

- Cocaine Addiction, T. R. Kosten and H. D. Kleber, Eds. (Guilford, New York, 1992), pp. 172–192.
2. R. N. Ehrman, S. J. Robbins, A. R. Childress, C. P. O'Brien, *Psychopharmacology* **107**, 59 (1992).
 3. H. de Wit and J. Stewart, *ibid.* **75**, 134 (1981).
 4. J. H. Jaffe, N. C. Cascella, K. M. Kumor, M. A. Sherer, *ibid.* **97**, 59 (1989); K. L. Preston, J. T. Sullivan, E. C. Strain, G. E. Bigelow, *J. Pharmacol. Exp. Ther.* **262**, 279 (1992).
 5. J. Stewart, *Pharmacol. Biochem. Behav.* **20**, 917 (1984); J. Stewart and P. Vezina, *Brain Res.* **457**, 287 (1988).
 6. D. C. S. Roberts, M. E. Corcoran, H. C. Fibiger, *Pharmacol. Biochem. Behav.* **6**, 615 (1977); D. C. S. Roberts, G. F. Koob, P. Klonoff, H. C. Fibiger, *ibid.* **12**, 781 (1980). Mesocortical dopamine systems also may be involved [N. E. Goeders and J. E. Smith, *Science* **221**, 773 (1983)].
 7. D. R. Sibley and F. J. Monsma Jr., *Trends Pharmacol. Sci.* **13**, 61 (1992).
 8. J. W. Kebabian and D. B. Calne, *Nature* **277**, 93 (1979).
 9. C. R. Gerfen *et al.*, *Science* **250**, 1429 (1990).
 10. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (NIDA, Research Triangle Park, NC) and dissolved in saline.
 11. Details for intravenous catheter implantation and the self-administration apparatus were described previously [D. W. Self, A. W. McClenahan, D. Beitner-Johnson, R. Z. Terwilliger, E. J. Nestler, *Synapse* **21**, 312 (1995)]. Briefly, rats self-administered cocaine during their light cycle under a fixed ratio 1:time out 5 s schedule (0.5 mg of cocaine per kilogram of body weight delivered in a 0.1 ml volume over 10 s) in daily 4-hour reinstatement test sessions. All stimuli associated with the infusion (for example, the cue light and pump noise) were the same during both the cocaine and saline phases. After three consecutive tests with low responding in the saline phase (less than five responses in the final hour), the animals were given an intraperitoneal priming injection of a dopamine agonist, or of the drug vehicle, 30 min into the saline phase. The number of nonreinforced responses was measured during the final hour of the saline phase. The response totals from the final hour were analyzed with the Wilcoxon signed-ranks test of related subjects. Priming test sessions were always preceded by baseline test sessions with less than five lever presses in the final hour, and the order of presentation of agonist and dose was counterbalanced across animals. All dopamine agonists were obtained from Research Biochemicals (Natick, MA), dissolved in saline, and injected in a volume of 2 ml per kilogram of body weight.
 12. D. L. Levesque *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8155 (1992). Although 7-OH-DPAT was purported originally to bind selectively at D₃ receptors under certain experimental conditions *in vitro*, recent evidence suggests that 7-OH-DPAT can bind with high affinity to both D₂ and D₃ (D₂-like) receptors and can produce effects at both receptors *in vivo* [C. H. Large and C. M. Stubbs, *Trends Pharmacol. Sci.* **15**, 46 (1994); J. E. Freedman, B. L. Waszczak, R. F. Cox, G. J. Greif, *ibid.*, p. 173; A. M. Gonzalez and D. R. Sibley, *Eur. J. Pharmacol.* **272**, R1 (1995)].
 13. K. Tsuruta *et al.*, *Nature* **292**, 463 (1981).
 14. P. H. Andersen and J. A. Jansen, *Eur. J. Pharmacol.* **188**, 335 (1990).
 15. C. B. Hubner and G. F. Koob, *Neuropsychopharmacology* **3**, 101 (1990); M. S. Kleven and W. L. Woolverton, *Psychopharmacology* **101**, 208 (1990).
 16. S. B. Caine and G. F. Koob, *Science* **260**, 1814 (1993).
 17. Horizontal locomotor activity was measured with 12 drug-naïve rats in a circular apparatus described previously [M. J. D. Miserendino, X. Guitart, R. Z. Terwilliger, S. Chi, E. J. Nestler, *Mol. Cell. Neurosci.* **4**, 440 (1993)]. The rats were habituated to the apparatus for 90 min/day for four consecutive days. On days 5, 7, 9, and 11, the rats were given an intraperitoneal injection of SKF 82958, 7-OH-DPAT, caffeine (Sigma), or the drug vehicle (saline) in a counterbalanced order, and horizontal locomotor activity was measured for the next 90 min. Totals were analyzed with the Wilcoxon signed-ranks test of related subjects. Drug-injection days were separated by baseline days on which no drugs were injected to reduce the possibility of conditioned locomotor effects.
 18. S. H. Snyder, J. J. Katims, Z. Annau, R. F. Bruns, J. W. Dalay, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3260 (1981).
 19. D. W. Self and L. Stein, *Brain Res.* **582**, 349 (1992); M. R. Weed, K. E. Vanover, W. L. Woolverton, *Psychopharmacology* **113**, 51 (1993).
 20. P. M. Callahan, J. B. Appel, K. A. Cunningham, *Psychopharmacology* **103**, 50 (1991); R. D. Spealman, J. Bergman, B. K. Madras, K. F. Melia, *J. Pharmacol. Exp. Ther.* **258**, 945 (1991); J. M. Witkin, D. E. Nichols, P. Terry, J. L. Katz, *ibid.* **257**, 706 (1991).
 21. Two groups ($n = 5$ per group) of rats that self-administered similar amounts of cocaine (0.5 mg/kg per infusion) in daily 2-hour self-administration test sessions were given intraperitoneal injections of SKF 82958 (1.0 mg/kg in a volume of 2 ml/kg) or saline twice daily; the first injection was given 30 min before the beginning of the test session and the second injection was given 8 hours later. Cocaine self-administration in rats treated with SKF 82958 was suppressed in the first hour of each test session ($F_{1,8} = 144.2$, $P < 0.001$) to an average of 0.46 self-infusions per hour over 12 test sessions (2 weeks) compared with an average of 15.8 self-infusions per hour for saline-treated rats. During the second hour, most rats treated with SKF 82958 initiated cocaine self-administration, presumably as the drug wore off. There was no change in the average number of self-infusions per session over the course of the treatments for either group (saline-treated, $F_{11,44} = 1.150$, $P = 0.349$; SKF 82958-treated, $F_{11,44} = 0.525$, $P = 0.876$).
 22. F. H. Gavin, *Science* **251**, 1580 (1991).
 23. R. H. Belmaker and D. Wald, *Br. J. Psychiatry* **131**, 222 (1977); T. S. Shippenberg and A. Herz, *Brain Res.* **436**, 1987; T. S. Shippenberg, R. Bals-Kubik, A. Herz, *Psychopharmacology* **103**, 209 (1991); S. Siris, *Schizophr. Bull.* **17**, 75 (1991).
 24. C.-E. Johanson and C. R. Schuster, in *Psychopharmacology: The Fourth Generation of Progress*, F. E. Bloom and D. J. Kupfer, Eds. (Raven, New York, 1995).
 25. R. A. Wise, A. Murray, M. A. Bozarth, *Psychopharmacology* **100**, 355 (1990).
 26. Supported by grants DA08227, DA00203, and DA05603 from NIDA, by the Abraham Ribicoff Research Facilities of the Connecticut Mental Health Center, and by a generous gift from the William Benter Foundation.

6 October 1995; accepted 16 January 1996

Coordination of Three Signaling Enzymes by AKAP79, a Mammalian Scaffold Protein

Theresa M. Klauck,* Maree C. Faux,* Kirstin Labudda, Lorene K. Langeberg, Susan Jaken, John D. Scott†

Multivalent binding proteins, such as the yeast scaffold protein Sterile-5, coordinate the location of kinases by serving as platforms for the assembly of signaling units. Similarly, in mammalian cells the cyclic adenosine 3',5'-monophosphate-dependent protein kinase (PKA) and phosphatase 2B [calcineurin (CaN)] are complexed by an A kinase anchoring protein, AKAP79. Deletion analysis and binding studies demonstrate that a third enzyme, protein kinase C (PKC), binds AKAP79 at a site distinct from those bound by PKA or CaN. The subcellular distributions of PKC and AKAP79 were similar in neurons. Thus, AKAP79 appears to function as a scaffold protein for three multifunctional enzymes.

Control of multiple cellular events by protein phosphorylation requires many levels of regulation in order to generate specific cellular responses. One regulatory mechanism is that kinases and phosphatases are maintained at discrete cellular locations through their interaction with targeting proteins (1). Enzymes may be positioned in close proximity to specific substrates, which then can be efficiently modified in response to the appropriate signals. Evidence supporting this model has shown that protein tyrosine kinases and phosphatases couple to downstream cytoplasmic enzymes through adapter proteins that contain SH2 and SH3 domains (2). Serine-threonine kinases and phosphatases are also maintained by scaffold proteins or anchoring proteins. In yeast, the scaffold

protein Sterile-5 (STE5) provides a framework to order successive members of a yeast mitogen-activated protein kinase cascade, thereby permitting sequential activation of each enzyme in the pheromone mating response (3). In neurons, PKA and CaN are both localized to postsynaptic densities (PSDs) by association with AKAP79, which positions both enzymes close to key neuronal substrates (4). Because other neuronal signaling enzymes are present at the PSD (5), we investigated their potential to associate with the anchoring protein AKAP79.

PKC, a family of serine-threonine kinases, is tethered to the PSD through association with binding proteins (6). We used a solid-phase binding assay (overlays) with PKC as a probe (7) on bovine brain extracts (8) to detect several PKC-binding proteins, including a protein that migrated with the same mobility as a prominent RII-binding protein of 75 kD (9). This band corresponds to AKAP75, the bovine homolog of AKAP79 (10), indicating that the anchoring protein could bind both RII and PKC. Indeed, recombinant AKAP79 bound to

T. M. Klauck, M. C. Faux, K. Labudda, L. K. Langeberg, J. D. Scott, Vollum Institute, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201, USA.
S. Jaken, W. Alton Jones Cell Science Center, 10 Old Barn Road, Lake Placid, NY 12946 USA.

*These authors contributed equally to this manuscript.

†To whom correspondence should be addressed.

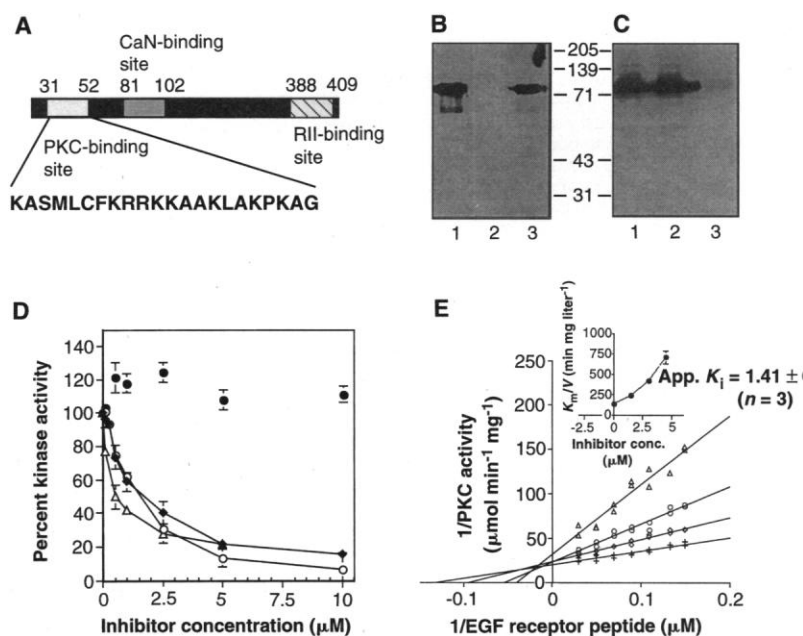
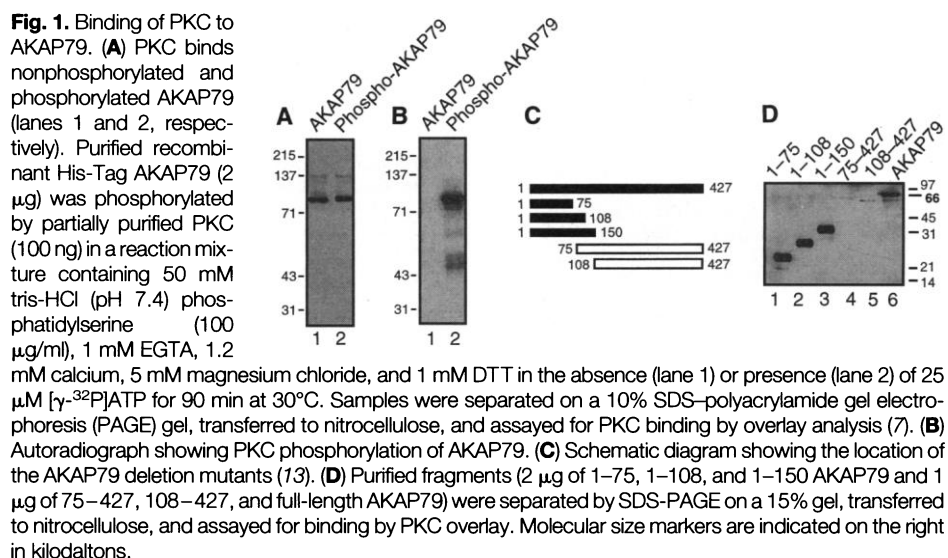
PKC in the presence of Ca^{2+} and phosphatidylserine (Fig. 1A). The α , β I, and β II isoforms of PKC interacted with AKAP79, whereas other signaling enzymes that are associated with the PSD, such as Ca^{2+} -calmodulin-dependent protein kinase II and the type I phosphatase, did not bind the AKAP (11). Phosphorylation of AKAP79 by PKC had no qualitative effect on binding (Fig. 1, A and B). This result suggests that the AKAP binds in a different manner from other PKC-substrate-binding proteins such as MARCKS (myristoylated alanine-rich C kinase substrate) and γ -adducin, for which phosphorylation regulates association with PKC (12). We mapped the PKC-binding region by screening a family of AKAP79 fragments (Fig. 1C) (13). Fragments encompassing the first 75 residues of AKAP79 bound PKC, whereas COOH-terminal fragments containing the RII- and CaN-binding regions did not (Fig. 1D). These data imply that PKC binds to AKAP79 at a site that is distinct from those bound by RII and CaN (4, 14).

Because basic and hydrophobic regions are determinants for binding of certain proteins to PKC (15), we focused on a region located between residues 31 to 52 of AKAP79 (Fig. 2A). A peptide encompassing this region specifically blocked the interaction of AKAP79 with PKC in the overlay assay (Fig. 2B) but did not affect RII binding to the AKAP (Fig. 2C) (16). Conversely, the RII anchoring inhibitor peptide [AKAP79(390–411)] did not affect PKC binding (Fig. 2B) but did block interaction with RII (Fig. 2C). This result indicates that residues 31 to 52 represent the principal determinants for PKC binding. Many kinases or phosphatases bound to anchoring proteins are maintained in an inactive state (1, 4). Accordingly, recombinant AKAP79 protein inhibited PKC activity (17) with a half-maximal inhibition (IC_{50}) of $0.35 \pm 0.06 \mu\text{M}$ ($n = 3$) (Fig. 2D). In addition, the AKAP79 peptide (residues 1 to 75) inhibited PKC activity with IC_{50} values of $2.0 \pm 0.6 \mu\text{M}$ ($n = 4$) and $1.6 \pm 0.3 \mu\text{M}$ ($n = 4$), respectively (Fig. 2D). In contrast, AKAP79(31–52) did not inhibit the activity of the catalytic subunit of PKA (Fig. 2D) (18). Inhibition of PKC activity by AKAP79(31–52) was mixed, with an apparent inhibition constant (K_i) of $1.41 \pm 0.28 \mu\text{M}$ ($n = 3$) (Fig. 2E). The secondary plot of the Michaelis constant divided by the maximal velocity (K_m/V_{max}) as a function of inhibitor concentration was nonlinear, suggesting binding at more than one site (Fig. 2E, inset). Preincubation of AKAP79(31–52) with excess calmodulin prevented inhibition of PKC activity (19). We propose that PKC is localized and inhibited by AKAP79 in a Ca^{2+} - and phosphatidylserine-depen-

dent manner, and additional signals, such as Ca^{2+} -calmodulin, may be required to release the enzyme.

The interaction of PKC and AKAP79 was independently confirmed by coprecipitation of the complex from human embryonic kidney (HEK) 293 cells in the presence of exogenous recombinant AKAP79

(Fig. 3A) (20). PKC was not coprecipitated with AKAP79 in the absence of exogenous AKAP79 probably because of low endogenous amounts of AKAP79 in the lysates (Fig. 3A). Similar results were obtained when AKAP79 was added to bovine brain extracts or incubated with purified PKC- β II (21) in vitro in the presence of Ca^{2+} and



phospholipid (19). Further evidence for the association of PKC and AKAP79 was provided by double-immunofluorescence staining (22) of HEK 293 cells overexpressing AKAP79 (23). PKC and AKAP79 exhibited limited staining at the cell periphery in mock-transfected cells (Fig. 3B), but the staining pattern was markedly enhanced in transfected cells, demonstrating that overexpression of AKAP79 increases PKC targeting to the periphery of the cell (Fig. 3B). Collectively, our data indicate that PKC binds AKAP79, and it is likely that this complex occurs in vivo.

AKAP79 may act as a signaling scaffold for the subcellular targeting of PKA, CaN, and PKC. To demonstrate that these enzymes bind to AKAP79 in mammalian cells, we isolated ternary complexes from bovine brain. We used affinity purification of the R subunit of PKA on adenosine 3',5'-monophosphate (cAMP)-agarose to

copurify AKAP75 (Fig. 4A) (24). If PKC were bound to the complex via the AKAP, then we would expect PKC to be present after elution of bound proteins with cAMP. Immunoblotting analysis demonstrated that PKC was indeed present (Fig. 4B), showing that RII and PKC can bind simultaneously to AKAP75. In separate experiments, a ternary complex of CaN, PKC, and AKAP75 was isolated from bovine brain extracts by immunoprecipitation with antibodies against CaN A subunit (25). Immunoblotting demonstrated that the precipitated complex contained PKC (Fig. 4C), whereas control experiments with nonimmune serum were negative. This result indicated that CaN and PKC can bind to the AKAP simultaneously; therefore, different combinations of PKA, CaN, and PKC can bind the AKAP in bovine brain. Isolation of a quaternary complex was not feasible because of the low stoichiometry of binding

for each enzyme to the AKAP (4, 26) and the probable transient nature of the protein-protein interactions.

AKAP79 appears to be a mammalian scaffold protein that coordinates the location and activity of three prominent signaling enzymes. Although AKAP79 functionally resembles the yeast scaffold protein STE5, there are differences between the two signaling scaffolds. The AKAP79 complex is likely to respond to three distinct activation signals, whereas a single upstream event, the activation of STE20, is sufficient to transduce signals from one kinase to the next in the STE5 scaffold (3). In addition, the AKAP signaling complex is targeted to sites near the postsynaptic membrane in neurons. Immunofluorescence studies demonstrate that PKC and the AKAP are concentrated at sites below the plasma membrane at the cell body and in dendritic bundles of hippocampal neurons (Fig. 4D). The dendritic staining pattern is consistent with the colocalization of both proteins at the PSD. Targeting to the PSD would ensure that each enzyme in the signaling complex is well placed to receive signals transduced across the synapse and that each enzyme is colocalized with specific substrate proteins. Potential substrates include α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-kainate and Ca^{2+} channels, which are modulated by anchored PKA, and N-methyl-D-aspartate (NMDA) receptors, which are activated by PKC and attenuated by CaN (27). PKC may also modulate the targeting of certain NMDA receptor subtypes to the postsynaptic membranes (28). Thus synaptic signaling events appear to be coordinated, and we propose that this occurs through a modular scaffold protein, AKAP79, that anchors two second messenger-regulated kinases and one phosphatase.

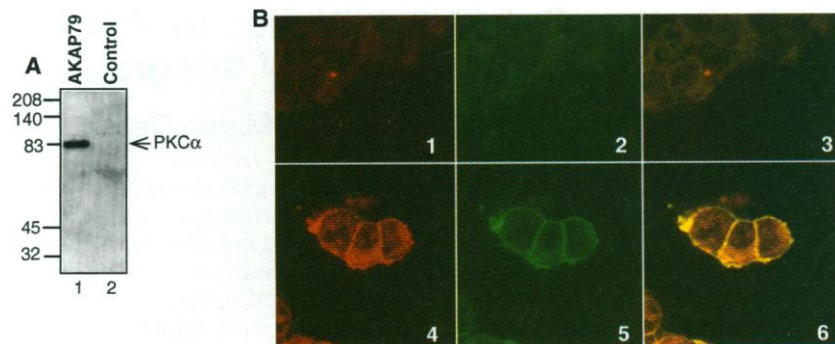
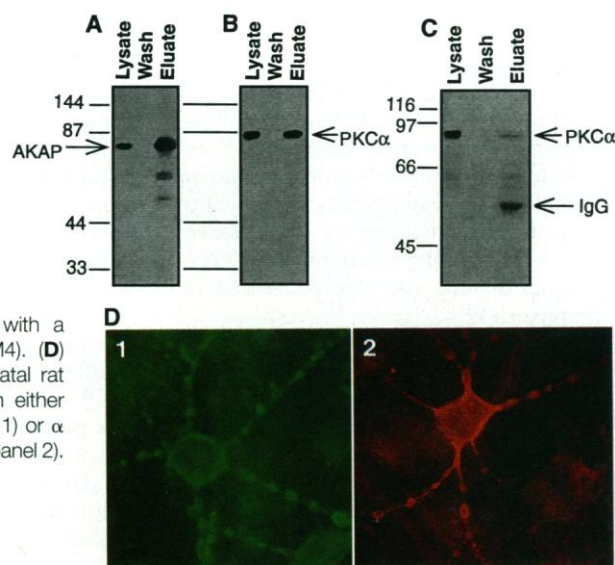


Fig. 3. Cellular association and colocalization of PKC and AKAP79. **(A)** Coprecipitation of AKAP79 and PKC from HEK 293 cell lysates (20). Lysates (1.2 mg) were incubated in the presence (lane 1) or absence (lane 2) of exogenous recombinant AKAP79 (2 μ g) and immunoprecipitated with a polyclonal antibody to AKAP79 (2503). Precipitates were blotted and probed with a monoclonal antibody to PKC α (M4) (7). **(B)** Stable HEK 293 cell lines expressing pcDNA3 (upper panels) and AKAP79 (lower panels) were stained with antibodies to PKC (M4) (panels 1 and 4) or AKAP79 (2503) (panels 2 and 5) and analyzed by confocal microscopy (22). Double staining for PKC and AKAP79 is displayed by superimposing the images (panels 3 and 6).

Fig. 4. The AKAP79 signaling scaffold. **(A and B)** The R subunit of PKA was purified from bovine brain by affinity chromatography with cAMP-agarose (24). Protein blots were probed with 32 P-RII α to detect AKAPs (A) or with a monoclonal antibody to PKC α (M4) (B). **(C)** CaN from bovine brain extracts was immunoprecipitated with affinity-purified antibodies to the CaN A subunit (25). Protein blots were probed with a monoclonal antibody to PKC α (M4). **(D)** Confocal microscopy (22) of neonatal rat hippocampal neurons stained with either antibody to AKAP79 (2503) (panel 1) or α and β PKC-specific antibody (M7) (panel 2).



REFERENCES AND NOTES

1. M. Hubbard and P. Cohen, *Trends Biochem. Sci.* **18**, 172 (1993); J. D. Scott and S. McCartney, *Mol. Endocrinol.* **8**, 5 (1994); D. Mochly-Rosen, *Science* **268**, 247 (1995).
2. T. Pawson, *Nature* **373**, 573 (1995); H. Sun and N. K. Tonks, *Trends Biochem. Sci.* **19**, 480 (1994).
3. K.-Y. Choi, B. Satterberg, D. M. Lyons, E. A. Elion, *Cell* **78**, 499 (1994); S. Marcus, A. Polverino, M. Barr, M. Wigler, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7762 (1994); J. A. Printen and G. F. Sprague Jr., *Genetics* **138**, 609 (1994); J. E. Kranz, B. Satterberg, E. A. Elion, *Genes Dev.* **8**, 313 (1994).
4. V. M. Coghlan *et al.*, *Science* **267**, 108 (1995).
5. D. P. Rich, R. J. Colbran, C. M. Schworer, T. R. Soderling, *J. Neurochem.* **53**, 807 (1989).
6. M. Wolfe and N. Sahyoun, *J. Biol. Chem.* **261**, 13327 (1986); M. Wolf, S. Burgess, U. K. Misra, N. Sahyoun, *Biochem. Biophys. Res. Commun.* **140**, 691 (1986).
7. PKC overlays were done as described [S. L. Hyatt, T. M. Klauck, S. Jaken, *Mol. Carcinog.* **3**, 45 (1990)]. Bound PKC was detected with α and β PKC-specific monoclonal antibody M7 [K. L. Leach *et al.*, *J. Biol. Chem.* **263**, 13223 (1988)].
8. Crude extracts of bovine brain were prepared by

- grinding frozen tissue with a mortar and pestle in liquid N₂ and homogenizing in a hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5% Triton X-100, leupeptin (2 µg/ml), pepstatin (2 µg/ml), and 1 mM each of dithiothreitol (DTT), benzamide, and 4-(2-aminoethyl)-benzenesulfonyl fluoride) in a 15-ml Dounce homogenizer. Extracts were centrifuged at 25,000g at 4°C for 30 min. Unless indicated otherwise, the cleared lysate was used in the experiments.
9. T. Klauck, unpublished data.
 10. D. Sarkar, J. Erlichman, C. S. Rubin, *J. Biol. Chem.* **259**, 9840 (1984).
 11. PKC isoform-specific overlays were done by T. Klauck and M. Faux (unpublished data). Ca²⁺-calmodulin-dependent protein kinase II and type I phosphatase overlays were done by J. D. Scott (unpublished data).
 12. C. Chapline, K. Ramsay, T. Klauck, S. Jaken, *J. Biol. Chem.* **268**, 6858 (1993); S. L. Hyatt, L. Liao, A. Aderem, A. C. Nairn, S. Jaken, *Cell Growth Differ.* **5**, 495 (1994).
 13. PCR-generated fragments corresponding to the specified regions of AKAP79 were cloned into a pET-16b vector (Novagen) in frame with a histidine₁₀-Tag (His-Tag) following standard cloning protocols. The complete nucleotide sequence of each fragment was confirmed by automated sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 cells and purified from the soluble fraction under nondenaturing conditions by nickel affinity chromatography (Novagen). Protein concentrations were determined by the DC protein assay (Bio-Rad).
 14. D. W. Carr, R. E. Stofko-Hahn, I. D. C. Fraser, R. D. Cone, J. D. Scott, *J. Biol. Chem.* **267**, 16816 (1992).
 15. L. Liao, S. L. Hyatt, C. Chapline, S. Jaken, *Biochemistry* **33**, 1229 (1994).
 16. RII overlays were carried out as described [S. M. Lohman, P. DeCamilli, I. Einig, U. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6723 (1984)].
 17. PKC was assayed as described [C. House, R. E. H. Wettenhall, B. E. Kemp, *J. Biol. Chem.* **262**, 772 (1987)] in a reaction containing 50 mM tris-HCl (pH 7.4), 10 mM MgCl₂, 2 mM CaCl₂, 1 mM DTT, 100 µM [γ -³²P]adenosine triphosphate (ATP) (200 to 500 cpm/pmol), phosphatidylserine (20 µg/ml), and either vertebrate growth factor receptor peptide (VRKRLRLRL) (29) or myelin basic protein (residues 4 to 14) peptide as substrates at 30°C. PKC (50 nM) was diluted in 20 mM tris (pH 7.7), 1 mM DTT, and bovine serum albumin (BSA; 1 mg/ml). Inhibition constants (I_{C₅₀}) were determined over an inhibition concentration range of 0.1 to 10 µM. K_i values were obtained from secondary plots of K_m/V_{max} versus inhibitor concentration, after determination of K_m and V_{max} for four concentrations of inhibitor from Lineweaver-Burk plots assayed over the same substrate concentration range.
 18. PKA was assayed as described [J. D. Corbin and E. M. Reiman, *Methods Enzymol.* **38**, 287 (1974)].
 19. M. C. Faux, unpublished data.
 20. HEK 293 cells were collected in hypotonic buffer containing protease inhibitors and sonicated three times for 5 s. Total lysates (1.2 mg in 300 µl) were incubated overnight in the absence or presence of 2 µg of purified recombinant AKAP79. Samples were incubated with 4 µg of affinity-purified polyclonal antibody to AKAP79 (2503) and precipitated with 40 µl of a 50% solution of protein A-Sepharose (Sigma). Complexes were washed twice in hypotonic buffer, twice in low-salt buffer [50 mM Hepes (pH 7.4), 0.15 M NaCl, 0.1% NP-40, 1 mM EDTA, and 0.1% SDS], twice in high-salt buffer (50 mM Hepes, 0.5 M NaCl, 0.1% NP-40), and twice in low-salt buffer. The precipitated complexes were boiled for 5 min in 20 µl of SDS-sample preparation buffer and immunoblotted.
 21. J. W. Orr, L. M. Keranen, A. C. Newton, *J. Biol. Chem.* **267**, 15263 (1992).
 22. For confocal microscopy, HEK 293 stable cell lines expressing pcDNA3 or AKAP79 were grown on cover slips overnight, rinsed in phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS, treated with acetone (-20°C) for 1 min, rehydrated, and pre-blocked with 0.1% BSA in PBS. An antibody mixture containing mouse antibody to PKC (M4; 1:200 dilution) and rabbit antibody to AKAP79 (2503; 1:200

- dilution) was applied for 1 hour. The cover slips were then washed three times in 0.1% BSA in PBS. Fluorescein isothiocyanate-conjugated donkey antibody to rabbit immunoglobulin G (IgG) (1:50) and Texas red-conjugated donkey antibody to mouse IgG (1:100) were applied for 1 hour. The cover slips were then washed three times in 0.1% BSA in PBS, mounted with Slow Fade Antifade (Molecular Probes), and analyzed with the Leitz Fluovert FU confocal photomicroscope with a 63/1.4 N.A. OEL PL APO lens. Specific staining was not detected in control experiments with secondary antibody alone.
23. HEK 293 cells were transfected with Lipofectin Reagent (Life Technologies) with either pcDNA3 or pcDNA3 containing a 1312-base pair Hind III-Not I fragment encoding AKAP79. After 24 hours, G418 (0.5 mg/ml; Sigma) was added to select for stable cells overexpressing AKAP79. Cells were maintained in media containing G418 (0.2 mg/ml). Protein immunoblot analysis demonstrated increased expression of AKAP79, whereas PKC α expression remained constant.
 24. Regulatory subunits of PKA were purified with the use of cAMP-agarose as described [R. L. Potter, P. H. Stafford, S. S. Taylor, *Arch. Biochem. Biophys.* **190**, 174 (1978)] with modifications (4). The complex from bovine brain lysates (8) was eluted from cAMP-agarose with 75 mM cAMP.
 25. Immunoprecipitation of CaN was achieved by incu-

bating bovine brain lysates (1 mg protein) (8) with affinity-purified antibodies to the CaN A subunit (8 µg) for 4 hours at 4°C. Proteins were precipitated as described (20).

26. V. M. Coghlan, L. B. Lester, J. D. Scott, *Adv. Protein Phosphatases* **8**, 51 (1995).
27. C. Rosenmund *et al.*, *Nature* **368**, 853 (1994); B. D. Johnson, T. Scheuer, W. A. Caterall, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11492 (1994); D. N. Lieberman and I. Mody, *Nature* **369**, 235 (1994); G. Tong, D. Shepherd, C. E. Jahr, *Science* **267**, 1510 (1995).
28. M. D. Ehlers, W. G. Tingley, R. L. Huganir, *Science* **269**, 1734 (1995).
29. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; T, Thr; and V, Val.
30. We thank A. Newton for purified PKC β II, R. Maurer for purified PKC β I, J. Engstrom for assistance with confocal microscopy, and our colleagues at the Vollum Institute for critical evaluation of the manuscript and helpful discussion. Supported in part by National Institutes of Health grant GM48231 (to J.D.S.) and funding from Icos Corporation and grant CA538841 and GM50152 (to S.J.). T.M.K. is the recipient of training grant DK07680.

10 November 1995; accepted 12 January 1996

Similarities Between Initiation of V(D)J Recombination and Retroviral Integration

Dik C. van Gent, Kiyoshi Mizuuchi, Martin Gellert*

In the first step of V(D)J recombination, the RAG1 and RAG2 proteins cleave DNA between a signal sequence and the adjacent coding sequence, generating a blunt signal end and a coding end with a closed hairpin structure. These hairpins are intermediates leading to the formation of assembled antigen receptor genes. It is shown here that the hairpins are formed by a chemical mechanism of direct trans-esterification, very similar to the early steps of transpositional recombination and retroviral integration. A minor variation in the reaction is sufficient to divert the process from transposition to hairpin formation.

Functional immunoglobulin and T cell receptor genes are assembled during vertebrate lymphoid development from separate gene segments. This DNA rearrangement, called V(D)J recombination, takes place at recombination signal sequences (RSSs) that specify the border of the coding segments (1). Double strand breaks (DSBs) at the RSS border depend on expression of the RAG1 and RAG2 genes (2) and are probably intermediates in this recombination reaction (3, 4). After DSB formation, a pair of signal ends or coding ends is coupled to form a signal joint or coding joint, respectively. These joining reactions require several genes that are also involved in general DSB repair (4).

We recently developed a cell-free assay in which specific cleavage at RSSs requires only the RAG1 and RAG2 proteins (5, 6). An oligonucleotide containing one RSS

serves as a substrate and is cut efficiently. A DNA species containing a nick in the top strand (as drawn in Fig. 1) at the border of the RSS and the flanking ("coding") sequence was found to be the precursor to a DSB. The products of the cleavage reaction are a blunt, 5'-phosphorylated signal end and a coding end with a hairpin structure. These are the same species detected in recombinationally active cells.

Formation of the new phosphodiester bond at the tip of the hairpin requires energy. Because no adenosine triphosphate (ATP) or other high-energy cofactor is present in the cleavage reaction, the energy is likely to be derived from one of the broken phosphodiester bonds. The energy of the bond broken in the initial nicking reaction does not appear to be conserved: DNA substrates with a preexisting nick at this position are efficiently converted into hairpins (6). Thus, the energy of the phosphodiester bond in the bottom strand opposite the nick must be conserved, either through a covalent protein-DNA intermediate or by direct trans-esterification. A

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0540, USA.

*To whom correspondence should be addressed.