Expression and Functional Analysis of a Novel Isoform of Gicerin, an Immunoglobulin Superfamily Cell Adhesion Molecule*

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We have cloned a novel cDNA of gicerin, a cell adhesion molecule belonging to the immunoglobulin superfamily. Both gicerin isoforms share the same extracellular domain, which has five immunoglobulin-like loop structures and a transmembrane domain as s-gicerin, but differ in the cytoplasmic tail domain. As the newly identified form has a larger cytoplasmic domain than the previously reported form, we refer to them as l-gicerin and s-gicerin, respectively. l-gicerin is transcribed from a distinct mRNA containing an inserted sequence not found in s-gicerin mRNA which caused a frameshift for the coding region for a cytoplasmic domain. Previous studies demonstrated that gicerin showed a doublet band of 82 and 90 kDa in chicken gizzard smooth muscle. We report that the 82-kDa protein corresponds to s-gicerin and the 90-kDa protein to l-gicerin. We also found that the two gicerin isoforms are expressed differentially in the developing nervous system. Functional analysis of these gicerin isoforms in stable transfectants revealed that they had differ in their homophilic adhesion properties, as well as in heterophilic cell adhesion assayed with neurite outgrowth activity. In addition, these isoforms have neurite-promoting activity by their homophilic adhesion, but differ in their ability to promote neurite outgrowth.

Cell adhesion molecules are widely expressed in the nervous system and play a crucial role in the organization of neural networks during development (1, 2). Members of the immunoglobulin (Ig) superfamily are homophilic cell adhesion molecules, expressed in the nervous systems of a variety of animal species (3–5) that participate in neurite extension, fasciculation of nerve fibers, and neuronal migration (6–10). Recent studies provide evidence that they are also involved in synaptic plasticity (11) and programmed cell death of neurons (12). These findings underscore the importance of Ig superfamily molecules in cell-cell communication during nervous system development. Cell adhesion molecules of the Ig superfamily are divided into several subgroups. Members of one group have only Ig-like domains in their extracellular domains; the others form a subgroup that have fibronectin type III-related domains between lg-like and transmembrane domains (13). Members of the first group include fasciclin III (14), neuromusculin (15), and SCI/DM-GRASP/Ben (16, 17) that are mainly expressed in neurons, as well as MAG1 (3, 18) and SMP (19) that are found in glial cells. Ig superfamily molecules have also been classified into two groups by their mode of membrane association, with some containing a phosphatidylinositol anchor (20, 21) and others a transmembrane domain (8, 22). Interestingly, some Ig superfamily members undergo alternative splicing to generate multiple forms that differ in the type of membrane association used (23, 24). The alternatively spliced products are differentially expressed spatially and temporally and expected to play different roles (4). MAG, the molecule most homologous to gicerin, is known to have two isoforms that differ in the length of their cytoplasmic domains (3, 25). These forms are differentially regulated during development with only l-MAG found to be associated with Fyn, a nonreceptor tyrosine kinase (26).

Gicerin is a cell adhesion molecule with five Ig-like domains (27). It was first identified as a binding protein for neurite outgrowth factor (NOF), a member of the laminin family (28, 29). In the nervous system gicerin expression is transient, restricted to early stages of development when neurons extend neurites or undergo migration (30), whereas constitutive expression is found in muscles and kidney. Introduction of gicerin into L929 fibroblast cells in vitro indicate that gicerin exhibits homophilic cell adhesion activity in addition the heterophilic adhesion to NOF (27). In previous studies, we noted that Western blot analysis of endogenous gicerin in chicken gizzard reveals a doublet band, perhaps reflecting the presence of alternatively spliced products. In the present study, we identified and characterized a novel longer isoform of gicerin termed l-gicerin, which contains a different cytoplasmic tail than that found in the previously reported form of gicerin (s-gicerin (accession number D38559)). We have also investigated possible functional differences between these two gicerin isoforms and compared their expression during nervous system development.

MATERIALS AND METHODS

cDNA Cloning and Sequencing—A 1.4 kb 11 chicken gizzard cDNA library (Clontech) was used for screening using the plaque hybridization method. A cDNA probe encoding gicerin (27) was prepared by the random primer labeling method with [32P]dCTP, and screening was carried out with standard procedures (31). Purified cDNA inserts were subcloned into a Bluescript II SK(–) phagemid vector (Stratagene), and the sequences were determined by the dideoxy chain termination method (32) using Sequenase (U. S. Biochemical Corp.). Both strands were fully sequenced by obtaining endonuclease-digested fragments.

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1 The abbreviations used are: MAG, myelin-associated glycoprotein; NOF, neurite outgrowth factor; RT-PCR, reverse transcription polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; CG, ciliary ganglion; bp, base pair; NCAM, neural cell adhesion molecule.
RT-PCR and Southern Blot Analysis—Total RNA was isolated from chicken gizzard smooth muscle by the AGPC method (33) and a 1-μg aliquot was reverse-transcribed by murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The products were then used for PCR templates with synthetic oligonucleotide primers which contained EcoRI restriction site: G-3′, TATAGAATCTTGAATTCCGGC- GGGCGG. Resultant single-stranded DNA templates were ligated into Blunt-Cloning vector pGEM3Zf+ (Promega) and transformed into E. coli DH5α. Using restriction analysis, clones were isolated and the positive clones were re-screened. Three clones were confirmed as containing the insert sequence, was used instead of G-3′. The degree of cell aggregation was estimated by the index calculated as 100/T, where T is the total particle number at the incubation time t. Experiments were performed four times and the scores averaged. Photographs were taken of cells in suspension after 1 h of incubation.

Naurite Extension of Ciliary Ganglion Cells on the Feeder Layer of Gicerin Transfectants—Ciliary ganglia (CGs) were dissected from the 8-day chick embryo. They were treated with 0.05% trypsin and 0.53 mM EDTA for 20 min at room temperature, then reuspended in Dulbecco's modified Eagle's medium containing 10% FCS and dissociated by gentle pipetting. Resultant single cells were plated on a plastic dish for 1 h to let non-neuronal cells attach to the dish (25, 28). The nonattached ciliary ganglion neurons were plated on feeder layers of confluent gicerin transfectants or parental L929 cells grown in 30-mm dishes (~500 CG cells/dish) and co-incubated for 24 h (39). The cultures were fixed with Zamboni's solution, and the CG neurons were detected by immunocytochemistry with anti-MAP2 antibodies (40). The relative length of the neurites from the MAP2-positive cells were judged under microscopy, and the same experiments were performed at least three times. In some experiments, anti-gicerin polyclonal antibodies were added both to the preincubated CG cells and the feeder cells 1 h before starting the co-culture, and the anti-MAP2 monoclonal antibodies were employed for detection of neurons with neurites.

Cell Adhesion Assays—Cell adhesion experiments were carried out as reported previously (27). Four-ml aliquots of each test sample containing 10 ng/ml of purified NOF (28) were spotted on a 35-mm test dish coated with nitrocellulose and incubated for 1 h in a humidified CO2 incubator at 37 °C. The dishes were washed with Dulbecco's modified Eagle's medium containing 10% FCS three times, and then placed on the test dishes. To assess the inhibition of adhesion activity by antibodies against gicerin, the CG cells were preincubated with the antibodies at a final concentration of 0.2 mg/ml on ice for 30 min before plating on the test dishes. The dishes were kept in a CO2 incubator at 37 °C for 30 min, then washed twice with Hank's solution containing Ca2+ and Mg2+ and fixed with Zamboni's solution. Adhesion to the dishes was then assessed by phase-contrast microscopy.

RESULTS

Molecular Cloning of a Novel Isoform of Gicerin—An s-gicerin cDNA clone was used to prepare a probe for screening a P11 chicken gizzard cDNA library. We obtained seven independent positive clones and determined their sequences, after subcloning into phagemid Bluescript II SK (−). We obtained cDNA clones that encoded sequences that differed from that reported for s-gicerin and rescreened the same library to obtain four clones that encoded a novel protein. We found that the novel l-gicerin cDNA (EMBL/DDB/GSD/NICB accession number D49849) had a 121-bp insert in the cytoplasmic domain that encoded 63 amino acid residues and produced a frameshift to form a larger tail with a distinct sequence (Figs. 1 and 2). The cytoplasmic domain of l-gicerin showed no significant homology with known molecules. Analysis of the extracellular domain did not reveal any difference between these gicerin isoforms.

Characterization of Gicerin Isoform mRNAs—To confirm the existence of these two distinct gicerin mRNA species, total RNA was extracted from chicken gizzard smooth muscle and analyzed by reverse transcription-PCR (Fig. 3A). As expected, two sizes of RT-PCR products were detected when the G-5′ and G-3′ primers that span the insert were used. Furthermore, only one product was amplified when the I-3′ primer, based on the insert sequence, was used instead of G-3′. We also obtained a single product when the 5′ primer I-5′, based on a segment of the insert, was used with G-3′. Digestion of these PCR products with SacI, which has one site conserved in both isoforms (underlined in Fig. 1), generated products with the expected sizes (Fig. 3B).

Identification of l-gicerin by Western Blot Analysis—To produce an antisera specific for l-gicerin, we utilized two multi- ple antigenic peptides found in the cytoplasmic domain of l-gicerin but not s-gicerin (Fig. 2). Immunoprecipitation was carried out with anti-gicerin antibodies prepared previously or with antibodies prepared against the l-gicerin-specific peptides. To examine the selectivity of the anti-l-gicerin antisera, we first immunoprecipitated both gicerin isoforms from Non-
idet P-40 gizzard extracts with anti-gicerin antibodies and then performed immunoblot analysis. Only the upper band of the doublet detected by the anti-gicerin antisem was recognized by anti-l-gicerin antiserum (Fig. 4). Conversely, the upper band was detected by both antisera in Western blot analysis, when immunoprecipitation was performed with anti-l-gicerin antibodies. We performed immunoblot analysis following digestion of the Nonidet P-40 extracts of the gizzard with N-glycosidase F, since the predicted amino acid sequence of gicerin has eight potential N-linked glycosylation sites. The digested gicerins were detected as a doublet band of 70 and 64 kDa, and only the upper band of the doublet was recognized with anti-l-gicerin antiserum (Fig. 5). The predicted molecular masses of l- and s-gicerin were calculated to be 69.0 and 63.9 kDa, respectively, which matches that observed for the deglycosylated gicerins.

These studies demonstrate that the l-gicerin transcript encodes the larger isoform of gicerin. Next, we compared the expression of gicerin isoforms in the nervous system by Western blot analysis with these two antisera. Previous studies revealed that gicerin was highly expressed in the embryonic retina, cerebellum, and optic tectum and thereafter levels gradually decrease during development. We found that s-gicerin was expressed in the retina, l-gicerin in the optic tectum, and both in the cerebellum (Fig. 6, A and B).

Establishment of l-gicerin-expressing Cell Lines—The l-gicerin cDNA was subcloned into the mammalian expression vector pcDNA1 to construct pcDNA1-lG. The construct was co-transfected into the mouse fibroblast cell L929 with a neomycin resistance vector pMAM-neo, and cells were selected with G418. Cell lines were screened for high levels of l-gicerin expression by immunofluorescent cytochemistry and Western blot analysis with anti-gicerin antiserum. We chose three clones (l-lG-1, -3, and -5) for further experiments. While s-gicerin transfectants exhibited a single band of 86 kDa (27), l-gicerin lines exhibited a doublet band of 92 and 84 kDa (Fig. 7A). Appearance of a lower band of l-gicerin might reflect proteolytic degraded fragments, since only the upper band was detectable by l-gicerin-specific antiserum, and the intensity of the upper band decreased as the lower band increased following prolonged storage (data not shown). Following digestion with N-glycosidase F, the molecular size of the recombinantly

Fig. 1. DNA and deduced amino acid sequences of l-gicerin. Cysteine residues are marked with asterisks, and the single transmembrane domain is underlined. Sequences employed for RT-PCR primers and the Sac I consensus sequence are underlined by thinner bars. The primer names are indicated above the sequences.

Fig. 2. Comparison of cytoplasmic sequences of l-gicerin and s-gicerin. Amino acid sequences of cytoplasmic domains of both s-gicerin and l-gicerin are indicated with cDNA sequences. The numbers shown correspond to the amino acids indicated in Fig. 1. Amino acid residues employed for constructing multiple antigen peptides for generating l-gicerin-specific antisem are shown in bold and underlined. The oligopeptide names are indicated above the sequences.
expressed proteins decreased to 64 kDa for s-gicerin and 70 and 66 kDa for l-gicerin. Only the upper band of l-gicerin transfectants was detected by anti-l-gicerin antiserum before and after N-glycosidase F digestion. The deglycosylated product of s-gicerin corresponded to the predicted molecular mass of 63.9 kDa (Fig. 7B). Only the upper band of the deglycosylated products from l-gicerin transfectants corresponded to the predicted mass of 69.0 kDa, while the lower band may be due to degradation after extraction.

Aggregation Activity of Cells Expressing Single Gicerin Isoform—In previous studies, we have shown that s-gicerin possesses homophilic cell aggregation activity. To investigate possible differences in the functional activities of s- and l-gicerin, we first compared their ability to mediate homophilic adhesion...
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Analysis of multiple transfectants of both types revealed that cell lines expressing the same gicerin isoform exhibited nearly identical cell aggregation activity (Fig. 9). Previous studies of multiple s-gicerin transfectants revealed a similar pattern across cell lines (27).

Comparison of the degree of cell aggregation activity revealed that s-gicerin transfectants aggregated more strongly than l-gicerin transfectants. These findings suggest that the cytoplasmic domain influences homophilic binding activity.

Neurite Extension from Ciliary Ganglion Cells Grown on a Layer of Gicerin Transfectants—We next examined the effect of gicerin on neurite outgrowth. As CG neurons of 8-day chick embryos express gicerin, these were co-cultured on a layer of the gicerin transfectants or parental L929 fibroblasts. We measured the relative length of neurites by MAP2 immunocytochemistry, and more than 1000 neurons per each clone were counted. We scored cells with neurite length longer than the cell body diameter as neurite-positive (Fig. 10). CG neurons showed little neurite extension when grown on the parental L929 cells for 24 h, but exhibited extensive outgrowth on s-gicerin transfectants (Fig. 11). Neurite extension was blocked by preincubating with anti-gicerin antibodies, indicating that neurite extension from embryonic CG neurons was mediated by gicerin. Compared with s-gicerin transfectants, l-gicerin was less active in promoting neurite extension. As l-gicerin was also less active in the homophilic adhesion assay, these findings provide additional evidence that the cytoplasmic domain plays an integral role in gicerin's functional activity.

Cell Adhesion Activity of Gicerin Transfectants—We have demonstrated previously that s-gicerin transfectants adhere to NOF in a specific fashion. In comparing the ability of s- and l-gicerin transfectants to NOF, we found that s-gicerin trans-
The products. Southern blot analysis indicated the presence of s-gicerin (l-gicerin) which differed from s-gicerin only in the cytoplasmic coding region. The predicted molecular mass was calculated to be 69.0 kDa, corresponding to the larger band (70 kDa) seen following N-glycosidase F digestion. These two molecules did not co-precipitate when immunoprecipitated with l-gicerin-specific antibodies: l-sG-1 (B) and l-Ig-1 (D). No adhesion of l-Ig-1 to laminin (E) or parental L929 to NOF (F) was observed. The same results were obtained with other transfectants. Bar, 120 mm.

DISCUSSION

In the present paper, we isolated a novel isoform of gicerin (l-gicerin) that differs from the previously reported isoform s-gicerin in the cytoplasmic tail domain. Comparison of the functional activity of these isoforms revealed that l-gicerin was not as effective as s-gicerin in assays of both homophilic and heterophilic adhesion. These findings indicate that the cytoplasmic domain is not merely a passive structural feature but exerts an important influence on the adhesive properties of the extracellular domain.

Gicerin was originally purified from chicken gizzard as a doublet with molecular sizes of 82 and 90 kDa (29). Recently, we have reported the molecular cloning and functional analysis of gicerin, which we renamed s-gicerin here (27). However, these studies did not clarify the origin of the doublet band. Since gicerin mRNA from gizzard exhibited a broad single band of about 5.2 kilobases, we considered two likely possibilities: 1) the two bands on SDS-PAGE reflect different post-transcriptional modification such as glycosylation or 2) they were translated from different mRNA species with similar size.

Following digestion of gizzard membrane extracts with N-glycosidase F, two bands migrating as 70 and 64 kDa proteins were detected by Western blot analysis. The predicted molecular mass of s-gicerin was 63.9 kDa, matching the size of the smaller digested product. The presence of the 70-kDa gicerin immunoreactive band favored the existence of two different gicerin proteins. Of note, precedent for this alternative is present in the cerebellum. Differential expression among cell types may help account for different responses to NOF and gicerin seen in these regions. We have reported that neurons in cultured retinal explants that express s-gicerin selectively extend neurites in response to NOF (29), while the cerebellar explants cultured on NOF display migration of neurons (probably granular cells) in addition to neurite extension (30).

To examine functional differences between these two gicerin isoforms, we established cell lines expressing each alone. Although the degree of l-gicerin expression differed a little among three cell lines tested, similar cell aggregation activities were observed among the l-gicerin transfectants. Presumably, the amount of gicerin expressed was over a threshold amount needed to achieve a maximal cell adhesion activity. A similar result was obtained in previous experiments examining s-gicerin transfectants (27). Experiments investigating NCAM and cadherin activity have revealed that the NCAM displays a threshold value of expression for neurite extension, while cadherin shows a graded concentration-dependent activity. It is worth noting that the molecular mass of gicerin's cytoplasmic tail also influence its NOF binding activity.

Fig. 11. Comparison of neurite extension. Dissociated CG neurons from embryonic day 8 chick retina were cultured for 24 h on confluent monolayers of control L929 or gicerin transfectants (l-sG-1 and -2 and l-Ig-1 and -3). To assess inhibition of neurite extension by anti-gicerin antibodies, both the CG neurons and feeder cells were preincubated with antibodies 30 min before mixing the cultures. The cultures were fixed and immunostained with anti-MAP2 antibodies. Neurons with neurites longer than the cell body were scored as neurite positive. More than 1000 neurons were examined microscopically for each condition shown. Only a single gicerin gene in the genome (data not shown). We, therefore, concluded that these two mRNA species were generated by alternative splicing.

We then examined whether the doublet band detected with SDS-PAGE originates from these two mRNA species, as expected. An antiserum specific for l-gicerin was produced and reacted with only the upper 90-kDa band of the doublet seen in gizzard extracts and the upper 70-kDa band following N-glycosidase F treatment. These two molecules did not co-precipitate when immunoprecipitated with l-gicerin-specific antisera, even under mild condition in the presence of 0.5% Nonidet P-40, suggesting that they do not form a tight dimer. The distribution of both subtypes was examined in the developing nervous system by Western blot analysis. In the retina, only s-gicerin was detected. In contrast, only l-gicerin was found in the optic tectum. Both gicerins were expressed in the cerebellum. Differential expression among cell types may help account for different responses to NOF and gicerin seen in these regions. We have reported that neurons in cultured retinal explants that express s-gicerin selectively extend neurites in response to NOF (29), while the cerebellar explants cultured on NOF display migration of neurons (probably granular cells) in addition to neurite extension (30).
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