Atypical Soluble Guanylyl Cyclases in Drosophila Can Function as Molecular Oxygen Sensors*  

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Conventional soluble guanylyl cyclases are heterodimeric enzymes that synthesize cGMP and are activated by nitric oxide. Recently, a separate class of soluble guanylyl cyclases has been identified that are only slightly activated by or are insensitive to nitric oxide. These atypical guanylyl cyclases include the vertebrate β2 subunit and examples from the invertebrates Manduca sexta, Caenorhabditis elegans, and Drosophila melanogaster. A member of this family, GCY-35 in C. elegans, was recently shown to be required for a behavioral response to low oxygen levels and may be directly regulated by oxygen (Gray, J. M., Karow, D. S., Lu, H., Chang, A. J., Chang, J. S., Ellis, R. E., Marletta, M. A., and Bargmann, C. I. (2004) Nature 430, 317–322). Drosophila contains three genes that code for atypical soluble guanylyl cyclases: Gyc-88E, Gyc-89Da, and Gyc-89Db. COS-7 cells co-transfected with Gyc-88E and Gyc-89Da or Gyc-89Db accumulate low levels of cGMP under normal atmospheric oxygen concentrations and are potently activated under anoxic conditions. The increase in activity is graded over oxygen concentrations of 0–21%, can be detected within 1 min of exposure to anoxic conditions and is blocked by the soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ). Gyc-88E and Gyc-89Db are co-expressed in a subset of sensory neurons where they would be ideally situated to act as sensors. This is the first demonstration of a soluble guanylyl cyclase that is activated in response to changing oxygen concentrations.

The intracellular messenger, cGMP, is synthesized by guanylyl cyclases (1). There are two families of guanylyl cyclase, integral membrane proteins known as receptor guanylyl cyclases and the cytoplasmic soluble guanylyl cyclases (1). Conventional soluble guanylyl cyclases are heterodimeric enzymes that are activated by nitric oxide (NO)† (1). Another class of soluble guanylyl cyclase has been identified that have a similar structure but are only slightly activated by or are insensitive to NO (2, 3). Examples of these atypical guanylyl cyclases include the β2 subunit in mammals and MsGC-β3 from the insect Manduca sexta (3). We have also predicted that all the soluble guanylyl cyclases in Caenorhabditis elegans, and three of the soluble guanylyl cyclase subunits in Drosophila are part of this subfamily (3). The Drosophila genome contains five genes that code for soluble guanylyl cyclase subunits, named according to their chromosomal locations; Gyc-99B, Gycβ-100B, Gyc-88E, Gyc-89Da, and Gyc-89Db (3). Gyc-99B and Gycβ-100B form a conventional, heterodimeric enzyme that is potently activated by NO (6). When transiently expressed in COS-7 cells, Gyc-88E is active in the absence of additional subunits whereas Gyc-89Da and Gyc-89Db are only active when co-expressed with Gyc-88E and all three combinations are slightly activated by some, but not all, NO donors (5). Two biochemical data in combination with sequence and phylogenetic analysis of Gyc-88E, Gyc-89Da, and Gyc-89Db suggested that they are also members of a family of atypical guanylyl cyclases (3). One of the members of this family in C. elegans, GCY-35, has recently been demonstrated to be required for oxygen sensation (4). When placed in an oxygen gradient, C. elegans preferentially congregate at 5–12% oxygen whereas individuals that have a deletion in gcy-35 distribute themselves evenly across the gradient (4). This behavior appears to require cGMP production and unlike any other guanylyl cyclase, the heme-binding domain of GCY-35 binds oxygen (4). This suggested that the activity of GCY-35 is regulated by oxygen concentration, although it was not possible to directly demonstrate this as recombinant GCY-35 showed no enzyme activity (4). In this report we show that the Drosophila atypical soluble guanylyl cyclase subunits are activated in the absence of oxygen and hence could act as oxygen sensors,alerting the animals to hypoxic environments.

EXPERIMENTAL PROCEDURES

Plasmids containing the open reading frames of the Drosophila soluble guanylyl cyclase subunits were transiently transfected into COS-7 cells in 12-well tissue culture plates as described previously (5). 72 h after transfection, the culture medium was removed from the cells and replaced with physiological saline (composition in mM: NaCl, 120; KCl, 5.4; CaCl₂, 2; MgCl₂, 2; Tris, 25; glucose, 15; pH 7.4) that had been saturated with a mixture of oxygen and nitrogen. The culture plate was then placed in a plexiglass chamber with the same oxygen/nitrogen mixture flowing through it at ~10 cubic feet/h. The composition of the gas mixture was varied according to the experiment and continuously monitored with an oxygen monitor (model 5120, Ohmeda, Helsinki, Finland). The cells were rapidly lysed after a 60-min incubation period by replacing the saline with acidified ethanol (100:1, ethanol: HCl). The lysed cells were centrifuged to remove cell debris and the supernatant dried under vacuum. The residue was assayed for cGMP content by enzyme-linked immunosorbent assay (7).

RESULTS AND DISCUSSION

To determine whether any of the Drosophila soluble guanylyl cyclase subunits were regulated by oxygen, different combinations of subunits were transiently expressed in COS-7 cells and the cGMP content of the cells determined after exposure to a variety of conditions. The three atypical guanylyl cyclase subunit combinations that exhibit guanylyl cyclase activity, Gyc-88E, Gyc-88E + Gyc-89Da, and Gyc-88E + Gyc-89Db, all

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1 The abbreviations used are: NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; ANOVA, analysis of variance.
showed higher accumulation of cGMP in the presence of 100% nitrogen compared with 21% oxygen/79% nitrogen (Fig. 1). By contrast, the conventional soluble guanylyl cyclase, Gyc-98E/89Da, showed no such change. To confirm that the conventional soluble guanylyl cyclase subunits were capable of being activated under these conditions, a NO donor was added directly to the medium while the cells were exposed to 21% oxygen. In this case the cells showed the expected robust increase in cGMP levels (Fig. 1). It was interesting to note that the cGMP levels that accumulated when cells transfected with Gyc-88E/89Da were exposed to anoxic conditions were comparable to the levels accumulated by cells transfected with conventional guanylyl cyclase subunits that were exposed to an NO donor.

To determine the concentration range of oxygen that affected the activity of Gyc-88E/89Da and Gyc-88E/89Db, cells that were transfected with these combinations of subunits were exposed to varying oxygen concentrations for 60 min and their cGMP accumulation measured (Fig. 2). These data showed that both subunit combinations exhibited a graded increase in activity from 21 to 0% oxygen. A small difference was seen between the two subunit combinations; there was no difference in the cGMP accumulation in cells transfected with Gyc-88E/89Da or Gyc-88E/89Db in response to anoxic conditions.

The graded effect from 0–21% oxygen is consistent with the model in *C. elegans* that these atypical guanylyl cyclases act as oxygen sensors (4). One of the requirements for a molecular oxygen sensor that can signal hypoxic conditions is that it binds oxygen with low affinity (8). If Gyc-88E/89Da or Gyc-88E/89Db was inhibited by oxygen and bound oxygen with a high affinity, it would be expected that only a low concentration of oxygen would be sufficient to fully inhibit cGMP accumulation. As there was a graded decrease in cGMP accumulation over the concentration range of 0–21%, the data suggest that both Gyc-88E/89Da and Gyc-88E/89Db bind oxygen with a relatively low affinity. The time course of cGMP accumulation in response to anoxia was also determined. Cells transfected with either Gyc-

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**Fig. 3.** Time course of activation of Gyc-88E/89Da and Gyc-88E/89Db in response to anoxic conditions. COS-7 cells were transiently transfected with the subunits shown and exposed to 21% oxygen/79% nitrogen and then switched at different times to 100% nitrogen. The total incubation time was 60 min in each case. The cells were then lysed and their contents assayed for cGMP content. Data represent the mean ± S.E. of four determinations. *, values significantly different (p < 0.05) from 21% oxygen: ANOVA followed by Dunnett’s post-test.

**Fig. 4.** Activation of Gyc-88E/89Da and Gyc-88E/89Db under anoxic conditions is blocked by the soluble guanylyl cyclase inhibitor, ODQ. COS-7 cells were transiently transfected with the subunits shown and exposed to 21% oxygen/79% nitrogen (open bars) or 100% nitrogen (solid bars) in the absence (non-stippled) or presence of 100 μM ODQ (stippled bars) for 60 min and assayed for cGMP content. Data represent mean ± S.E. of four determinations. *, values significantly different (p < 0.05) from 0% oxygen: ANOVA followed by Bonferroni post-test.
8E/89Da or Gyc-88E/89Db were switched from 21% oxygen/79% nitrogen to 100% nitrogen at different times within the 60-min exposure period and then assayed for cGMP content (Fig. 3). This experiment showed that within 1 min of exposure to an anoxic environment there was a significant accumulation of cGMP in cells expressing Gyc-88E/89Db, which continued to accumulate throughout the 60-min exposure time. Cells that expressed Gyc-88E/89Da took longer to show a significant increase in cGMP levels (30 min compared with 1 min), but this could have been due to the lower level of activity of this subunit combination.

Conventional soluble guanylyl cyclases are activated by NO through the binding of NO to the prosthetic heme group (9). The heme-binding domain of the C. elegans GCY-35 was the first guanylyl cyclase demonstrated to be able to bind oxygen (4). To determine whether the heme group of Gyc-88E/89Db was likely to mediate the anoxia activation, the cells were incubated in ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one), a soluble guanylyl cyclase inhibitor that acts by oxidizing the iron in the heme group (10). Exposure of COS-7 cells transiently expressing Gyc-88E/89Db to 100 μM ODQ completely abolished the anoxia activation (Fig. 4), suggesting that the heme group is necessary for activation.

These data suggest that Gyc-88E/89Da and Gyc-88E/89Db can act as oxygen sensors in Drosophila. If these enzymes exhibit the same properties in vivo as they do in COS-7 cells, when the cells are exposed to reduced oxygen concentrations, there will be an increase in the levels of cGMP. The co-localization of these subunits in putative chemosensory neurons supports this notion (5). Sensory neurons that express both Gyc-88E and Gyc-89Db and send projections to the surface of Drosophila embryos are found in the head, terminal segments, and lateral margins (5). They are in an ideal position to rapidly signal to the CNS when the animal encounters reduced oxygen concentrations to respond quickly (seconds to minutes) to reduced oxygen concentrations (12, 14). The recent report on the role of GCY-35 in the behavioral response of C. elegans to hypoxia supports the model of atypical guanylyl cyclases as molecular oxygen sensors (4). In vertebrates, the glomus cells of the carotid body detect short-term changes in oxygen concentration leading to a variety of physiological changes (8). The molecular nature of the oxygen sensor in these cells is unclear, although it is thought to be either a heme protein or a potassium channel that directly binds oxygen (8, 15). There are several candidate heme proteins, including NADPH oxidase, NO synthase, and a low affinity mitochondrial cytochrome (15). The atypical guanylyl cyclase, Gyc-88E/89Db, in the peripheral nervous system of Drosophila may prove to be a valuable model system to study this critical biological pathway.

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