6. D. J. Grainger, T. R. Hesketh, P. L. Weissberg, J. C. Metcalfe, *Biochem. J.* **283**, 403 (1992).
7. T. Nakamura *et al.*, *Nature* **342**, 440 (1989); D. P. Bottaro *et al.*, *Science* **251**, 802 (1991).
8. M. B. Sporn, A. B. Roberts, L. M. Wakefield, R. K. Assoian, *Science* **233**, 532 (1986); G. K. Owens, A. T. Geisterfer, Y. W. Yang, A. Komoriya, *J. Cell Biol.* **107**, 771 (1988); S. Bjorkerud, *Arterioscl. Thromb.* **11**, 892 (1991); G. H. Gibbons, R. E. Pratt, V. J. Dzau, *J. Clin. Invest.* **90**, 456 (1992).
9. L. M. Wakefield *et al.*, *Growth Factors* **1**, 203 (1989).
10. R. M. Lyons, L. E. Gentry, A. F. Purchio, H. L. Moses, *J. Cell Biol.* **110**, 1361 (1990).
11. D. D. Danielpour *et al.*, *J. Cell. Physiol.* **138**, 79 (1989).
12. A. A. Colletta *et al.*, *Br. J. Cancer Res.* **82**, 132 (1990); D. J. Grainger, P. L. Weissberg, J. C. Metcalfe, in preparation.
13. E. J. Battegay, E. W. Raines, R. A. Seifert, D. F. Bowen-Pope, R. Ross, *Cell* **102**, 1217 (1990).
14. M. A. Rath *et al.*, *Arteriosclerosis* **9**, 579 (1989); G. L. Cushing *et al.*, *ibid.*, p. 593; A. M. Niendorf *et al.*, *Virchows Archiv. A.* **417**, 105 (1988).
15. G. G. Rhoads, G. Dahlen, K. Berg, N. Morton, A. L. Dannenberg, *J. Am. Med. Assoc.* **256**, 2540 (1986); A. Rosengren, L. Wilhelmsen, E. Eriksson, B. Risberg, H. Wedel, *Br. Med. J.* **301**, 31248 (1990).
16. S. Kojima, P. C. Harpel, D. B. Rifkin, *J. Cell Biol.* **113**, 1439 (1991).
17. B. L. Knight, Y. F. Perombelon, A. K. Soutar, D. P. Wade, M. Seed, *Atherosclerosis* **87**, 227 (1991).
18. M. L. Koschinsky *et al.*, *Biochemistry* **30**, 5044 (1991).
19. We thank C. Witchell for assistance with cell cultures and the Papworth Hospital transplant surgeons for their cooperation. Supported by Wellcome Prize Fellowship (to D.J.G.), British Heart Foundation (to P.L.W. and J.C.M.), Glaxo Group (to H.L.K.), American Heart Association (California Affiliate) (to D.P.W.), and the Institute of Biological and Clinical Investigation (to R.M.L.).

7 December 1992; accepted 4 March 1993

Complexes of Ras·GTP with Raf-1 and Mitogen-Activated Protein Kinase Kinase

Shonna A. Moodie,* Berthe M. Willumsen, Michael J. Weber, Alan Wolfman

The guanosine triphosphate (GTP)–binding protein Ras functions in regulating growth and differentiation; however, little is known about the protein interactions that bring about its biological activity. Wild-type Ras or mutant forms of Ras were covalently attached to an insoluble matrix and then used to examine the interaction of signaling proteins with Ras. Forms of Ras activated either by mutation (Gly12Val) or by binding of the GTP analog, guanylyl-imidodiphosphate (GMP-PNP) interacted specifically with Raf-1 whereas an effector domain mutant, Ile36Ala, failed to interact with Raf-1. Mitogen-activated protein kinase (MAP kinase) activity was only associated with activated forms of Ras. The specific interaction of activated Ras with active MAP kinase kinase (MAPKK) was confirmed by direct assays. Thus the forming of complexes containing MAPKK activity and Raf-1 protein are dependent upon the activity of Ras.

The majority of cellular Ras in quiescent cells is in the inactive guanosine diphosphate (GDP)–bound conformation. Stimulation of cells with mitogens or differentiation factors increases the abundance of the active GTP-bound form of Ras (*1*). The activity of Ras is required for regulatory signaling by plasma membrane tyrosine kinase oncogenes and receptors (*2*). Blocking the action of Ras, either by microinjection of antibodies or through the expression of a dominant negative mutant, prevents the activation of the cytosolic serine-threonine protein kinases Raf-1 and MAP kinase (*3, 4*). The expression of activated Ras, either in whole cells or in vitro activates these cytosolic serine-threonine protein kinases (*5–7*). These cytosolic enzymes can function as part of a kinase cascade, in which MAP kinases are activated by a MAPKK (*8*), which in turn can be phosphorylated and activated by the Raf-1 protein kinase (*9*). The components of this pathway may not always be coupled to each other, however, because neither Ras or Raf-1 activates MAP kinases in Rat1a cells (*10*).

Though it is clear that Ras participates in signaling between the cell membrane and the cytosol, the biochemical interactions that mediate the action of Ras are poorly understood. To determine if Ras might physically interact with cytosolic signaling components in a GTP-dependent manner, we covalently bound recombinant c-Ha-Ras (referred to as Ras) to silica beads activated by an *N*-hydroxyl succinimide group (*11, 12*). The coupled Ras was active in binding guanine nucleotides and has been used to examine interactions between Ras and neurofibromin, a regulator of Ras function (*12*). The immobilized Ras was used to probe rat brain cytosol for signaling proteins that specifically interacted with Ras·GTP.

Immobilized Ras bound to GMP-PNP (a nonhydrolyzable GTP analog) formed a stable complex with Raf-1 (Fig. 1A). Approximately 15% of the total cytosolic Raf-1 associated with immobilized Ras·GMP-PNP. The transforming Ras mutant, Ras^{Val12} bound to GMP-PNP, was about 10% as effective as wild-type Ras in forming stable complexes with Raf-1 (Fig. 1A). An even smaller amount of Raf-1 associated with the GDP-bound form of Ras^{Val12}. A form of Ras with a mutation in the effector domain, Ras^{Ala36}, bound to either GDP or GMP-PNP failed to bind detectable amounts of Raf-1 (Fig. 1B). Thus, the stable association of Raf-1 with immobilized Ras correlated well with the formation of the active GTP-bound form of Ras.

We investigated the GTP-dependent association of Ras with MAPKK and MAP kinase, which have been reported to function downstream of Raf-1. Immunoprecipitates of MAP kinase from the rat brain lysate did not catalyze the phosphorylation of myelin basic protein (MBP) in the presence of [γ-^{32}P]adenosine triphosphate (ATP) (*13*). This enabled us to use the activation of endogenous MAP kinase activity as an assay for MAPKK. Incubation of cytosolic proteins bound to immobilized Ras·GMP-PNP with ATP produced soluble kinase activity with a high substrate specificity for MBP as compared with either histone or casein (Fig. 2A) (*14*). The soluble kinase activity was also detected with

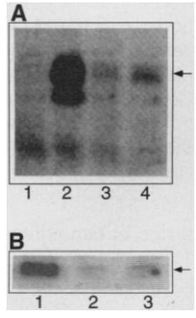

Fig. 1. Association of Raf-1 with immobilized Ras. (**A**) Lysate was incubated with immobilized Ras·GDP (lane 1), Ras·GMP-PNP (lane 2), Ras^{Val12}·GDP (lane 3), or Ras^{Val12}·GMP-PNP (lane 4) for 60 min at 4°C (*15*); 2× Laemmli sample buffer was added to the sedimented material and the eluted proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred, and immunoblotted with a rabbit polyclonal antibody to Raf-1. The position of Raf-1 is denoted by the arrow. (**B**) As in (A) except that lysate was incubated with immobilized Ras·GMP-PNP (lane 1), Ras^{Ala36}·GDP (lane 2), or Ras^{Ala36}·GMP-PNP (lane 3). Control immunoprecipitation of lysate with the antibody revealed a single band of 68 kD whereas the rabbit non-immune serum did not react with this protein. Quantitation of Raf-1 bound to immobilized Ras was done on a phosphorimager and expressed as the percentage of Raf-1 protein immunoprecipitated from identical amounts of crude lysate. Comparable amounts of antibody and lysate were used to those in the experiment described in Table 1.

S. A. Moodie and A. Wolfman, Department of Cell Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44106.
B. M. Willumsen, Institute of Microbiology, University of Copenhagen, Øster Farimagsgade 2A, DK 1353 Copenhagen, Denmark.
M. J. Weber, Department of Microbiology, Health Sciences Centre, University of Virginia, Charlottesville, VA 22908.
*To whom correspondence should be addressed.

Ras^{Val12}·GMP-PNP but not with either Ras^{Ala36}·GDP or Ras^{Ala36}·GMP-PNP. We also detected kinase activity associated with immobilized Ras^{Val12}·GDP (Fig. 2B).

Proteins of 98, 68, 47, and 42 kD became phosphorylated in samples of cellular proteins associated with immobilized Ras·GMP-PNP and incubated in the presence of [γ-^{32}P]ATP (*13*). The phosphorylated 42- and 47-kD proteins were, however, the only phosphorylated proteins that were released into the soluble reaction mixture (Fig. 2C). The proportion of these proteins detected in the soluble fraction was not altered by increasing the ionic strength of the sample after the phosphorylation reaction. The soluble kinase activity was not released from immobilized Ras·GMP-PNP in the presence of 1 μM adenylyl-imidodiphosphate (AMP-PNP), but 1 μM ATP did cause release (*13*). Thus, the production of soluble MBP kinase activity from immobilized Ras·GMP-PNP appears to depend on phosphorylation and coincides with the appearance of the phosphorylated 42- and 47-kD proteins.

Soluble MBP kinase activity that had associated with immobilized Ras·GMP-PNP was removed by immunoprecipitation with a polyclonal antibody to a 42-kD member of the MAP kinase family, $p42^{mapk}$. The remaining supernatant activated exogenously added unphosphorylated recombinant $p42^{mapk}$ (Fig. 3A). Protein immunoblot analysis of $p42^{mapk}$ and a 44-kD MAP kinase, $p44^{mapk}$, detected approximately equal amounts of both proteins associated with Ras, Ras^{Val12}, or Ras^{Ala36} bound to GDP or GMP-PNP (Fig. 3B) (*13*). These data suggest that the activation of $p42^{mapk}$ that occurred when cytosol was incubated with Ras·GMP-PNP resulted from a MAPKK activity. The activity of MAPKK was examined by measuring the amount of endogenous $p42^{mapk}$ phosphorylated by proteins isolated with the different Ras mutants in either of their guanine nucleotide–bound forms. MAPKK activity was detected in samples incubated with Ras·GMP-PNP, Ras^{Val12}·GDP, or Ras^{Val12}·GMP-PNP (Fig. 3C). Neither Ras·GDP nor Ras^{Ala36} samples contained active MAPKK. Phosphorylation of $p42^{mapk}$ was not detected in samples incubated with a protein related to Ras, immobilized RalA, bound to GMP-PNP (*13*). In a separate assay, the phosphorylation of a catalytically inactive mutant of $p42^{mapk}$ was detected in samples prepared with either Ras·GMP-PNP, Ras^{Val12}·GDP, or Ras^{Val12}·GMP-PNP (Fig. 3D). We have been unable to detect specific bands corresponding to the published molecular size of MAPKK (47 to 49 kD) by protein immunoblot analysis.

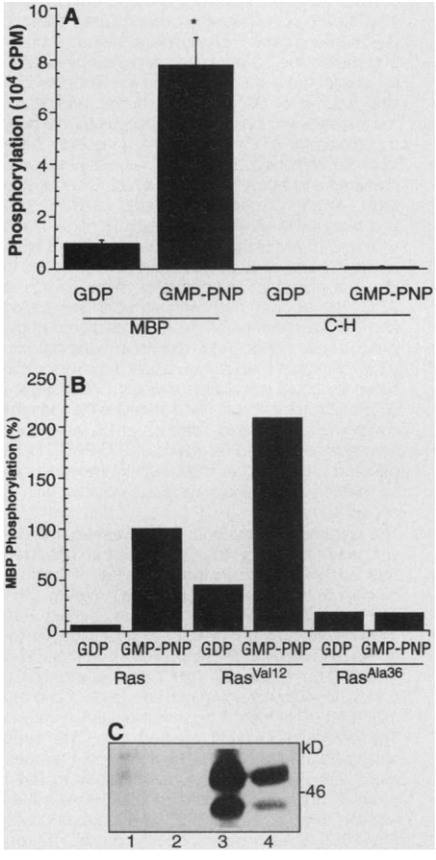

Fig. 2. Detection of MBP kinase activity associated with wild-type and mutant forms of immobilized Ras·GMP-PNP (**A**) Lysate was incubated with immobilized Ras·GDP or Ras·GMP-PNP for 60 min at 4°C. The washed sedimented material was assayed for phosphorylation of MBP or casein and Histone (C-H) as described (*14*). Bars represent mean ± SE (n = 3); *, P < 0.05. (**B**) Samples prepared from immobilized Ras, Ras^{Val12}, and Ras^{Ala36} bound to either GDP or GMP-PNP were assayed for MBP kinase activity as described in (A). The data are expressed as a percent of the amount of activity associated with Ras·GMP-PNP. These data are representative of three separate experiments. (**C**) Proteins isolated with either Ras·GDP (lanes 1 and 2) or Ras·GMP-PNP (lanes 3 and 4) were incubated with 50 μl of p21 buffer containing 10 μM [γ-^{32}P]ATP (10 μCi) for 20 min at 37°C. The soluble reaction mixture (lanes 2 and 4) and the proteins that remained associated with the immobilized Ras (lanes 1 and 3) were analyzed by SDS-PAGE for phosphoproteins (*16*).

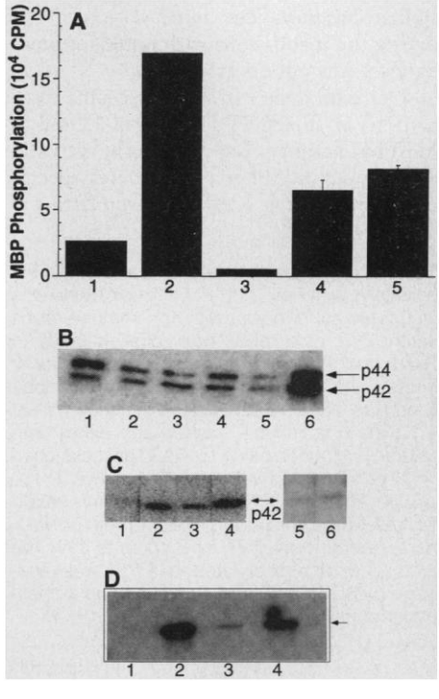

Fig. 3. MAP kinase and MAP kinase kinase activity associated with immobilized Ras. (**A**) Lysate was incubated with immobilized Ras·GDP (lane 1) or Ras·GMP-PNP (lanes 2 to 5) for 60 min at 4°C. Supernatants (Fig. 2) were removed and assayed for MAP kinase activity as described (lanes 1 and 2). The supernatants eluted with ATP were immunoprecipitated with polyclonal antibody to $p42^{mapk}$ (*17*) and the immunoprecipitates (lane 4) were assayed for MAP kinase activity. Supernatants cleared of $p42^{mapk}$ were assayed for MAP kinase activity in the absence (lane 3) or presence (lane 5) of recombinant inactive $p42^{mapk}$ (0.5 μg). Incubation of the recombinant inactive $p42^{mapk}$ by itself did not produce any MBP phosphorylation. The immunoprecipitates and supernatants prepared from immobilized Ras·GDP did not result in the activation of recombinant inactive $p42^{mapk}$. Bars represent mean ± SE (n = 3). (**B**) Samples from immobilized Ras·GDP (lane 1), Ras·GMP-PNP (lane 2), Ras^{Val12}·GDP (lane 3) or Ras^{Val12}·GMP-PNP (lane 4) prepared as described (*11*). Silica beads without covalently bound Ras (lane 5) and cytosolic extract (10 μg, lane 6) were also analyzed for the presence of $p42^{mapk}$ and $p44^{mapk}$ with a mouse monoclonal antibody (*18*). (**C**) Lysate was incubated with immobilized Ras·GDP (lane 1), Ras·GMP-PNP (lane 2), Ras^{Val12}·GDP (lane 3), or Ras^{Val12}·GMP-PNP (lane 4), Ras^{Ala36}·GDP (lane 5), or Ras^{Ala36}·GMP-PNP (lane 6) for 60 min at 4°C as described. Proteins eluted with [γ-^{32}P]ATP were immunoprecipitated with antibody to $p42^{mapk}$, analyzed by SDS-PAGE, and detected with a phosphorimager. (**D**) Lysate was incubated with immobilized Ras·GDP (lane 1), Ras·GMP-PNP (lane 2), Ras^{Val12}·GDP (lane 3), or Ras^{Val12}·GMP-PNP (lane 4) for 60 min at 4°C. The washed sedimented material was incubated for 20 min with 50 μl of p21 buffer containing ATP (10 μM). The supernatants were immunoprecipitated with polyclonal antibody to $p42^{mapk}$ for 2 hours at 4°C. The supernatants cleared of $p42^{mapk}$ were assayed for MAP kinase activity and found to contain amounts comparable to that bound by Ras·GDP. After a 20-min incubation at 37°C in the presence of catalytically inactive $p42^{mapk}$ (0.5 μg) and 10 μM [γ-^{32}P]ATP (10 μCi), the phosphorylated $p42^{mapk}$ was immunoprecipitated and analyzed by SDS-PAGE and detected with a phosphorimager (*17*). Arrow, catalytically inactive $p42^{mapk}$.

However, we did find that the association of MAPKK activity with immobilized Ras·GMP-PNP occurred independently of the association of Raf-1. Rat brain lysates were cleared of Raf-1 by incubation with antibody to Raf-1 and protein A–Sepharose. These cleared lysates were probed with immobilized Ras·GMP-PNP. No Raf-1 was detected associated with immobilized Ras, but there was no reduction in amount of ^{32}P incorporated into the endogenous $p42^{mapk}$ (Table 1).

All three determinations of MAPKK activity (MBP phosphorylation, phosphorylation of endogenous $p42^{mapk}$, and the phosphorylation of the catalytically inactive $p42^{mapk}$) detected activity associated with Ras^{Val12}·GDP. Immobilized Ras·GDP is prepared in the presence of excess GDP and exchange between bound and free guanine nucleotides did not occur during the course of a typical experiment (*13*). These two considerations make it unlikely that the signals observed with Ras^{Val12}·GDP result from exchange with endogenous GTP and suggest that proliferative signals generated by the transforming Val12 mutant may be less guanine nucleotide–dependent than those mediated by wild-type protein. Under similar circumstances, farnesylation of Ras during the incubations with crude rat brain extracts was not detected (*13*).

Our data demonstrate the specific association of Ras·GTP with Raf-1 and a MAPKK activity. Ras apparently activates endogenous MAPKK or associates specifically with active MAPKK. Our data are consistent with previous studies that suggest that Ras might function as a switch between plasma membrane tyrosine kinases and soluble serine-threonine kinases (*2*). It is unclear whether the Raf-1 or MAPKK proteins interact directly with Ras·GMP-PNP or through an as yet unidentified target protein. We have been unable to determine whether the association of Raf-1 with Ras·GMP-PNP alters any of the biochemical properties of Raf-1. The consistency in the interactions of Raf-1 protein and MAPKK activity with wild-type Ras and mutant Ras proteins suggest that Raf-1 and a MAPKK activity may associate with activated Ras in signaling complexes.

Table 1. Independent association of MAPKK and Raf-1 with Ras·GMP-PNP. Crude lysate was incubated either alone, with non-immune rabbit serum (NI), or a rabbit polyclonal antibody to Raf-1 coupled to protein A–Sepharose for 2 hours at 4°C and then incubated with immobilized Ras·GDP or Ras·GMP-PNP for 60 min at 4°C. The beads were washed and either analyzed by immunoblotting for Raf-1 association (n = 2) or incubated with 10 μM [γ-^{32}P]ATP (10 μCi) for 20 min at 37°C. The supernatants eluted with ATP were immunoprecipitated with polyclonal antibody to $p42^{mapk}$ for 2 hours at 4°C. The amount of phosphorylated $p42^{mapk}$ was analyzed by SDS-PAGE and quantitated on a phosphorimager. Values are mean ± SE (n = 4).

	Raf-1 binding (%)	Phosphorylation of $p42^{mapk}$ (%)
Ras·GDP + lysate	4	1.5 ± 0.6
Ras·GMP-PNP + lysate	100	100
Ras·GMP-PNP + NI lysate	106	145 ± 21
Ras·GMP-PNP + Raf-1–depleted lysate	0	104 ± 8

REFERENCES AND NOTES

1. T. Satoh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7926 (1990); J. B. Gibbs, M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, U. S. Vogel, *J. Biol. Chem.* **265**, 20437 (1990); A. P. R. M. Osterop *et al.*, *ibid.* **267**, 14647 (1992); K. Muroya, S. Hattori, S. Nakamura, *Oncogene* **7**, 277 (1992); K. Zhang, A. G. Papageorge, D. R. Lowy, *Science* **257**, 671 (1992).
2. L. R. Mulcahy, M. R. Smith, D. W. Stacey, *Nature* **313**, 241 (1985); M. R. Smith, S. J. DeGudicibus, D. W. Stacey, *ibid.* **320**, 540 (1986).
3. K. W. Wood, C. Sarnecki, T. M. Roberts, J. Blenis, *Cell* **68**, 1041 (1992).
4. S. M. Thomas, M. DeMarco, G. D'Arcangelo, S. Halegoua, J. S. Brugge, *ibid.*, p. 1031; A. M. M. de Vries-Smits, B. M. T. Burgering, S. J. Leevers, C. J. Marshall, J. L. Bos, *Nature* **357**, 602 (1992); D. J. Robbins *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6924 (1992).
5. N. G. Ahn *et al.*, *J. Biol. Chem.* **266**, 4220 (1991); M.-S. Qiu and S. H. Green, *Neuron* **9**, 705 (1992); S. L. Pelech and J. S. Sanghera, *Science* **257**, 1355 (1992); *Trends Biochem. Sci.* **17**, 233 (1992); N. G. Anderson, *Cell. Signalling* **4**, 239 (1992).
6. S. J. Leevers and C. J. Marshall, *EMBO J.* **11**, 569 (1992); M. Pomerance, F. Schweighoffer, B. Tocque, M. Pierre, *J. Biol. Chem.* **267**, 16155 (1992).
7. E. K. Shibuya, A. J. Polverino, E. Chang, M. Wigler, J. V. Ruderman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9831 (1992); S. Hattori *et al.*, *J. Biol. Chem.* **267**, 20346 (1992).
8. J. Wu *et al.*, *Biochem. J.* **285**, 701 (1992); N. G. Ahn *et al.*, *J. Neurochem.* **59**, 147 (1992); A. Rossomando, J. Wu, M. J. Weber, T. W. Sturgill, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5221 (1992); K. Shirakabe, Y. Gotoh, E. Nishida, *J. Biol. Chem.* **267**, 16685 (1992); R. Seger *et al.*, *ibid.*, p. 14373; P. D. Adams and P. J. Parker, *ibid.*, p. 13135; S. Matsuda *et al.*, *EMBO J.* **11**, 973 (1992); N. Gomez and P. Cohen, *Nature* **353**, 170 (1991); H. Kosako, Y. Gotoh, S. Matsuda, M. Ishikawa, E. Nishida, *EMBO J.* **11**, 2903 (1992); S. Nakielny, P. Cohen, J. Wu, T. W. Sturgill, *ibid.*, p. 2123; C. M. Crews and R. L. Erikson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8205 (1992).
9. J. M. Kyriakis *et al.*, *Nature* **358**, 417 (1992); L. R. Howe *et al.*, *Cell* **71**, 335 (1992); P. Dent *et al.*, *Science* **257**, 1404 (1992).
10. S. K. Gupta, C. Gallego, G. L. Johnson, L. E. Heasley, *J. Biol. Chem.* **267**, 7987 (1992).
11. Recombinant Ras, Ras^{Val12}, and Ras^{Ala36} were purified from soluble extracts of *Escherichia coli*. Ras^{Ala36} was active in binding GMP-PNP. Crude rat brain cytosol was prepared as described (*19*). Preparation, assay, and formation of immobilized Ras·GMP-PNP were described (*12*). Immobilized Ras (25 μg) was incubated in the presence of 250 μl of crude rat brain extracts (15 to 20 mg/ml) for 60 min at 4°C with constant mixing. The rat brain extracts containing the immobilized Ras were layered onto 5× p21 buffer [100 mM MOPS (pH 7.4), 1 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA]. The immobilized protein was allowed to settle and the remaining supernatant was removed to the interface. The buffer containing sucrose was overlayed with 2M urea to wash the walls of the microcentrifuge tubes of nonspecifically bound proteins. The entire supernatant was removed and the sedimented material was washed twice with 1× p21 buffer containing 1 mM dithiothreitol and NP-40 (0.02%) and three times with p21 buffer alone.
12. D. DiBattiste, M. Gobulic, D. W. Stacey, A. Wolfman, *Oncogene* **8**, 637 (1993).
13. S. A. Moodie and A. Wolfman, unpublished data.
14. The washed immobilized Ras (*11*) was incubated at 37°C for 20 min in the presence of 10 μM ATP and 10 μM each of the following phosphatase inhibitors: β-glycerophosphate, *O*-phospho-L-serine, *O*-phospho-L-threonine, *O*-phospho-L-tyrosine, *p*-nitrophenyl phosphate (tris salt), and sodium vanadate in a total volume of 50 μl of p21 buffer. Uridine 5′-triphosphate and cytidine 5′-triphosphate were poor substitutes for ATP in eluting the phosphoproteins from the immobilized Ras. The supernatants were collected and assayed as described. Either bovine MBP (10 μM) or dephosphorylated casein (2 μg) and histone H1 (2 μg) with 10 μM [γ-^{32}P]ATP (10 μCi) and 10 μM of the phosphatase inhibitors listed above in a total of 50 μl of p21 buffer were added to 50 μl of eluted phosphoproteins. After 20 min at 37°C, 120 μl of 10% phosphoric acid was added and the reaction mixtures were spotted onto phosphocellulose paper. After extensive washing with 0.5% phosphoric acid, the radioactivity was measured by liquid scintillation counting. Alternatively, 50 μl of 2× sample buffer was added to the samples containing casein and histone, which were then heated for 5 min and resolved by SDS-PAGE (10% gel) and transferred to Immobilon-P. Phosphorylated histone and casein were quantitated with a phosphorimager.
15. The sedimented material was washed as described (*11*) except that 300 μg of immobilized Ras was incubated in the presence of 3 ml of crude rat brain extract (15 to 20 mg/ml). The proteins associated with the immobilized Ras were resolved by SDS-PAGE (12% gel) and transferred to Immobilon-P. Nitrocellulose was blocked in TBS [20 mM tris-HCl (pH 7.4), 150 mM NaCl, 0.01% NP-40] containing calf serum (1%) and dry milk (5%) for at least 1 hour at room temperature. The nitrocellulose was washed with TBS three times and incubated for 2 hours at room temperature with a rabbit polyclonal antibody to Raf-1 (from R. Shatzman). The nitrocellulose was then washed three times with TBS and incubated with 5 μCi ^{125}I-labeled goat antibody to rabbit $F(ab)_2$ fragment for 1 hour at room temperature. The nitrocellulose was washed three times with TBS for 20 min, dried, and analyzed either by autoradiography or by imaging on a Molecular Dynamics Phosphorimager.
16. The washed immobilized Ras was incubated at 37°C in the presence of 10 μM [γ-^{32}P]ATP (10 μCi) and phosphatase inhibitors (10 μM) (*14*) in a total volume of 50 μl of p21 buffer. After a 20-min incubation at 37°C, the kinase reactions were terminated by the addition of 50 μl of 2× sample buffer and boiled for 5 min. The phosphorylated proteins were resolved by SDS-PAGE (8 or 10% gels), transferred to Immobilon-P, and analyzed with a phosphorimager. Maximal incorporation of ^{32}P into protein was observed in the presence of 5 to 10 μM ATP and was linear up to 20-min incubation at 37°C.
17. Rabbit polyclonal antiserum to $p42^{mapk}$ (10 μl) was coupled to protein A–Sepharose (100 μl) in a total volume of 500 μl of p21 buffer at 4°C for one hour with constant mixing. The coupled complex was washed once with p21 buffer. Immunoprecipitation of $p42^{mapk}$ was initiated by the addition of the protein A–coupled antibody to the phosphoproteins eluted from immobilized Ras. Samples were then incubated for 2 hours at 4°C with constant mixing. Unphosphorylated recombinant $p42^{mapk}$ (0.5 μg) was added in the presence of

MBP (25 μM) and assayed for MAP kinase activity or mutant catalytically inactive recombinant $p42^{mapk}$ (0.5 μg) was added and examined for in vitro phosphorylation. After a 20-min incubation at 37°C in the presence of [γ-^{32}P]ATP, the phosphorylated catalytically inactive $p42^{mapk}$ was immunoprecipitated for 2 hours at 4°C. The sedimented material was extensively washed, and the proteins were resolved by SDS-PAGE (10% gel), transferred to Immobilon-P, and detected by autoradiography or a phosphorimager.

18. Samples were analyzed for $p42^{mapk}$ and $p44^{mapk}$ by SDS-PAGE and by protein immunoblotting. The blots were probed with a monoclonal antibody to $p42^{mapk}$ or $p44^{mapk}$, a rabbit antibody to mouse IgG, and ^{125}I-labeled goat antibodies to rabbit F(ab)$_2$. Signals were detected and quantitated with a phosphorimager.
19. A. Wolfman and I. G. Macara, *Science* **248**, 67 (1990).
20. Supported by NIH grants GM 41220 to A.W. and CA 40042, CA 39076, and GM47332 to M.J.W.; the Danish Cancer Society (91-034); and Danish Biotechnology (B.M.W.). S.A.M. is a recipient of a Wellcome Trust (United Kingdom) travel scholarship. We thank L. Feig for the RalA protein, J. Lang for photography, and D. W. Stacey and C. M. Weyman for critically reading the manuscript and helpful suggestions. This manuscript is dedicated to the memory of Dr. Edgar Cummings Henshaw.

8 January 1993; accepted 26 March 1993

Effects of cAMP Simulate a Late Stage of LTP in Hippocampal CA1 Neurons

U. Frey,* Y.-Y. Huang, E. R. Kandel

Hippocampal long-term potentiation (LTP) is thought to serve as an elementary mechanism for the establishment of certain forms of explicit memory in the mammalian brain. As is the case with behavioral memory, LTP in the CA1 region has stages: a short-term early potentiation lasting 1 to 3 hours, which is independent of protein synthesis, precedes a later, longer lasting stage (L-LTP), which requires protein synthesis. Inhibitors of cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKA) blocked L-LTP, and analogs of cAMP induced a potentiation that blocked naturally induced L-LTP. The action of the cAMP analog was blocked by inhibitors of protein synthesis. Thus, activation of PKA may be a component of the mechanism that generates L-LTP.

Behavioral and cellular studies of learning and memory in both invertebrates and vertebrates indicate that there are stages in memory storage. Long-term memory, lasting days or even weeks, can be distinguished from short-term memory, lasting minutes (or hours), with inhibitors of protein synthesis (*1–3*). Although LTP in the hippocampus is thought to be a candidate mechanism for explicit forms of memory (*4*), most cellular analyses of LTP have been done within hippocampal slices that typically survive only 1 to 2 hours. As a result, these analyses have focused only on an early phase of LTP. Hippocampal LTP in the CA1 region also has a later phase (*5–7*). The early phase (E-LTP) is initiated typically with a single train of high-frequency stimuli, starts immediately after the post-tetanic potentiation induced by the tetanus, and lasts about 1 to 3 hours. The late phase typically requires three or more trains of tetanic stimuli separated by 10 min, begins only slowly after the first 1 to 3 hours, and lasts for at least 10 hours. Only the late component of LTP (L-LTP) requires protein synthesis (*5, 6, 8*).

E-LTP is induced by activation of the *N*-methyl-D-aspartate (NMDA) receptor, which leads to Ca^{2+} influx, and requires the activation of serine-threonine and tyrosine protein kinases (*9–11*). Little is known about the second messengers necessary for the induction and maintenance of L-LTP. The only clues to a second messenger potentially important for L-LTP come from the finding that L-LTP is blocked by antagonists of dopamine receptors (*12, 13*), particularly by blockers of the D_1 type of dopamine receptors (*14*).

Because the D_1 receptor stimulates adenylyl cyclase, we explored the possibility that L-LTP is dependent on the cAMP-dependent protein kinase (PKA). We examined the effects on L-LTP of inhibiting PKA with Rp–cyclic adenosine 3′,5′-monophosphorothioate (Rp-cAMPS) (100 μM), a membrane-permeable cAMP analog and a competitive inhibitor of PKA. Application of Rp-cAMPS for 15 min before inducing LTP had no significant effect on normal synaptic transmission during the first 3 hours and only a small effect on E-LTP at 30 to 60 min, but the inhibitor completely blocked the late phase of LTP as evident in both the field excitatory postsynaptic potential (EPSP) and in the popula-

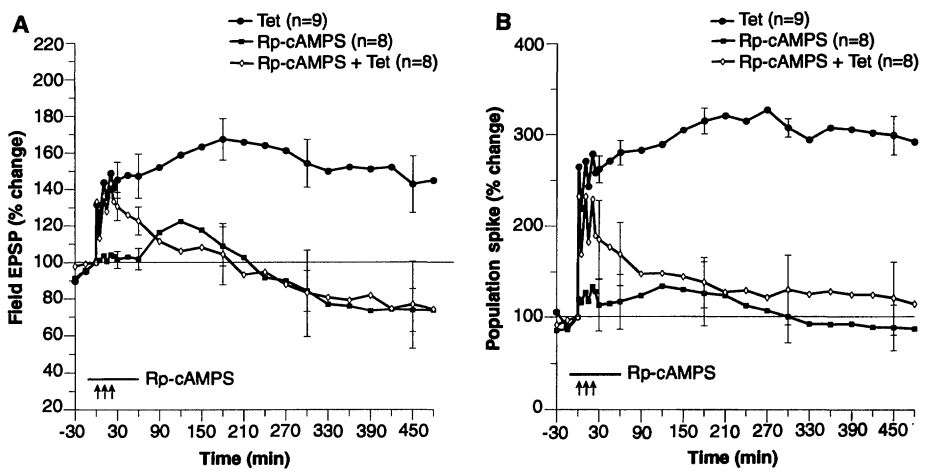

Fig. 1. Effect of Rp-cAMPS (100 μM) on LTP in hippocampal region CA1. (**A**) Time course of changes in the field EPSP measured as the slope function (SF). (**B**) Percentage change of the population spike amplitude (PS). Rp-cAMPS was applied 15 min before LTP induction and the drug was washed out 60 min after the first tetanization. LTP was induced (arrows) by giving three tetanic trains (Tet) (each train was 100 Hz for 1 s; 10-min intervals between trains)—a stimulation pattern that initiates stable L-LTP for at least 8 hours. The group treated with Rp-cAMPS was statistically significant different from control LTP at 45 min (PS) and 1.5 hours (SF) after LTP induction ($P < 0.05$). Hippocampal slices from 7-week-old male Sprague-Dawley rats were prepared for conventional electrophysiological recordings as described (*25*). Instead of a modified Krebs-Ringer solution, a medium was prepared containing 50% basal Eagle's medium (BME), 25% Hanks' balanced salt solution (HBSS), 25% horse serum, and glucose (6.5 mg/ml) (Specialty Media Inc. or Gibco). Under these conditions, the slices survived for at least 3 weeks in an O_2 incubator at 37°C. The superfused slices were incubated in a static bath (2.5 ml). The bath volume was exchanged every 2 to 3 hours with 7.5 to 10 ml of fresh medium. After incubation for 3 to 6 hours, a base line was recorded for at least 30 min. Four biphasic constant current pulses (0.2 Hz) were used for sampling at 10-min intervals in the first hour and at 30-min intervals in the following hours after LTP induction. The slope of the EPSP (mV/ms) was measured from the average waveform from four consecutive responses. Stainless steel electrodes were used (A-M Systems) for stimulation and recording. Error bars indicate ± SEM. The data were statistically evaluated with the two-tailed Mann Whitney U test.