A Metabotropic Glutamate Receptor Family Gene in Dictyostelium discoideum*

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Metabotropic glutamate receptors (mGluRs) are a class of G-protein-coupled receptors that possess a seven transmembrane region involved in the modulation of excitatory synaptic transmission in the nervous system. mGluR orthologs have been identified in Drosophila, Caenorhabditis elegans, and higher organisms. Drosophila possesses two mGluR genes, DmGluRα and DmXR. We screened the Dictyostelium genome data base using the ligand binding domain of rat mGluR1 as bait, and identified a new receptor, DdmGluPR, belonging to the mGluR family. Similar to Drosophila DmXR, the residues of mGluRs involved in the binding of the α-carboxylic and α-amino groups of glutamate were well conserved in DdmGluPR, but the residues interacting with the γ-carboxylic group of glutamate were not. The phylogenetic analysis suggests that DdmGluPR diverged after the mGluR family-GABABRs split but before mGluR family divergence. DdmGluPR mRNA was expressed in vegetative cells and throughout starvation-induced development, but the level of the expression was relatively high until 4 h after starvation. DdmGluPR was localized to the plasma membrane of axenically grown Ax-2 cells expressed as a green fluorescent protein fusion protein. DdmGluPR-null cells grew faster at high cell density and reached higher densities than wild-type cells. DdmGluPR-null cells exhibited delayed aggregates formation upon starvation and impaired chemotaxis toward cAMP. Although expressions of cAR1 and caca, CAM-signaling components, were rapidly induced and peaked at 2–4 h in wild-type cells, DdmGluPR-null cells displayed sustained and peaked at 8 h of the expressions of these genes. Our findings suggest the involvement of DdmGluPR in the early development of Dictyostelium discoideum.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) DQ447637.

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important roles of the DdmGluPR gene in aggregates formation during Dictyostelium development.

MATERIALS AND METHODS

Cell Growth and Development—Vegetative cells of D. discoideum Ax-2 were grown axenically in PS-medium (1% peptone, 0.7% yeast extract, 1.5% D-glucose, 0.11% KH2PO4, 0.05% Na2HPO4, 40 ng/ml vitamin B12, 80 ng/ml folic acid) at 22 °C. DdmGluPR-null cells were grown in PS-medium supplemented with 10 μg/ml blasticidin S (Invitrogen). To allow cells to differentiate, cells were washed in 10 mM Na-potassium phosphate buffer (10 mM Na-potassium phosphate, 0.2 mM MgSO4, and 1mM CaCl2, pH 6.1), plated on non-nutrient agar (1.5% agar in 10 mM Na-K phosphate buffer) at a density of 10⁷ cells/ml, and incubated at 22 °C. Conditioned medium was prepared by starving wild-type or DdmGluPR-null cells at 5 x 10⁶ cells/ml in 10 mM Na-potassium phosphate buffer. To examine the effect of conditioned medium on cell aggregation, cells were starved in submerged culture at densities 8, 4, 2, 1, and 0.5 × 10⁵ cells/ml in 10 mM Na-potassium phosphate buffer.

Sequence Analysis—BLAST searches were performed using the data base for Dictyostelium and for other organisms (http://ncbi.nlm.nih.gov/). The signal sequence and transmembrane regions of DdmXR were predicted by using PSORTII and SOSUI. The sequence alignment between DdmGluPR, the Drosophila DmXR, and the mouse mGluR3 was produced using ClustalW. The phylogenetic tree was constructed using class III GPCR sequences from various species retrieved from the data base. Sequences were aligned using ClustalW. The resulting multi-alignment was then used for the construction of an evolutionary tree by the Neighbor Joining method using MEGA2 program.

Northern Blot Analysis—cDNAs for DdmGluPR, cAR1, aca, psA, spiA, discoidin, and Ig7 were obtained by RT-PCR, cloned into pT7 Blue (Novagen) or pBluescript SK (Stratagene) and confirmed by sequencing. The ecmB gene was kindly provided from Dr. R. Escalante at Instituto de Investigaciones Biomedicas, Spain. All of the cDNAs were transcriptionally labeled by T3 or T7 RNA polymerases to make the antisense digoxigenin-labeled riboprobe. The extracted total RNA (5 μg) were resolved on 1% formaldehyde-agarose gels and transferred onto positively charged nylon transfer membranes (Hybond N+, Amersham Biosciences). Hybridizations were carried out using digoxigenin-labeled riboprobe.

Expression of GFP-DdmGluPR—The expression vector pDXA-GFP2 was kindly provided by Dr. Thomas T. Egelhoff at Case Western Reserve School of Medicine (15). GFP was fused at the N terminus of DdmGluPR. The construct was introduced into Dictyostelium by electroporation, and stable transformants were selected on G418.

DdmGluPR Gene Inactivation—The DdmGluPR-null strains were generated by gene targeting. cDNA encompassing the entire coding region (~2.5 kbp) with restriction sites EcoRI and XbaI at 5’ and 3’, respectively, was obtained by RT-PCR and cloned into pBluescript SK. The targeting construct contains the digested fragment with EcoRI and using restriction enzymes. The targeting construct was linearized with NotI and introduced into DdmGluPR-null cells by electroporation. Stable transformants were selected on G418.
HindIII (~1.7 kbp), and the Bsr cassette was inserted at the KpnI site. The Bsr cassette was kindly provided by Drs. Y. Tanaka and T. Morio at University of Tsukuba, Japan. The targeting construct was digested with EcoRI and HindIII and introduced into wild-type Ax-2 cells. Transforms were selected for blasticidin resistance. Clone C2 was identified as the DdmGluPR-null strain and used for the experiments.

Assay for Chemotaxis toward cAMP—Prior to assay, Dictyostelium cells were starved in 10 mM Na-K buffer, pH 6.1, at 22 °C for 4 h. Small wells in the center of 1.5% non-nutrient agar plates were filled with cAMP solution (5 \( \times \) 10^{-9} M solution), and the starved cells were spotted on the agar plates 5 mm from the edge of the well at 10^7 cells/ml. The plates were further incubated for 5 h at 22 °C, and the resultant chemotaxis toward the wells was observed under a phase contrast microscope. To examine the effects of most of natural amino acid including Glu, Arg, Lys, Asp, His, Gln, Pro, Ser, Ala, Thr, Leu, Met, Val, and GABA on starvation-induced chemotactic response toward cAMP, all of the reagents were dissolved in water at 100 mM (pH 6.1). The cells were starved, and the chemotactic response to cAMP was observed in the presence of each reagent at 1 mM.

RESULTS

Sequence Analysis of Dictyostelium DdmGluPR Homologous to mGluRs—We screened the Dictyostelium genome data base using the BLAST program with the rat mGluR1 LBD (amino acid residue 33–522) as a bait and identified a new receptor homologous to mGluRs, called DdmGluPR (Dictyostelium mGlu precursor receptor), in D. discoideum (DDB0231976 GenBankTM accession number DQ447637). The DdmGluPR gene encodes an 816-amino-acid protein as shown in Fig. 1A. The N terminus of the predicted amino acid sequence of DdmGluPR contained a potential signal sequence, and seven possible transmembrane regions were predicted from hydrophobicity algorithms for a plasma membrane protein (Fig. 1B). These structural features suggest that DdmGluPR is a class of GPCR. The mGluRs share sequence similarity with the GABAB receptors (GABABRs), the calcium-sensing

![FIGURE 2. Multiple alignment of DdmGluPR, mouse mGluR3, and Drosophila DmXR. A, amino acid sequences of DdmGluPR, mouse mGluR3, and Drosophila DmXR were aligned using ClustalW. Identical residues are shaded black and similar residues are shaded gray. The domain presumed for the binding of the \( \eta \)-carboxylic and \( \gamma \)-amino groups of glutamate in mGluRs is grouped by a square. B, comparison of the conserved residue in mouse mGluR3 and the residues at equivalent positions in DdmGluPR for the glutamate binding. Numbers show the position in mouse mGluR3 conserved residues for the glutamate binding.](http://www.jbc.org/)
receptor, and pheromone receptors and constitute the class III GPCR family. The extracellular region of DdmGluPR showed the closest sequence similarity to mouse mGluR3 and Drosophila, and DdmGluPR were aligned, and an evolutionary tree was constructed by the Neighbor Joining method. Bootstrap values for branch are indicated. DdmGluPR and Drosophila DmXR are shown with bold.

FIGURE 3. Phylogenetic analysis among DdmGluPR, Drosophila DmXR, and class III GPCRs. The sequences of the indicated class III GPCRs (accession numbers indicated) from human, rat, mouse, bovine, chick, C. elegans, goldfish, salmon, fugu, sea urchin, Drosophila, and DdmGluPR were aligned, and an evolutionary tree was constructed by the Neighbor Joining method. Bootstrap values for branch are indicated. DdmGluPR and Drosophila DmXR are shown with bold.
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FIGURE 4. Northern blot analysis of DdmGluPR mRNA expression during development. A, total RNA (5 μg) was prepared from either growing cells or from cells developed on non-nutrient agar plates for 0, 4, 8, 16, 20, and 24 h. The blot was hybridized with the probes shown, cAR1; a cAMP receptor, aca; an adenyl cyclase, psA; the spore-specific marker, etcB; the prestalk/stalk-specific marker, psA; the prespore-specific marker. Mitochondrial rRNA Ig7 was used as a loading control. B, localization of GFP-DdmGluPR was visualized under fluorescence microscope (right panel, GFP-DdmGluPR). The left panel shows the phase image of the same field of cells (Phase). Scale bar: 50 μm.

of the coding region with an insertion of a blasticidin cassette (Fig. 5A) was introduced into the Dictyostelium wild-type strain Ax-2. Individual clones were selected, and positive clones were identified by the presence of an ~3-kb PCR product that is ~1.5 kb in wild-type cells (Fig. 5B, left panel). The disruption of DdmGluPR was further confirmed by the absence of a PCR product from an oligo(dT)-primed RT-PCR (Fig. 5B, right panel).

Cell Growth and Prestarvation Response in DdmGluPR-null Mutant—Because DdmGluPR was expressed in vegetative phase cells, we examined the cell growth of the DdmGluPR-null mutant. DdmGluPR-null cells grew faster at high cell density compared with wild-type Ax-2 cells (Fig. 6A). The absence of DdmGluPR appeared to enhance vegetative growth. A mGluR Gene in Dictyostelium

FIGURE 5. Isolation of DdmGluPR-null mutant. A, schematic representation of the DdmGluPR gene disruption. cDNA encompassing the entire coding region (~2.5 kb) with restriction sites EcoRI and XbaI at 5′ and 3′, respectively was obtained by RT-PCR. The construct contains the digested fragment with EcoRI and HindIII (~1.7 kb), and the Bsr cassette was inserted into at KpnI site. The arrows denote two primers (primer U and L) for PCR analysis. Primer L recognizes part of the region that is not present in the targeting construct. B, identification of DdmGluPR disruptants by PCR analysis. Genomic DNA was isolated from several clones after transformation. PCR analysis using primers U and L is predicted to yield an ~3-kb product for a DdmGluPR gene disruptant and a product ~1.5 kb for an intact gene (left panel). To detect the DdmGluPR transcript, total RNA was prepared from the transformants and RT-PCR analysis was performed using the same primers U and L (right panel). W, wild-type Ax-2; C2, C7, C8, and C9, blasticidin-resistant clones.

Development of DdmGluPR-null Mutant—To investigate the defect in the developmental process of the DdmGluPR-null mutant, development on non-nutrient agar plates was monitored microscopically. As shown in Fig. 7, DdmGluPR-null cells exhibited delayed aggregates formation compared with wild-type Ax-2 cells. We observed faint aggregates in wild-type cells at 5 h after starvation, and the aggregates grew larger and increased density thereafter, whereas the aggregates in Ddm-
GluPR-null cells were not prominent even after 7 h and showed low density at 8 h. To analyze the relation of delayed aggregates formation in DdmGluPR-null cells to early gene expressions such as cAR1 and aca, we examined the expression patterns of these genes during development in DdmGluPR-null cells and wild-type Ax-2 cells by Northern blot analysis (Fig. 8A). cAR1 and aca were rapidly induced and peaked 2–4 h upon starvation and decreased thereafter in wild-type cells, whereas the expressions of these genes were reduced, sustained, and peaked at ~8 h in DdmGluPR-null cells compared with wild-type cells. We next examined chemotaxis in response to cAMP of DdmGluPR-null cells. The movement of cells toward a source of cAMP was observed microscopically. Fig. 8B showed directional movement of DdmGluPR-null cells toward cAMP, but it was inefficient. The migration distance from the spot of DdmGluPR-null cells was 66% of that of wild-type cells. Our findings suggest that the delayed aggregate formation of DdmGluPR-null cells phenotype is caused by inefficient chemotaxis toward cAMP with aberrant expression pattern of cAR1 and aca during early development. We further tested the ability of DdmGluPR expression to rescue the phenotype of DdmGluPR-null cells. The null cells that expressed GFP-DdmGluPR showed restorations of the delayed aggregates formation and the inefficient chemotaxis toward cAMP almost similar to those of Ax-2 cells (Fig. 9, A and B).

Our analysis of the primary structure of DdmGluPR suggests that DdmGluPR may be able to bind to an amino acid. To test the possibility of agonist or antagonist effect of an amino acid on DdmGluPR, we examined the effects of most of natural amino acids including Glu, Arg, Lys, Asp, His, Gly, Gin, Pro, Ser, Ala, Asn, Thr, Leu, Met, Val, and GABA on the starvation-induced chemotactic response toward cAMP. As shown in Fig. 9C, we could not detect any alteration of chemotactic response toward cAMP in the presence of an amino acid as indicated or GABA at 1 mM, suggesting that a simple amino acid is not a ligand for DdmGluPR. Aggregate formation on non-nutrient agar was also not affected in the presence of these amino acids or GABA (data not shown). Aggregation of starved cells requires the presence of extracellular molecules, the CMF involved in a cell density-sensing mechanism. CMF receptors appears to mediate the G-dependent and G-independent signal transductions (13, 14). To test the sensitivity to CMF, we starved wild-type and DdmGluPR-null cells at various densities in submerged culture in the presence or absence of CMF (Table 1). Both wild-type and DdmGluPR-null cells starved in the absence of CMF were able to aggregate in 24 h at densities 4 × 10^5 cells/ml or above. The same cells starved for 24 h in the presence of CMF prepared from either wild-type or DdmGluPR-null cells formed aggregates at lower densities. Thus, the cell density-sensing mechanism by CMF does not require DdmGluPR.

**DISCUSSION**

Glutamate receptors are categorized into ionotropic glutamate receptors (iGluRs) and mGluRs. The iGluRs internally contain ligand-gated ion channels. Chen et al. (16) have identified an iGluR, GluR0,
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from a prokaryote, the cyanobacterium *Synechocystis*. GluR0 has a hybrid structure between iGluRs and potassium channels and is thought to be a common ancestor for these molecular families. The mGluRs are structurally and evolutionally distinct from the iGluRs.

Here, we identified a new receptor DdmGluPR in *Dictyostelium* belonging to the mGluR family. The BLAST search showed that the extracellular region of DdmGluPR was closest in sequence similarity to mouse mGluR3 and *Drosophila* DmXR. Miti et al. (8) has found the receptor called mXR as belonging to the mGluR subclass in insects such as *Anopheles*, *Apis*, and *Drosophila*. They could not find any orthologues of mXR in *C. elegans* or mammalian genomes. The residues of mGluts involved in the binding of the α-carboxyl and α-amino groups of glutamate were well conserved in both of DdmGluPR and *Drosophila* DmXR but the residues interacting with the γ-carboxylic group of glutamate were not. Ligands for Drosophila DmXR and DdmGluPR are not determined yet, but this suggests that the natural ligand might be an amino acid-like molecule. However, a simple amino acid is unlikely as a ligand. The GABABR and the mGluR families appeared to be derived from a common ancestral receptor. The phylogenetic analysis suggests that DdmGluPR diverged after the mGluR family-GABA<sub>B</sub>Rs split but before mGluR family divergence. Although, *Drosophila* DmXR diverged after mGluR calcium-sensing and pheromone receptors split. This is in good agreement with the sequence in which the transmembrane region of DdmGluPR is similar to GABA<sub>B</sub>Rs while the region of DmXR is similar to mGluRs. Thus, we propose that DdmGluPR is an ancestor leading to the mGluR family divergence, and *Drosophila* DmXR is a direct ancestor for mGluRs divergence. mXR-type receptors would disappear during evolution in higher organisms except insects.

We also described the functional involvement of DdmGluPR in the early development of *D. discoideum*. Prestarvation factors and conditioned medium factors were not the ligands for DdmGluPR, because the responses against these factors showed no difference between wild-type cells and DdmGluPR-null cells. We also noted that the DdmGluPR-null mutant was not affected in sensing folate (data not shown), although folate contains a glutamate structure. DdmGluPR mRNA was expressed in vegetative cells and throughout development, but the level of the expression was relatively high until 4 h and peaked at 2 h (data not shown) after starvation, suggesting that an unknown ligand is released at early development and affects the expressions of cAR1 and aca through DdmGluPR.

The mGluRs are known to modulate synaptic properties in vertebrates. The *Drosophila* genome possesses another mGluR gene, *DmGlura*, an mGluR2/3 ortholog (7). *DmGlura* is the only functional mGluR in *Drosophila*. Bogdanik et al. (17) have described that *DmGlura* is expressed at the glutamatergic neuromuscular junction and the null mutants display an increase in synaptic facilitation during short stimulus trains. The mutant phenotype cannot be replicated by an acute application of mGluR antagonists, suggesting that *DmGlura* regulates the development of presynaptic properties rather than directly controlling short term modulation. To understand the functional roles of DmmGluPR and mXRs will provide information for developmental roles of mGluRs in the nervous system or other tissues.

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REFERENCES

FIGURE 9. Restoration of DdmGluPR-null mutant phenotype by ectopic expression of GFP-DdmGluPR and the effect of most of natural amino acids and GABA on cAMP chemotaxis. A, wild-type Ah-2 (Wild), DdmGluPR-null (KO), and the null mutant expressed GFP-DdmGluPR (KO + DdmGluPR) cells were harvested, washed, and spotted at a density of 1 × 10<sup>4</sup> cells/ml on non-nutrient agar plates. Phase contrast micrographs were taken after 6 h of starvation. Scale bar: 250 μm. B, wild-type Ah-2, DdmGluPR-null, and the null mutant expressed GFP-DdmGluPR cells were starved in 10 mM Na-K buffer, pH 6.1, at 22 °C for 4 h and spotted on the agar plates at 1 mm from the edge of the well, which was filled with cAMP solution (5 μM of 10 mM solution). Chemotaxis response to cAMP was quantified by measuring the distance of cell migration. Each value represents the mean ± S.E. (n = 3). *p* < 0.001 compared with KO cells. C, starvation-induced chemotactic response toward cAMP on non-nutrient agar in the presence of an amino acid as indicated or GABA at 1 mM concentration. Each value represents the mean ± S.E. (n = 3).

### TABLE 1

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Ability to aggregate at different cell densities

Wild-type (Wild) and DdmGluPR-null (KO) cells were starved at various cell densities in submerged culture in the presence or absence of CMF. The fields of cells were examined after 24 h. The presence of aggregates is denoted with a plus, whereas the absence of aggregates is represented with a minus. CMF-A and CMF-B were prepared from wild-type and DdmXR-null cells, respectively.