Cytosolic rat brain synapsin I is a diacylglycerol kinase

(signal transduction/inositol phospholipids)

DAVID W. KAHN AND JEFFREY M. BESTERMAN

Department of Cell Biology, Glaxo Inc., Research Triangle Park, NC 27709

Communicated by George H. Hitchings, April 22, 1991

ABSTRACT The phosphorylation of diacylglycerol (DG), a reaction catalyzed by DG kinase, may be critical in the termination of effector-induced signals mediated by protein kinase C. Synapsin I is a principal target of intracellular protein kinases and is thought to be involved in the release of neurotransmitter from axon terminals. We present several lines of evidence which indicate that rat brain synapsin, in addition to this role, may function as a DG kinase. Purified rat brain DG kinase was digested with trypsin, which produced three major fragments whose sequence was identical to three regions in synapsin I. Using a rabbit anti-synapsin polyclonal antiserum, the elution profile of synapsin immunoreactivity coincided exactly with that of DG kinase activity in column fractions from the final step in the DG kinase purification procedure. As is the case with synapsin, the purified enzyme was a strongly basic protein with an isoelectric point greater than 10.0. Finally, incubating the DG kinase with highly purified bacterial collagenase, an enzyme that partially degrades the proline- and glycine-rich synapsin, resulted in the simultaneous loss of DG kinase activity and synapsin immunoreactivity. We conclude that cytosolic rat brain synapsin is capable of functioning as a DG kinase.

The roles of individual components of several signal transduction pathways in the physiology of the nervous system are being identified rapidly. The phosphoproteins synapsins Ia and Ib were initially identified as principal targets for cAMP- and later the Ca^{2+} -calmodulin-dependent protein kinases (ref. 1; see ref. 2 for review). It is thought that the cycling of synapsins between a phosphorylated and dephosphorylated state is coupled to the release of neurotransmitter from presynaptic axon terminals. Synapsins possess distinct domains capable of associating with (synaptic) vesicles and microfilaments (3). These allow the protein to act as a "tether," linking synaptic vesicles to the neuronal cytoskeleton. On depolarization of the presynaptic neuron, an increase in intracellular Ca^{2+} leads to activation of Ca^{2+} -calmodulin-dependent protein kinases. These catalyze phosphorylation of synapsin, which results in decreased affinity for the vesicles, allowing the vesicles to release their contents (4).

The generation of DGs within neuronal membranes is also believed to be involved in the transduction of extracellular signals by affecting the translocation and activation of protein kinase C (see ref. 5 for review). The termination of DG-mediated signals is thought to primarily involve DG kinase, as studies in intact cells revealed that inhibitors of this enzyme are capable of prolonging the effector-mediated response (6–8). We now present evidence of a new function for rat brain synapsin I as a DG kinase, establishing a point of convergence between the depolarization-induced, Ca^{2+} -mediated and effector-induced, DG-mediated signal transduction pathways.

MATERIALS AND METHODS

Purification of DG Kinase. The DG kinase was purified by using 20 adult female Long-Evans rats (45–55 days old), which were anesthetized with CO_2 and then decapitated. The forebrains were removed and rapidly homogenized, in pairs, in 160 ml of 20 mM Tris-HCl/1.0 mM dithiothreitol/3 mM magnesium chloride/5 mM benzamidine hydrochloride/1.6 mg of leupeptin and antipain adjusted to pH 7.4. Homogenization was performed in a Dounce homogenizer (A pestle) with 10–12 strokes at 4°C. The homogenate was then centrifuged at $100,000 \times g$ for 1 h at 4°C. The supernatant was removed and applied to a column (1.5 $\times$ 15 cm) of phosphocellulose equilibrated in 20 mM Tris-HCl, pH 9.5/1.0 mM dithiothreitol/250 mM sucrose/5 mM benzamidine hydrochloride/leupeptin and antipain added to 1 $\mu\text{g}/\text{ml}$ (buffer A). The flow rate was 0.9 ml/min and 3.6-ml fractions were collected. In all column chromatography steps, the columns were eluted at 4°C and collected in tubes that contained 25 μg of phosphatidylserine and 15 μg of diolein that was dispersed by sonication in 250 mM Mes (phosphocellulose) or buffer A [blue agarose and Sephacryl S300 (Pharmacia)], respectively, distributed in 100- μl aliquots prior to column elution. After washing with 40 ml of buffer A to remove nonbinding proteins, the phosphocellulose column was washed with a linear gradient created by mixing 100 ml of buffer A with 100 ml of buffer A containing 1 M NaCl. DG kinase activity was eluted at concentrations of 100 and 600 mM NaCl and designated peaks 1 and 2, respectively. The central three to five fractions containing DG kinase activity in each peak were pooled and applied separately to a column (1.6 $\times$ 1.0 cm) of Blue A agarose (Amicon) equilibrated in buffer A (pH 7.4) (buffer B). The flow rate was 0.5 ml/min and 2.0-ml fractions were collected. After washing with 10 ml of buffer B to remove nonbinding proteins, the column was washed in the reverse direction with a linear gradient created by mixing 20 ml of buffer B with 20 ml of buffer B containing 1.5 M NaCl (for peak 1) or by mixing 20 ml of buffer with 20 ml of buffer B containing 2.0 M NaCl (for peak 2). The central three to five fractions containing DG kinase activity in peaks 1 and 2 were pooled separately, concentrated using Centrprep 30 concentrators (Amicon), and applied directly to a column (26 $\times$ 84 cm) of Sephacryl S300 (Pharmacia) equilibrated in buffer B containing 1.5 M NaCl. The column was eluted with a flow rate of 1.5 ml/min and 2.5-ml fractions were collected. Peak fractions were pooled and concentrated using Centrprep 30 concentrators.

DG Kinase Assay. Mixtures contained 100 μg of phosphatidylserine, 31 μg of diolein, 50 mM potassium phosphate (pH 7.4), 10 mM NaF, 10 mM MgCl_2 , 5 mM ATP, 2 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), and 0.3–300 μg of protein in a total vol of 250 μl . The lipids were dispersed by sonication in the buffer prior to addition of ATP and enzyme. After 10 min of incubation at 37°C, the reaction was stopped by addition of 1.0 ml of chloroform/methanol/

concentrated HCl (100:200:2). Samples were Vortex mixed, and then 300 μ l of CHCl_3 and 300 μ l of 2 M KCl were added and the samples were Vortex mixed again (9). After 10 min at -20°C , the samples were centrifuged at $50 \times g$ for 2 min. The organic (lower) phase was removed and evaporated under a stream of N_2 . Dried samples were resuspended in 50 μ l of chloroform/methanol/water (3:1:0.08) and spotted on Whatman LK6D thin-layer chromatography plates. The plates were developed in chloroform/methanol/glacial acetic acid/water (65:43:1:3) (10). Greater than 95% of the radioactivity comigrated with a phosphatidic acid standard and these areas were scraped and radioactivity was determined by liquid scintillation counting.

Western Blot Analysis. SDS/PAGE was performed by the method of Laemmli (11) using 8% gels. After SDS/PAGE of aliquots from the column fractions, protein was transferred to nitrocellulose by the method of Towbin *et al.* (12) as modified by Szewczyk and Kozloff (13) to increase recovery of basic proteins. Transfer was overnight at 30 V and 4°C using 25 mM 3-[1,1-dimethyl-2-hydroxyethyl]amino]-2-hydroxy-1-propane-sulfonic acid (pH 9.5) as transfer buffer. After transfer, synapsin was detected using a rabbit polyclonal antiserum raised against bovine synapsin and an anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad). Protein content was quantified by the method of Bradford (14).

Amino Acid Analysis. Purified peak 2 enzyme was concentrated prior to digestion by reverse-phase HPLC using a polymer PRP column (2.1×30 mm; Brownlee). Protein was eluted at 80°C with a linear gradient of acetonitrile (20–90% in 20 min) in 0.1% trifluoroacetic acid. Digestion with sequence grade trypsin (Boehringer Mannheim) was performed in 0.1 M Tris-HCl (pH 8.5) for 16 h at room temperature with an enzyme/substrate ratio of 1:100. Peptides were isolated by HPLC with an Aquapore OD300 column (1×250 mm; Brownlee) and eluted with a linear gradient (0–60%; 15 min) of acetonitrile/isopropanol (1:1; vol/vol) in 0.1% trifluoroacetic acid. Automated Edman degradations were performed using the Applied Biosystems 477A liquid-pulse sequencer. Phenylthiohydantoin-derivatized amino acids were identified on a PTH analyzer (Applied Biosystems; model 120A).

RESULTS

Purification and Characterization of DG Kinase. Soluble rat brain DG kinase was resolved into two peaks of activity (peaks 1 and 2), a result consistent with previous reports of multiple DG kinase isoforms (15–18). The enzyme was purified such that both forms were bound and eluted as single peaks from a reverse-phase HPLC column when the eluant was monitored for absorbance at 280 nm. The purified isoforms had specific activities of 7900 and 1400 nmol per min per mg of protein for peaks 1 and 2, respectively, values comparable with those previously published for purified DG kinase (19, 20). The apparent native molecular weights of the two forms, as determined by gel-filtration chromatography, were identical ($139,600 \pm 9200$ and $127,500 \pm 20,300$ Da for peak 1 and peak 2, respectively; $n = 3$). (In the absence of 1.5 M NaCl, both peak 1 and peak 2 eluted in the void volume of the Sephacryl S300 column.)

Tryptic digests were prepared from purified peak 2 DG kinase and the amino acid sequences of three major peptide fragments were determined. A search of protein sequences in the Protein Identification Resource data base showed that the sequence determined with each of the three peptides was identical to three separate portions of rat brain synapsin I (see Fig. 1A). SDS/PAGE of purified peak 1 and peak 2 DG kinase revealed a major band at 73–80 kDa, a molecular mass that corresponded with that of synapsin. Analysis of the published sequence of synapsin I (2, 21) revealed the presence of three sites that are homologous to a consensus sequence for ATP

FIG. 1. Possible ATP binding regions in synapsin I and sequences identified in purified rat brain DG kinase corresponding to sections of rat brain synapsin I. (A) Purified DG kinase (peak 2) was digested with trypsin, and three of the major fragments produced were sequenced. The sequences determined corresponded to three areas contained in rat brain synapsin I. These areas in synapsin are indicated by shading. (B) Three areas of synapsin that contain the consensus sequence Gly-Xaa-Gly-Xaa-Xaa-Gly found in ATP binding sites of kinases.

binding sites of kinases (see Fig. 1B) originally described by Rossman (22, 23). We therefore hypothesized that rat brain synapsin might function catalytically as a DG kinase. Several additional lines of evidence are consistent with this hypothesis.

Elution Profile of Synapsin Immunoreactivity Versus DG Kinase Activity. A polyclonal antiserum raised against purified bovine synapsin I, and known to cross-react with rat brain synapsin (24), was used to determine the elution profile of immunoreactive material in relation to the position of DG kinase activity in column fractions from the final step in the DG kinase purification. As shown in Fig. 2, Western blot analysis demonstrated the presence of synapsin immunoreactivity in both peaks of DG kinase (Fig. 2A and C), and in each case the location of immunoreactivity correlated exactly with the appearance of DG kinase activity (Fig. 2B and D).

Basicity of the DG Kinase. An unusual property of synapsin is its basic isoelectric point, which has been determined to be 10.2 (1). In a survey of >800 proteins, <2% of the proteins had isoelectric points of >10.0 (25). We have determined that peak 1 DG kinase consisted solely of protein with an isoelectric point of ≥ 10.0 by isoelectric focusing (data not shown). Moreover, the ability of both peak 1 and peak 2 DG kinase to bind to a cation-exchange resin at pH 9.5 (step 1 of the purification procedure described in Fig. 2) confirms that the DG kinase activity is comprised of a highly basic protein.

Inhibition of DG Kinase by Bacterial Collagenase. Another distinctive property of synapsin is its susceptibility to cleavage by bacterial collagenase (1), an enzyme that catalyzes hydrolysis of the Pro-Xaa-Gly-Pro sequence in collagen or artificial substrates containing the proper substrate site (26). We therefore tested the effect of collagenase from *Clostridium histolyticum* on DG kinase catalytic activity. Digestion with bacterial collagenase for 2 h at 30°C resulted in loss of >80% of the DG kinase catalytic activity (Fig. 3A). Aliquots from these incubations simultaneously lost synapsin immunoreactivity on Western blot analysis (Fig. 3B). The collagenase had no effect on DG kinase activity or synapsin immunoreactivity when the 2-h incubation step was omitted, demonstrating that inhibition of activity was not the result of a rapidly acting (e.g., competitive) inhibitor present in the collagenase preparation. Control incubations also revealed that the bacterial collagenase had no effect on the integrity of bovine serum albumin, ovalbumin, and carbonic anhydrase (data not shown), nor did it have an effect on the catalytic activity of yeast alcohol dehydrogenase (see Fig. 3A), indicating that the effect on DG kinase activity was not produced

FIG. 2. Correlation of synapsin immunoreactivity with DG kinase activity. Sephacryl S300 column fractions from peak 1 (A and B) and peak 2 (C and D) were assayed for DG kinase activity and synapsin immunoreactivity. This experiment was repeated three times with identical results.

by a contaminating protease with broad specificity. The possibility of nonspecific protease activity producing decreases in DG kinase activity was further minimized by performing these experiments in the presence of protease inhibitors (leupeptin, antipain, and benzamidine; see Fig. 2). These data indicate a functional association of DG kinase activity with synapsin. As an additional control, the effect of a recombinant mammalian collagenase, an enzyme highly specific for a sequence within native, triple-helical collagen only (27), was also investigated. The mammalian enzyme had no effect on either the DG kinase activity or the synapsin immunoreactivity (Fig. 3).

DISCUSSION

Several reports in the literature are consistent with the hypothesis that rat brain synapsin is a DG kinase. Subcellular fractionation studies in which DG kinase was localized by catalytic activity or by immunoreactivity established the presence of this enzyme in synaptosomal preparations (28, 29). These studies also indicate that, within the synaptosomal preparations, as is the case for synapsin (30, 31), DG kinase activity is greatly enriched in synaptic vesicles. Additional similarities include the ability of both synapsin I and DG kinase to associate with microtubules (32, 33), as well as with phospholipids, with both molecules showing high affinity for acidic phospholipids (18, 34). Finally, both molecules exist in

soluble and membrane-associated forms and have the ability to undergo translocation between these subcellular locations (18, 35).

Most protocols for the purification of synapsin utilize steps involving protein denaturing reagents (1, 24). Bovine synapsin, purified by one of these methods (24), is devoid of DG kinase activity (data not shown). This is not surprising, however, as we, and others, have found brain DG kinase to be particularly unstable, requiring special efforts to preserve activity during purification (18–20). Denatured bovine synapsin was also used to raise the polyclonal antiserum used in this study. Attempts to immunoprecipitate the purified brain DG kinase activity, as well as authentic bovine synapsin I, using this antiserum were not successful, a result probably explained by the inaccessibility of epitopes in the native protein conformation that had been exposed in the denatured protein.

Recently, the first full-length cDNA sequence of a mammalian DG kinase has been reported (36). The sequence was derived from a pig thymus DG kinase which, although cross-reactive with an antibody against a porcine brain DG kinase (28), appears to be distinct from the rat brain DG kinase isoforms, which are the focus of this study. Unlike the rat brain DG kinase, the porcine thymus enzyme has an acidic isoelectric point (37) and possesses an amino acid sequence distinct from that of synapsin.

FIG. 3. The effect of collagenase on DG kinase and yeast alcohol dehydrogenase activity and synapsin immunoreactivity. (A) The effect of collagenase on DG kinase (solid bars) and yeast alcohol dehydrogenase (YADH; hatched bars) activity. (B) Western blot analysis of corresponding incubations from A using anti-synapsin antibodies. Incubation mixtures containing 25 mM Hepes and 10 mM CaCl_2 (pH 7.4) and 50 μg of peak 1 DG kinase purified through the phosphocellulose step were incubated for 2 h at 30°C. The incubation mixtures also contained the following: control (6.2 mM Tris-HCl, pH 7.4) (lane a); 111 units of type VII collagenase from *C. histolyticum* (Sigma) in 6.2 mM Tris-HCl (pH 7.4) (lane b); same as lanes a and b but incubations were prepared immediately prior to enzyme assay (lanes c and d); 100 nM recombinant mammalian collagenase in 6.2 mM Tris-HCl (pH 7.4) (lane e); bacterial collagenase with no DG kinase (lane f). The effect of mammalian collagenase on yeast alcohol dehydrogenase activity was not determined. The smaller molecular mass doublet of synapsin immunoreactivity is a product of synapsin degradation. This experiment was repeated twice, in duplicate, with identical results.

In conclusion, since the purified rat brain DG kinase (both peaks 1 and 2) had an apparent native molecular mass of 130–140 kDa, we hypothesize that one form of rat brain DG kinase consists of either (i) a homodimer of synapsin I, consistent with the known ability of synapsin to self-associate (1, 38), or (ii) a heterodimer of synapsin I and a second, catalytically essential, subunit with a molecular mass and isoelectric point similar to that of synapsin. Further work is needed to distinguish between these two possibilities. In either case, by functioning as a potential regulator of the

attenuation of protein kinase C-mediated signals, synapsin is in a position capable of modulating “cross-talk” between several signal transduction pathways of great importance in neuronal function.

We thank Dr. Steve R. Goodman (University of South Alabama) for his generous gifts of rabbit anti-bovine synapsin antiserum and authentic bovine synapsin and Dr. Gerard M. McGeehan for recombinant mammalian collagenase. We thank Drs. Gordon D. McIntyre and Harry LeVine for many useful discussions. We also thank Will Burkhardt for performing the amino acid sequence determinations and Linda B. Gilmore for technical assistance in the Western blot analyses.

- Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 5155–5163.
- Sudhof, T. C., Czernik, A. J., Kao, H.-T., Takei, K., Johnston, P. A., Horiuchi, A., Kanazir, S. D., Wagner, M. A., Perin, M. S., De Camilli, P. & Greengard, P. (1989) *Science* **245**, 1474–1480.
- Benfenati, F., Bahler, M., Jahn, R. & Greengard, P. (1989) *J. Cell Biol.* **108**, 1863–1872.
- Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3035–3039.
- Huang, K.-P. (1989) *Trends NeuroSci.* **12**, 425–432.
- de Chaffoy de Courcelles, D., Roevens, P. & Van Belle, H. (1985) *J. Biol. Chem.* **260**, 15762–15770.
- Bishop, W. R., Ganong, B. R. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 6993–7000.
- Muid, R. E., Penfield, A. & Dale, M. M. (1987) *Biochem. Biophys. Res. Commun.* **143**, 630–637.
- Hauser, G., Eichberg, J. & Gonzales-Sastre, F. (1971) *Biochim. Biophys. Acta* **248**, 87–95.
- Carney, D. H., Scott, D. L., Gordon, E. A. & LaBelle, E. F. (1985) *Cell* **42**, 479–488.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Towbin, L., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
- Szewczyk, B. & Kozloff, L. M. (1985) *Anal. Biochem.* **150**, 403–407.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Yamada, K. & Kanoh, H. (1988) *Biochem. J.* **255**, 601–608.
- Kato, M. & Takenawa, T. (1990) *J. Biol. Chem.* **265**, 794–800.
- Lemaitre, R. N., King, W. C., MacDonald, M. L. & Glomset, J. A. (1990) *Biochem. J.* **266**, 291–299.
- Besterman, J. M., Pollenz, R. S., Booker, E. L., Jr., & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9378–9382.
- Kanoh, H., Kondoh, H. & Ono, T. (1983) *J. Biol. Chem.* **258**, 1767–1774.
- Lin, C.-H., Bishop, H. & Strickland, K. P. (1986) *Lipids* **21**, 206–211.
- McCaffrey, C. A. & DeGennaro, L. J. (1986) *EMBO J.* **5**, 3167–3173.
- Rossman, M. G., Moras, D. & Olsen, K. (1974) *Nature (London)* **250**, 194–199.
- Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
- Krebs, K. E., Zagon, I. S. & Goodman, S. R. (1986) *Brain Res. Bull.* **17**, 237–241.
- Pharmacia Fine Chemicals (1982) *Isoelectric Focusing: Principles and Methods* (Ljungforetagen AB, Örebro, Sweden).
- Soberano, M. E. & Schoellmann, G. (1972) *Biochim. Biophys. Acta* **271**, 133–144.
- Gross, J., Harper, E., Harris, E. D., Jr., McCroskery, P. A., Highberger, J. H., Corbett, C. & Kang, A. H. (1974) *Biochem. Biophys. Res. Commun.* **61**, 605–612.
- Kanoh, H., Iwata, T., Ono, T. & Suzuki, T. (1986) *J. Biol. Chem.* **261**, 5597–5602.
- Strosznajder, J., Wikiel, H., Kelleher, J. A., Leu, V. S. & Sun, G. Y. (1986) *Neurochem. Int.* **8**, 213–221.
- Bloom, F. E., Ueda, T., Battenberg, E. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5982–5986.
- Huttner, W. B., Schiebler, W., Greengard, P. & DeCamilli, P. (1983) *J. Cell Biol.* **96**, 1374–1388.
- Goldenring, J. R., Lasher, R. S., Vallano, M. L., Ueda, T., Naito, S., Sternberger, N. H., Sternberger, L. A. & DeLorenzo, R. J. (1986) *J. Biol. Chem.* **261**, 8495–8500.

33. Daleo, G. R., Piras, M. M. & Piras, R. (1976) *Eur. J. Biochem.* **68**, 339–346.
34. Benefenati, F., Greengard, P., Brunner, J. & Bahler, M. (1989) *J. Cell Biol.* **108**, 1851–1862.
35. Sihra, T. S., Wang, J. K. T., Gorelick, F. S. & Greengard, P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8108–8112.
36. Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C. & Tanabe, T. (1990) *Nature (London)* **344**, 345–348.
37. Kanoh, H., Yamada, K., Sakane, F. & Imaizumi, T. (1989) *Biochem. J.* **258**, 455–462.
38. Font, B. & Aubert-Foucher, E. (1989) *Biochem. J.* **264**, 893–899.