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10. Propagation of spindle oscillations was observed in experiments on thalamic slices [U. Kim, T. Bal, D. A. McCormick, *J. Neurophysiol.* **74**, 1301 (1995)] and in computational models of thalamic slices [D. Golomb, X. J. Wang, J. Rinzel, *ibid.* **75**, 750 (1996); A. Destexhe, T. Bal, D. A. McCormick, T. J. Sejnowski, *ibid.* **76**, 2049 (1996)]. In the present in vivo experiments, we have only observed propagation of spindles in the thalamus after decortication as an exception. Spindle sequences occasionally showed synchrony even among widely spaced thalamic territories (Fig. 1, bottom; Fig. 2, decorticated spectra).
11. Power spectra were calculated according to W. H. Press, B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling [*Numerical Recipes. The Art of Scientific Computing* (Cambridge Univ. Press, Cambridge, 1986)].
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13. Intracellular recordings were obtained from the thalamus of decorticated animals with glass microelectrodes filled with 3 M potassium acetate and dc resistances of 35 to 45 megohms. Pipettes were held with two independent micromanipulators, and the distance between the tip was adjusted according to the point of penetration in the tissue. Cells recorded in the VL nucleus were reached by descending through the head of the caudate nucleus. The more posterior pipette penetrated through the exposed surface of the dorsal thalamus at stereotaxic coordinates corresponding to the lateral posterior (LP) nucleus. A total of eight stable couples was studied at distances of less than 1 to 2 mm and 12 couples at distances of 3 mm or more from six different animals. Stable recordings had resting membrane potentials more negative than −60 mV, overshooting action potentials and input resistances between 17 and 24 megohms. To ensure stability of intracellular recordings, we paralyzed the animals with galamine triethiodide (33 mg per kilogram of body weight, intravenously) and artificially ventilated them, with control of the end-tidal CO_2 concentration at around 3.7%. Further stability was obtained by performing cisternal drainage, bilateral pneumothorax, and hip suspension, and by filling the hole left by the decortication with a 4% agar solution. Body temperature was maintained at 37° to 38°C. A constant state of deep anesthesia was obtained by additional doses of barbiturate and continuous monitoring of the electroencephalogram (EEG) from the contralateral hemisphere. A high-impedance amplifier with active bridge circuitry was used to record and inject current in the cells. The signals were recorded on an eight-channel tape with bandpass of 0 to 9 kHz and digitized off-line at 10 kHz for analysis and display.
14. Intracellular recordings in barbiturate-anesthetized cats have shown that, during spindles, the GABA-containing RE cells generate rhythmic spike-bursts within the frequency range of spindling, superimposed on a slowly rising and decaying depolarizing envelope (*1*). Spike-bursts of RE cells, particularly those in the rostral pole and rostrolateral sector of the nucleus, impose rhythmic IPSPs onto a large number of TC cells through their divergent connections in the dorsal thalamus [M. Steriade, A. Parent, J. Hada, *J. Comp. Neurol.* **229**, 531 (1984)]. TC rebound bursts are generated at the offset of the IPSPs and transmitted back to RE cells, where they generate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate)–kainate excitatory postsynaptic potentials (EPSPs) [T. Bal, M. von Krosigk, D. A. McCormick, *J. Physiol. (London)* **483**, 641 (1995)], and to neocortical cells, where glutamatergic EPSPs are at the basis of the spindle oscillations observable in the EEG [M. Steriade and M. Deschênes, in *Cellular Thalamic Mechanisms*, M. Bentivoglio and R. Spreafico, Eds. (Elsevier, Amsterdam, 1988), pp. 51–76].
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18. We thank D. Drolet, P. Giguère, and G. Oakson for technical assistance. Supported by the Medical Research Council of Canada, Human Frontier Science Program, Fonds de la Recherche en Santé du Québec, and the Howard Hughes Medical Institute.

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PIN: An Associated Protein Inhibitor of Neuronal Nitric Oxide Synthase

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The neurotransmitter functions of nitric oxide are dependent on dynamic regulation of its biosynthetic enzyme, neuronal nitric oxide synthase (nNOS). By means of a yeast two-hybrid screen, a 10-kilodalton protein was identified that physically interacts with and inhibits the activity of nNOS. This inhibitor, designated PIN, appears to be one of the most conserved proteins in nature, showing 92 percent amino acid identity with the nematode and rat homologs. Binding of PIN destabilizes the nNOS dimer, a conformation necessary for activity. These results suggest that PIN may regulate numerous biological processes through its effects on nitric oxide synthase activity.

Nitric oxide (NO) is a major messenger molecule in the cardiovascular, immune, and nervous systems. In the brain, NO is responsible for the glutamate-linked enhancement of 3′,5′ cyclic guanosine monophosphate (cGMP) levels (*1*) and may be involved in apoptosis (*2*), synaptogenesis (*1, 3*), and neuronal development (*1*). Because NO cannot be stored in vesicles like other neurotransmitters, its release is regulated by the activity of the enzyme that makes it, NO synthase (NOS).

To search for associated proteins that might alter nNOS activity, we used the yeast two-hybrid system (*4, 5*). Yeast expressing a fusion protein consisting of amino acids 2 to 377 of nNOS and the Gal4 DNA-binding domain (BD) were transformed with a rat hippocampal cDNA library fused to the Gal4 activation domain (AD). Screening of ~3 × 10^6 clones resulted in the isolation of a cDNA that encodes a protein, designated PIN (protein inhibitor of nNOS), that interacts with nNOS (*6*). This interaction was specific because PIN binds to nNOS but not to distinct domains of another protein, the rapamycin and FKBP target (RAFT) (Fig. 1A). Expression of several truncated fragments of nNOS (*7*) as Gal4 BD fusions revealed that amino acids 163 to 245 of nNOS are sufficient for PIN binding in yeast (Fig. 1B). This region lies outside of the nNOS PDZ domain, a protein-binding module that may target nNOS to synaptic structures (*8, 9*), and it does not overlap with regions of nNOS previously implicated in binding to calmodulin or cofactors.

Northern (RNA) blot analysis with the PIN cDNA as a probe revealed an abundant 0.9-kb transcript present at highest levels in the testes, intermediate levels in the brain,

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and lowest levels in most peripheral tissues (*10*). We screened a rat brain cDNA library (*11*) with the PIN cDNA and obtained a 615–base pair (bp) cDNA that contained a 270-bp open reading frame, preceded 87 bp upstream by an inframe stop codon. This start codon was located in a context that conformed to the Kozak consensus sequence (*12*). These data revealed that the clone obtained in the yeast two-hybrid screen coded for the complete PIN sequence and 25 amino acids from the 5′ untranslated region.

PIN is predicted to be an 89–amino acid protein (Fig. 1C), with no recognizable structural motifs but with numerous highly conserved (*13*) homologs across species. PIN has 92% amino acid sequence identity to a hypothetical protein identified in the *Caenorhabditis elegans* genome sequencing project (*14*) and has 92% amino acid sequence identity to a *Chlamydomonas reinhardtii* protein of unknown function recently identified as a component of a macromolecular complex that includes flagellar dynein (*15*). The search also identified expressed sequence tags (ESTs) from several species, some of which span the entire or nearly the entire coding sequence of PIN. Rat PIN displays 100% identity with the human and mouse sequences, 63% with the *Schistosoma mansoni* homolog, and 62% identity with the *Arabidopsis thaliana* homolog (*16*). This high degree of conservation is reminiscent of other protein families such as FKBP-12 (*17*), cyclophilin (*18*), and the 14-3-3 families (*19*), and suggests that PIN serves important, biologically conserved functions. Preliminary experiments indicate that PIN associates with several other proteins besides nNOS (*10*). Thus, PIN's biological functions may involve association with numerous proteins.

We next examined the ability of nNOS, endothelial NOS (eNOS), and inducible NOS (iNOS) in lysates from transfected human embryonic kidney (HEK) 293 cells (*20*) to bind to an immobilized glutathione-S-transferase (GST)–PIN fusion protein. Only nNOS specifically associated with PIN (Fig. 2A), a result predicted by our observation that the PIN-binding domain of nNOS (amino acids 163 to 245) is absent from eNOS and iNOS. Lysates from nNOS-transfected HEK 293 cells were mixed with bacterial lysates containing either GST-PIN or GST and then applied to a NOS affinity resin containing 2′,5′-adenosine diphosphate (ADP) ribose (*20*). GST-PIN bound to the resin in the presence of nNOS, whereas GST did not bind (Fig. 2B). In a blot overlay assay, radiolabeled GST-PIN selectively bound nNOS from lysates of nNOS-transfected, but not mock-transfected, HEK 293 cells (*20*) (Fig. 2C). Finally, physiologic complexes of PIN and nNOS were detected in rat cerebellum extracts by immunoprecipitation with an antibody to nNOS (*21*) (Fig. 2D).

PIN did not alter the subcellular localization of nNOS in transfected HEK 293 and N1E-115 mouse neuroblastoma cells overexpressing nNOS and PIN (*10*). We also assessed the effect of PIN on NO-dependent cGMP formation in HEK 293 cells cotrans-

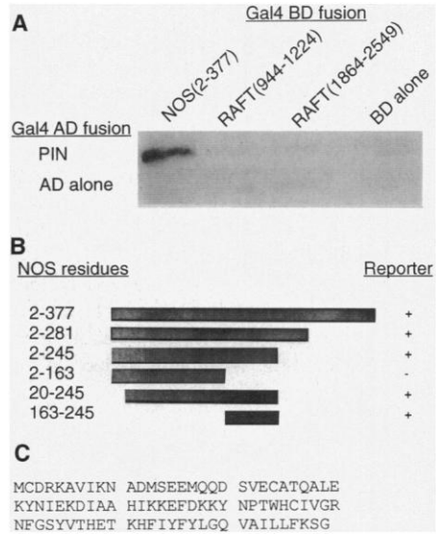

Fig. 1. Interaction of nNOS and PIN in the yeast two-hybrid system. (**A**) Yeast was transformed with the indicated Gal4 AD and Gal4 BD plasmids and grown on plates containing histidine. A typical filter lift (*5*) is shown in which β-galactosidase activity was detected by the appearance of a dark blue precipitate. pAD-PIN activated *lacZ* transcription in the presence of the pBD-NOS(2–377) but not control proteins derived from RAFT. β-Galactosidase activity correlated with growth on histidine-deficient plates (*10*). (**B**) Mapping of the PIN-binding domain of nNOS. The Gal4 BD was fused to regions of NOS, and the ability of these proteins to interact with PIN was assayed with the yeast two-hybrid assay. The relative β-galactosidase activity is indicated in the column on the right. (**C**) Deduced amino acid sequence of PIN. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

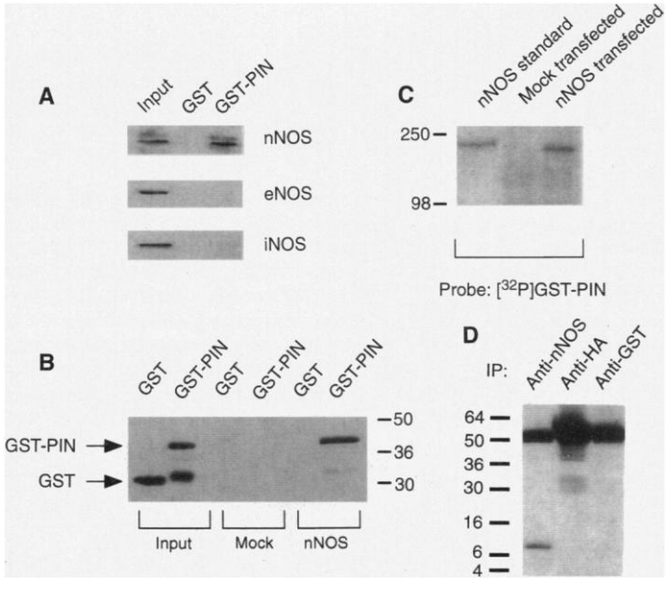

Fig. 2. Interaction of nNOS and PIN. (**A**) PIN binds to nNOS but not to iNOS or eNOS. Bacterially expressed GST-PIN and GST were bound to glutathione-agarose. Lysates of HEK 293 cells transfected with expression plasmids for nNOS, iNOS, and eNOS were applied to the GST fusion protein columns and incubated for 1 hour at 4°C (*20*). The resin was washed extensively and then eluted with SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer and immunoblotted with the indicated isoform-specific antibodies (Transduction Labs). Input is 10% of the protein loaded onto the columns. (**B**) GST-PIN binds to nNOS immobilized on 2′,5′-ADP–Sepharose, a NOS affinity resin. Bacterial lysates containing the indicated recombinant proteins were mixed with lysates from nNOS-transfected HEK 293 cells or mock-transfected cells. The mixture was applied to 2′,5′-ADP–Sepharose, and the bound protein was eluted with 10 mM NADPH and detected by immunoblotting with an antibody to GST (anti-GST) (*20*). Samples of the bacterial lysates are included to show the mobilities of the different recombinant proteins. (**C**) GST-PIN recognizes nNOS in a blot overlay assay. Two cyclic AMP–dependent protein kinase A (PKA) sites were inserted between the GST moiety and PIN to create a GST-PIN fusion protein that was labeled in vitro with [γ-^{32}P]-adenosine triphosphate (ATP) by PKA. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the radiolabeled GST-fusion protein (*20*). The radiolabeled protein binds to purified nNOS and to nNOS in transfected HEK 293 cells but not in mock-transfected cells. (**D**) An antibody to nNOS specifically coprecipitates PIN. Rat cerebellar lysate was immunoprecipitated (IP) with 1 μg of an antibody to nNOS (*30*) or the indicated control antibody and protein A–agarose. HA, hemagglutinin. The immunoprecipitates were washed (*21*) and eluted in SDS-PAGE sample buffer. PIN, the lower band, was detected by immunoblot with an antiserum to PIN (*21*). Molecular sizes in (B) to (D) are indicated in kilodaltons. The upper band is the immunoglobulin heavy chain.

fected with PIN and nNOS (*22*). These experiments revealed that PIN suppressed calcium ionophore–stimulated cGMP formation in a concentration-dependent manner (Fig. 3A). Transfection of PIN alone did not alter basal cGMP levels or cGMP levels induced by calcium ionophore (*10*).

To determine whether PIN directly inhibits nNOS, we examined the effect of recombinant GST-PIN on nNOS activity in lysates of HEK 293 cells stably transfected with nNOS (Fig. 3B). As little as 250 nM PIN substantially reduced nNOS activity relative to the GST control, with 50% inhibition evident at about 1 μM. Similar quantities of GST had little effect on nNOS activity (*10*). A preparation of PIN lacking the GST moiety (*23*) showed a similar dose-dependent inhibition of nNOS activity, with 50% inhibition at about 5 μM (Fig. 3B). This reduction in affinity may be due to thermal denaturation during preparation (*23*).

We explored the possibility that PIN affects nNOS dimerization, which is thought to be essential for NOS activity (*24*, *25*). The subunits of nNOS form a dimer that remains intact during low-temperature SDS-PAGE in the presence of tetrahydrobiopterin (BH_4) and arginine (*25*). In this assay, nNOS migrated as a monomer after incubation with concentrations of GST-PIN that inhibit >90% of nNOS activity (Fig. 4A). Incubation with GST resulted in minimal loss of dimerized nNOS. We next determined the apparent molecular mass of nNOS by gel filtration. The 320-kD nNOS dimer has been shown previously to migrate with an apparent molecular mass of ~600 kD (*25*). After incubation of nNOS with GST, nNOS migrated at ~600 kD, whereas after incubation with GST-PIN, nNOS appeared at the monomeric position (Fig. 4B). These data suggest that PIN destabilizes the nNOS dimer.

BH_4 and arginine are thought to contribute to the stability of the NOS dimer. Thus, iNOS dimerizes in the presence of BH_4 and arginine and monomerizes when these cofactors are removed by dialysis (*24*). The nNOS inhibitor 7-nitroindazole noncompetitively reduces the affinity of BH_4 and arginine (*26*) and causes nNOS to migrate as a monomer in SDS-PAGE (*25*). We found that neither BH_4 nor arginine alters nNOS binding to PIN in vitro (*10*), implying that PIN may prevent dimerization through a mechanism distinct from that of 7-nitroindazole. In the SDS-PAGE stability assay, nNOS migrates as a monomer in preparations that include 7-nitroindazole or that lack BH_4 and arginine, but it still migrates as a dimer in gel filtration assays (*25*). A second, SDS-sensitive dimerization domain within nNOS may permit dimerization in the presence of 7-nitroindazole. Using the yeast two-hybrid system, we have recently found that the first 165 amino acids of nNOS, which include the PDZ domain, can dimerize (*10*). This region does not bind to BH_4 or arginine but is adjacent to the PIN-binding site in NOS. It remains to be determined whether PIN regulates dimerization by affecting this domain or through some other function.

While this report was under review, a screen for genes required for *Drosophila* oogenesis led to the cloning of *Drosophila* and human PIN homologs (*27*). In *Drosophila*, homozygous loss-of-function mutations are embryonic lethal (*27*). These data, along with the extraordinary evolutionary conservation of PIN and our preliminary evidence that it interacts with multiple proteins, suggest that it may be a major regulatory protein influencing numerous physiological processes.

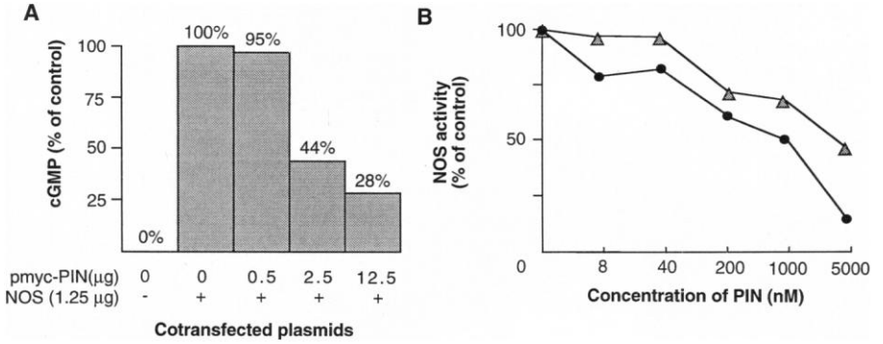

Fig. 3. Inhibition of NO generation by PIN. (**A**) PIN reduces NO-dependent cGMP elevation in transfected HEK 293 cells. HEK 293 cells were transfected with a nNOS expression vector and the indicated concentrations of pmyc-PIN (*22*). The pRK5 parent vector was also transfected to adjust the total DNA quantity transfected to 13.75 μg per experiment. In each experiment, nNOS amounts were detected by immunoblot and were unchanged (*10*). Cells were treated with 10 μM calcium ionophore A23187 for 1 hour, and cGMP levels were measured by radioimmunoassay (Amersham). (**B**) PIN inhibits the conversion of arginine to citrulline by nNOS in a concentration-dependent manner. GST-PIN (circles) and thrombin-cleaved PIN (triangles) were incubated with transfected cell lysates containing nNOS for 1 hour at 37°C. NOS assays were initiated by the addition of $CaCl_2$, NADPH, and [^{3}H]arginine. The percentage of nNOS activity remaining is indicated relative to activity measurements with control proteins at the indicated final concentrations (*29*). Control activity levels were determined with either GST or thrombin-cleaved BIRK (*23*).

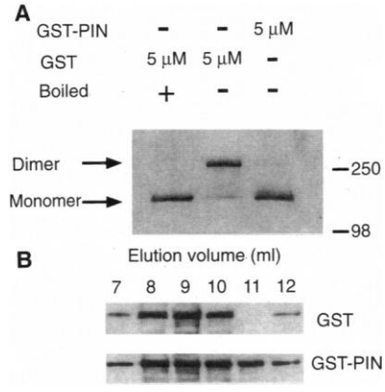

Fig. 4. Inhibition of nNOS dimerization by PIN. (**A**) nNOS migrates as a monomer after treatment with recombinant PIN. The nNOS dimer is stable in SDS-PAGE sample buffer but denatures and migrates at the expected monomeric molecular mass of 160 kD if the sample is boiled before electrophoresis (*25*). nNOS preparations from transfected HEK 293 cells similar to those in Fig. 3B were assayed for dimerization by SDS-PAGE. A boiled sample is included to show the expected mobility of the nNOS monomer. Molecular sizes are indicated in kilodaltons. (**B**) The apparent molecular mass of nNOS by gel filtration is reduced after treatment with recombinant PIN. Samples were resolved by fast performance liquid chromatography with a Superose 12 gel filtration column (Pharmacia). Fractions were concentrated and the material was immunoblotted with an antibody to nNOS. The column was calibrated with the following standards: thyroglobulin (relative molecular mass M_r 670,000) 8.1 ml, gamma globulin (M_r 158,000) 10.9 ml, ovalbumin (M_r 44,000) 12.6 ml, myoglobin (M_r 17,000) 14.3 ml, and cyanocobalamin (M_r 1350) 18.8 ml.

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6. Two-hybrid screens and parent vectors pPC97 and pPC86 were as described (*5*). Plasmid pBD-NOS(2–377) was constructed by insertion of a nNOS polymerase chain reaction (PCR) product encoding amino acids 2 to 377 into the Sal I–Bgl II sites of pPC97, resulting in an open reading frame encoding a Gal4 BD–NOS fusion protein. The nNOS fragment was constructed by PCR with the following primers: 5′-GACTAGTCGACTGAAGAGAACACGTTTGGG-3′ (coding strand) and 5′-TCTGCAGATCTCAGTGGGCCTTGGAGCCAAA-3′ (noncoding strand). A

rat hippocampal cDNA library in pPC86 [X.-J. Li *et al.*, *Nature* **378**, 398 (1995)] was amplified once in DH10B (Gibco BRL) (*28*) and transformed into yeast containing pBD-NOS(2–377). pAD-PIN was identified as a 0.5-kb clone that activated *lacZ* transcription and conferred histidine protorophy in the presence of pBD-NOS(2–377). Plasmids were sequenced by automated fluorescent sequencing. The PIN sequence has been deposited in GenBank (accession number U66461).

7. pPC97 derivatives containing fragments of RAFT were constructed by PCR and cloned into the Sal I and Sac I sites of pPC97. Truncated NOS fragments comprising amino acids 2 to 163 and 2 to 281 were generated by restriction of the initial NOS(2–377) PCR fragment with Nco I and Ava I, respectively, followed by blunt-end ligation into pPC97. Other truncated NOS fragments were prepared by PCR.
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20. The cDNA for PIN was excised from pAD-PIN with Sal I and Not I and cloned into those sites in pGEX-4T2 (Pharmacia). Fusion proteins were prepared in *Escherichia coli* BL21(DE3) (Novagen) with glutathione-agarose (Sigma) [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)], except that bacterial pellets were lysed in lysis buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl, and 2 mM EDTA], supernatants were adjusted to 1% Triton X-100, and protein was purified with elution buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl, 10 mM reduced glutathione, and 2 mM EDTA]. HEK 293 cells were transfected with plasmids for nNOS [D. S. Bredt *et al.*, *Nature* **351**, 714 (1991)], eNOS [S. Lamas, P. A. Marsden, G. K. Li, P. Tempst, T. Michel, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6348 (1992)], and iNOS (C. J. Lowenstein, C. S. Glatt, D. S. Bredt, S. H. Snyder, *ibid.*, p. 6711). Transfections were performed with 10 μg of each plasmid with the calcium phosphate method (*28*). After transfection, cells were sonicated in buffer A [50 mM tris-HCl (pH 7.7), 100 mM NaCl, 2 mM EDTA, and 1% Triton X-100] and cleared by centrifugation. This cellular lysate was incubated with GST or GST-PIN immobilized on glutathione-agarose and then washed extensively in HNTG buffer [20 mM Hepes (pH 7.4), 500 mM NaCl, 10% glycerol, and 0.1% Triton X-100]. For assays testing PIN binding to immobilized NOS, 20 μg of bacterial lysate was added to 200 μg of transfected HEK 293 cell lysate and bound to 2′,5′-ADP-Sepharose 4B (Pharmacia) and subsequently washed and eluted with 10 mM NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) as described [D. S. Bredt and S. H. Snyder, *ibid.* **87**, 682 (1990)]. The eluate was immunoblotted with a rabbit antibody to GST (NovaCastra, Burlingame, CA). For blot-overlay analysis, pGEX-4T2 was modified such that two sites for protein kinase A (PKA) encoded on complementary synthetic oligonucleotides (5′-AATTCGTCGTGCATCTGTTGAACTA-CGTCGAGCTTCAGTTGCG-3′, upper strand) were ligated into the Eco RI–Sal I sites to generate plasmid pGEX4T-2K. Kinase reactions and blot overlays were performed as described [W. M. Kavanaugh and L. T. Williams, *Science* **266**, 1862 (1994)].
21. Rat cerebella were homogenized in IP buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl] and clarified by centrifugation. Washes were performed with wash buffer 1 [50 mM tris-HCl (pH 7.7), 500 mM NaCl] and wash buffer 2 [50 mM tris-HCl (pH 7.7), 500 mM LiCl]. The rabbit polyclonal antibody to the hemagglutinin epitope (HA) was from BAbCO (Richmond, CA). A rabbit antiserum to PIN was generated to a hexahisitidine-tagged fusion protein containing the full-length PIN sequence (*23*). The antiserum was used in immunoblots at a dilution of 1:1000 and recognizes a band of the expected molecular size. Preincubation of the antiserum with GST-PIN confirmed the specificity of this antiserum (*10*).
22. A Sal I–Bgl II fragment comprising the entire translated sequence of the insert in pAD-PIN was generated by PCR and subcloned into the Sal I–Bam HI site of the cytomegalovirus (CMV)-driven eukaryotic expression vector pCMV-myc to generate a fusion protein consisting of an NH_2-terminal Myc tag followed by a pentaglycine linker and the PIN insert.
23. Fusion proteins were prepared as in (*20*) except that proteins were eluted from glutathione agarose by cleavage with thrombin in thrombin cleavage buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl, 2.5 mM $CaCl_2$, and 1% Triton X-100] for 16 hours at 37°C. The eluate was adjusted to 5 mM EGTA, 4 μM leupeptin, and 400 nM aprotinin. Dilutions were made with thrombin cleavage buffer adjusted in this manner. A GST-BIRK fusion consisting of amino acids 347 to 442 of BIRK2 [D. S. Bredt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6753 (1995)] was cleaved with thrombin as above and used as a control protein in NOS assays. A different preparation with a bacterially expressed hexahistidine-tagged PIN fusion protein inhibited nNOS activity and dimerization in a similar concentration-dependent manner (*10*).
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29. Lysates from transfected HEK 293 cells (20 μl) were incubated with fusion protein (50 μl) at 37°C for 1 hour. Enzymatic activity assays were initiated by the addition of $CaCl_2$, NADPH, and [^{3}H]arginine to a final volume of 250 μl. Citrulline accumulation was measured as described [D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9030 (1989)]. Background levels were determined in the presence of 2 mM EGTA and no added $CaCl_2$.
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Change of a Catalytic Reaction Carried Out by a DNA Replication Protein

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The RepA protein of plasmid pC194 initiates and terminates rolling circle replication. At initiation, it forms a 5′-phosphotyrosyl DNA link, whereas at termination, a glutamate residue directs hydrolytic cleavage of the newly synthesized origin, and the resulting 3′-hydroxyl group undergoes transesterification with the phosphotyrosine link. The protein is thus released from DNA, and the termination is uncoupled from reinitiation of replication. Replacement of the glutamate with tyrosine in RepA altered this mechanism, so that termination occurred by two successive transesterifications and became coupled to reinitiation. This result suggests that various enzymes involved in DNA cleavage and rejoining may have similar mechanistic and evolutionary roots.

DNA strand transfer during site-specific recombination can occur by two different mechanisms. In the first, a covalent protein-DNA intermediate is formed, similar to that formed in reactions catalyzed by the DNA topoisomerases (*1*). Strand transfer proceeds through two successive transesterifications as exemplified in reactions catalyzed by the λ integrase and by the γδ resolvase (*2*, *3*). A phosphotyrosyl (*1*, *2*) or phosphoserine linkage (*3*) is formed, and a hydroxyl group is left on the cleaved DNA. This group then initiates a second transesterification reaction, forming a phosphodiester link and resolving the protein-DNA complex. The second mechanism, demonstrated in phage Mu transposition and hu-

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