means that the conductance was saturated with increasing salt concentrations. The ion selectivity of the channel was probed with other cations. Relative permeability ratios were determined to be approximately 1:1:0.5:0.3:0.25:0.2 for K^+:Cs^+:Na^+:Ca^{2+}:Li^+:Mg^{2+}, respectively (Fig. 4). TEA-chloride was impermeable.

Because of the small number of active channels (one to two at a given time), the observed inhibition of the K^+ conductance by TEA^+ could not be reliably evaluated. Single channel conductance was identical with potassium acetate instead of KCl. This demonstrates that the conductance is specific for monovalent cations, with a preference for K^+. With voltages of up to 150 mV applied to the bilayer, the range of channel open times was voltage-independent whereas single channel conductance increased linearly. Enzymatic activity of adenylyl cyclase and pore-forming activity were strictly interdependent; any decrease of enzyme activity resulted in a comparable loss of pore-forming activity. Addition of column fractions lacking adenylyl cyclase activity or containing heat-inactivated enzyme or addition of elution buffer alone (Fig. 4A) did not elicit channel activity. This excluded artifacts due to the use of Brij 35 (0.05%) as a detergent. The channel properties of the adenylyl cyclase were distinct from those reported for cation-specific channels of the ciliary membrane (*14*); in those experiments only a few active channels were detected in the lipid bilayer although 10^{10} channels were added per experiment (*14*). The pore-forming activity of the protozoan adenylyl cyclase equaled that of bacterial porins, which have the highest reconstitution capacity of all membrane proteins (*15*).

The adenylyl cyclase assayed for pore-forming activity was a homogenous protein which was purified according to its adenylyl cyclase activity. The pore-forming activity copurified through six distinct separation steps and was absolutely dependent on enzymatic activity of the protein. This suggests that the protozoan adenylyl cyclase is not only regulated by the resting membrane potential but is itself the transmembrane ion channel responsible for setting the resting potential. Thus, it has an intrinsic secondary regulatory function. This adenylyl cyclase may, therefore, be an evolutionary ancestor of the metazoan enzyme which contains 12 membrane-spanning regions of unknown function (*2*). Cloning of the gene corresponding to the protozoan enzyme may specify its exact relationship to metazoan adenylyl cyclases.

The data also shed new light on the relationship between cyclases, hormone receptors, and ion channels. All combinations can possibly exist: (i) receptor and cyclase in one protein as in the guanylyl cyclase–atrial natriuretic factor receptor (*16*); (ii) receptor and ion channel coupled in an oligomere as in the nicotinic acetylcholine receptor; and (iii) an ion channel intrinsic to an adenylyl cyclase as in the protozoan enzyme reported here. Such an ion channel–adenylyl cyclase unit might also operate in metazoans, possibly as a class of K^+ pores that are responsible for the resting conductance. Recently, a close functional coupling between a voltage-independent K^+ conductance, probably the resting conductance, and the cAMP system has been demonstrated in *Drosophila* (*17*).

REFERENCES AND NOTES

1. M. Schramm and Z. Selinger, *Science* **225**, 1350 (1984); H. G. Dohlman, M. G. Caron, R. J. Lefkowitz, *Biochemistry* **26**, 2657 (1987); A. G. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987); T. Pfeuffer and E. J. M. Helmreich, *Curr. Top. Cell. Regul.* **29**, 129 (1988).
2. J. Krupinski *et al.*, *Science* **244**, 1558 (1989).
3. S. Klumpp, D. Gierlich, J. E. Schultz, *FEBS Lett.* **171**, 95 (1984).
4. J. E. Schultz, R. Grünemund, R. von Hirschhausen, U. Schönefeld, *ibid.* **167**, 113 (1984); N. M. Bonini, M. C. Gustin, D. L. Nelson, *Cell Motil. Cytoskeleton* **6**, 256 (1986).
5. H. Machemer, in *Paramecium*, H.-D. Görtz, Ed. (Springer-Verlag, Berlin, 1988), pp. 185–215; *J. Protozool.* **36**, 463 (1989); S. Machemer-Röhnisch and H. Machemer, *Eur. J. Protistol.* **25**, 45 (1989); Y. Naitoh, in *Electrical Conduction and Behaviour in "Simple" Invertebrates*, G. A. B. Shelton, Ed. (Clarendon, Oxford, 1982), pp. 1–48.
6. T. Oka, Y. Nakaoka, F. Oosawa, *J. Exp. Biol.* **126**, 111 (1986); T. Majima, *Biophys. Chem.* **11**, 101 (1980).
7. E. A. Richard, R. D. Hinrichsen, C. Kung, *J. Neurogenet.* **2**, 239 (1985).
8. S. Klumpp and W. J. Ch. Schürhoff-Goeters, unpublished data.
9. G. Yellen, *Annu. Rev. Biophys. Biophys. Chem.* **16**, 227 (1987).
10. R. D. Hinrichsen and Y. Saimi, *J. Physiol. (London)* **351**, 397 (1984); Y. Saimi and B. Martinac, *J. Membr. Biol.* **112**, 79 (1990).
11. Y. Saimi and C. Kung, *Annu. Rev. Genet.* **21**, 47 (1987); R. R. Preston, J. A. Kink, R. D. Hinrichsen, Y. Saimi, C. Kung, *Annu. Rev. Physiol.* **53**, 309 (1991); R. R. Preston, Y. Saimi, E. Amberger, C. Kung, *J. Membr. Biol.* **115**, 61 (1990).
12. Paramecia wild-type 51s were grown axenically in 20 liter fermenters (*18*). Cilia were prepared by the Ca^{2+} shock method and purified by differential centrifugation [J. Thiele, S. Klumpp, J. E. Schultz, C. F. Bardele, *Eur. J. Cell Biol.* **28**, 3 (1982)]. Adenylyl cyclase in cilia isolated from 40 liters of culture was solubilized with Lubrol PX (2%) [J. E. Schultz and S. Klumpp, *Methods Enzymol.* **195**, 466 (1991)]. The enzyme was purified on the following FPLC columns: (i) DEAE-Trisacryl (IBF biotechnics), equilibrated and run with 20 mM tris-HCl (pH 7.5), 5% glycerol (v/v), 0.1% Brij 35 (v/v), and 0.2% thioglycerol (v/v) (buffer A). The enzyme was eluted with 600 mM sodium acetate in buffer A. Yield was 80%. (ii) Phenyl-Superose (Pharmacia) equilibrated with 20 mM tris-HCl (pH 7.5), 500 mM sodium acetate, 0.2% thioglycerol. The column was washed with buffer containing 100 mM sodium acetate and protein was eluted with 20 mM tris-HCl (pH 8.0), 0.2% thioglycerol, 1% Brij 35, and 20% glycerol. Recovery was 80%. (iii) Hydroxylapatite Biogel HTP (Bio-Rad) equilibrated with 20 mM tris-HCl (pH 7.5), 15% glycerol, 1% Brij 35, and 0.2% thioglycerol (buffer B). Adenylyl cyclase was eluted in 3 ml with 300 mM sodium phosphate and

applied to (iv) a Sephacryl-S300 HR column (320 ml) run with buffer B. The yield through steps iii and iv was >55%. (v) ATP-Sepharose (2 ml) (Pharmacia) run with buffer B containing Brij 35 (0.05%) and 2 mM $MnCl_2$. For elution, $MnCl_2$ was omitted from the buffer and 300 mM sodium acetate was added. Yield was 45%. (vi) Chromatography on Phenyl-Superose HR 5/5 as in step ii (60% yield). (vii) Rechromatography on ATP-Sepharose (1 ml) as in step v with a recovery of 30%. Final yield of adenylyl cyclase activity was 1%. The enzyme was assayed as described (*3*). For SDS–polyacrylamide gel electrophoresis, adenylyl cyclase eluting in 1 ml was precipitated with four volumes of methanol-acetone (1:1). Upon storage at −20°C, approximately 50% of enzyme activity was lost per month.

13. E. Pfeuffer, S. Mollner, T. Pfeuffer, *EMBO J.* **4**, 3675 (1985); M. D. Smigel, *J. Biol. Chem.* **261**, 1976 (1986).
14. B. E. Ehrlich, A. Finkelstein, M. Forte, C. Kung, *Science* **225**, 427 (1984); G. Boheim *et al.*, in *Transport in Biomembranes: Model Systems and Reconstitution*, R. Antolini, A. Gliozzi, A. Gorio, Eds. (Raven, New York, 1982), pp. 87–97.
15. R. Benz, K. Janko, W. Boos, P. Läuger, *Biochim. Biophys. Acta* **551**, 305 (1978); R. Benz, *Annu. Rev. Microbiol.* **42**, 359 (1988). About 3×10^{10} molecules of purified porins per milliliter are needed to observe single channel events. The low efficiency of the bilayer system in reconstitution experiments is probably due to unspecific adsorption of the hydrophobic membrane proteins to teflon surfaces of the measuring cell (10 cm^2). Calculating with a molecular size of 100 kD for the adenylyl cyclase, about 6×10^9 molecules (1 ng/ml) were required to cause channel formation in an identical cuvette. If the adenylyl cyclase was diluted (0.1 ng/ml), no activity was observed.
16. D. G. Lowe *et al.*, *EMBO J.* **8**, 1377 (1989).
17. R. Delgado, P. Hidalgo, F. Diaz, R. Latorre, P. Labarca, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 557 (1991).
18. U. Schönefeld, A. W. Alfermann, J. E. Schultz, *J. Protozool.* **33**, 222 (1986).
19. M. A. Delaage, D. Roux, H. L. Cailla, in *Molecular Biology and Pharmacology of Cyclic Nucleotides*, G. Falco and R. Paoletti, Eds. (Elsevier, Amsterdam, 1978), pp. 155–170.
20. T. Sonneborn, *Methods Cell Physiol.* **4**, 241 (1970).

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The *Son of sevenless* Gene Product: A Putative Activator of Ras

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The *Son of sevenless* (*Sos*) gene functions in signaling pathways initiated by the sevenless and epidermal growth factor receptor tyrosine kinases. The *Sos* gene has now been isolated and sequenced. Its product is a 1595–amino acid protein similar to the CDC25 protein in *Saccharomyces cerevisiae*, a guanine nucleotide exchange factor that activates Ras. These results imply a role for the *ras* pathway in *Drosophila* neuronal development.

IN THE DEVELOPING *DROSOPHILA* EYE, a cluster of eight photoreceptor neurons develop autonomously in each facet or ommatidium (*1, 2*) with the central cell R8 being the first to express neuronal markers (*3*). R8 directly induces a neighboring cell to develop as the R7 neuron (*4*). This induction is mediated by the products of the *bride of sevenless* (*boss*) and *sevenless* (*sev*) genes (*4–8*). The *boss* gene product is a transmembrane protein that is localized to the R8 cell (*5*), and the *sevenless* gene encodes a tyrosine kinase receptor expressed on the membranes of many cells in the developing cluster (*9, 10*). Extensive genetic and molecular analysis of this system suggests that the boss protein on R8 binds to and activates the sevenless receptor on the R7 precursor cell, leading to its eventual differentiation into a neuron (*5*). In *sev* or *boss* mutants, R7 cells are missing from all facets of the eye.

Recent genetic analysis has shown that a gene called *Son of sevenless* (*Sos*) functions in a signal transduction pathway initiated by the sevenless receptor (*11*). This gene was first identified as a dominant mutant allele called Sos^{JC2}, which suppresses the sev^{E4} allele. This suppression is allele-specific, suggesting an interaction between the proteins encoded by these genes. Genetic analysis further demonstrated that Sos^{JC2} product is necessary autonomously in the R7 cell for this cell to develop in a sev^{E4} background. The function of the *Sos* gene product is not limited to the development of the R7 cell; in weak loss of function alleles, other photoreceptor cells are missing, and complete loss of function leads to recessive lethality. The *Sos* gene also functions downstream of the *Drosophila* epidermal growth factor receptor (*11*). We now describe the isolation and characterization of the *Sos* gene and show that it encodes the *Drosophila* homolog of CDC25, an activator of Ras in *Saccharomyces cerevisiae*.

The *Sos* gene maps to the 34D5 region of the second chromosome. This region has been saturated for mutations and a deficiency map has been reported (*12*). We have confirmed the mapping results (relevant loci and breakpoints are shown in Fig. 1A).

Starting with a clone in 35A, we initiated a chromosomal jump to 34D3 and followed it with a chromosomal walk in the region of the *Sos* gene. In all, about 150 kb of genomic fragments were cloned as cosmid and phage DNA. Genomic fragments were used in chromosomal in situ hybridization experiments to delimit the *Sos* region (see below). A molecular map of this region was constructed (Fig. 1B) and four transcription units were identified through cDNA clones isolated from a cDNA library from eye imaginal discs.

Molecular characterization of the many chromosomal rearrangements mapping to the region allowed us to discriminate between these cDNAs. Three deletions, Df(2L)*b*71ka, Df(2L)*b*84h1, and Df(2L)-*Sco*RV7, genetically uncover *Sos* and loci to its right (Fig. 1A). In situ hybridization

Table 1. Interaction of *Dras*-1 with *Sos* in flies homozygous for sev^{E4}. Df(3R)by62 and Df(3R)by10 overlap in the 85D11-F1 region and include the transcription unit for *Dras*-1 (*20*). One copy of either of these deletions eliminates the suppression of sev^{E4} by Sos^{JC2}. This is a specific effect in that over twenty deletions mapping outside the region [for example, Df(3R)D1BX12] have been tested in this assay and do not have any effect on the suppression. The ommatidia were screened by the optical technique called pseudopupil (*6*); *n* is the number of ommatidia screened. The $boss^1$ allele is a null allele at the *boss* locus.

Genotype	Ommatidia with R7 cells
sev^{E4}/sev^{E4}; Sos^+/Sos^+; +/+	0% (n = 2239)
sev^{E4}/sev^{E4}; Sos^{JC2}/Sos^+; +/+	16% (n = 2392)
sev^{E4}/sev^{E4}; Sos^{JC2}/Sos^+; $boss^1/boss^+$	0% (n = 2337)
sev^{E4}/sev^{E4}; Sos^{JC2}/Sos^+; Df(3R)by62/+	0% (n = 2175)
sev^{E4}/sev^{E4}; Sos^{JC2}/Sos^+; Df(3R)by10/+	0% (n = 2180)
sev^{E4}/sev^{E4}; Sos^{JC2}/Sos^+; Df(3R)D1BX12/+	17% (n = 898)

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