Biochemical Properties and Cellular Localization of the Drosophila DG1 cGMP-dependent Protein Kinase*

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The protein encoded by the Drosophila cGMP-dependent protein kinase gene, DG1, was expressed in Sf9 cells. cGMP (10 μM) stimulated histone H2B phosphorylation by the DG1 protein kinase 20-fold. Maximal activity was observed at 40–50 mM Mg2+. The concentrations of cGMP, cAMP, cIMP, 8-bromo-cGMP, and 8-bromo-cAMP that gave 50% activation were 0.19 ± 0.06, 11.7 ± 2.8, 5.3 ± 1.5, 0.04 ± 0.01, and 0.62 ± 0.06 μM, respectively. cGMP activation was cooperative with a Hill coefficient (nH) of 1.28 ± 0.10, whereas activation by cAMP was not cooperative.

DG1 kinase expressed in Sf9 cells was found to be a dimer with an amino-terminal dimerization domain. It also autophosphorylated in a reaction stimulated by cGMP and cAMP. Immunoadsorbed DG1 protein from fly extracts was also capable of autophosphorylation, and this assay was used to quantitate the DG1 kinase in extracts from heads and bodies of adults and whole embryos. Activity was highest in heads of either sex and male bodies, intermediate in female bodies, and lowest in embryos. These results were in accord with DG1 mRNA abundance.

Tissue distribution of the DG1 kinase was investigated by immunohistochemistry. In embryos, specific immunoreactivity was observed in large cells scattered along the anterior-posterior axis at stage 13. Prominent staining of adult heads was restricted to the proximal level of the lamina cortex.

In mammals cGMP-dependent protein kinase is a mediator of the regulatory effects of the ubiquitous second messenger, cGMP. Unlike the nucleotide, however, the enzyme has a limited tissue distribution (1). The highest levels of mammalian type I cGMP-dependent protein kinase are found in smooth muscle, pericytes, platelets, and Purkinje neurons (2–4). The enzyme is presumed to play physiologically significant roles in each of these cell types, and in fact, physiological roles have been determined for type I cGMP-dependent protein kinase in chloride transport in the small intestine (9) and several regions of the brain, particularly the thalamus (10). A regulatory role for type II cGMP-dependent protein kinase in chloride transport in the small intestine has been suggested (9).

Assessing a possible role of cGMP-dependent protein kinase in cells where its expression is low is difficult, since the effects of cGMP could be carried out by cGMP-regulated phosphodiesterases or cGMP-gated channels (1). Nonetheless, there are reports of a number of cell types in which cGMP-dependent protein kinase is likely to mediate some of the effects of cGMP, despite being present in low amounts. For example, physiologically significant roles for type I cGMP-dependent protein kinase in neutrophils and macrophages have been suggested by Lincoln and co-workers (11, 12). cGMP-dependent protein kinase may also regulate the stimulation of potassium channel activity by atrial natriuretic peptide in rat pituitary tumor cells (13), induction of long term potentiation in guinea pig hippocampal slices (14), and somatostatin-induced inhibition of Ca2+-channel opening in chick ciliary ganglion neurons (15). In contrast to these advances in knowledge of the mammalian cGMP-dependent protein kinases, nothing is known about the physiological roles of the Drosophila cGMP-dependent protein kinases.

There are two Drosophila cGMP-dependent protein kinase genes (DG1 and DG2) (16, 17). Sequence comparisons with mammalian cGMP-dependent protein kinases indicate that DG1 and certain forms of DG2 protein contain a kinase inhibitory domain, two cGMP binding domains, an unconserved connecting sequence, an ATP binding site, and a catalytic domain (7, 8, 17–20). Based on the same comparisons, it has also been suggested that some forms of DG2, but perhaps not DG1, contain an amino-terminal dimerization domain similar to the mammalian enzymes (17, 21, 22).

The protein products of the DG1 and DG2 genes have not been studied. As a result, even the name, cGMP-dependent protein kinase, for the DG1 and DG2 gene products is based solely on sequence homology (17). In order to gain insights into the physiological role of cGMP-dependent protein kinase in Drosophila, we have undertaken a biochemical characterization of DG1 protein and examined its tissue distribution in embryos and adults.

EXPERIMENTAL PROCEDURES

Materials and Miscellaneous Methods—Molecular biology reagents, Escherichia coli strain TB1, and the pMAL-c expression vector were obtained from New England BioLabs. pVL1393 was from Invitrogen. Miracloth was purchased from Calbiochem, lysozyme from Boehringer Mannheim, histone H2B from Worthington Biochemicals, horseradish peroxidase-conjugated donkey anti-rabbit IgG from Jackson Laboratories, and radioactive compounds from DuPont NEN. Oligonucleotides were synthesized by the Molecular Resource Center at St. Jude Children’s Research Hospital. DNA sequencing, RNA preparation, Northern blot analysis, cDNA library screening, and histone kinase assays were carried out as described previously (16, 23, 24). Protein was

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Characterization of a DG1 cDNA Clone—A near full-length DG1 cDNA was isolated from the library of Ishi et al. (26) and subcloned into the EcoRI site of pUC19 to produce pG8. Sequence analysis revealed that the cDNA of 2551 bases was longer than cDNA2a (17) by 24 bases at the 5’ end and 93 bases at the 3’ end (Accession number U59901). Despite 19 polymorphisms between the protein coding sequences, there are only two differences in the amino acid sequences encoded by cDNA2a and cDNA2b. The Gln at 438 instead of His and Arg at 438 instead of Gln. Neither the Gln nor the Arg in the cDNA2a sequence is conserved between DG1 and DG2 or mammalian proteins.

Production of DG1 Expression Vectors—An adaptor strategy was used to clone the entire DG1 sequence into pMAL-c (Stul, EcoRI) in frame with maltE coding sequence. The adaptor placed coding sequence for the first five amino acids of DG1 in frame with maltE followed by an Hpai site. The Sall site in the 3’-untranslated region in pG8 was destroyed, and then the HincII, EcoRI fragment was cloned into the adapted pMAL-c vector.

This construct was then digested with XhoI and XbaI, blunted with Klenow, and religated to yield an expression plasmid encoding DG1 amino acids 2–457 and maltE. To express the carboxy-terminal domain of DG1 (amino acids 428–768), the NheI (blunt)-EcoRI fragment of pG8 was cloned into pMAL-c (Stul, EcoRI). The NheI-EcoRI and the Apal (blunt)-EcoRI fragments were also cloned into pMAL-c (Stul, EcoRI) to yield vectors expressing DG1 amino acids 228–768 and 286–768, respectively. The expression vectors encoding the full-length DG1 amino acid sequence, as well as amino acids 228–768 or 286–768, expressed MBP/DG1 fusion proteins of expected length as determined by Western blot, but the amount of undergraded protein was substantially reduced compared with MBP/DG1N (amino acids 2–457) and MBP/DG1C (amino acids 428–768) fusion proteins.

To construct an expression vector for DG1 in SF9 cells, the S EcoRV-NheI and the 3’ NheI-EcoRI fragments of pG8 were triple-ligated with pVL1393 (Smal, EcoRI) to make pVLG.

Purification of MBP Fusion Proteins and Production of Anti-DG1 IgG—Preliminary studies determined that a rich broth without glucose was the best media for expression of MBP/DG1C in TB1 bacteria. Published methods were used for the purification of MBP fusion proteins (27) with several modifications. Buffer L contained 0.05% Tween 20, and 10% polyacrylamide gel. The gel was rinsed in water at room temperature for 15 min and then incubated in 2% KCl at 4 °C for 30 min. The proteins were then detectable as white bands by side illumination against a black background. The undegraded antigen was cut from the gel, and the gel strip was rinsed in phosphate-buffered saline for 3 min. The acrylamide strips were stored at −70 °C until use.

To inject rabbits, the frozen strips were lyophilized and then ground to a fine powder with a mortar and pestle. The antigen was then emulsified with Freund’s adjuvant. The initial intramuscular and subcutaneous injections were with complete Freund’s adjuvant, while additional injections at 6-week intervals were with incomplete Freund’s adjuvant. Plate bleeds were analyzed by Western blot analysis using extracts from DG1-expressing SF9 cells (see below). Antibody titer reached a maximum after four or five injections and the rabbits were bled and sacrificed.

IgG was prepared using reagents from Pierce. The final volume of the purified IgG was adjusted to equal the volume of the initial serum sample.

Expression of DG1 Protein in SF9 Cells—Recombinant baculovirus expressing DG1 was produced by cotransfecting SF9 cells with 2 μg of pVLG and 1 μg of replication-defective baculovirus DNA (Pharmigen) in a T25 flask using the supplier’s transfection buffers and protocol. After 4 days the media were removed and spun at 3000 × g for 3 min. The transfection supernatants were stored at 4 °C. Four independent virus isolates were obtained by plaque purification, and high titer virus stocks were prepared for each.

To assay DG1 kinase synthesized in SF9 cells by the histone kinase assay, a single T25 flask was infected with hirer titer virus and incubated at 27 °C for 2–3 days. The cells were dislodged from the flask by pipetting, centrifuged, washed with 5 ml of phosphate-buffered saline, and lysed in 1 ml of 20 mM potassium phosphate (pH = 6.8), 2 mM EDTA, 25 mM benzamidine, 1 mM PMSF, 10 mM 2-mercaptoethanol, and 0.5% Triton X-100. The lysate was centrifuged at 10,000 × g for 30 min, and 2-μl aliquots were used for each assay.

For sucrose density gradient analysis, SF9 cells were infected and lysed as for the histone kinase assays. Bovine serum albumin and yeast alcohol dehydrogenase were added to 0.2 ml of lysate that was centrifuged through a 4-m1 5–20% sucrose gradient for 15 h at 55,000 × g. Samples were collected by puncturing the bottom of the centrifuge tube and collecting 5 drop fractions.

Autophosphorylation Assays—Sixty flies, either live or frozen at −70°C, were homogenized in 1.5 ml of 20 mM potassium phosphate (pH = 6.8), 2 mM EDTA, 25 mM benzamidine, 1 mM PMSF, 5 mM 2-mercaptoethanol in a 5-ml Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 × g for 30 min, and two 0.4-ml duplicate samples of supernatant were each added to 0.4 ml of homogenization buffer plus 25 μl of anti-MBP/DG1 IgG. An 80-μl aliquot of Protein A-Sepharose (2 μg/ml) in homogenization buffer (1:1) was added, and DG1 antigen was adsorbed for 1 h at 4 °C. The Sepharose beads were washed at 4 °C with 1 ml of Wash Buffer A (10 mM Tris (pH = 7.5), 2 mM EDTA, 0.5 mM PMSF, 0.2% Nonidet P-40, and 0.5 mM NaCl) three times, followed by 2 washes with 1 ml of Wash Buffer B (Wash Buffer A, except 0.15 M NaCl), and finally with 1 ml of Autophosphorylation Buffer (20 mM potassium phosphate (pH = 6.8), 0.05 mg/ml bovine serum albumin, 10 mM MgCl2, 5 mM 2-mercaptoethanol). Beads were then resuspended in 0.1 ml of Autophosphorylation Buffer (20 mM potassium phosphate (pH = 6.8), 0.05 mg/ml bovine serum albumin, 10 mM MgCl2, 5 mM 2-mercaptoethanol). Beads were pelleted, resuspended in 0.1 ml of 1.5 × SDS Sample Buffer and boiled for 3 min and analyzed by SDS-PAGE. Results were recorded by exposure of the dried gel to x-ray film, and the autophosphorylation reaction initiated with 2 μl of a 1:1 mixture of [γ-32P]ATP (10 μCi/ml, 7000 Ci/mmol) and appropriate cyclic nucleotide or water. The reaction was continued for 10 min and stopped with 25 μl of 0.5 M EDTA (pH = 8.0). The beads were pelleted, the supernatant removed, and the beads eluted with 0.1 ml of 1.5 × SDS Sample Buffer. The eluted material was boiled for 3 min and analyzed by SDS-PAGE. Results were recorded by exposure of the dried gel to x-ray film, and the autophosphorylation reaction was quantified by PhosphorImager analysis (Molecular Dynamic).

Immunohistochemistry—Anti-MBP/DG1 IgG was used to detect DG1 antigen in embryos and adults. Embryos were collected on grape plates smeared with brewers’ yeast paste. The embryos from 1 plate were rinsed in water, dechorionated with 50% bleach, washed with water, and fixed in 30 ml of phosphate-buffered saline that contained 0.2% Triton X-100 and 1% formaldehyde and was equilibrated with 0.5 ml of heptane. After a 15 min fixation at room temperature, the embryos were spun at 100 × g for 10 s and the fixation medium removed. The embryos were washed in PBT (phosphate-buffered saline plus 0.2% Triton X-100) and aliquoted to 1.5 ml microcentrifuge tubes. Embryos were devitellinized by shaking for 2 min at room temperature in 10% heptane, 90% methanol (initially at −70 °C). The embryos were then washed 3 times with 1 ml of PBT, 1 time with 1 ml of PBT containing 10% heat-inactivated goat serum (PBT-10%HIGS), and incubated overnight at 4 °C in 0.5 ml of PBT-10%HIGS and anti-MBP/DG1 IgG or control IgG (1:500). To detect bound antibody, the embryos were washed at room temperature 3 times for 30 min each with 1 ml of PBT, 1 time for 30 min with PBT-5%HIGS, and incubated 2 h in 0.5 ml of PBT-5%HIGS and donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:500). The embryos were then washed 3 times for 30 min each with PBT and stained using the Sigma DAB Peroxidase Substrate Tablet Set according to the supplier’s instructions. Staining was monitored under a dissecting microscope and the reaction stopped by washing the embryos with water. The embryos were equilibrated in 80% glycerol and mounted on slides.

For immunohistochemistry in adults, flies were frozen in OCT compound (Tissue-Tek, Miles, Inc.) and 10-μm sections were collected on subbed slides. The slides were air-dried for 1 h and fixed for 15 min in 2% formaldehyde in phosphate-buffered saline at room temperature. The slides were then washed 2 times for 15 min in phosphate-buffered saline and 1 time for 5 min in PBT. Anti-MBP/DG1 IgG or control IgG (1:250) was applied to the slides in PBT-5%HIGS and incubated overnight at 4 °C. The slides were then washed three times for 15 min each with PBT at room temperature, and bound antibody was detected using the Vectastain ABC (Vector Laboratories) kit according to the supplier’s instructions with second antibody at a dilution of 1:250.

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.
A 20-fold stimulation was seen after the addition of 10 nM nucleotide-specificity of the baculovirus-expressed DG1 kinase. As shown to be appropriate by determining the Mg$^{2+}$ content of histone H2B as substrate. In the presence of 10 nM cGMP, extracts of Sf9 cells containing DG1-expressing virus contained 50–100 times the activity of cells infected with control recombinant virus (Fig. 1).

Results

MBP/DG1 Fusion Proteins—Two DG1 fusion proteins, MBP/DG1$^N$ and MBP/DG1$^C$, were expressed in E. coli strain TB1 from pMAL-c vectors (see “Experimental Procedures”). Both fusion proteins were highly soluble and readily purified and were injected into rabbits to produce antisera. As previously reported for the mammalian type I protein (28), bacterially expressed DG1 catalytic domain was devoid of kinase activity (not shown).

Baculovirus-expressed DG1—Recombinant baculovirus containing the entire DG1 coding sequence from pG8 was produced. Four separate plaques were chosen and each grown to high titer (see “Experimental Procedures”). All four preparations of high titer virus induced high levels of cGMP-dependent protein kinase activity in infected Sf9 cells at 48 h. SDS-PAGE analysis of extracts from infected cells revealed a prominent 84-kDa band not present in extracts of Sf9 cells infected with a control recombinant virus (Fig. 1).

To investigate the kinetic properties of the baculovirus-expressed DG1 kinase, histone kinase assays were performed as described previously for bovine lung extracts (24). These assays contained 50 mM Mg$^{2+}$, reflecting the unusual Mg$^{2+}$-dependence of mammalian cGMP-dependent protein kinase when using histone H2B as a substrate. In the presence of 10 μM cGMP, extracts of Sf9 cells infected with DG1-expressing virus contained 50–100 times the activity of cells infected with control virus. Therefore, protein kinase assays were performed directly on Sf9 cell extracts. The use of 50 mM Mg$^{2+}$ was subsequently shown to be appropriate by determining the Mg$^{2+}$-dependence of the baculovirus-expressed enzyme. As shown in Fig. 2, histone kinase activity is maximal at elevated [Mg$^{2+}$].

Histone kinase assays were employed to determine the cyclic nucleotide specificity of the baculovirus-expressed DG1 kinase. A 20-fold stimulation was seen after the addition of 10 μM cGMP. Assays were performed on extracts from Sf9 cells infected with virus from three of the four plaques initially chosen. The concentrations of five cyclic nucleotides (cGMP, cAMP, cIMP, 8-bromo-cAMP, and 8-bromo-cGMP) that gave 50% activation (A$_{0.5}$) are listed in Table I.

Unexpectedly, cGMP, cIMP, and 8-bromo-cGMP gave evidence of cooperativity in the histone kinase assay; therefore, Hill coefficients ($n_H$) are also reported for the five cyclic nucleotide monophosphates. Although cooperativity is observable in the direct binding assay of cGMP to the bovine lung enzyme, cooperativity has not been observed in the histone kinase assay (24, 29, 30).

The same kinetic properties were observed for enzyme derived from three of the four viral plaques. The fourth baculovirus plaque produced enzyme with a high basal level of histone kinase activity (>50% of maximally stimulated). The cause of this high basal activity was not further investigated, but it underscores the importance of using more than one plaque as a source of virus.

The Quaternary Structure of DG1 Kinase—Induction of large amounts of active DG1 kinase in Sf9 cells made it possible to determine if the enzyme is a dimer. Extracts of Sf9 cells containing the fly kinase were analyzed by sucrose gradient centrifugation (Fig. 3). The major peak of DG1 protein was seen in sucrose gradient fractions corresponding to $M_r = 170,000$ (84-kDa subunits) with a minor peak at $M_r = 65,000$ (Fig. 3, A and B). This result is reminiscent of the holoenzyme dimer and the monomeric 65-kDa proteolytic fragment described for the bovine lung enzyme (31). Histone kinase analysis of the sucrose gradient fractions confirmed this interpretation for the fly cGMP-dependent protein kinase by demonstrating that the $M_r = 65,000$ monomer retained catalytic activity (Fig. 3C). It was also determined that intact DG1 kinase did not form a cysteinelinked dimer during SDS-PAGE under nonreducing conditions (not shown).
Interestingly, antibody against the amino-terminal portion of DG1 kinase (anti-MBP/DG1N) readily detected the 84-kDa intact subunit, but only faintly detected the 65-kDa fragment (not shown). This suggests that the antibody’s epitopes are predominantly near the amino terminus and that this end of the intact subunit was lost by proteolysis.

DG1 Autophosphorylation—The ability of the baculovirus-expressed DG1 enzyme to autophosphorylate was investigated. There was no detectable autophosphorylation in the absence of cyclic nucleotide, but both cGMP and cAMP induced a specific phosphorylation of the 84-kDa band (not shown). The availability of antisera to the bacterial fusion proteins afforded the opportunity to determine if the in vivo synthesized DG1 kinase could autophosphorylate. As shown in Fig. 4, DG1 from extracts of whole flies immunoadsorbed with anti-MBP/DG1N IgG could undergo autophosphorylation in the presence of 10 μM cGMP. In contrast, phosphorylation was not observed using anti-MBP/DG1C IgG (not shown). Both antisera efficiently recognize the baculovirus-expressed enzyme on Western blots (see above).

The Mg\(^{2+}\) dependence of autophosphorylation for the bovine lung cGMP-dependent protein kinase is markedly different from that seen in the histone kinase assay (24). Therefore, the Mg\(^{2+}\) dependence of autophosphorylation for the fly enzyme in the immunoadsorption assay was determined. The maximal activity for autophosphorylation occurs at approximately 10 mM Mg\(^{2+}\) (Fig. 2).

Quantitation of DG1 Activity in Fly Extracts—The activity of cGMP-dependent protein kinase in crude extracts of Drosophila is too low to measure by standard assays. In addition, DG1 antigen immunoadsorbed from extracts of 100 adult flies is barely detectable by Western blot analysis using Vectastain ABC and other sensitive Western blot detection methods. Therefore, relative quantitation of DG1 by the autophosphorylation assay was investigated using the conditions established in Fig. 2. The assay was shown to be linear for 10 min (but not 20 min) at 30°C, and dilution of the extract prior to immunoadsorption by 2-, 4-, and 8-fold gave 49, 21, and 10% of the initial activity. Thus, the autophosphorylation assay was used to estimate the relative levels of DG1 activity in fly extracts.

### Table I: Kinetic constants for DG1 protein kinase

| cNMP   | \(A_{0.5}\) (μM) | \(n_H\)       |
|--------|------------------|---------------|
| cGMP   | 0.19 ± 0.06 (9)  | 1.28 ± 0.10 (6) |
| cAMP   | 11.7 ± 2.8 (3)   | 0.92, 0.95     |
| cIMP   | 5.3 ± 1.5 (4)    | 1.24, 1.25     |
| 8BrcGMP| 0.04 ± 0.01 (4)  | 1.24 ± 0.05 (3) |
| 8BrcAMP| 0.62 ± 0.06 (4)  | 1.10, 1.16     |

![Fig. 3. Sucrose density gradient centrifugation of the baculovirus-expressed DG1 cGMP-dependent protein kinase.](http://www.jbc.org/)

**Fig. 3.** Sucrose density gradient centrifugation of the baculovirus-expressed DG1 cGMP-dependent protein kinase. Gradient fractions were collected and analyzed by SDS-PAGE, Western blot, and assays for histone kinase activity. The bottom of the gradient tube is to the left. A, Coomassie Blue stain of SDS-PAGE of fractions 13–25. The 43- and 67-kDa bands are yeast alcohol dehydrogenase (a tetramer) and bovine serum albumin, respectively. B, Western blot analysis of gradient fractions 13–25 using antibody against the carboxyl-terminal domain of DG1 (anti-MBP/DG1N). C, histone kinase activity of gradient fractions 12–27 in the presence of 10 μM cGMP, presented as cpm.

![Fig. 4. Autophosphorylation of DG1 from adult fly extracts.](http://www.jbc.org/)

**Fig. 4.** Autophosphorylation of DG1 from adult fly extracts. Autophosphorylation reactions were performed on extracts from whole flies as described under "Experimental Procedures." cGMP was present at 10 μM. Lane 1, autophosphorylation activity obtained after immunoadsorption with normal rabbit IgG; lane 2, autophosphorylation activity obtained after immunoadsorption with anti-MBP/DG1N IgG.

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J. L. Foster and O. M. Rosen, unpublished observations.

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The first important observation was that the specific activity (arbitrary PhosphorImager units/min/mg protein) of fly extracts differed by severalfold from different genetic backgrounds. Despite this variation, a consistent sex difference was noted. The specific activity of extracts from females was only 35 ± 16% (n = 16) of the specific activity of that seen in comparable male extracts. To further characterize this difference, extracts of heads and bodies of both sexes were assayed. Normalizing the specific activity of the four extracts to 1.00 for the extracts from female heads gave specific activities for the extracts from female bodies, male heads, and male bodies of 0.38, 0.96, and 1.03, respectively (averages of two determinations). The specific activity of extracts from 0- to 24-h embryos was a tenth that of extracts from adult heads.

The results of these autophosphorylation assays were unexpected because the previous Northern blot analysis by Kalderon and Rubin (17) had indicated that DG1 mRNA levels were higher in 0–24-h embryos than in heads and low in bodies. Therefore, we re-examined the abundance of DG1 mRNA in heads, bodies, and 0–24-h embryos, and the results are presented in Fig. 5. In the present study, DG1 mRNA abundance was highest in heads, somewhat lower in bodies, and apparently lowest, although easily detectable, in embryos. These results are in accord with the levels of DG1 autophosphorylation activity found in comparable samples.

Spatial Expression Pattern of DG1 Protein—To determine which tissues and cell types contain the DG1 kinase, we employed anti-MBP/DG1\(^{11}\) IgG. The embryonic expression pattern of DG1 was characterized in whole mounts of embryos representing all developmental stages. Surprisingly, immunoreactive product was observed only in stage 13 embryos (Fig. 6A) and not at any other stage of embryogenesis (not shown). At this embryonic stage, prominent staining was observed near the anterior edge of the amnioserosa (open arrow) and in cephalic regions (filled arrow). This staining was specific for the DG1 antibody, since it was not seen in the absence of anti-MBP/DG1\(^{11}\) IgG, with anti-MBP IgG, or with IgG from an antisera to an unrelated fly protein. As shown in higher magnification in Fig. 6B, the immunoreactive cells were round or irregular in shape and scattered along the anterior-posterior axis of the embryos. The shape and positions of these cells are characteristic of hemocytes/macrophages, which have been observed to migrate through the anterior amnioserosa and cephalic regions at this stage of embryogenesis (32).

The postdevelopmental pattern of DG1 expression is also spatially restricted to a limited number of cell types. In sections of head tissue, specific antibody staining was detected only in the lamina of the optic lobes, and within this neural structure signal was limited to the proximal level of the cortex (Fig. 7).

Although it appeared that immunoreactive product was localized in neural processes (Fig. 7A) and cell bodies (Fig. 7B), the pattern of staining did not exactly match that of any known cell type. This pattern of staining was not seen in the absence of anti-MBP/DG1\(^{11}\) IgG or with the control antibodies described above.

Most tissues of the adult body did not exhibit immunoreactive staining. The testis was one structure that did show consistent, albeit weak, staining (not shown). In contrast, ovarian tissues did not stain with anti-MBP/DG1\(^{11}\) IgG (not shown).

**DISCUSSION**

**Biochemical Properties of DG1 Kinase**—We have expressed the DG1 protein in baculovirus-infected Sf9 cells and characterized its biochemical properties. Expression yielded an 84-kDa protein that exhibited cGMP-dependent protein kinase activity.
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FIG. 7. Immunohistochemical staining for DG1 in the Drosophila nervous system. Fixed, stained, and frozen sections of fly heads incubated with anti-MBP/DG1N IgG (see “Experimental Procedures”). Panels A and B illustrate the pattern of lamina staining in different planes of section in two different individuals. Stained processes can be observed in panel A, whereas cell bodies and processes are evident in panel B. l, lamina; r, retina.

activity. The initial characterization of DG1 kinetic properties is interesting in light of the model for cGMP-dependent protein kinase activation presented by Hofmann et al. (33). This model, which is based on mammalian type Ia, includes an autoinhibitory domain (amino acids 60–68, conserved in the mammalian types Ib and II and in DG1 and DG2) that blocks the kinase activity of the catalytic domain. The block can be partially relieved by the binding of cGMP to a high affinity site, or a low affinity site, or by the interaction of arginine-rich proteins with a “poly(L-arginine) site” (34). Binding of cGMP to the high affinity site partially activates the enzyme and increases the affinity of the low affinity site for cGMP. Subsequent binding of cGMP to the low affinity site fully activates the enzyme. The cooperativity inherent in this model is observed with cGMP binding studies but not with histone phosphorylation assays, since the arginine-rich substrate enhances activation by binding to the poly(L-arginine) site, to the catalytic site with MgATP, or to both. This interaction obviates the cooperativity found in the cGMP binding assay and results in enzyme activation at lower cGMP concentrations relative to assays using non-histone substrates (24, 30).

DG1 differs from mammalian type Ia cGMP-dependent protein kinase by exhibiting cooperativity in the histone kinase assay. In addition, the $K_d$ in this assay (0.19 $\mu$m) is high relative to the type Ia kinase. One possible explanation for these observations is that the poly(L-arginine) site is altered or absent in DG1. Conversely, similarity between the cGMP binding sites of DG1 and mammalian type Ia cGMP-dependent protein kinase is suggested by the 5-fold lower activation constant of 8-bromo-cGMP relative to cGMP (Table I and Ref. 1). The DG1 protein substrate binding site also appears to be similar to that of the mammalian type Ia kinase. This conclusion is based on the [Mg$^{2+}$] dependence of kinase activity with histone H2B as the substrate. H2B binds tightly to cGMP-dependent protein kinase, and the dependence on high [Mg$^{2+}$] may reflect an increased rate of dissociation of phosphorylated H2B from the enzyme active site (35).

DG1 kinase autophosphorylation is stimulated by cGMP and cAMP. This is similar to type Ib autophosphorylation but contrasts with the autophosphorylation of Ia, which is stimulated by cAMP but not cGMP (1, 24, 36). Thus, the fly enzyme shares properties of both the mammalian type Ia and type Ib cGMP-dependent protein kinases.

DG1 Kinase Is a Dimer—DG1, like the three mammalian forms of cGMP-dependent protein kinase, is a dimer (Fig. 3 and Refs. 21 and 22). The ability of cGMP-dependent protein kinases to form dimers is thought to depend on amphipathic helical sequences present in the five different cGMP-dependent protein kinases (Fig. 8). These sequences are 29–47% identical and are proposed to form leucine/isoleucine zippers (1, 8, 37). The DG1 sequence, however, contains valine residues at three of the six expected hydrophobic positions. Thus, either valine can substitute for leucine/isoleucine in the predicted zipper structure or the mechanism of DG1 dimerization is different from the mammalian kinases.

None of the DG2 proteins has been shown to dimerize, but sequence similarities suggest that some do (17). Thus, it is
likely that all known eumetazoan cGMP-dependent protein kinases dimerize and that dimerization is necessary for physiological function. MacMillan-Crow and Lincoln (38) have previously proposed that dimerization is required for the enzyme to bind to, and hence localize with, cellular substrates.

Developmental and Tissue Expression of DG1 Protein—Exact comparisons between the results of Northern blots, enzymatic assays, and immunohistochemistry are not possible. Immunohistochemistry is not strictly quantitative, since it only reveals accessible antigen present above the limit of detection and best identifies cells that express high levels of antigen. Although enzymatic assays and Northern blot analysis may reflect widespread, but low, levels of expression not detected by immunohistochemistry, they are also subject to ambiguities and lack precision of localization. Nonetheless, the results from immunohistochemical staining, Northern analysis, and autophosphorylation assays were consistent with one another. Taken together these observations suggest physiologically significant roles for DG1 in hemocytes/macrophages, some cells of the optic lamina, and perhaps testis.

In embryos DG1 protein can be detected by immunohistochemistry at developmental stage 13. Immunoreactivity is restricted to a few large, irregular cells having the appearance of hemocytes or macrophages (32). During embryogenesis, a large percentage of hemocytes differentiate into macrophages that engulf the cellular debris that arises during embryonic cell death (32). There are about 700 hemocytes after stage 11 of embryogenesis. At stage 13, the hemocyte population migrates from its site of origin in the head mesoderm throughout the organism (32). Since we observed immunoreactivity primarily in cephalic regions and near the amnioserosa, it is likely that the DG1 kinase is expressed in only a subpopulation of hemocytes/macrophages. An alternative explanation for this observed pattern of expression is that antigen is being detected from cells that degenerate early during development and are then phagocytosed by macrophages.

In the adult nervous system immunoreactive staining is detected within cell bodies and/or processes of the optic lamina and is not seen in other regions of the adult head. The identity of these cells is at present unknown, although based on their positions they could represent the L5 monopolar neurons of the lamina. Alternatively, they might be marginal glia or some other cell type within the lamina.

In mammals cGMP-dependent protein kinases may have important functions in cells that express rather small amounts of the protein (11–15). That this may be the case in Drosophila as well is suggested by the readily detectable DG1 autophosphorylation activity and DG1 mRNA in female bodies, despite the lack of a clear signal by immunohistochemical staining. It remains to be determined if cGMP-dependent protein kinases have homologous functions in flies and mammals. Of particular importance will be the characterization of the several DG2-encoded proteins and determination of their tissue distribution.

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