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Identification of γA-Like Protocadherin Expressed During Chick Development

Anthony A. Capehart[1]* and Christine B. Kern[2]

[1]Department of Biology, East Carolina University, Greenville, North Carolina 27858
[2]Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425

Abstract The protocadherins are calcium-dependent cell adhesion molecules of the cadherin superfamily that have been described in numerous species. Although less well characterized than classical cadherins, the protocadherins are also thought to facilitate critical cell–cell interactions during embryonic development. We have cloned a novel protocadherin from the embryonic chick utilizing a monoclonal antibody produced against a peanut agglutinin-binding fraction of cultured chick limb tissue to screen a λZAP cDNA expression library from the stage 25 limb. A 2.8 kb cDNA clone was obtained that encoded multiple cadherin-like ectodomains. Northern blotting revealed a single 4.6 kb RNA transcript that was highly enriched in the stage 43 chick brain. Utilization of 3′ Rapid amplification of cDNA ends (RACE) identified the entire 2.4 kb reading frame. The chick protocadherin contained five cadherin-like extracellular repeats and a highly conserved cytoplasmic domain. Amino acid alignment of the extracellular domains revealed marked identity to the human γA protocadherin subfamily. In situ hybridization showed low levels of mRNA localization in several developing chick tissues, but stronger expression in the neural tube and dorsal root ganglia at stage 27. In the stage 43 chick brain, protocadherin mRNA was noted in discrete regions, particularly within the developing optic lobe. As for protocadherins described in other species, these results suggest that this novel γA-like protocadherin may also play a role in chick neural development. J. Cell. Biochem. 90: 608–618, 2003.

Key words: cadherin superfamily; cell adhesion; cell–cell interaction; neural development; organogenesis

Cell adhesion represents a critical mechanism for successful initiation and completion of organogenesis during development. The cadherins represent a major group of calcium-dependent cell adhesion molecules that participate in this process at multiple sites in the vertebrate embryo. The search for new members of the cadherin superfamily led to the discovery of the protocadherins [Sano et al., 1993] which were characterized as integral membrane proteins bearing five or more cadherin-like extracellular repeats and conserved cytoplasmic domains that did not interact directly with β-catenin as described for the classical cadherins [Suzuki, 2000].

Recent evidence has shown that the developing brain is particularly enriched in protocadherins [Hirano et al., 1999a,b] and it has been hypothesized that they provide a homophilic mechanism for adhesive attachment to ensure the appropriate sorting and arrangement of neurons and their synaptic connections. Immunolocation of protocadherins, such as protocadherin α (cadherin-related neural receptor), at synaptic junctions supports this proposed function [Kohmura et al., 1998]. Furthermore, it has also been reported that other protocadherins may have additional roles in the central nervous system as receptors in the reelin signal transduction pathway [Senzaki et al., 1999] and in long-term potentiation [Yamagata et al., 1999].

Following their initial identification, subsequent discovery of additional protocadherins permitted their grouping into several subfamilies. The largest of these is the protocadherin

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*Correspondence to: Anthony A. Capehart, Department of Biology, East Carolina University, Greenville, NC 27858. E-mail: capehartt@mail.ecu.edu

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γ (protocadherin 2) subfamily described in human and mouse [Wu and Maniatis, 1999, 2000; Wu et al., 2001; Hirano et al., 2002]. Genomic characteristics of this subfamily include exons grouped along a single chromosome, with each single exon encoding the extracellular, transmembrane, and a portion of the cytoplasmic domain for each subfamily member. All members of the γ subfamily share exons encoding the terminal portion of the cytoplasmic domain. The presence of potentially functional redundant cell adhesion molecules has led to the idea that expression of different members of the cadherin superfamily may facilitate formation and maintenance of the extensive neural network required for central nervous system function [Wu and Maniatis, 1999].

In addition to a role in the nervous system, protocadherins have been implicated in other key developmental events in the frog, mouse, and zebrafish, including ectodermal differentiation [Bradley et al., 1998], gastrulation [Kim et al., 1998], and somitogenesis [Yamamoto et al., 1998; Kim et al., 2000; Rhee et al., 2003]. To date, however, published reports of protocadherins in the chick developmental model have been absent. In an effort to identify novel molecules expressed during embryonic chick development, in the present study we have identified a cDNA encoding a novel protocadherin γA-like family member and demonstrate its expression in the developing chick nervous system.

METHODS

Monoclonal Antibody (Mab) IG3

High density micromass cultures derived from stage 22 to 23 [Hamburger and Hamilton, 1951] whole chick limb buds were prepared as described previously [Capehart and Biddulph, 1991]. After 18 h, cultures were washed with phosphate buffered saline (PBS) and incubated 1 h on ice with 150 μg/ml biotinylated peanut agglutinin (bPNA, Vector Labs, Burlingame, CA). Plates were washed with PBS and extracted 30 min on ice in 0.1% NP-40 (Sigma, St. Louis, MO) in 50 mM sodium borate, pH 8.6, containing protease inhibitors. Extracts were clarified and incubated overnight at 4°C with avidin–agarose D beads (Vector Labs) with gentle agitation. Beads were washed thoroughly with extraction buffer and eluted in 2% sodium dodecyl sulfate (SDS). SDS was precipitated with 20 mM potassium chloride, supernatant concentrated and exchanged to 0.05% NP40 in PBS using Centricon 10 filters (10,000 MWCO; Amicon, Beverly, MA), and dialyzed against PBS. Dialysates were used as immunogen for monoclonal antibody production in mice utilizing standard techniques [Harlow and Lane, 1988]. Hybridomas were screened by Western blotting against limb mesenchyme extracts and Mab IG3 was subcloned by limiting dilution.

Western Blot Analysis

PNA affinity-purified extracts were dissolved in SDS–PAGE buffer and electrophoresed on 8% reducing polyacrylamide gels [Laemmli, 1970]. Proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA) and blocked with 5% BSA in 50 mM Tris, pH 7.5, 150 mM sodium chloride containing 0.05% Tween-20 (TBS-T; Sigma). Membranes were incubated 1 h at room temperature with undiluted IG3 hybridoma supernatant, washed, and incubated an additional 1 h with alkaline phosphatase-conjugated goat and mouse IgG (Promega, Madison, WI) diluted 1:1,000 in TBS-T. Samples were washed thoroughly and developed using NBT/BCIP substrate (Promega).

IG3 antibody was also affinity purified by incubation of hybridoma supernatants with IPTG-induced polypeptide expressed by the plaque purified cDNA clone (see below) and bound to nitrocellulose filters in a modification of the method described by Smith and Fisher [1984]. Bound antibody was eluted from filters in 0.1 M glycine, pH 2.5, neutralized with Tris, and exchanged into PBS using Centricon 10 filters.

Isolation of cDNA and Sequencing

The IG3 antibody was used to screen a λZAP II (Stratagene, La Jolla, CA) expression library prepared from stage 25 chick limb tissue according to manufacturer's instructions. Approximately 2×10^5 PFU were screened and a single positive clone was plaque purified and the insert retrieved as Bluescript SK plasmid by in vivo excision per manufacturer's protocol (Stratagene). Both strands of the 2.8 kb insert were sequenced by fluorescent dye termination on an automated DNA sequencer (Applied Biosystems, Foster City, CA). The complete chick protocadherin coding sequence was obtained by

utilizing the SMART RACE cDNA kit (Clontech, Palo Alto, CA). Briefly, first strand cDNA synthesis utilized stage 43 chick total brain RNA and the 3′CDS primer A and PowerScript Reverse Transcriptase. Following first-strand synthesis, Rapid amplification of cDNA ends (RACE) was performed with two different gene specific primers: 1913F 5′TCGTTCAGGCACACGATGGAGG 3′ and 2469F 5′CCTACGTGTCGGTGCAGGCGG 3′ and Advantage GCTM PCR (Clontech) was employed for the SMART amplification. A PCR product generated from the 2469F primer was subcloned into the pGEMT Easy vector (Promega) and sequenced.

Sequence Analysis

Protocadherin ectodomain DNA sequences were translated and aligned using GAP and PRETTY sequence alignment tools from the SeqWeb interface to the GCG Wisconsin sequence analysis package [Genetics Computer Group, 1999]. Following alignment of chick and human EC1–EC5 ectodomains in CLUSTAL, a neighbor-joining phylogenetic tree was reconstructed using PHYLIP (Phylogeny Inference Package, Version 3.6) and the phenogram visualized with TREEVIEW using default parameters [Page, 1996]. The following GenBank amino acid sequence entries of human γA, B, and C protocadherins were used for comparisons: AAD43733, NP002579, NP003726, NP003727, NP061735-NP061751, NP114477, and chick γC3: BAA20457.

Northern Analysis

Total RNA was isolated from stage 43 brain tissue using the RNeasy Midikit (Qiagen, Valencia, CA) and 10 μg of total RNA was electrophoresed on a 1% denatured gel at 45 V. RNA was transferred to a Genescreen Plus membrane (DuPont, Wilmington, DE) according to manufacturer's instructions. PCR was used to generate a 620 bp probe template from the original chick cDNA clone with primers: 1868F 5′GCGATAAGGCTGGTGAGAAATCTG 3′ and 2487R 5′CCGCCTGCACCGACACGTAGG 3′. The PCR-generated DNA fragment was gel purified, sequenced, and 100 ng used to generate ^{32}P-labeled probe per manufacturer's protocol (Pharmacia, Uppsala, Sweden). Probe hybridization was performed ON at 42°C in 5× SSPE, 50% formamide, 5× Denhardt's reagent, 1% SDS, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA. Washes were performed in 2× SSPE at 65°C and 0.1% SSPE at RT. X-OMAT autoradiography film (Kodak) was exposed to the membrane 5 days before development.

In Situ Hybridization

Samples were fixed ON in 85% ethanol, 10% formaldehyde, 5% glacial acetic acid, subsequently dehydrated, and embedded in paraffin. Sections were cut at 7–8 μm and in situ hybridization was performed essentially as described by Breitschopf et al. [1992]. Briefly, PCR was utilized to generate a 500 bp cDNA probe template with primers: 1305F 5′GGCGAGATGAAGGTTTATCCAA 3′ and 1805R 5′TTTCACGTCCCCGTTGGTT 3′, which was subcloned into pGEMT Easy (Promega). Digoxygenin-labeled RNA probes were prepared from Nco I or Nde I linearized plasmids with the DIG RNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions and labeled probe quantified by dot blot. Hybridizations were performed ON at 55°C in 2× SSC, 50% formamide, 10% dextran sulfate, 0.02% SDS, and 0.01% yeast tRNA containing either 200 ng/ml anti-sense and sense (negative control) probes. Specimens were washed thoroughly at 55°C in 1× SSC and 50% formamide. Sections were incubated 1 h at RT in blocking buffer (Roche) containing 10% fetal bovine serum and 1% sheep serum and 1h in 1:1,000 sheep anti-digoxygenin–alkaline phosphatase in blocking buffer. Following TBS washes, labeled probe was visualized using NBT/BCIP substrate (Roche). For identification of stage 43 brain structure, we consulted Romanoff [1960].

RESULTS

A PNA-binding fraction of chick limb tissue was utilized to generate the IG3 monoclonal antibody in an effort to identify novel molecules expressed during embryonic chick limb development. While Mab IG3 did not work for immunohistochemistry on paraffin-embedded sections, Western blotting with the antibody identified a protein of approximately 78.3 kD in PNA-binding extracts of cultured limb tissue (Fig. 1). Immunoblotting results reported above were verified by use of plaque affinity-purified IG3 preparation (see below). Mab IG3 was used to screen a chick limb cDNA expression library in order to clone and identify the IG3 antigen. A single 2.8 kb clone was plaque purified and

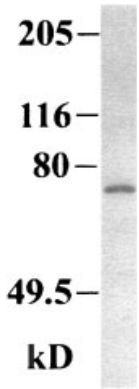


Fig. 1. Immunoblot analysis of IG3 antigen in peanut agglutinin-binding extracts of cultured stage 22–23 chick limb tissue. The IG3 antibody recognizes a band of approximately 78.3 kD.

sequenced (Fig. 2). Sequencing of the 5′ end showed a reading frame with a putative signal peptide beginning at nucleotide 968 followed subsequently by sequence encoding five cadherin-like ectodomains. Basic Local Alignment Search Tool (BLAST) analysis revealed similarity to the protocadherin family, but also showed that the clone represented a truncated cDNA lacking a protocadherin cytoplasmic domain. 3′ RACE of stage 43 (day 17) chick neural tissue generated a 1.6 kb product from the internal 2469F primer that was subcloned and sequenced. This clone shared an identical overlap of approximately 400 bp with the original chick protocadherin cDNA and thus added 1.2 kb of additional sequence that encoded the putative cytoplasmic domain and also contained a translational stop codon. Based on these data, the entire reading frame of this chick protocadherin was determined to 2.43 kb in length. Subtracting the putative signal peptide, the deduced amino acid sequence was predicted to have a molecular mass of approximately 84.5 kD, close to the estimated molecular weight for the IG3 antigen as detected by Western blot.

BLAST alignment of the deduced amino acid sequence showed highest homology with members of the human [Wu and Maniatis, 1999] and mouse [Wu et al., 2001] protocadherin γA subfamily. Because of the extensive homology between human and mouse orthologues, comparisons of this reported chick sequence were limited to human protocadherin family members. Amino acid similarity and identity between chick and human protocadherin γA subfamily members over the entire deduced amino acid sequence ranged between 62–63% and 55–57%, respectively, and declined to 54–55% and 48–49% for human γB and γC subfamily members, respectively. Alignment of the putative calcium-binding terminal regions of the five cadherin-like ectodomains [EC; Overduin et al., 1995; Shapiro et al., 1995], showed strong identity between chick and human γ subfamily members in the characteristic D(R/Y/F)E and DXNDNXP peptide sequences (Fig. 3A). Other highly conserved amino acids of particular note were a cysteine in EC1, leucines in EC2–4, aspartates and glycines in the DXG motif of EC2–5, additional asparagines in EC4 and 5, and phenyalanines at the carboxy termini of EC1, EC2, and EC4. Interestingly, much of the EC6 ectodomain (Fig. 2), which in other protocadherins does not contain the cadherin sequence signature, was absent in this chick γA-like cDNA. However, within the deduced amino acid sequence for the EC6 region prolines conserved in other γ family members were maintained at several positions (#566, 567, 572, and 599).

GAP alignment was also performed against the only other related chick protocadherin located by BLAST, (*Gallus gallus*) similar to pcdh 2 (γC3) sequence (BAA20457). This analysis showed an overall amino acid similarity and identity of 57 and 52%, respectively, which was lower than that found between chick γA-like peptide and human γA subfamily members.

GAP alignment of the cytoplasmic polypeptide sequence of chick γA-like protocadherin showed a similarity and identity of 73–74% and 71–72% to members of the human γA group and also terminated with the highly conserved lysine-rich motif (KKKSGKKEKK). Consideration of the amino acid sequence of the entire cytoplasmic domain revealed 77% similarity and 76% identity to the (*Gallus gallus*) similar to pcdh 2 protein, however, from amino acid 691 to 811, the cytoplasmic domains were 100% identical (Fig. 3B). Over this same terminal cytoplasmic amino acid sequence, the chick

Fig. 2. Nucleotide sequence of chick γA-like protocadherin cDNA (GenBank Accession # AY325274) and its deduced amino acid sequence. The putative signal peptide sequence is shaded and the beginning of each cadherin-like extracellular domain is indicated by EC1–EC6. The putative hydrophobic transmembrane segment is underlined and potential N-glycosylation sites are shown in bold.

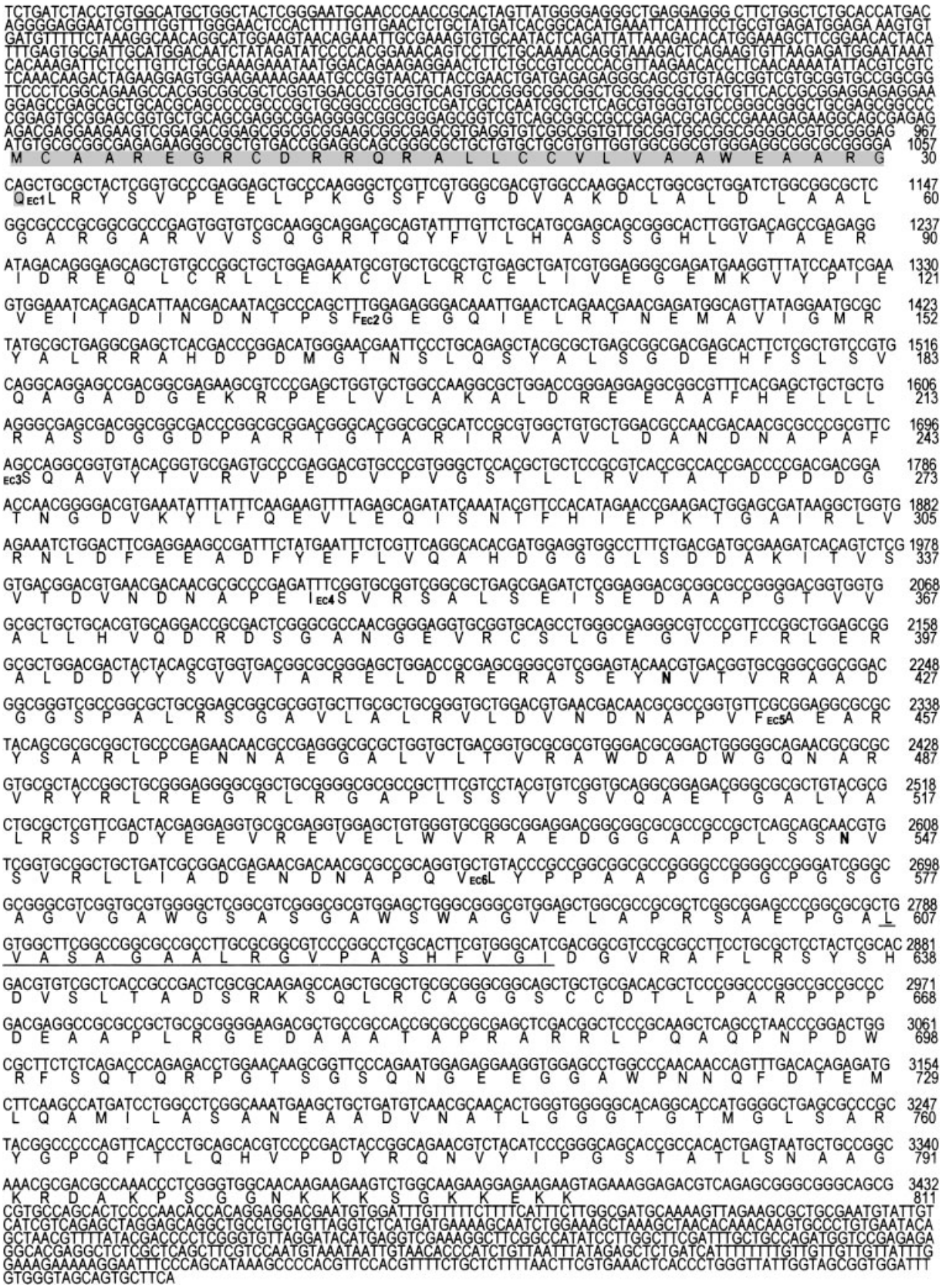


Fig. 2

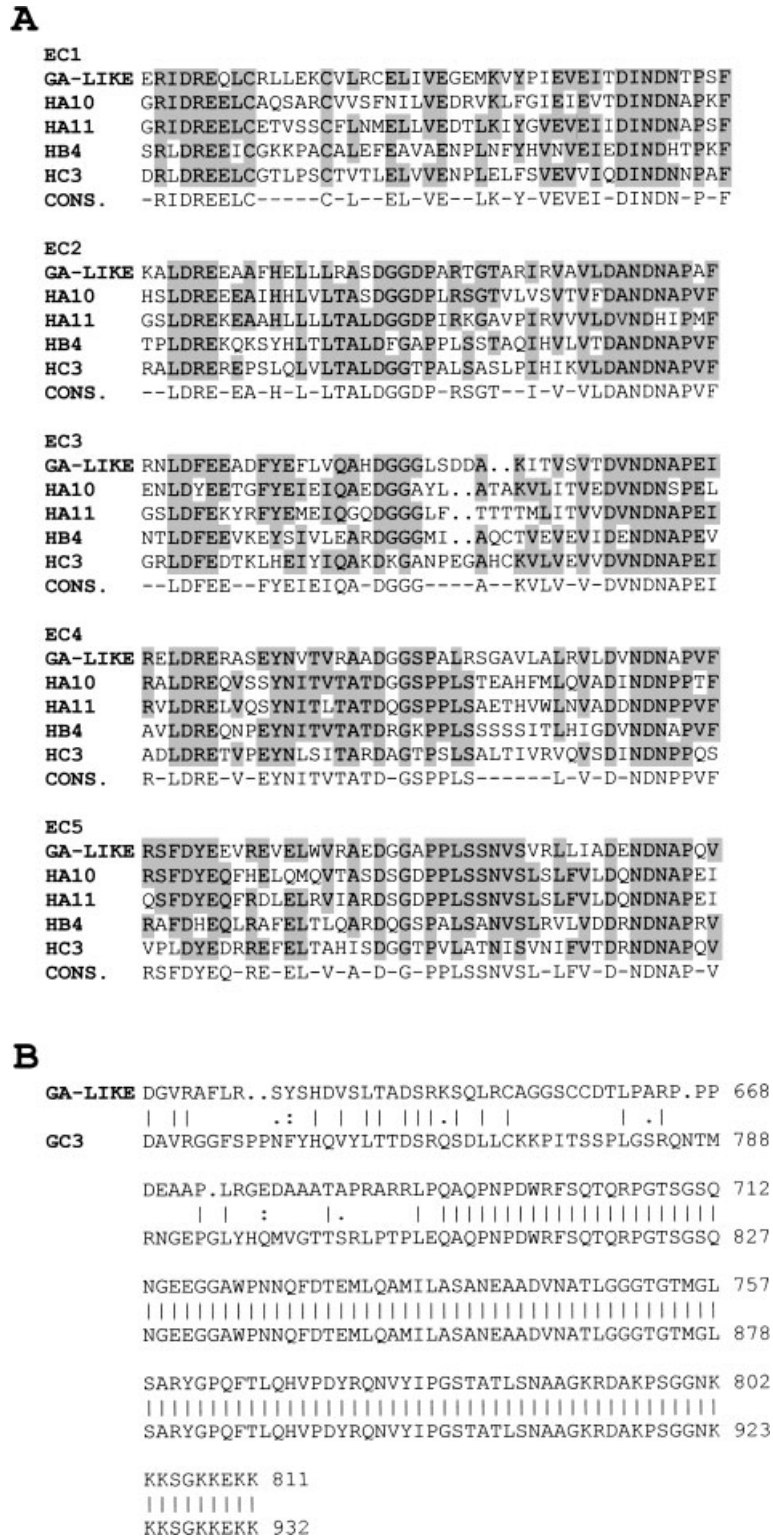


Fig. 3. Alignment of chick γA-like protocadherin deduced amino acid sequence with representative γA, B, and C protocadherins. **A**: Alignment of the terminal region of the five cadherin-like repeats (EC1–EC5) with human γA, B, and C protocadherins is shown. The consensus was determined by amino acid identity (shaded regions) in at least three out of five sequences. GA-like-chick γA-like (AY325274); HA10-γA10 (NP061736); HA11-γA11 (NP061737); HB4-γB4 (NP003727); HC3-γC3 (NP002579). **B**: Alignment of deduced amino acid sequence of the putative cytoplasmic domains of chick γA-like protocadherin and (*Gallus gallus*) similar to pcdh 2 (BAA204570). Vertical lines indicate sequence identity. Note sequence identity over the terminal 120 amino acid residues between the two chick sequences.

γA-like protocadherin was 88% similar and 87–88% identical to the human γA subfamily (not shown).

Shown in Figure 4 is the phylogenetic analysis in which chick protocadherin EC1–5 peptide sequences were compared with members of the human γA, B, and C subfamilies [Wu and Maniatis, 1999]. The resulting phenogram suggested that chick γA-like protocadherin was most related to human γA11.

Because protocadherins in other species have consistently exhibited high levels of expression in brain tissue, Northern blot analysis was performed using total RNA from stage 43 (17 day) chick brain. Northern analysis revealed the presence of a single, approximately 4.6 kb transcript in late stage brain tissue (Fig. 5). In order to localize chick γA-like protocadherin mRNA, in situ hybridization was performed at stage 27 (5.0–5.5 days; Fig. 6) and later in stage 43 brain tissue (Fig. 7). At stage 27, in situ hybridization analysis showed only low levels of γA-like protocadherin expression in the developing limb bud, primarily in organizing skeletal muscle masses and prospective dermal tissue (not shown), however, increased expression was noted in the dorsal aspect of the neural tube and in dorsal root ganglia at stage 27 (Fig. 6). In stage 43 chick brain tissue, protocadherin γA-like expression was particularly marked in neuronal cell bodies of the developing optic lobe (Fig. 7). Since to our knowledge this is the first report of chick protocadherin expression, in order to minimize potential cross reactivity we chose regions that in other family members exhibit greatest variability to generate probes for both Northern blotting and in situ hybridization. Probes were derived from cDNA encoding the variable extracellular domains and nucleotide alignment of these probe template regions with the (*Gallus gallus*) similar to pcdh 2 (chick γC3) sequence showed only 55–57% identity.

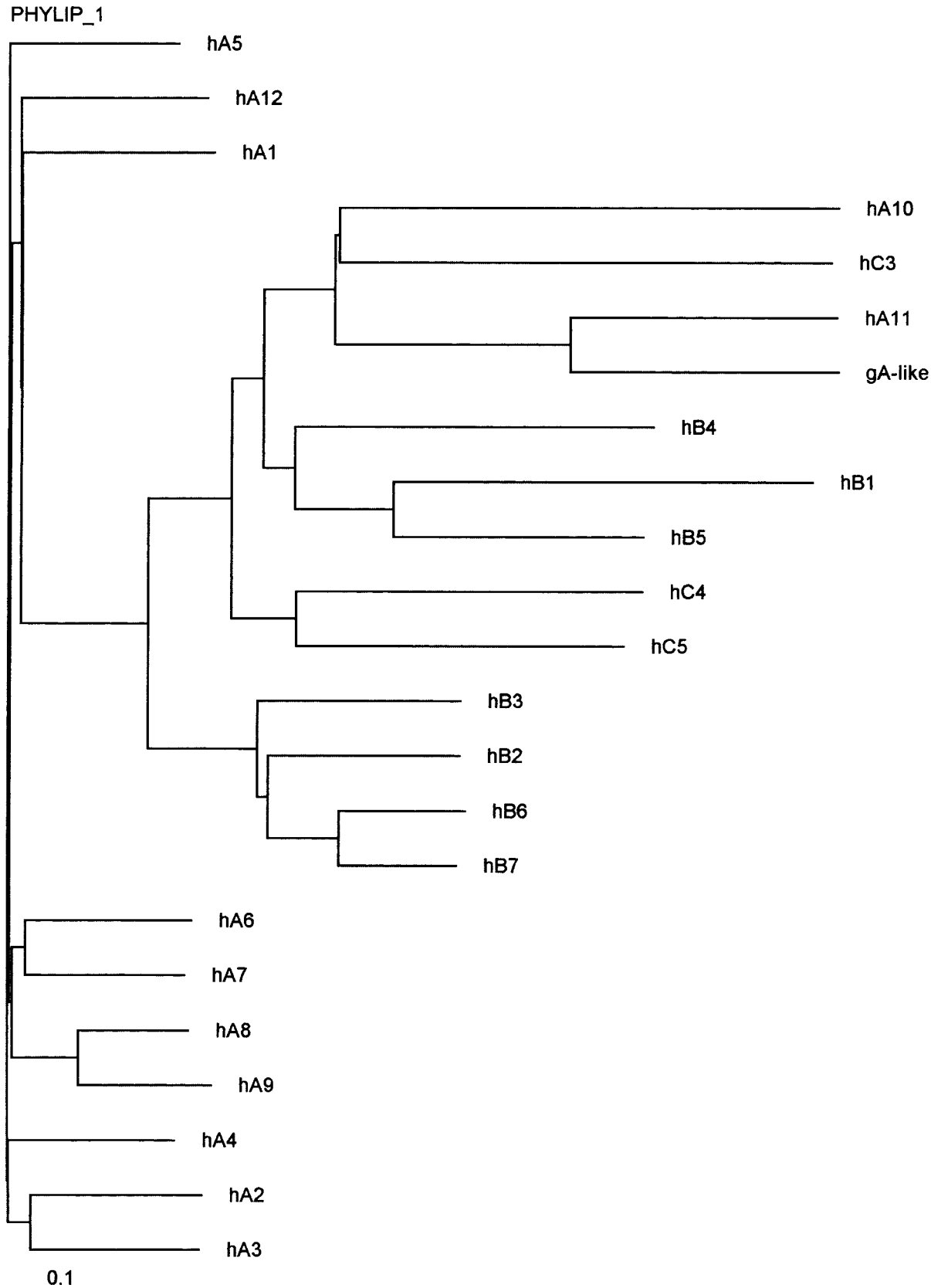
DISCUSSION

In the present study, we report a novel protocadherin expressed in the developing chick embryo. Although an unpublished chick γC3 orthologue (BAA20457) has previously been submitted directly to GenBank, to the best of our knowledge this is the first reported characterization of a γA-like protocadherin in this species.

Sequence analysis of the cDNA described in this report suggested assignment to the protocadherin γA subfamily [Wu and Maniatis, 1999] and its cadherin-like extracellular domain was most similar to human protocadherin γA11. The first five cadherin-like ectodomains contained features largely conserved in other γA subfamily members, suggesting that the repetitive motifs in the chick protocadherin also correspond to the characteristic structural folding unit of the extracellular domain of the molecule as suggested for other cadherin superfamily members [Overduin et al., 1995; Shapiro et al., 1995]. Interestingly, much of the EC6 domain, which lacks the typical cadherin-repeat motif seen in mammalian members of this family, is missing in this chick γA-like protocadherin. It is not known at present whether this is a normal feature of the chick γA subfamily or whether it represents an alternatively processed variant. As only a single RNA transcript was predominant in tissues examined, the latter possibility appears less likely, although nothing is yet known about alternatively expressed chick protocadherin isoforms. An additional notable feature of the extracellular domain of the chick sequence was the presence of two putative N-glycosylation sites that could potentially bear the galactose-*N*-galactosamine disaccharide recognized by the PNA lectin.

A high degree of conservation with the human γA subfamily was seen in the putative cytoplasmic domain of this chick γA-like protocadherin, which also ended with the characteristic lysine-rich motif. This finding further supports the observation of Wu and Maniatis [1999] that all proteins of the same protocadherin family have a high degree of cytoplasmic identity. This was found to be true in comparison of the chick γA-like and chick γC3 sequences, which shared 100% identity over the terminal 120 amino acids. The identical composition over this cytoplasmic sequence range is not unexpected as previously described mammalian protocadherin γ families

Fig. 4. Phenogram of chick γA-like and known human γA, B, and C subfamily protocadherins. The tree was constructed with the PHYLIP program through comparison of the first five extracellular domains of each protocadherin by the neighbor-joining method. The chick γA-like protocadherin is most similar to human γA11 (NP061737). Bar indicates 0.1 substitutions per site.


PHYLIP_1
hA5
hA12
hA1
hA10
hC3
hA11
gA-like
hB4
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hB5
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hC5
hB3
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Fig. 4.

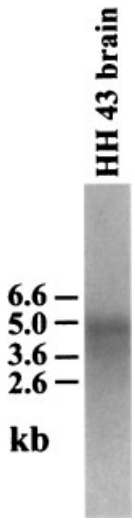

Fig. 5. Northern blot analysis of chick γA-like protocadherin detects a 4.6 kb transcript. Total RNA was isolated from stage 43 chick brain (HH 43 brain) and 10 μg loaded per lane. The membrane was hybridized with probe derived from the chick protocadherin cDNA encoding the extracellular domain.

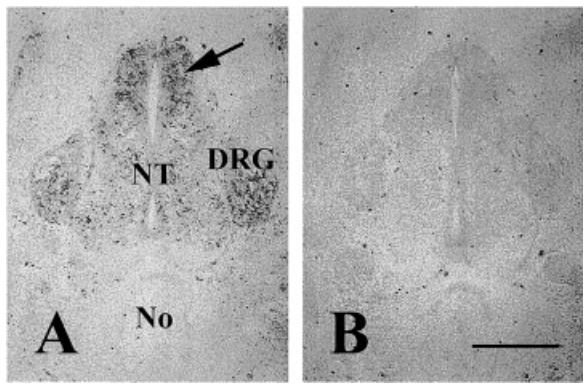

Fig. 6. Expression of γA-like protocadherin mRNA in cross sections of the stage 27 chick neural tube (NT) and dorsal root ganglia (DRG). **A**: The dorsal aspect (arrow) of the NT and DRG show strong hybridization with anti-sense probe. **B**: Tissue section of this same region of the stage 27 chick shows little or no hybridization with sense probe. No, notochord. Scale bar = 250 μm for both panels.

consist of one exon encoding extracellular, transmembrane, and part of the cytoplasmic domains followed subsequently by three small, constant exons that conclude the carboxy terminal portion of the cytoplasmic domain [Wu and Maniatis, 1999]. Up-stream of the identical cytoplasmic region, there was limited homology between the two chick sequences and similarity was overall less than that observed between the chick γA-like and human γA subfamily. This limited homology (41% identity) between the chick γA-like and the chick γC3 cDNAs in peptide sequence upstream of the cytoplasmic domain further supports identification of the γA-like clone as a novel chick protocadherin.

Of particular interest was the preferential expression of γA-like transcripts in the developing chick nervous system, which correlated with increasing neural complexity and organization. Expression of γA-like transcripts was noted at stage 27 in both dorsal root ganglia and dorsal spinal cord at the corresponding spinal level. At this developmental time point, the dorsal horn and dorsal root ganglia are well populated with interneurons and sensory neurons, respectively [Romanoff, 1960], and it is interesting to speculate that γA-like protocadherins may help facilitate organization of the neural network responsible for integration of sensory information between these neuronal populations. Alternatively, in mutant mice lacking protocadherin γ family members it was demonstrated that early stages of axonal outgrowth and synapse formation were not adversely affected, but that neurodegeneration occurred due to loss of spinal interneurons [Wang et al., 2002], suggesting that γ protocadherins may be required for the survival of this specific neuronal suppopulation. Expression of γA-like protocadherin in the dorsal neural tube of the chick opens interesting avenues for future exploration of its role in the developing spinal cord.

In agreement with Northern analysis, in situ hybridization showed that γA-like mRNA was also expressed in the late stage chick brain. Interestingly, at this time the predominant signal was in the mesencephalon, located in discrete strata of the optic lobe. By processing visual information and facilitating its transfer to other parts of the central nervous system, the optic lobe represents a particularly highly developed region of the chick brain and reflects the high degree of neural organization responsible for avian visual acuity [Gunturkun, 1991]. In mammalian species examined thus far few reports have cited protocadherin γ family localization in midbrain structures, but protocadherin 2A (γC3) mRNA has been localized in the roof plate of the cerebral aqueduct in the mouse [Hirano et al., 2002]. Expression of γA-like transcripts in neuronal laminae of the chick optic lobe at stage 43 correlates with a developmental period of extensive axonal growth and

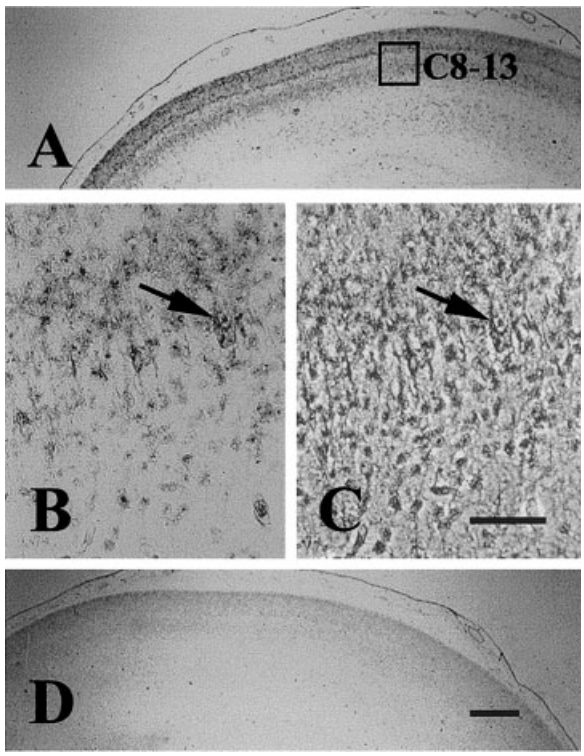

Fig. 7. Expression of γA-like protocadherin mRNA in sagittal sections of the stage 43 chick brain. **A**: Strong hybridization with anti-sense probe is located within laminae of the optic lobe. Boxed area outlines Cajal layers 8–13 (C8–13) shown in panel B. **B**: Higher magnification of boxed area in panel A illustrating anti-sense probe hybridization associated with large ganglion cell neuron (arrow). **C**: Phase contrast image of panel B. **D**: Tissue section of this same region of the stage 43 chick brain shows little or no hybridization with sense probe. Scale bars = 25 μm, panels B & C. 250 μm, panels A & D.

synapse formation [LaVail and Cowan, 1971; Pereya-Alfonso et al., 1997] and suggests that this novel protocadherin may participate in establishment of proper neural organization in this specific brain compartment. It will be interesting to determine what other protocadherins are expressed in this same region and to assess their potential role in facilitating the many different adhesive interactions that must be orchestrated for successful construction of the chick central nervous system.

Whether this novel member γA-like member of the protocadherin family functions to mediate cell:cell adhesion during neuronal organization or provides a mechanism for ensuring synaptic plasticity during formation of neural projections [Frank and Kemler, 2002] as hypothesized for other clustered protocadherins is not yet known. Work is currently under way to assess further the spatial and temporal expression of this novel γA-like protocadherin in the embryonic chick and to examine its function during neural development.

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