Innervation and Target Tissue Interactions Induce Rab-GDP Dissociation Inhibitor (GDI) Expression during Peripheral Synapse Formation in Developing Chick Ciliary Ganglion Neurons *In Situ*

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Regulated exocytosis of neurotransmitter from synaptic vesicles involves the function of a small GTP-binding protein, Rab3A. Rab-GDP dissociation inhibitor (GDI) is an important modulator of Rab function and subcellular distribution. We have characterized the respective roles of innervation and target tissue interactions in regulating GDI expression during synapse formation in chick ciliary ganglion (CG) neurons developing *in situ*. Here we report the first full-length chick GDI cDNA sequence. It is highly homologous to mammalian GDI isoforms and includes all of the sequence-conserved regions critical for Rab3A binding. This chick GDI mRNA is predominantly expressed in neurons as judged by Northern blot analysis of tissue distribution and by *in situ* hybridization of CG sections. Developmental increases in CG GDI mRNA levels occur in two phases as determined by reverse transcription (RT)–PCR and by Northern analysis of both normal-developing and input- or target tissue-deprived ganglia. The initial phase appears to be independent of cell–cell interactions. In contrast, the second, larger increase is induced by both presynaptic inputs and postganglionic target tissues but does not occur until target tissue innervation. Synaptic interaction with the target seems necessary for the regulatory response to both inputs and target tissues. GDI protein levels show similar changes. The developmentally delayed ability of inputs and targets to influence GDI levels differs from the regulation of neurotransmitter receptor expression in CG neurons. These results suggest that distinct extrinsic regulatory signals influence the expression of synapse-related components at the presynaptic axon terminal versus postsynaptic membrane in an individual neuron.

Key words: chick Rab-GDP dissociation inhibitor (GDI); embryonic chick ciliary ganglion; neuronal synapse formation; neuron-specific gene expression; induction; regulation; innervation; target tissue interactions; presynaptic terminal differentiation; synaptic vesicle exocytosis

Synapses are specialized contact sites between neurons and their targets that function in intercellular communication. *In vivo* cellular and molecular mechanisms that regulate neuronal synapse formation are primarily undefined. Neurons engage in two distinct types of synaptic interactions; they receive innervation and form synapses on target tissues. An individual neuron must differentiate both postsynaptic and presynaptic specializations that are composed of some similar, as well as numerous distinct, proteins (Apel and Merlie, 1995; Burns and Augustine, 1995; Sudhof, 1995). Synapse formation and maintenance depend on appropriate developmental expression of multiple genes and trafficking of the encoded proteins to the proper region, which is the specialized postsynaptic membrane of the soma and dendrites or the axonal presynaptic terminal. Expression of synapse-associated genes may be controlled by an intrinsic differentiation program as well as by extrinsic signals derived from the pre- and postsynaptic tissues.

The chick parasympathetic ciliary ganglion (CG) is uniquely well-suited for defining the separate roles of inputs and target tissues in regulating neuronal gene expression during synaptogenesis *in vivo* because of the feasibility of surgical manipulations that selectively prevent these interactions. Taking this approach, we have shown that innervation and target tissues have unique, as well as redundant, regulatory effects on the expression of a postsynaptic membrane component, nicotinic acetylcholine receptor (nAChR) subunits (Levey et al., 1995). nAChRs mediate excitatory synaptic transmission in the CG.

Retrograde signals from the target tissues may also influence presynaptic terminal differentiation. During normal development, increases in mRNA levels for several presynaptic proteins, including soluble *N*-ethyl maleimide-sensitive factor attachment protein 25, synaptotagmin I, synaptophysin IIa and IIb, Rab3A, and Rab-GDP dissociation inhibitor (GDI), correlate with axon terminal differentiation and target tissue innervation (Catsicas et al., 1991; Motoike et al., 1993; Lou and Bixby, 1995). These proteins function in the regulated exocytosis of synaptic vesicles. For example, GDI regulates the subcellular distribution and function of Rabs, a family of GTPases that regulate vesicular membrane trafficking (Pfeffer et al., 1995; Schalk et al., 1996). Rabs cycle between an active GTP-bound, specific membrane-associated form and an inactive GDP-bound cytosolic state. GDI forms a stable soluble complex with Rab-GDP. Thus, GDI plays an im-

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portant role in recycling Rabs between the cytoplasm and target membranes. In neurons, GDI recycles Rab3A, which associates specifically with synaptic vesicles (Fischer von Mollard et al., 1992). Rab3A regulates the efficiency of Ca^{2+}-triggered exocytotic fusion of synaptic vesicles with the presynaptic terminal membrane (Castillo et al., 1997; Geppert et al., 1997). Although mRNA levels of the indicated presynaptic proteins increase during peripheral synaptogenesis, the respective role of innervation and target tissues in regulating their expression is not known.

In this paper, we define the *in vivo* regulatory effects of inputs and target tissues on GDI expression in the embryonic chick CG. We report the first chick GDI cDNA full-length sequence and establish that this isoform is predominantly expressed in neurons. We show that both innervation and target tissues induce increases in GDI mRNA and protein levels, but at a later developmental stage of synapse formation than that at which the inductive effects on nAChRs, which function in the postsynaptic membrane, are seen.

MATERIALS AND METHODS

Chick embryos, staging, and surgical manipulations. White Leghorn embryonated chick eggs (Spafas, Norwich, CT) were incubated at 37°C in a forced-draft turning incubator until use. Embryos were staged according to the classification scheme of Hamburger and Hamilton (1951). Surgical micromanipulations to prevent preganglionic innervation or postganglionic target tissue interactions were performed as described in detail previously (Arenella et al., 1993; Dourado et al., 1994). Briefly, the sole source of presynaptic input, the accessory oculomotor nucleus, was ablated at embryonic day 4 (E4), or the developing optic vesicle, which contains the target muscles, was unilaterally removed at E2, all before synaptogenesis. To obtain ganglia deprived of both innervation and target tissues, we removed the developing eye at E2 and the preganglionic nucleus at E4 in the same embryo (Levey et al., 1995). To ensure the complete removal of pre- and postganglionic tissues, we dissected ganglia only from embryos lacking visible preganglionic connections to the CG and residual eye structures. The complete removal of all preganglionic neurons and the absence of aberrant innervation from other sources or intraganglionic contacts were established by paraffin histological examination of the brain of operated embryos, by immunocytochemical labeling with monoclonal antibodies to synaptic vesicle antigens, and by ultrastructural analysis (Engisch and Fischbach, 1992; Arenella et al., 1993). CGs from normal-developing, operated, and sham-operated embryos were dissected at selected stages of synapse formation, ranging from E4.5 to E19. CGs and other dissected tissues were immediately frozen on dry ice and stored at −80°C until use for reverse transcription (RT)–PCR or for Northern or Western blot analysis.

Isolation and sequencing of full-length chick GDI cDNA. Mouse GDI-2 primers (Shisheva et al., 1994) that target the coding region 5′-end were used to generate chick GDI cDNA by RT–PCR amplification of CG total RNA. The identity of the PCR product as GDI was confirmed by sequencing. Two cDNAs that are highly homologous to mammalian GDIs were isolated, having sizes of 373 and 975 bp. The 975 bp GDI cDNA contained the smaller 373 bp sequence at its 5′-end. The two products were obtained, instead of one, because of the unforeseen ability of the mouse antisense primer to anneal to two regions in the chick GDI sequence.

The larger GDI cDNA was used as a probe to screen a total of 500,000 plaques from a White Leghorn E18 chick CG cDNA library constructed in λgt10 (kindly provided by Dr. Tom Boyd, Ohio State University) by the use of standard procedures (Sambrook et al., 1989). Seven positive plaques were identified. They all showed the same insert size (2.3 kb) after PCR amplification with primers recognizing the phage polylinker sequence. The cDNA was subcloned (PCR 2.1 cloning vector; Invitrogen, San Diego, CA) as a full-length sequence and as smaller clones by taking advantage of convenient restriction sites. Sequence analysis was performed by using the dideoxy chain-termination method with SP6 and T7 promoter primers [Sequenase Version 2.0 DNA sequencing kit (Sanger et al., 1977)]. A full-length chick GDI sequence, including 5′- and 3′-untranslated regions (UTRs) (2.3 kb), was isolated (see Fig. 1).

To test for GDI isoforms, we rescreened the embryonic chick CG cDNA library at lower stringency (50°C as opposed to a washing temperature of 60°C) with a coding region probe [nucleotides (nt) 111–1457 of the chick GDI sequence]. The filters were then stripped and hybridized to a radiolabeled chick GDI 3′-UTR probe (nt 1555–1994). Nucleotide and predicted amino acid sequences were tested for homology to known sequences in the DNA and protein databases of the National Center for Biotechnology Information by Blast search.

Northern blot analysis. Total CG RNA was extracted by three different scaled-down guanidinium thiocyanate protocols (Feramisco et al., 1982; Chomczynski, 1993; Levey et al., 1995), separated by agarose gel electrophoresis (Sambrook et al., 1989), and transferred to nylon membrane (Zeta-probe) by alkaline blotting. Filters were hybridized with cDNA probes, which were labeled with [α-^{32}P]dCTP to high specific activity by random priming (Life Technologies, Gaithersburg, MD) and purified using Chroma Spin-100 columns (Clontech, Cambridge, UK). Hybridization and washing conditions were as specified by the membrane manufacturer (Bio-Rad, Hercules, CA). The coding region and 3′-UTR probes were identical to those used for the cDNA library screening. For quantitation, signals in the different lanes were compared by densitometric scanning (PDI densitometer; Protein DNA Image Ware Systems, Huntington Station, NY) and normalized to the signal obtained after stripping the filter and rehybridizing it with a chick 18S ribosomal cDNA (375 bp) probe.

Relative RT–PCR. In addition to Northern blot analysis, relative RT–PCR was used to measure GDI mRNA levels in total RNA extracted from CGs of normal-developing, operated, and sham-operated embryos, using an amount of RNA equivalent to that present in a single CG. Two different sets of specific primers were used to amplify different segments of the GDI coding sequence. The first specific pair (5′-primer, atgaatgaggagtacgacgtg; 3′-primer, CTGcagtggaaggaactttg) amplified a 367 bp sequence from the beginning of the coding region (nt 111–477). The second pair [5′-primer, CCGgatatctacgtctgcatg; 3′-primer, CCGTCTAGAttactgctgctcctcctc (with the sequence in capital letters improving primer efficiency and/or providing a convenient restriction site for cloning)] amplified a 357 bp sequence from the end of the coding sequence (nt 1101–1457). RT–PCR was done as described previously (Levey et al., 1995; Ikonomov and Jacob, 1996) with the exception of two modifications that were introduced to improve priming specificity. First, reverse transcription was performed using Superscript II RNase H-reverse transcriptase (Life Technologies) and a higher temperature (50°C instead of 42°C). Second, the PCR-annealing temperature was increased to 63°C. This optimized protocol resulted in a single band corresponding to the targeted-GDI sequence with either set of primer pairs. Amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. The identity of the PCR product as GDI was confirmed by restriction endonuclease mapping and by the sequencing of six minipreps.

To account for changes in neuron number caused by the surgical manipulations, we normalized GDI mRNA levels per CG to those of cβ4-tubulin mRNA. The levels of this neuron-specific transcript are not significantly affected by the absence of synaptic interactions (Levey et al., 1995). Aliquots of the same CG total RNA were simultaneously amplified with cβ4-tubulin–specific primers. Care was taken to remain in the linear phase of amplification for both sequences. Usually, 20 cycles were used for cβ4-tubulin, whereas 30 cycles were used to amplify GDI mRNA. For quantitation, PCR was performed in the presence of [α-^{32}P]dCTP, and the radiolabeled PCR products were separated on a 2% agarose gel. Visible bands, as well as corresponding areas of gel from control lanes (samples minus reverse transcriptase), were excised and transferred into liquid scintillator (5 ml), and their radioactivity was counted in a liquid scintillation analyzer (Tri-carb 2250CA; Packard, Meridian, CT). The ratio between the amount of GDI cDNA in the test versus control sample for chick GDI was normalized to the ratio for cβ4-tubulin.

In situ *hybridization.* To establish the cellular distribution of GDI mRNA in the CG, we processed E14–E19 ganglia for *in situ* hybridization as described previously (Boyd et al., 1988, 1991). The only modification is the use of digoxigenin-labeled cRNA probes instead of ^{35}S-labeled probes. Briefly, CGs were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, overnight at 4°C and were embedded in paraffin. Sections (8 μm thick) were cut on a Reichert-Jung Biocut microtome and mounted on glass slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma, St. Louis, MO). Sections were treated with proteinase K, acetylated with 0.25% acetic anhydride, dehydrated with ethanol, prehybridized, hybridized with probe at a concentration of 1–2 μg/ml, treated with RNase, and washed with SSC. Digoxigenin-labeled sense and antisense cRNA probes corresponding to either 213 bp of the 3′-UTR (nt 1555–1767) or 367 bp of coding region (nt 111–477) were synthesized using a linearized cloning vector containing the appropriate insert and SP6 or T7 RNA

polymerase binding sites, respectively, according to the recommended manufacturer's protocol (Genius 4 RNA labeling kit; Boehringer Mannheim, Indianapolis, IN). After RNase treatment and SSC washing, CG sections were preincubated with 10% normal goat serum (Sigma) for 1 hr and then incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Manheim) at a 1:500 dilution for 1 hr. To localize the bound alkaline phosphatase, we incubated sections overnight in nitroblue tetrazolium chloride (0.33 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate 4-toluidine salt (0.16 mg/ml) in 0.1 M Tris buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$ (Fields-Berry et al., 1992), rinsed the sections with 0.1 M PBS, followed by 0.01 M Tris and 0.02 M EDTA buffer, mounted the sections with coverslips, and viewed them by bright-field microscopy (Zeiss Axioskop).

Myc-tagged chick GDI protein expression in cos-7 cells. For expression studies of chick GDI protein, cos-7 cells (kindly provided by Dr. Alonzo Ross, Worcester Foundation for Biomedical Research) were transfected with myc-tagged GDI coding sequence. A myc tag (encoding the peptide epitope EQKLISEEDL) was attached to the 3′-end of the chick GDI coding sequence using PCR. PCR primers were also used to add restriction sites (*Eco*RI and *Xba*I to the 5′- and 3′-ends of the GDI-myc sequence, respectively) for cloning in PCMV5 transfection vector. In addition, the primers were designed to eliminate an *Eco*RV restriction site at the 3′-end of GDI by changing a single bp (nt 1435) from T to C, which does not create an amino acid change but reduces the number of restriction digestion fragments generated during construction of the myc-tagged GDI sequence. The presence of only the desired sequence changes in the PCR product was verified by sequencing. The GDI 5′-end fragment digested with *Eco*RI-*Hin*dIII and the myc-tagged 3′-end fragment digested with *Eco*RV-*Xba*I were ligated to the middle section of the chick GDI coding region, which was isolated by *Hin*dIII-*Eco*RV digestion of full-length GDI. The resulting construct was cloned into *Eco*RI-*Xba*I–digested PCMV5 vector.

Cos-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml gentamycin, in a humidified 5% CO_2 incubator at 37°C. For transfections, the cells, at a plating density of 1–2 × 10^5 per ml, were grown overnight in a six-well culture plate and then incubated with either 2 μg of plasmid DNA containing the chick GDI myc-tagged construct, chick GDI without the myc tag, or mouse GDI-2 myc-tagged construct (Shisheva et al., 1994) or of PCMV5 plasmid-lacking insert, together with 2 or 20 μl of Lipofectin Reagent (Life Technologies) in 1 ml of serum-free medium for 6 hr, according to the manufacturer's instructions. The medium was then replaced with 2.0 ml of normal growth medium, and the cells were harvested 60 hr later. The apparent electrophoretic mobility of the protein encoded by the chick GDI sequence was determined by Western blot analysis.

Western blot analysis. GDI protein levels in CGs from operated and control embryos were compared by immunoblotting. Frozen CGs were homogenized at 4°C in 20 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, 250 mM sucrose, and a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin), incubated for 5 min on ice, and centrifuged for 15 min at 16,000 × *g*. Aliquots of the cytosol fraction, set to correspond to an equal number of neurons (Levey et al., 1995), were solubilized in Laemmli buffer (Laemmli, 1970) and separated on 10% polyacrylamide gels by SDS-PAGE. After transfer to polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA), blots were saturated with blocking buffer using previously specified conditions (Shisheva et al., 1994) and were incubated with anti-mouse GDI or anti-myc antibodies. Anti-mouse GDI-2 rabbit polyclonal antibody (Shisheva et al., 1994) was used at a 1:10,000 dilution to detect chick GDI because 15 amino acids of the chick sequence are identical to the mouse 18 amino acid peptide (amino acids 387–404) used to generate the pAb. Mouse anti-myc mAb American Type Culture Collection (ATCC) 1729 (ATCC, Rockville, MD) (Munro and Pelham, 1988) was used to detect the myc-tagged GDI protein. After washing the blots with Tris-buffered saline containing 0.1% Tween-20, bound antibodies were detected using horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse IgG for GDI or myc primary antibody, respectively) and chemiluminescence (Renaissance kit; DuPont, Billerica, MA). Immunoreactivity was quantified by densitometric scanning of the protein bands, after selecting exposure times within the linear range of the x-ray film.

RESULTS

Chick GDI cDNA is highly homologous to mammalian GDI sequences in the coding regions but has unique 5′- and 3′-UTRs

The full-length chick GDI cDNA clone was isolated from an E18 chick CG cDNA library constructed in λgt10 (generously provided by Dr. Tom Boyd, Ohio State University). The probe used to screen the library was a 975 bp chick GDI cDNA generated by RT–PCR amplification of CG total RNA using mouse GDI-2 specific primers (see Materials and Methods) (Shisheva et al., 1994). A 2.3 kb clone was obtained. Subcloning and DNA sequencing identified a contiguous open reading frame of 1345 nucleotides, which is flanked by 110 nucleotides of 5′-untranslated sequence and 831 nucleotides of 3′-untranslated sequence (Fig. 1). The initiation codon is preceded by a consensus sequence that fulfills the criteria of a eukaryotic translation start site (Kozak, 1987). The coding region shows high homology (ranging from 75 to 82% identity at the nucleotide level) to the coding sequences of mammalian GDI isoforms (Table 1). In contrast, the 5′- and 3′-UTRs of chick GDI have no significant homology with the mammalian sequences. GDI is a highly conserved protein in eukaryotes from yeast to human, having been cloned and characterized in yeast, plants, nematode, fruit fly, and several mammalian species, including bovine, rodent, and human (Matsui et al., 1990; Zahner and Cheney, 1993; Garret et al., 1994; Nishimura et al., 1994, 1995; Sedlacek et al., 1994; Shisheva et al., 1994; Yang et al., 1994; Araki et al., 1995; Bachner et al., 1995; Janoueix-Lerosey et al., 1995; Zarsky et al., 1997). In mammals, up to three GDI isoforms have been characterized; these are GDI-1/α, GDI-2, and GDI-β. The isoforms are encoded by highly homologous but distinct genes. For example, in the mouse, GDI-β shows 75% sequence identity with GDI-1/α and 88% sequence identity with GDI-2 at the nucleotide level, but the isoforms have distinct 5′- and 3′-UTRs (Janoueix-Lerosey et al., 1995; D. Post and A. C. Shisheva, unpublished observations). In comparison, there appears to be only one predominant GDI isoform expressed in the embryonic chick CG. Using low-stringency conditions, we isolated 32 additional plaques by screening the CG cDNA library, and they all correspond to this isoform.

The protein predicted from the open reading frame of chick GDI consists of 448 amino acid residues, with a calculated molecular mass of 50,681 Da. Chick GDI is one amino acid longer than mammalian GDI-1/α and three amino acids longer than GDI-2 or GDI-β at the 3′-end of the coding region. At the amino acid level, chick GDI is 91–94% identical to mammalian GDI-2 and GDI-β and 87% identical to GDI-1/α (Table 1). Importantly, all of the amino acids that are critical for binding Rab3A [tyrosine, glutamate, arginine, threonine, and methionine at positions 39, 233, 240, 248, and 250, respectively (Schalk et al., 1996)] are conserved in chick GDI, further confirming its identity as GDI.

Chick GDI mRNA is predominantly expressed in neurons

Analysis of the tissue distribution of this chick GDI mRNA suggests it is a neuron-specific isoform. A single 2.3 kb band was observed when total RNA from E19 chick CG and brain was analyzed by Northern blotting with either a radiolabeled noncoding (3′-UTR) or coding region probe under high-stringency conditions (Fig. 2). Only weak signals were detected when the same amount of total RNA from E19 chick liver and skeletal muscle were blotted with the same probes. In comparison, two GDI

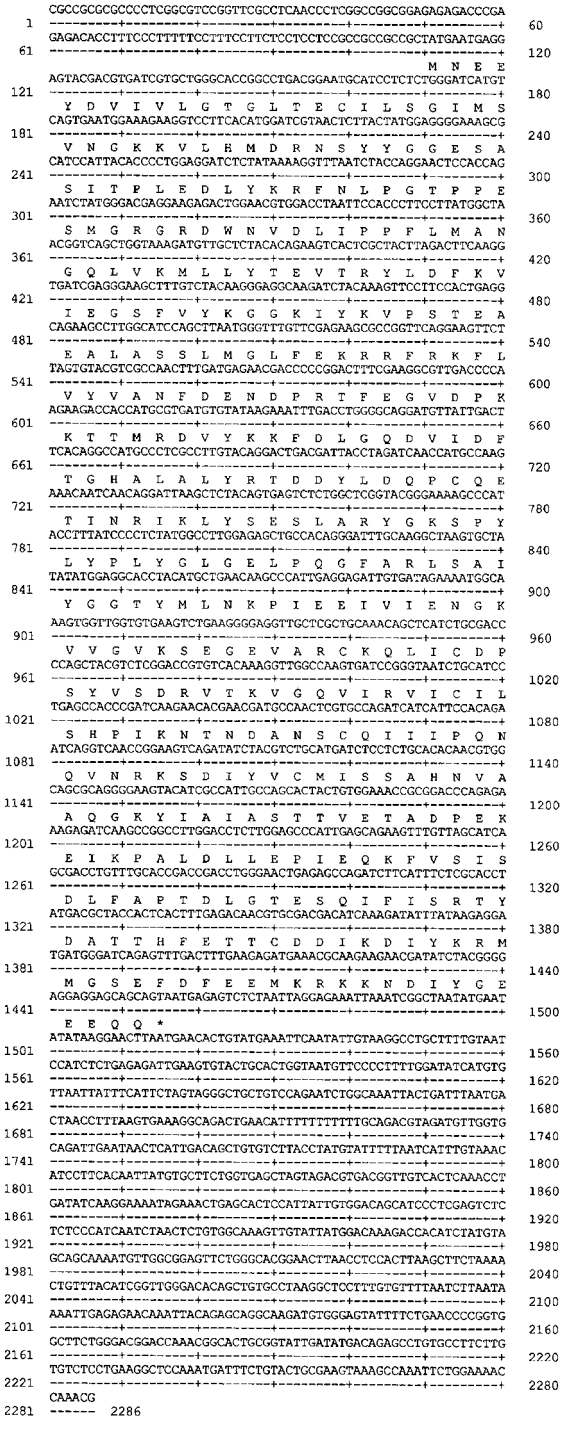

Figure 1. Nucleotide and protein sequences of chick GDI. Full-length chick GDI cDNA has a contiguous open reading frame of 1345 nucleotides. The deduced amino acid sequence is indicated *below* the codons. An *asterisk* denotes the 3′-terminal stop codon. The GDI cDNA encodes a protein of 448 residues, with a calculated, unglycosylated molecular mass of 50,681 Da. Chick GDI is one amino acid longer than mammalian GDI-1/α and three amino acids longer than GDI-2 and GDI-β at the 3′-end of the coding region. The chick sequence has been identified as belonging to the GDI gene family because of high homology with the coding regions of mammalian GDI isoforms (see Table 1) and the presence of the conserved amino acids essential for Rab3A binding [tyrosine, glutamate, arginine, threonine, and methionine at positions 39, 233, 240, 248, and 250, respectively (Schalk et al., 1996)]. In contrast, chick GDI 5′- and 3′-untranslated sequences (110 and 831 nucleotides, respectively) have no significant homology with mammalian sequences.

Table 1. Percent identity between chick GDI coding sequence and GDI isoforms from other species

		Percent identity	
Species	Type	Nucleotide	Amino acid
Mouse[3]	GDI-2	81%	94%
Mouse[4]	GDI-β	82%	93%
Human[1]	GDI-β	81%	93%
Rat[6]	GDI-β	81%	91%
Human[2]	GDI-1/α	78%	87%
Bovine[8]	GDI-1/α	77%	87%
Rat[7]	GDI-1/α	76%	87%
Mouse[5]	GDI-1/α	75%	85%
Drosophila[9]	GDI	66%	65%
Nematode[10]	GDI	63%	65%
Yeast[11]	GDI-1	N.A.	62%
Plant[12]	GDI	N.A.	49%

Accession numbers: 1, D13988; 2, X79353; 3, U07951; 4, L36314; 5, U07950; 6, X74401; 7, X74402; 8, D90103; 9, L03209; 10, U00002; 11, U18916; and 12, Y07961. N.A., Not available in database.

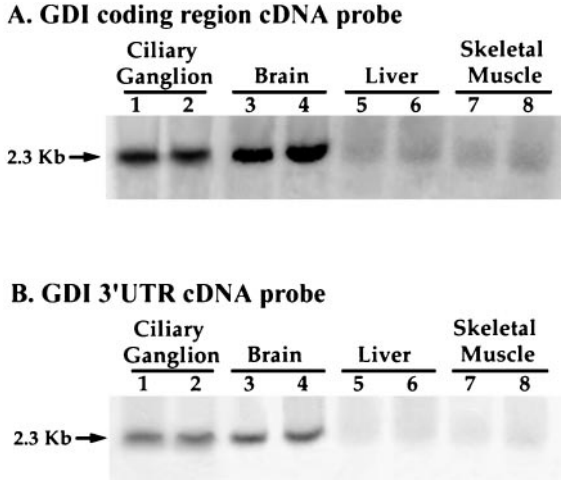

Figure 2. Chick GDI mRNA is predominantly expressed in neural tissue as established by Northern blot analyses. Duplicate samples of total RNA (20 μg per *lane*) extracted from E19 chick CG, brain, liver, and skeletal muscle were separated by gel electrophoresis, blotted, and hybridized with a [α-^{32}P]dCTP-labeled GDI coding region probe (960 bp; *A*) or 3′-UTR probe (440 bp; *B*) under high-stringency conditions. A single 2.3 kb band was detected in the CG and brain with both probes (*lanes* 1, 2, CG; *lanes* 3, 4, brain). Only a weak signal was detected in liver and skeletal muscle (*lanes* 5, 6, liver; *lanes* 7, 8, skeletal muscle). Ribosomal RNA was used to check that RNA levels were similar in all *lanes* (data not shown).

messages of different sizes were detected in rat brain by blotting with a bovine GDI coding region cDNA probe (Matsui et al., 1990; Nishimura et al., 1994).

In situ hybridization studies of the cellular localization of chick GDI mRNA demonstrate that this isoform is expressed in neurons of the embryonic CG (Fig. 3). Using digoxigenin-labeled antisense and sense cRNA probes corresponding to 213 bp of 3′-UTR or 367 bp of coding sequence, we hybridized E14–E19 CG paraffin sections and washed the sections under high-stringency conditions. For comparison, alternate serial sections were hybridized with a neuron-specific nicotinic cholinergic receptor α7 subunit cRNA probe, corresponding to 400 bp of coding sequence (Jacob and Berg, 1983; Couturier et al., 1990;

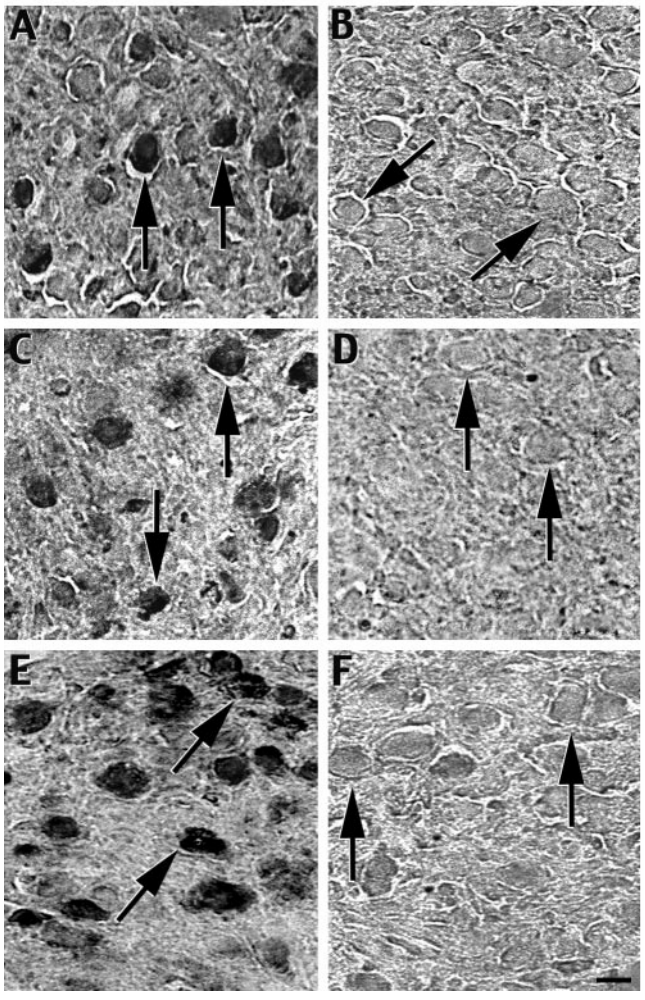

Figure 3. Chick GDI mRNA is present in ciliary ganglion neurons as determined by *in situ* hybridization. Paraffin sections of CGs from E14–E19 chicks were hybridized with digoxigenin-labeled antisense or sense cRNA probes, incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase, reacted for alkaline phosphatase staining, and examined by bright-field light microscopy. *A*, GDI coding region antisense cRNA probe. *B*, GDI coding region sense cRNA probe; *C*, GDI 3′-UTR antisense probe. *D*, GDI 3′-UTR sense probe. *E*, α7 coding region antisense cRNA probe. *F*, α7 coding region sense cRNA probe. The nicotinic cholinergic receptor α7 subunit antisense cRNA probe is included as a positive control for the localization of a neuron-specific mRNA in CG alternate serial sections. GDI and α7 have a similar distribution, with label being concentrated in neuronal cell bodies. α7 mRNA staining is stronger, suggesting that GDI mRNA is less abundant than α7 mRNA in CG neurons. Nonspecific labeling is low with all three sense probes (*B*, *D*, *F*). *Arrows* indicate neuronal somata. Scale bar, 20 μm.

Vernallis et al., 1993). GDI and α7 mRNAs have a similar distribution, with specific labeling being confined to neuronal somata (Fig. 3). However, staining intensity is weaker with GDI than with α7 antisense probes, as also observed with Northern blot analysis (data not shown), suggesting that GDI mRNA is less abundant than α7 mRNA in CG neurons. Altogether, these results suggest that there is one major isoform of GDI mRNA expressed in neurons of the avian CNS and PNS.

Presynaptic inputs and retrograde signals from the target tissues induce increases in GDI mRNA levels during, but not before, peripheral synapse formation

GDI transcript levels increase during pre- and postganglionic synapse formation in the embryonic chick CG, as determined by both RT–PCR with specific primers to the chick GDI coding sequence and Northern blot analysis with a 3′-UTR probe. For relative quantitation of GDI mRNA levels by RT–PCR, cβ4-tubulin mRNA, a form of β-tubulin that is exclusively expressed in neurons (Sullivan et al., 1986; Lee et al., 1990), was used as a standard. Both transcripts were amplified in the exponential range from aliquots of the same CG total RNA sample (Ikonomov and Jacob, 1996). GDI mRNA levels were normalized to that of cβ4-tubulin to account for reductions in neuron number during naturally occurring cell death and after surgical manipulations. cβ4-tubulin transcript levels are not altered in the absence as compared with the presence of synaptic interactions in age-matched CGs, as previously demonstrated (Levey et al., 1995).

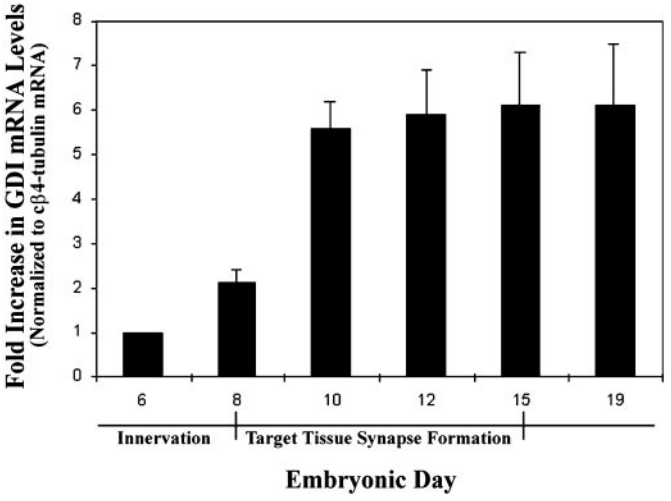

Figure 4. Histogram demonstrating developmental increases in ciliary ganglion GDI mRNA levels during neuronal differentiation and synapse formation. A histogram based on relative RT–PCR analyses of GDI mRNA levels in the chick CG at selected developmental ages ranging from E6 to E19 is shown. Two sets of specific primers that amplify different segments of the chick GDI coding sequence were used to measure GDI mRNA levels in total RNA equivalent to the amount present in a single CG. PCR was performed in the presence of [α-^{32}P]dCTP, and the radiolabeled PCR products were separated by agarose gel electrophoresis. The single band, which corresponded to the targeted GDI sequence (see text), was excised, and the level of radioactivity was established by liquid scintillation counting. E6 CG GDI mRNA levels are arbitrarily set at 1. Values are expressed as the mean ± SEM; $n = 4–7$ separate experiments for each age indicated. To account for changes in neuron number during normal development and naturally occurring cell death, we normalized GDI mRNA levels to transcript levels of cβ4-tubulin, a neuron-specific form of β-tubulin, which is not regulated by synaptic interactions (Levey et al., 1995). Similar changes were observed by Northern blot analysis with a GDI 3′-UTR probe after the amount of RNA was normalized for changes in neuron number (data not shown). GDI transcript levels increase during the period of synaptogenesis and then plateau.

During normal development, all ciliary ganglion neurons are functionally innervated by E8, with innervation beginning at E4.5 (Landmesser and Pilar, 1972; Jacob, 1991). CG neurons establish synapses with their target muscle tissues from E8.5 to E14 (Meriney and Pilar, 1987; Pilar et al., 1987).

GDI mRNA levels were barely detectable before synaptogenesis, at E4.5. Transcript levels increase 5.6-fold during neuronal differentiation and synapse formation, from E4.5 to E15, and then level off up to E19, the latest age examined (Fig. 4). The mRNA levels rise twofold from E4.5 to E7 (data not shown). The greatest developmental increase in GDI mRNA levels, 2.8-fold, was ob-

served between E8 and E10, coinciding with target tissue synapse formation and maturational changes in synaptic efficacy and morphology of the presynaptic inputs (Landmesser and Pilar, 1972). Similar results were found by Northern blot analysis when the data were normalized per neuron number (data not shown). Thus, innervation and target tissue interactions both seem to regulate GDI transcript levels in developing CG neurons.

To determine the respective roles of inputs and target tissues in inducing these developmental increases, we measured GDI mRNA levels in CG neurons deprived of synaptic interactions. To prevent innervation or target tissue interactions, we surgically removed the sole source of presynaptic inputs, the accessory oculomotor nucleus in the midbrain, or the developing optic vesicle, which contains the target tissues, respectively (Levey et al., 1995). Surgeries were performed before synaptogenesis and cause no direct damage to the neurons or transection of their processes. Neuron numbers are reduced because of the removal of sources of trophic support (Landmesser and Pilar, 1974; Furber et al., 1987). Importantly, surviving neurons are healthy based on ultrastructural and electrophysiological criteria and the demonstration of developmental increases in the levels of specific mRNAs and proteins (Engisch and Fischbach 1990, 1992; Arenella et al., 1993; Dourado et al., 1994; Levey et al., 1995). Input-deprived CG neurons form synapses on their target tissues, whereas innervation is established and maintained on target-deprived neurons (Landmesser and Pilar, 1974; Furber et al., 1987).

At E8, GDI mRNA levels are not significantly different in CGs deprived of either input or target tissues as compared with normal-developing control CGs (Fig. 5). In contrast, at E10, GDI mRNA levels are twofold lower in either input- or target-deprived neurons relative to age-matched control values. Interestingly, decreases in GDI mRNA levels in target-deprived CGs are already present at E9, whereas effects of input deprivation are delayed and not detectable until E10.

To test for additive effects, we prevented both innervation and target tissue interactions from forming in single embryos. GDI transcript levels are reduced fourfold at E10 (Fig. 5). In support of these quantitative RT–PCR results, similar declines in GDI mRNA levels were observed in three separate Northern blot experiments. CG total RNA from E10 normal-developing and operated embryos (n = 20–25 CGs per sample) was blotted with a GDI 3′-UTR probe, and the resulting signals were normalized to 18S ribosomal RNA content (Fig. 5).

These findings suggest that the increase in GDI transcript levels is independent of cell–cell interactions, up to E8. In contrast, the subsequent larger increase from E8 to E10 is dependent on the establishment of synaptic contact with the target tissue and the maintenance of presynaptic inputs.

Regulatory changes in GDI protein levels correlate well with GDI mRNA levels

The protein encoded by chick GDI mRNA seems to be the avian homolog of mammalian GDI-2 or GDI-β. At the amino acid level, chick GDI mRNA is 93% identical to these two mammalian isoforms and 87% identical to GDI-1/α (Table 1). Heterologous expression of myc-tagged chick GDI coding sequence in cos-7 cells results in a single band that has the same electrophoretic mobility as mouse GDI-2-myc, as determined by Western blot analysis with an anti-mouse GDI-2 polyclonal antibody (Fig. 6*A*) (Shisheva et al., 1994). Chick GDI and mammalian GDI-2 proteins have an apparent size of 46 kDa. In contrast, mammalian GDI-1 proteins are larger, 55 kDa, despite the small difference in

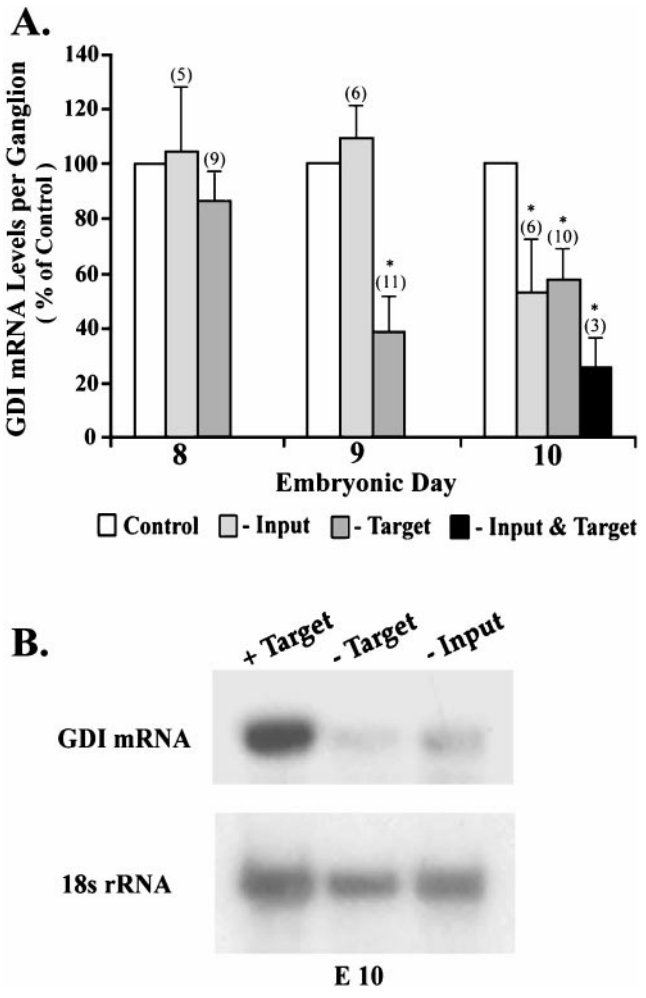

Figure 5. Innervation and target tissue interactions induce increases in ciliary ganglion GDI mRNA levels but only during peripheral synapse formation, as established by RT–PCR and Northern blot analyses. *A*, Histogram based on relative RT–PCR analyses of GDI mRNA levels in individual CGs (see Fig. 4 legend) developing in the absence versus the presence of pre- or postganglionic synaptic interactions is shown. GDI mRNA levels in CGs deprived of target tissue because of its removal unilaterally are compared with the contralateral CG value that serves as an internal control. Input-deprived CGs are compared with age-matched normal-developing ganglia. GDI mRNA levels are not significantly different in normal-developing CGs as compared with that in contralateral control CGs. GDI transcript levels are normalized to cβ4-tubulin mRNA levels to control for decreases in neuron number caused by the surgeries. GDI mRNA levels in the control CG are arbitrarily set at 100%. Values are expressed as the mean ± SEM. The *numbers* in *parentheses above* the *bars* indicate the number of individual ganglia analyzed for each surgical manipulation. An *asterisk* indicates a statistically significant difference relative to controls based on Student's two-sided t test, $p < 0.05$. Declines in GDI mRNA levels in ganglia developing in the absence of the target tissue are seen at E9 and E10 relative to levels in contralateral control ganglia from the same embryo. Reductions in GDI mRNA levels in the absence of innervation are seen at E10 but not earlier. The greatest declines occur in the absence of both inputs and targets at E10, suggesting additive effects. *B*, Similar declines in GDI mRNA levels in E10 CGs from operated embryos were observed by quantitative Northern blot analysis with a 3′-UTR cDNA probe (440 bp). The amounts of total RNA loaded per *lane* were corrected to compensate for changes in neuron number caused by the operations (Levey et al., 1995). For normalization, the same filter was stripped and reprobed with a [^{32}P]dCTP-labeled 18S ribosomal cDNA probe (375 bp). *Left lane*, Contralateral control CGs; *middle lane*, target tissue-deprived CGs from the same embryo; *right lane*, input-deprived CGs from age-matched embryos.

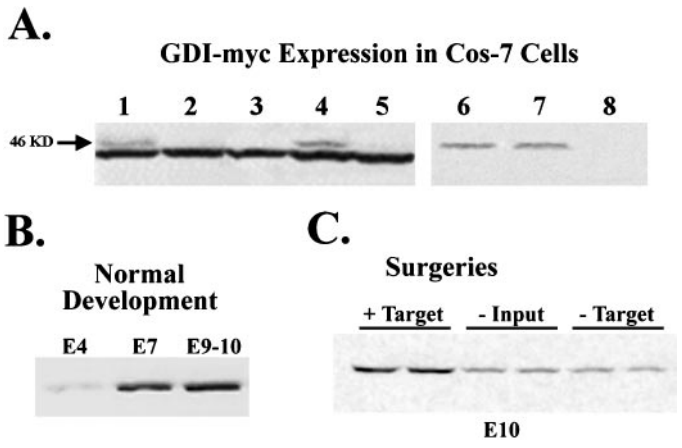

Figure 6. Regulatory changes in GDI protein levels resemble GDI mRNA levels in the ciliary ganglion as determined by Western blot analysis. *A*, The identity of the chick GDI coding sequence was further confirmed by Western blot analysis. Cos-7 cells were transfected with either myc-tagged chick GDI cDNA or myc-tagged mouse GDI-2 subcloned into pCMV5. Cytosolic fractions of the transfected cells were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-mouse GDI-2 polyclonal antibody (Shisheva et al., 1994). For both constructs, a single 46 kDa band was observed. Shown are extracts from cos-7 cells transfected with chick GDI myc-tagged (*lane 1*), mouse GDI-2 myc-tagged (*lane 4*), chick GDI lacking the myc tag (*lanes 2, 5*), and pCMV5 transfection vector lacking insert (*lane 3*). The *lower band* in all *lanes* is the endogenous cos-7 cell GDI and shows similar amounts of protein were loaded in all *lanes*. Myc-tagged GDI (*lanes 1*, *4*) runs slightly higher than the endogenous GDI. A single band of the expected size (*lanes 6*, *7*) is detected by immunoblotting with an anti-myc mAb (ATCC 1729); pCMV5 transfection vector lacking insert is shown in *lane 8*. *B*, Immunoblotting shows increases in GDI protein levels in the normal-developing CG during synapse formation. Protein extracts of CGs at selected developmental ages ranging from E4 to E10, adjusted to correspond to equal numbers of neurons, were analyzed by Western blotting with anti-GDI-2 antiserum and chemiluminescence detection. *C*, GDI protein levels are lower in CGs developing in the absence of input (*lanes 3*, *4*) or targets (*lanes 5*, *6*) compared with that in contralateral control CGs (*lanes 1*, *2*) at E10.

their deduced molecular mass compared with that of GDI-2 proteins (Shisheva et al., 1994). Moreover, a single band of the expected size (46 kDa) was detected in CG protein extracts by Western blot analysis with the anti-mouse GDI-2 polyclonal antibody (Fig. 6*B*).

To compare regulatory changes in GDI protein and mRNA levels during normal development and after surgical manipulations, we analyzed aliquots of CG protein extracts corresponding to equal numbers of neurons. GDI protein levels increase fourfold from E4.5 to E9–E10, resembling the 5.6-fold increase in GDI mRNA levels (Fig. 6*B*). Moreover, GDI protein levels are decreased twofold in E10 CGs that have developed in the absence of innervation or target tissues (Fig. 6*C*), correlating well with the twofold declines observed in GDI mRNA levels by both RT–PCR and Northern blot analysis. These quantitatively similar changes in GDI protein and mRNA levels in developing CG neurons suggest that the regulation of GDI expression occurs at the level of gene transcription.

DISCUSSION

Major findings reported here are (1) the identification of a chick neuron-specific Rab GDI mRNA and (2) the regulation of expression of this mRNA by both innervation and target tissues after the onset of peripheral synapse formation in CG neurons. Developmental increases in CG GDI mRNA levels occur in two phases. The first phase appears to be independent of cell–cell interactions and occurs as the neurons differentiate. The second larger increase is induced by synaptic interactions. Innervation precedes target tissue synapse formation in CG neurons (Landmesser and Pilar, 1972; Jacob, 1991). However, the ability of inputs to regulate GDI expression is developmentally delayed and correlates with the onset of target tissue innervation. Target-induced increases in GDI expression also occur at this time, slightly preceding the regulatory response to input. These results demonstrate that retrograde signals from the targets initiate a sequence of molecular events that are required to achieve mature levels of functionally important presynaptic terminal components. Thus, target tissue plays a major role in controlling the differentiation and function of the presynaptic inputs that drive it.

Neuron-specific GDI is the predominant isoform expressed in the chick ciliary ganglion

The chick GDI clone we have isolated and characterized has similar features to all three mammalian GDI isoforms. The mammalian isoforms differ from one another in their apparent size and distribution. GDI-1/α, a 55 kDa protein, is predominantly expressed in the nervous system, whereas GDI-2 and GDI-β, 46 kDa proteins, are ubiquitously expressed (Nishimura et al., 1994; Bachner et al., 1995). Based on size and sequence, chick GDI closely resembles GDI-2 and GDI-β, having an apparent electrophoretic size of 46 kDa and sharing 93% sequence identity with these isoforms, compared with 87% identity with GDI-1/α at the amino acid level (Table 1). However, similar to GDI-1/α, chick GDI mRNA is predominantly expressed in neurons, based on *in situ* hybridization and Northern blotting. Moreover, the chick GDI sequence includes all of the amino acids that are essential for binding Rab3A, a neuron-specific protein that is associated with synaptic vesicles (Fig. 1) (Schalk et al., 1996).

Based on the existence of multiple mammalian GDI isoforms, we looked for additional GDI isoforms in the chick CG. This is an important issue for our studies on the regulation of GDI expression in these neurons. All of the data suggest that we are studying the expression of a single gene. All 33 GDI clones isolated by low-stringency screening of the embryonic CG cDNA library correspond to this isoform. Northern blotting with two different GDI probes from the coding region and a unique 3′-UTR has revealed a single band of identical size and similar tissue distribution, showing a strong signal in the chick CG and brain but only a weak signal in skeletal muscle and liver. There is a strong correlation between quantitative measurements of GDI mRNA levels in the CG using RT–PCR with specific primers to the coding sequence compared with Northern analysis with a GDI 3′-UTR probe. GDI coding and noncoding region cRNA probes show a similar neuron-specific distribution in *in situ* hybridization. Finally, a single protein band is observed in CG extracts by immunoblotting. Taken together, these results show that there is only one major GDI isoform expressed in embryonic CG neurons.

Preliminary data suggest the presence of a distinct GDI isoform in other chick tissues. Southern blotting of chick genomic DNA with a cDNA probe (370 bp) from the chick GDI coding sequence 5′-end revealed the existence of another hybridizing band. RT–PCR amplification of liver RNA with chick GDI specific primers produced a cDNA fragment that has 83% sequence identity to the neuronal GDI isoform. Thus, at least two GDI isoforms exist in chick.

GDI expression in ciliary ganglion neurons is regulated by synaptic interactions but only after the onset of target tissue innervation

Developmental increases in GDI expression in the CG occur in two phases. The first increase occurs during neuronal differentiation, from E4 to E8, and is independent of synaptic interactions. In contrast, the subsequent larger increase is induced by both inputs and target tissues during peripheral synapse formation, from E8 to E14. Similarly, GDI-α mRNA is not detectable in proliferating neuronal precursor cells of the mouse embryo, as determined by *in situ* hybridization (Bachner et al., 1995). GDI-α mRNA is expressed later in differentiated neurons of the embryonic and adult mouse CNS and PNS, with the highest levels present in regions of greatest synaptic activity. We report in this paper the separate role of innervation and target tissues in regulating GDI expression in neurons.

GDI mRNA and protein levels are not altered in CGs, up to E8, when surgical manipulations are used to prevent presynaptic innervation or retrograde signaling from the immature target tissues. However, after E8, GDI mRNA and protein levels are reduced to a similar extent when the neurons are prevented from establishing synaptic contact with their targets. Declines in GDI levels in the absence of inputs occur slightly later, at E10. The greatest decrease in GDI expression occurs in CG neurons deprived of both types of synaptic interaction.

These results demonstrate that the physiological target tissue is necessary, but not sufficient, to induce mature GDI levels in neurons. Regulatory signals from the presynaptic input are also needed. However, inductive effects of the inputs are developmentally delayed and seem to require innervation of the postganglionic targets and/or maturation of the preganglionic inputs. Synaptic contact with the peripheral target tissues may provide retrograde signals that induce the expression or release of unique regulatory factors from the presynaptic inputs. Alternatively, changes in the CG neurons after peripheral synapse formation may be necessary for the cells to respond to regulatory signals from the inputs. Precedence exists for CG neuron dependence on a target-derived factor before response to other regulatory signals. A 50 kDa factor from the eye, which contains the CG target tissues, is required for the neurons to respond to cAMP, which, in turn, causes an increase in the number of functional nAChRs (Margiotta et al., 1987; Halvorsen et al., 1991). Interestingly, responsiveness to cAMP is seen only after E10, coinciding with the time when inputs regulate GDI expression (Margiotta and Gurantz, 1989). Synaptic activity is a candidate for a presynaptic signal that regulates GDI mRNA levels. Greater synaptic activity is likely in CG neurons at the later embryonic stages, from E10 and up, because of cAMP-induced increases in the number of functional nAChRs, as well as increases in the number of synaptic vesicles in presynaptic terminals, in the number of nAChRs in the postsynaptic membrane, and in the relative abundance of high conductance to low conductance nAChRs (Margiotta and Gurantz, 1989; Jacob, 1991). Based on its time course, the regulatory effect of the target tissue on GDI expression seems to be contact-mediated or attributable to a factor released by synaptic transmission. The specific factors that mediate the regulatory effects of inputs and targets on GDI levels and the molecular mechanisms of their action remain to be determined. Our results demonstrate that both inputs and target tissues have important inductive effects on GDI expression, and these regulatory changes occur during peripheral synapse formation.

The expression of presynaptic terminal proteins is regulated differently from that of postsynaptic membrane components within a single neuron

Similar to expression of GDI, the expression of other presynaptic terminal proteins seems to be regulated by target tissue synapse formation. Increases in mRNA levels of SNAP-25, synapsin I, synaptotagmin I, and synaptophysin IIa and IIb correlate well with the time of target tissue synaptogenesis (Catsicas et al., 1991; Zurmohle et al., 1994; Lou and Bixby, 1995). A direct demonstration of the regulatory role of the target was obtained by showing specific declines in synaptotagmin I mRNA in chick spinal motoneurons deprived of their targets by limb bud removal (Campagna et al., 1997). Together with our findings, these data suggest that there is a program of presynaptic terminal differentiation that is stimulated by target tissues during peripheral synapse formation, thereby coordinating the expression of particular presynaptic terminal proteins. Our results demonstrate that inputs also induce the expression of synaptic terminal proteins in the neurons being contacted, but only after the innervated neurons have established connections with their own peripheral targets. Thus, target tissue plays a major role in activating molecular events that are required to achieve the mature functional state of its innervating presynaptic terminals.

Importantly, the regulation of GDI expression differs from that of nicotinic cholinergic receptors in CG neurons. Increases in receptor subunit mRNA levels induced by inputs and target tissues occur earlier, during preganglionic innervation, and before, as well as during, synapse formation with target tissues (Brumwell et al., 1995; Levey et al., 1995). These temporal differences in the induction of GDI and nicotinic cholinergic receptors suggest that distinct extrinsic factors and/or intracellular mechanisms regulate the differentiation of the two types of synaptic specializations formed within an individual neuron, the axonal presynaptic terminal and the somatodendritic postsynaptic membrane.

Our data demonstrate the complexity of regulatory events that influence neuronal synapse formation and the impressive responsiveness of neurons to signals provided by their synaptic partners. Innervation and target tissues act in a developmentally regulated, combinatorial manner to control the expression of synapse-related genes in neurons developing *in vivo*.

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