Developmental Expression of TERM-1 Glycoprotein on Growth Cones and Terminal Arbors of Individual Identified Neurons in the Grasshopper

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The chemoaffinity theory postulates the existence of cell-specific molecular signals that uniquely identify individual developing neurons. Such molecules are thought to promote both accurate axon outgrowth and the formation of correct synaptic connections. To identify candidates for such neuron-specific recognition molecules, we generated monoclonal antibodies that recognize surface-associated antigens expressed by individual identified neurons in the grasshopper embryo. Here we report on a molecular label that is expressed exclusively by two pairs of sibling interneurons in the developing CNS. Our experiments indicate that during axogenesis, this molecule is expressed at the surface of the growth cones of these cells, while during subsequent synaptogenesis, it becomes concentrated at the cells' developing terminal arbors. In both cases the molecule appears to be secreted by the labeled structures. This molecule, which we call TERM-1, is a glycoprotein with a molecular weight of approximately 48 kDa. The highly restricted spatiotemporal expression pattern of TERM-1 implies that individual developing neurons can acquire and retain unique molecular labels that may be important for neuron-specific outgrowth and target recognition.

[Key words: process outgrowth, growth cone, axogenesis, synaptogenesis, identified neurons, chemoaffinity, insect, TERM-1, sprouting]

One of the major questions in neurobiology concerns the molecular mechanisms that are responsible for the formation of precise neuronal connectivity during embryonic development. A number of investigations on axonal pathfinding, neuronal recognition, and synapse formation and maintenance indicate that a multiplicity of molecular signals is involved in specifying circuit formation (Goodman et al., 1984; Dodd and Jessel, 1988; Harrelson and Goodman, 1988; Patterson, 1992). These signals include cell surface molecules on outgrowing neurites and on glia, molecules localized in the extracellular matrix, and factors that are secreted by growth cones during neurite outgrowth and by developing terminals during synapse formation (Jessel, 1988; McMahan and Wallace, 1989; Sanes, 1989; Grenningloh et al., 1990; Reichardt and Thomaselli, 1991).

The biochemical and cell biological analysis of many of these cell surface and diffusible recognition molecules in invertebrates and in vertebrates has led to a marked resurgence of interest in the general concept of chemoaffinity (Sperry, 1963, 1965; Hunt and Cowan, 1990). According to this concept, individual developing neurons are thought to acquire and retain unique cell-specific molecular signals that promote both accurate axon outgrowth and the formation of correct synaptic connections. With the advent of hybridoma technology to the field of neurobiology (Barnstable, 1980; Zipser and McKay, 1981), numerous molecular labels with a restricted regional expression in the nervous system have been identified. New molecules of this type, as well as their encoding genes, have also been discovered using P-element-mediated enhancer detection in *Drosophila* (O'Kane and Gehring, 1987; Bier et al., 1989; Bellen et al., 1990). In the developing insect nervous system, molecules that label the axons of subsets of identifiable developing neurons have been described (Bastiani et al., 1987; Patel et al., 1987; Snow et al., 1988; Zinn et al., 1988; Bieber et al., 1989), and some of these molecules have been shown to be important in axonal pathfinding and synapse formation (Grenningloh et al., 1991; Halpern et al., 1991; Nose et al., 1992). However, despite the prevailing influence of the chemoaffinity theory, neuron-specific molecules that selectively label the growth cones and the developing synapses of individual identified neurons in the CNS have not yet been described.

Here we report the discovery of a molecular signal that is expressed exclusively by two pairs of identified interneurons in the developing CNS of the grasshopper. Our experiments indicate that during axogenesis the molecular signal accumulates at the growth cones of these cells while during subsequent synaptogenesis it becomes concentrated at the developing terminal arbors of these two neurons. This highly restricted spatiotemporal expression pattern suggests that the identified molecule, a glycoprotein called TERM-1, may play a role both in cell-specific axonal guidance and in cell-specific target recognition.

Materials and Methods

Antibody production. In order to search for candidate molecules that might specifically label the growth cones and developing synapses of individual neurons in the grasshopper embryo, we generated monoclonal antibodies (mAbs) against an enriched membrane fraction derived from isolated developing central nervous systems of embryos. For this, ventral nerve cords (1200 in total) comprising all thoracic and abdominal ganglia from 50–55% *Schistocerca gregaria* embryos (Bentley et al., 1979) were dissected in a solution of 3.7% formaldehyde in 10 mM PBS (pH 7.4) and fixed for 45–60 min. The nerve cords were washed three times for a total of 60 min in ice cold triethanolamine (TEA; 10 mM, pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml of each of the following protease inhibitors: antipain, leupeptin,

Received Aug. 10, 1992; revised Oct. 6, 1992; accepted Oct. 8, 1992.

We thank D. Hoessli for help in immunoprecipitation techniques and H. Schwarz and M. Dürrenberger for help with immunoelectron microscopy and laser confocal microscopy. We also thank C. S. Goodman for the anti-fasciclin antibody, D. Vonlanthen for excellent technical assistance, and K. Furukubo-Tokunaga for comments on the manuscript. This work was supported by the Swiss National Science Foundation.

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pepstatin, chymostatin, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and Na-*p*-tosyl-L-lysine-chloromethyl ketone. The tissue was then sonicated in the same TEA/PMSF/protease inhibitor cocktail, and subsequently centrifuged at $1200 \times g$ for 10 min at 4°C. The supernatant was stored on ice, and the pellet was resuspended in TEA/PMSF/protease inhibitor cocktail, sonicated, and centrifuged again. This procedure was repeated a second time and finally the three supernatants were combined and centrifuged at $100,000 \times g$ for 2 hr at 4°C. The pellet containing the enriched membrane fraction was resuspended in a small volume of PBS/PMSF, and aliquots were taken for quantitative protein determination (Lowry et al., 1951; Harlow and Lane 1988). This enriched membrane fraction was stored at -70°C.

Hybridoma techniques were carried out according to Harlow and Lane (1988). For immunization of BALB/c mice, three aliquots of the enriched membrane fraction were mixed with equal volumes of Freund's adjuvant and injected intraperitoneally at intervals of 4 weeks. The first three injections contained 570 μ g, 1080 μ g, and 610 μ g of total protein, respectively. The last injection (boost) was performed 5 d after the third injection and contained 320 μ g of total protein mixed in an equal amount of PBS instead of adjuvant. Three days later the spleen cells were fused with NS-2 myeloma cells. After an initial screening, monoclonal cell lines were obtained by the limiting dilution technique. The mAb 4G5 antibody (IgM) was further concentrated and purified from cell culture supernatant by ammonium sulfate precipitation and gel filtration using Sephacryl 200 HR (Pharmacia).

Screening of hybridoma cell lines. Hybridoma supernatants were screened immunocytochemically on whole-mount, 50–55% grasshopper embryos. After fixation for 45–60 min in 3.7% formaldehyde solution in 0.1 M PIPES (pH 6.95) with 2.0 mM EGTA and 1.0 mM MgSO₄ (PIPES-FA), the dissected embryos were washed 30 min with a solution of 0.025% Triton X-100 in 0.01 M PBS (pH 7.3–7.4) and preincubated first with a solution of 0.4% Triton X-100 and 0.2% bovine serum albumin in 0.01 M PBS for 30 min and then with a solution of 5% normal goat serum, 0.2% bovine serum albumin, and 0.4% Triton X-100 in 0.01 M PBS for 60 min. After these preincubation steps, the embryos were incubated in hybridoma cell culture supernatant diluted 1:1 with the last preincubation solution for 12 hr at 4°C. After washing the embryos in a solution of 0.025% Triton X-100 in 0.01 M PBS for 3 hr, they were incubated in a goat anti-mouse antibody conjugated with fluorescein isothiocyanate (GAM-FITC; Jackson ImmunoResearch Labs) diluted 1:300 with a solution of 2% normal goat serum and 0.025% Triton X-100 in 0.01 M PBS for 5 hr at room temperature. After several washes in this solution and in PBS, the preparations were cleared in a mixture of glycerol and PBS (9:1) with *p*-phenylenediamine added (Johnson and Nogueira Araujo, 1981) and viewed under FITC optics on a compound microscope.

Immunocytochemistry with mAb 4G5. Further characterization of mAb 4G5 staining was performed on either whole-mount preparations or serial sections. The procedure for staining whole-mount preparations with mAb 4G5 was identical to that used in the screening procedure with the exception that we used a final concentration of cell culture supernatant diluted 1:20 in blocking solution for the primary antibody incubation. Either peroxidase-conjugated goat anti-mouse antibody (GAM-PO) or GAM-FITC was applied as secondary antibodies at final concentrations of 1:250 in a solution of 0.025% Triton X-100 and 2% normal goat serum in 0.01 M PBS. Embryos labeled with GAM-FITC were washed repeatedly and then viewed under FITC optics, whereas GAM-PO-labeled preparations were processed for 3,3'-diaminobenzidine (DAB) staining under standard conditions (Bourne, 1983) and viewed under Nomarski optics. For whole-mount immunocytochemistry on larval ganglia, antibody incubation was preceded by collagenase/dispase (Boehringer Mannheim) incubation at 0.5 mg/ml in PBS for 2 hr at room temperature. Some experiments were performed on non-permeabilized whole-mount embryos where detergent was omitted in all steps, in order to restrict the mAb 4G5 labeling to the outer cell surface. Fluorescence signals were viewed with the appropriate filters on a Zeiss Axioskop compound microscope or a Zeiss confocal laser scanning microscope.

mAb 4G5 staining and staining for the neurotransmitter 5-HT were compared immunocytochemically on 55–60% embryonic whole-mounts using mAb 4G5 and a rabbit anti-5-HT antibody (Incstar Corp.) in double staining experiments. In these experiments, mAb 4G5 cell culture supernatant was diluted 1:10 and anti-5-HT antibody 1:250 in preincubation solution. After appropriate washing, a tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary antibody was diluted 1:100 directly into the GAM-FITC incubation solution

described above. After clearing, both fluorescence signals were viewed with the appropriate filters on a Zeiss Axioskop compound microscope.

Neuroblast pattern and cell lineage analysis. Neuroblast positions and cell lineage analysis within the developing brain were determined in 30–45% embryos using the toluidine blue staining method (Altman and Bell, 1973), which is relatively selective for neuroblasts. Cell lineage studies were also performed according to Shepherd and Bate (1990), using bromodeoxyuridine (BrdU) labeling to monitor DNA replication and cell division. For this grasshopper eggs were sterilized in sodium hypochlorite solution, washed, and dissected in Mitsunashi and Maramorosch insect medium (Sigma), supplemented with 10% normal goat serum, 150 μ l/liter 20-hydroxyecdysone (Sigma), 7.8 mg/liter bovine insulin (Sigma), and 5 ml/liter penicillin/streptomycin (Sigma) (complete MM medium). Single embryos were transferred into small plastic wells containing 10 mM BrdU in complete Mitsunashi and Maramorosch medium and incubated at room temperature on an orbital shaker for variable times. Thereafter, embryos were fixed in PIPES-FA and the BrdU incorporation visualized by DAB staining following the protocols given by Gratzner (1982) and Shepherd and Bate (1990). In some experiments, the DAB-stained embryos were subsequently subjected to mAb 4G5 and GAM-FITC immunohistochemistry as described above, and the mAb 4G5-stained cells viewed under fluorescence optics. This allowed colocalization of mAb 4G5 staining and anti-BrdU staining.

Immunoelectron microscopy. Embryos and first instars were dissected in a solution of 0.1 M PIPES-FA (pH 6.95), fixed for 1 hr at room temperature, and washed with PBS for another hour. After preincubation with a solution of 5% normal goat serum and 0.2% bovine serum albumin in PBS, the embryos were incubated in a 1:20 dilution of the primary antibody (mAb 4G5) at 4°C overnight. In one series of experiments the primary antibody used was a 1:1 mixture of mAb 4G5 and an anti-fasciclin II mAb. Thereafter they were washed several times in PBS and incubated in a 1:250 dilution of GAM-PO for 6 hr and then washed again with PBS. The peroxidase signal was visualized using 10 μ l/ml DAB stock, 20 μ l/ml NiCl₂ (1% solution), 1 mg/ml β -D-glucose, 0.3 mg/ml NH₄Cl, 2 μ l/ml glucose oxidase (Sigma), in PBS for 20 min at room temperature (Watson and Burrows, 1981; Raper et al., 1983). After washing for 1 hr the preparations were postfixed in 2% glutaraldehyde in PBS for 30 min at room temperature, washed again, incubated in 1.5% OsO₄ in PBS, and then washed and dehydrated in an acetone series. The prepared embryos were embedded in Spurr's resin. Ultrathin sections were then cut and mounted on Formvar-coated slot grids and viewed on a Zeiss EM 109 electron microscope without further staining.

Protein labeling and immunoprecipitation. To isolate and characterize the molecule recognized by mAb 4G5, we used a nonradioactive immunoprecipitation method based on the combined biotin labeling and enhanced chemiluminescence (ECL) detection method as described by Meier et al. (1992). Ventral nerve cords were individually dissected in PBS, pooled, and stored at -20°C. The thawed tissue was then washed twice in biotinylation buffer [10 mM Na-borate (pH 8.8), 150 mM NaCl, 0.2% NP-40, 1 mM EDTA, 1 mM PMSF and 1 μ g/ml of TPCK, chymostatin, antipain, leupeptin, pepstatin], homogenized with a Dounce in 500 μ l of the same buffer and finally extracted for 30 min on ice. After centrifugation at $15,000 \times g$ to remove insoluble material, the total homogenate was biotinylated using 250 μ g of D-biotinyl-L-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim) for 15–30 min at room temperature. The reaction was stopped by adding 25 μ l of 1 M NH₄Cl and 25 μ l of 1 M Tris-Cl, pH 8.0, and then desalted on a Sephadex G25 column previously equilibrated with an immunoprecipitation buffer of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM MgCl, 1 mM CaCl, 0.2% NP-40. The protein-containing eluate (about 2 ml) was complemented with PMSF, TPCK, chymostatin, leupeptin, antipain, and pepstatin, as described above. For the following immunoprecipitation, the 4G5 antibody was coupled to Protein A-coated Sepharose CL-4B beads (Pharmacia) via rabbit anti-mouse IgG (Jackson) as a linking antibody. In some cases this preformed antibody complex was covalently linked using dimethylpimelimidate (Harlow and Lane 1988). Following at least 6 hr of incubation, the beads were washed first with immunoprecipitation buffer followed by 10 mM Tris-HCl (pH 7.5), 0.5 M LiCl, 0.1% SDS, and 0.2% NP-40 and finally with 10 mM Tris (pH 7.5). The immunoprecipitation complex and bound epitopes were heat denatured in the presence of SDS and dithiothreitol and separated on large-size SDS-gradient gel, followed by Western transfer to nitrocellulose membrane (Towbin et al., 1979). The subsequent preincubation and streptavidin-biotin-HRP binding was performed as described by Meier et al. (1992), the blot extensively washed in 0.25% milk powder in TTBS (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1%

Figure 1. mAb 4G5 immunostaining on two pairs of brain interneurons. *A*, Immunoreactivity at the 55% of embryogenesis. Immunocytochemical staining shows two pairs of brain interneurons, which have their cell bodies in the pars intercerebralis of the protocerebrum, labeled by mAb 4G5. *Arrows*, Cell bodies located near the brain midline (*vertical bar*). *Arrowheads*, Principal dendritic arbors with ramifications that invade all major areas of the developing brain hemispheres. *B*, Immunoreactivity at the 37–40% stage of embryogenesis. *Arrow*, The pair of neurons has already initiated axogenesis and its axons are immunolabeled by mAb 4G5. *C*, Decussation and begin of descending axonal projections at the 45% stage. The neurites of the two labeled cell pairs reach the primary embryonic commissure and decussate. Scale bars: *A*, 200 μ m; *B* and *C*, 20 μ m.

Tween 20), and finally washed with higher stringency using 0.25% milk powder in a TTBS solution that was completed with 350 mM NaCl and 0.1% NP-40. After final TTBS washes, biotinylated and immunoprecipitated proteins were detected using an ECL method on special Hyperfilm ECL (Amersham).

To check for glycosylation of the molecule recognized by mAb 4G5, ventral nerve cords were homogenized and treated as above, but the biotinylation step was omitted. After immunoprecipitation, SDS-PAGE on large-size gels and Western transfer to nitrocellulose, the blots were preincubated in 100 mM Tris-HCl (pH 7.5) containing 1 mM CaCl_2 , 1 mM MgCl_2 , and 1 mM MnCl_2 . During the following step, 10 μ g/ml

biotinylated concanavalin A (ConA; Boehringer Mannheim) was added to the same solution and incubated 3–5 hr at room temperature. All subsequent steps (streptavidin–biotin–HRP incubation, washes, and ECL detection) were as described above.

Results

mAb 4G5 recognizes an antigen expressed by two pairs of brain interneurons

Immunocytochemistry with mAb 4G5 on whole-mounts and sections of grasshopper embryos reveals staining associated with

Figure 2. Cell lineage analysis of the labeled interneurons. *A*, Camera lucida drawing of five clusters of brain neuroblasts in one half of the 35% developed embryonic brain. The dorsal group of neuroblasts is indicated by bold outline and asterisk. *PC*, Primary brain commissure. *B*, Highly schematic representation of the arrangement of the brain neuroblasts in the dorsally located anterior-medial cluster. Asterisk labels the brain neuroblast that generates the mAb 4G5-immunoreactive sibling neurons (solid circles). *C*, Camera lucida drawing of the mAb 4G5-immunoreactive sibling neurons (solid circles) and their neuroblast of origin (asterisk) at approximately 37% of embryogenesis. Small arrows denote the axons of the two immunoreactive neurons extending medially and crossing the midline in the primary embryonic commissure (double-headed arrow). The neighboring anterior and posterior neuroblasts and their initial progeny are also shown. All three neuroblasts are characterized by their larger cell diameter. Stippled cells, Anterior brain neuroblast and its progeny. Hatched cells, Posterior brain neuroblast and its progeny. *M*, Brain midline. *D*, Morphology of one pair of mAb 4G5-immunoreactive neurons with neurites, axons, and dendritic ramifications in the left half of the brain (50–55% stage). Small arrows, In this preparation, both axons were in very close proximity along the primary commissure. Large arrow, primary dendrites. Scale bars: *A* and *D*, 100 μ m; *C*, 25 μ m.

two pairs of bilaterally symmetrical interneurons in the brain. The immunostained cells are protocerebral descending interneurons, and all have a very similar morphology (Figs. 1*A*, 2*D*). Each interneuron's cell body is located near the midline of the protocerebrum. The two somata of each interneuron pair are invariably in close contact and the two neurites that emerge from the cell bodies of an interneuron pair remain in close proximity to each other as they cross the midline of the brain. The dendritic arbors that both cells of each pair form in the protocerebrum, deutocerebrum, and tritocerebrum are virtually indistinguishable, as is the course of the descending axons of the labeled cells through the brain. The axons of each interneuron project to all of the ventral ganglia. In each of these ganglia the axons give rise to collaterals that branch widely and form comparable arborizations.

In 250 embryos studied, the two bilaterally homologous pairs of brain interneurons were the only neuronal cells in the entire nervous system that are labeled by mAb 4G5. The onset of neuron-specific staining with mAb 4G5 occurs at the onset of neurite outgrowth in the labeled interneurons. During neurite outgrowth and axogenesis, the 4G5 epitope becomes concen-

trated at the growth cones of the two labeled interneurons. During the period of synapse formation, the 4G5 epitope becomes restricted to the terminal arbors that generate the synapses of the two interneurons. These terminal arbors of the two interneuron pairs continue to be labeled with mAb 4G5 throughout postembryonic development and into adulthood.

With the exception of the highly specific labeling of the two pairs of interneurons, no further 4G5 immunoreactivity is found within the brain or ventral nerve cord. The only other 4G5 immunostaining appears in embryos near the time of hatching at the level of pigment cells in differentiated parts of the retina. The nature of this non-neuronal staining is not described further here.

Cell lineage and identification of the labeled interneurons

The structural similarity of the labeled interneurons, their exclusive labeling with mAb 4G5, and the fact that the cell bodies of each pair of labeled interneurons are always in close proximity suggest that each interneuron pair might have a common developmental origin from the same neuroblast. To investigate this, we first determined the pattern of neuroblasts that give rise

to the brain of the grasshopper in 200 embryonic preparations. The immunoreactive neurons derive from a dorsal layer of brain neuroblasts (Fig. 2*A*). The identified brain neuroblast of origin of the 4G5-immunoreactive neurons is situated in the dorsally located, anterior-medial cluster just ventral to the embryonic brain commissure. This brain neuroblast is first observed at about the 30% stage of embryogenesis (Fig. 2*B*). As revealed by double staining with mAb 4G5 and BrdU/anti-BrdU, the first ganglion mother cell that derives from this neuroblast divides to produce two sister neurons, both of which express the epitope recognized by mAb 4G5 (Fig. 2*C*). None of the other neuronal progeny of this neuroblast express the 4G5 epitope.

The location of the somata of the labeled interneurons near the embryonic midline of the protocerebrum and the mode of decussation of the main axon of these interneurons are similar to a group of neurons described in the adult pars intercerebralis by Williams (1975). However, a detailed comparison of their dendritic arborizations shows that the 4G5-immunoreactive interneurons are morphologically distinct from these pars intercerebralis neurons. Tyrer et al. (1984) describe a group of six 5-HT-immunoreactive neuron pairs that have cell soma positions similar to the 4G5-immunoreactive interneurons. However, double staining experiments on 55–60% embryos with mAb 4G5 and an anti-5-HT antibody show that the six neurons that are stained with the anti-5-HT antibody are located more anterior to the interneurons recognized by mAb 4G5. Thus, although the 4G5-immunoreactive neurons may belong to a larger group of brain interneurons in the pars intercerebralis region, from which some have already been described morphologically or immunocytochemically, they are morphologically distinct from any of those that have already been reported.

Spatiotemporal expression of the 4G5 antigen during axonal outgrowth

Throughout embryogenesis, the expression of the epitope recognized by mAb 4G5 is restricted to the two pairs of identified interneurons described above. mAb 4G5 immunoreactivity is always seen in both cell pairs at the time of onset of neurite outgrowth at approximately 37% of embryonic development (Fig. 1*B*). As soon as the growth cone and trailing neurite of the neurons are formed, these structures invariably become labeled by mAb 4G5. During the 3–5% of development that follows the onset of neurite outgrowth, the neurites of the immunoreactive neurons join the single primary commissure in the embryonic brain. After contacting the anterior border of this primary commissure, the neurites of the two cell pairs turn posteriorly and then laterally to cross the brain midline along the commissure (Figs. 1*C*, 2*C*). After decussating, the 4G5-immunoreactive neurites descend via the contralateral embryonic deutero- and tritocerebral tracts. The main neurites leave the brain as descending axons that project to the neuromeres of the ventral ganglia.

Near the end of the 40% stage of embryogenesis, the growth cones of the descending axons reach the level of the embryonic stomatodeum and enter the presumptive circumesophageal connectives. At this stage, labeling of the axon with mAb 4G5 is most intense at the level of the growth cone and the part of the axon just proximal to the growth cone. Staining with mAb 4G5 is also often observed at punctate regions on the filopodia of the growth cone. The more rostral axon shaft generally shows reduced immunoreactivity along most of its length, although spatially localized accumulation at discrete points along the axon is also observed. The concentration of the 4G5 epitope near the

growing terminal of the axons and especially on the main core region of the growth cone is typical for all of the developmental stages during which the axonal growth cones extend in the ventral nerve cord (Fig. 3). It is consistently observed both in immunofluorescence preparations (100 preparations studied) and in DAB-stained preparations (30 preparations studied).

During the early phase of axogenesis (40–45%), the two growth cones of each pair of immunoreactive sibling neurons usually grow synchronously and in close proximity to one another. Indeed, they are often tightly apposed (Fig. 3*A*). In cases in which one of the growth cones of a pair leads the other, the second growth cone is usually found navigating along the immunostained axon that the first has generated. Beginning with the 45% stage, when the axons reach the level of the prothoracic ganglion, the growth cones of the sibling axons can often be found growing side by side but clearly separated from one another by several micrometers (Fig. 3*B*). At this and subsequent stages, the two growth cones can also diverge away from one another (Fig. 3*C*), presumably following different tracts, and desynchronize their rate of extension. As a consequence, one axon is sometimes found well ahead of its ipsilateral twin, for distances of up to several segments. This type of separation of the two ipsilateral growth cones occurs both in the embryonic ganglia, where the growth cones always grow through a very dorsal axonal layer, and in the interganglionic connectives. The resulting divergent axonal paths, which eventually converge again to form loop-like structures (Fig. 3*D*), indicate that the immunoreactive axons are not strictly confined to a spatially limited axon fascicle. This also shows that the expression of the 4G5 epitope on both growth cones does not confer strong cohesive properties to the two growth cones. The sites at which sibling growth cones separate as well as the sites of their subsequent rejoining are not correlated with major embryonic structural cues such as segmental borders. Regardless of whether the two sibling growth cones are growing together or apart, 4G5 immunostaining is most intense at the distal tip of the axons near the growth cones. At most of the more rostral axonal sites the intensity of staining is considerably lower (open arrows in Fig. 3*A,B*). The exceptions to this have to do with the formation of axonal arbors.

Spatiotemporal expression of the 4G5 antigen during terminal arbor formation

At approximately 55% of embryonic development, well before the main axonal growth cones reach the last of the ventral ganglia, axon collaterals begin to emerge from the main axon shaft about four to five segments behind (rostral to) the leading axonal growth cone. In the developing subesophageal and thoracic neuromeres, which are not yet fused at this stage, the appearance of axonal collaterals occurs in a stereotyped spatial and temporal sequence. A total of three sets of main collaterals are generated in each neuromere from the axonal shaft in an anterior to posterior sequence. In all of these neuromeres, the most anterior located collaterals start to emerge from the main axon trunk at the rostral edge of the anterior commissure. The second set of collaterals emerges slightly later at a more caudal region between the anterior and posterior commissures. The third set of posterior collaterals is last to emerge from the axon shaft and grows along the posterior ganglionic commissure. Following their emergence, all of these main axon collaterals grow first medially toward the ganglion midline and then into the main neuropil region, where they begin to ramify. At later stages, the density

Figure 3. Outgrowth and differentiation of mAb 4G5-immunolabeled axons: distribution of the immunolabeled epitope on growth cones at 55% of development. *A*, Growth cones in close apposition (solid arrows) are intensely stained by mAb 4G5. Open arrow, Axon shafts show reduced immunoreactivity. *B*, Similar distribution of immunolabel on sibling growth cones, which are side by side but clearly separated from one another. Arrow, Decline in the intensity of the immunostaining on the axon shaft. *C*, Beginning loop formation by the two ipsilateral growth cones. Arrow, Point of axon divergence. *D*, Convergence and formation of loop-like structure by the two ipsilateral axons. (*C* and *D* are from two independent preparations.) *E*, Ramification of the axonal ending in the terminal abdominal ganglion. Arrows show the secondary growth cones that are formed occurring just after the arrival of the growing axon in this target ganglion. *F*, Intense mAb 4G5 labeling at the axon shaft regions where secondary growth cones emerge: increased epitope expression (arrows) at the onset of collateral formation. *G*, mAb 4G5 labeling during ongoing collateral formation. Growth cones (arrows) are advancing in a medial direction. Scale bars: *A* and *B*, 10 μ m; *C*–*G*, 25 μ m.

of axonal ramifications increases dramatically and also some laterally directed sprouts are observed.

At the time of formation of each of these main axonal collaterals in the segmental neuromeres, local accumulation of 4G5 immunostaining is seen at discrete sites of swelling along the axonal trunk. These protrusions are invariably the site of formation of the secondary growth cones that generate the main axonal collaterals (Fig. 3*F,G*). Due to the limits in the temporal resolution of our investigation, we were not able to determine if the localized accumulation of the 4G5 epitope is coincident with or precedes the formation of subcellular specializations that signal the emergence of a secondary growth cone from the axon. However, approximately 1% after the appearance of spatially clustered immunostaining along the axon, a small secondary growth cone can clearly be observed at each of the labeled sites on the axon. As is the case for the main axonal growth cone, once the secondary growth cones are formed and begin to extend, they too show increased concentrations of the 4G5 immunoreactivity at their core regions and more discrete punctate staining on their filopodia.

At 60–63% of embryonic development, the main axonal growth

cone reaches the last abdominal neuromere. Upon entering this neuromere, the growing axonal ending begins to ramify. In this process, several new growth cones are rapidly formed by each axon (Fig. 3*E*). All of these growth cones are intensely labeled with mAb 4G5. Each of these secondary growth cones generates a discrete axonal branch that initially continues to extend caudally, in the direction of the main axonal trunk. They subsequently ramify further and extend laterally to form the terminal arbor in this neuromere. At about the time when these collaterals are first observed in the last abdominal neuromere, axonal collaterals are also present in all of the more anterior ganglionic neuromeres.

During the formation of axonal arbors in the segmental ganglia of the embryo, an interesting change in the pattern of immunostaining in the labeled interneurons is observed (Fig. 4*A,B*). At approximately 70% of embryogenesis, the 4G5 immunostaining of the main axon shaft becomes extremely weak. Indeed, in many cases it is no longer possible to visualize the main axon shaft (arrows in Fig. 4*A,B*). In contrast, strong immunolabeling is always observed on the most distal parts of the growing axonal terminals (15 preparations studied). This staining is

Figure 4. mAb 4G5 immunolabeling during terminal axonal formation. *A*, Immunolabeled terminal arbors in mesothoracic (top) and metathoracic (bottom) neuromeres at approximately 70% of embryogenesis. Main axonal shafts in the connectives (arrows) are not labeled. *B*, Immunolabeled axonal arbors in the terminal abdominal ganglia of a 70% stage embryo. Note the punctate aggregates of mAb 4G5 immunolabel on the endings. Main axonal shafts in the connectives are no longer stained; the extent of the connectives is indicated by arrows. *C*, mAb 4G5 immunolabeling on a collagenase/dispase-treated abdominal ganglion from a third instar larva. Scale bars: *A* and *B*, 50 μ m; *C*, 100 μ m.

most intense at discrete, bleb-like structures on the fine branches of terminal axonal arbors. Although these structures may be nascent synapses, we have not yet been able to verify this explicitly. This spatially restricted, intense staining of varicosity-like structures at the terminal axonal arbors persists postembryonically. During postembryonic development, the ramification of the axonal arbors and the total amount of immunolabeling of the axon terminals increase markedly and immunoreactive processes can be seen invading most parts of the neuropil (Fig. 4*C*). Immunostaining of the main axonal shafts remains very low postembryonically and is, for the most part, below the detection level of light microscopy.

In the brain, the development of the dendritic arbors occurs in a manner similar to that of the axonal arbors. At approxi-

mately 50% of embryonic developmental, small branches emerge from the primary neurites near the point where the neurites leave the embryonic commissure and join the descending axon tracts in the brain. Subsequently the growing terminals of these dendritic branches ramify extensively and project to many of the major brain areas in the proto-, deuto-, and tritocerebrum, such as the mushroom bodies, antennal lobes, optic lobes, and central complex (Figs. 1*A*, 2*D*). The vast majority of the dendrites of a given sibling pair of labeled interneurons are formed contralateral to the cell somata, on the same side as the descending axon trunks. During postembryonic development, the number of dendritic ramifications in the brain continues to increase.

Throughout embryonic and postembryonic development, the

Figure 5. mAb 4G5 immunolabeling on nonpermeabilized ganglia, 55% stage. The immunolabeled epitope is detected on the growth cones and to a variable extent along the proximal axonal shaft. *A*, Immunoreactivity is only seen at the growth cone level (arrows). Rostral to the growth cone (arrowheads) immunolabeling is barely detectable. *B*, Variability of mAb 4G5 immunoreactivity. In addition to growth cone labeling (black arrows), only weak rostral axonal staining is seen in the left connective (open arrow). In contrast, extensive axonal staining is seen in the right connective. Scale bars: *A*, 50 μ m; *B*, 100 μ m.

dendritic arbors in the brain are immunolabeled by mAb 4G5. As is the case for the developing axonal arbors, intense staining is always associated with the terminal branches of the dendritic arbors. Immunostaining of the primary dendrites and of most of the neurite that connects these dendrites to the cell body is much weaker. The cell body continues to express a high level of the 4G5 epitope throughout embryonic and postembryonic development. Moreover, the initial segment of the primary neurite immediately adjacent to the cell body also remains intensely immunolabeled.

Extracellular localization of the 4G5 epitope on growth cones and terminal arbors

The spatiotemporal pattern of expression of the 4G5 epitope suggests that it is synthesized and stored in the cell body and then transported to the growing terminals of the labeled interneurons where it accumulates. To determine if the 4G5 epitope becomes associated with the outer cell surface of the developing terminals, we carried out a series of immunocytochemical experiments on 70 nonpermeabilized preparations in which mAb 4G5 remains restricted to the outside (cell surface and extracellular matrix) of the labeled neurons.

In experiments of this type the growth cones of all four cells are always intensely stained, irrespective of whether they are growing in the brain, in the connectives, or in the segmental ganglia. In addition to this cell surface-associated expression of the 4G5 epitope on the growth cone, a variable region of the axon behind (rostral to) the growth cone is also immunolabeled in unpermeabilized preparations. Usually only the growth cone and the adjacent region of the axonal shaft are stained with mAb

4G5 (Fig. 5*A*). Sometimes, however, much longer parts of the distal axon become labeled in addition to the growth cone, and occasionally the staining of the axon segment is discontinuous, with immunostained axonal regions separated by nonstained regions (Fig. 5*B*). For 22 growing axon tips at the 55% stage of embryogenesis in which mAb 4G5 was restricted to the outside of the neurons, the mean value for the total length of cell surface-associated axonal staining measured from the tip of the leading growth cone was $113 \pm 54 \mu$ m. For seven of these the length of staining was less than 100 μ m, and for two the length of staining was more than 150 μ m.

An examination of the surface label at the level of the growth cone by confocal laser microscopy (three preparations) shows that the distribution of the immunostaining is not homogeneous. Rather the 4G5 epitope is concentrated in punctate aggregates at numerous sites on the growth cone. This punctate accumulation of 4G5 epitope on the cell surface is also seen on the parts of the adjacent axon. Since aggregates of punctate staining are sometimes spatially separated from the main immunolabeled axon, we assume that the 4G5 epitope is not tightly bound to the axonal membrane. This is in accordance with experiments that show that much of the cell surface-associated 4G5 immunoreactivity is lost if the fixed whole-mount preparations are washed for over 12 hr.

In order to analyze the cell surface-associated distribution of the 4G5 epitope at the ultrastructural level, we carried out an electron microscopical study of 60 immunolabeled nonpermeabilized preparations. Transverse sections at the level of the growth cone show intense, localized staining in the adjacent extracellular space surrounding the growth cone and filopodia (Fig. 6*A,B*).

Figure 6. Ultrastructural localization of TERM-1: immunoelectron microscopical labeling of nonpermeabilized ganglia with mAb 4G5 to reveal the expression of TERM-1. *A*, Transverse section through a connective at the level of the labeled growth cones, 55% stage embryo. TERM-1 labeling is very intense and highly localized (arrowhead). *B*, Higher magnification of the view shown in *A*. TERM-1 appears to be secreted into the adjacent extracellular space surrounding the two axon tips, where it probably becomes associated with the extracellular matrix. None of the surrounding axon profiles are labeled. *C*, Double labeling experiment. mAb 4G5 and an anti-fasciclin II mAb are used together to contrast the cellular localization of TERM-1 and fasciclin II. Transverse section through axon fascicles in an abdominal ganglion, 55% stage embryo. The outer cell surface of numerous fasciculated axons is labeled in a manner characteristic for the fasciclin expression pattern (Bastiani et al., 1987). The staining remains in close proximity to the outer cell membrane (arrowheads). In contrast to this membrane-coupled staining, the staining surrounding the TERM-1 expressing axons is not tightly membrane bound. Rather, it is found throughout the surrounding extracellular space adjacent to the labeled axons (large arrows). Small arrows, Axon profiles that are not labeled with the anti-Fas II antibody. *D*, TERM-1 expression at axonal terminals: transverse section through the last abdominal ganglion of a 95% stage embryo. Immunostaining is restricted to the space that separates the labeled terminal (arrowheads) from neighboring small-diameter profiles. Scale bars: *A* and *C*, 2 μ m; *B* and *D*, 0.5 μ m.

At levels several hundred micrometers behind (rostral to) the growth cones, much weaker immunostaining is observed. This is in accordance with the results of our light microscopic studies, which show weaker staining on the more rostral parts of the axon.

To investigate the cell surface association of the 4G5 epitope further, we carried out a double labeling experiment using mAb 4G5 and an mAb that recognizes fasciclin II, a membrane-bound cell surface molecule (Bastiani et al., 1987; Snow et al., 1988). Although the 4G5-labeled axons are incorporated into a bundle of fasciclin II-positive axons, the nature of the 4G5 labeling is clearly different from that of the fasciclin II labeling (Fig. 6*C*). In contrast to the membrane-bound fasciclin II staining, the 4G5 staining clearly extends into the extracellular space

adjacent to the labeled axons. On the basis of these ultrastructural studies, we postulate that the 4G5 epitope is secreted into the extracellular space, where it becomes associated with the extracellular matrix surrounding the growth cone of the labeled neurons.

The notion that the 4G5 epitope is secreted into the extracellular space adjacent to the labeled processes is supported by the ultrastructural analysis of cell surface-associated expression of the 4G5 epitope at developing axonal terminals. In nonpermeabilized preparations, highly localized intense staining is observed in the space that separates these labeled terminals from neighboring small diameter profiles (Fig. 6*D*). We do not yet know if these intensely labeled sites on the axonal terminals are involved in synapse formation.

TERM-1: biochemical characterization of the glycoprotein immunoprecipitated by mAb 4G5

For an initial biochemical analysis, mAb 4G5 was used to immunoprecipitate the specific recognized proteins from biotinylated total homogenates prepared from grasshopper CNS (see Materials and Methods). Following immunoprecipitation the proteins were analyzed by SDS-PAGE and Western blotting combined with an ECL technique for signal amplification (Meier et al., 1992). Our results indicate that mAb 4G5 recognizes a single protein with an apparent molecular weight of 48 kDa (Fig. 7, lane B).

Control immunoprecipitates using an IgM antibody with a different specificity as well as the immunomatrix alone (secondary rabbit anti-mouse antibodies coupled to Protein A-Sepharose beads) do not reveal a similar band. Moreover, if mAb 4G5 immunoprecipitation is carried out from adult muscle tissue homogenates, which do not express this epitope, no specific band can be detected (Fig. 7, lane A). To investigate if the immunoprecipitated protein is glycosylated, nonbiotinylated immunoprecipitates were transferred to nitrocellulose and the resulting Western blot was then incubated with biotinylated ConA lectin. The results show that biotinylated ConA does indeed bind to the immunoprecipitated molecule (Fig. 7, lane C), indicating its glycosylation.

Because this glycoprotein is expressed at the developing terminals of the labeled interneurons, we call it TERM-1.

Discussion

The purpose of this study was the identification of candidate molecules for the type of individual, neuron-specific molecular labeling postulated by the chemoaffinity hypothesis (Sperry, 1963). For this we generated mAbs that recognize surface-associated antigens and then used mAb 4G5 to characterize a neuron-specific molecule, called TERM-1, that is expressed by only two pairs of neurons in the CNS. During development, TERM-1 becomes concentrated first at the navigating growth cones of these neurons and subsequently accumulates at their terminal arbors where it continues to be expressed postembryonically. An ultrastructural characterization suggests that TERM-1 is secreted by the labeled growth cones and terminals and becomes incorporated into the surrounding extracellular matrix. The spatiotemporal expression pattern and unique cellular specificity of TERM-1 suggest that it may play a role in neuron-specific process outgrowth and target recognition. In the following, we shall discuss the implications of these findings.

TERM-1 is expressed by individual identifiable brain interneurons

In the CNS, TERM-1 is expressed by two sibling cells and their bilateral homologs, all of which develop in an extremely similar manner and acquire a virtually identical structure. None of the other neurons in the animal's CNS or PNS express TERM-1 either during embryogenesis or postembryonically. Thus, in the nervous system, expression of TERM-1 is highly restricted and can be correlated with cell identity.

The interneurons that express TERM-1 have several striking anatomical features. Their dendritic arbors ramify widely in the brain, and they form axonal arbors in all of the subesophageal, thoracic, and abdominal neuromeres. Brain interneurons with comparable structures have been described, and some of these have been shown to be higher-order interneurons involved in multimodal sensory integration (Williams, 1975; Tyrer et al.,

Figure 7. Biochemical characterization of TERM-1: analysis by SDS-PAGE and Western blot. Total homogenates were prepared from ventral nerve cord ganglia or thoracic flight musculature, detergent extracted, and biotinylated. Solubilized proteins were then immunoprecipitated using preformed antibody complexes with mAb 4G5. After separation on large-size gradient gels and Western transfer to nitrocellulose, biotinylated proteins were detected using the ECL method. The TERM-1 protein was immunoprecipitated from ganglia homogenates and migrated with an apparent molecular weight of 48 kDa under reducing conditions (lane B). Similar immunoprecipitation experiments using adult musculature homogenates as control tissue did not reveal a comparable band (lane A). Glycosylation of TERM-1. Immunoprecipitation of TERM-1 from ganglia homogenates was carried out without previous biotinylation. Reprobing of Western transferred proteins with biotinylated ConA and subsequent ECL detection revealed the 48 kDa band (lane C). The weak, higher weight band in lane C most likely represents immunoglobulin heavy chain molecules that are glycosylated and known to bind ConA.

1984; Hensler, 1988). It will be important to determine if these other interneurons also express unique neuron-specific molecular labels or if the TERM-1-expressing interneurons are the only ones of this class to do so.

An interesting consequence of our investigation is the finding that neuroblasts in the brain can be identified as individuals much like those in the segmental ganglia (Bate, 1976). Moreover, the manner in which these neuroblasts generate their neuronal progeny during early neurogenesis is similar to that shown by neuroblasts in the segmental ganglia (Goodman et al., 1984; Doe and Goodman, 1985). This makes it possible to construct

a neuroblast map for the entire developing grasshopper brain (D. Zacharias, L. Williams, T. Meier, and H. Reichert, unpublished observations) and then relate identified brain interneurons to their neuroblast of origin (Shepherd and Bate, 1990). This, in turn, should make it possible to uniquely identify many of interneurons in the brain on the basis of their cell lineage.

Expression of TERM-1 correlates with process outgrowth

As soon as process outgrowth commences, TERM-1 becomes concentrated at the growth cones of the labeled neurons. Whenever subsidiary growth cones are formed, for example during collateral formation, TERM-1 becomes concentrated at these subsidiary growth cones as well. TERM-1 may, in fact, accumulate at the specific sites of subsequent outgrowth in these cells even before fully differentiated growth cones are formed. This might imply a role of TERM-1 in the generation of growth cone structure as well as in growth cone-mediated process outgrowth. Although other growth-associated molecules have been described (Skene, 1989; Reichardt et al., 1990), none have the type of neuron-specific expression pattern of TERM-1.

Synthesis of TERM-1 in the neuronal somata commences at the time of initial process outgrowth and continues throughout embryogenesis and postembryonic development. Once process outgrowth begins, TERM-1 accumulates at the distal endings of the labeled interneurons. This suggests that it is transported from the cell body to these sites via the main axon. The level of TERM-1 in the main axon is, however, relatively low. Indeed, once terminal arbors are formed, immunocytochemical detection of TERM-1 in the main axonal shaft is often difficult. An explanation for this might be that TERM-1 is rapidly transported in the axon and then becomes concentrated at the more distal sites. For membrane glycoproteins, mechanisms for concentration by axonal transport and trapping in growth cones have been described (Sheetz et al., 1990). However, since TERM-1 appears to be secreted at the growth cone, other mechanisms are probably responsible for the higher levels of expression at growing tip compared with the more proximal axon. Diffusion, turnover, or active degradation of TERM-1 soon after its secretion into the extracellular space are all possible explanations for fact that the TERM-1 signal on the axon diminishes with increasing distance from the growth cone.

The most intense staining at the level of the growth cone is found at numerous punctate sites on the growth cone's core region. These could be sites of active secretion of TERM-1. Alternatively, these accumulations could be caused by structures in the extracellular matrix or by an inherent tendency of the TERM-1 glycoprotein to form aggregates, once secreted. Punctate extracellular accumulation of TERM-1 is also characteristic of the region of the axon immediately behind the growth cone. These axon-associated aggregates could be due to secretion of TERM-1 directly from the growing axonal shaft. However, it seems more likely that such aggregates of TERM-1 are initially secreted by the growth cone, and that they remain localized at their sites of initial secretion as the growth cone extends.

TERM-1 is generally found extracellularly on the distal 100–150 μm of the growing axon tips. Since the rate of axonal growth of the labeled interneurons is approximately 50 $\mu\text{m/hr}$ (Zacharias, unpublished observations), neural elements at any given region could have access to the glycoprotein for at least 2–3 hr. Several different types of possible growth-associated interactions might be envisaged. TERM-1 in the extracellular matrix surrounding the labeled neurons might be used by other axons

as a temporally dynamic guidance cue for directed outgrowth or selective fasciculation. Guidance mechanisms of this general type have been postulated by the labeled-pathway hypothesis (Raper et al., 1983, 1984; Bastiani et al., 1984). Alternatively, since TERM-1 does not appear to be tightly bound and may diffuse away from the labeled axons, the glycoprotein could function as a cell-specific chemoattractant for other neuronal processes. Some neuronal cell-surface adhesion molecules are known to occur in a secreted form, and some of these may be capable of regulating the growth of neuronal processes (Karagoeos et al., 1991). Finally, secreted TERM-1 might interact biochemically with the extracellular matrix so as to alter the growth properties of the labeled axons through the extracellular matrix. Several proteins that are secreted by growth cones and alter neurite growth by acting on the extracellular environment are known (Pittman, 1990). *In vivo* antibody block experiments with polyclonal antibodies against TERM-1 will be important in order to decide among these different possibilities.

Although the expression of TERM-1 correlates well with process outgrowth, other possible functional roles for the molecule that are not directly related to growth should also be considered. For example, TERM-1 could accumulate at the growing tips of the labeled neurons during the phase of neuronal pathfinding as a consequence of axonal transport, but act primarily during the subsequent phases of synapse formation or synapse maintenance.

TERM-1 may be involved in synapse formation

As terminal arbors are formed, TERM-1 accumulates at these endings and becomes concentrated at discrete sites on the fine branches of the arborizations. This occurs on terminal branches that are formed by ramification of the main axonal growth cone and on branches formed by secondary growth cones from the axon shaft ("backbranching"; e.g., Liu and Westerfield, 1990). The discrete sites of intense labeling may be the sites of synapse formation. However, we have not yet been able to demonstrate this explicitly. Nevertheless, since the entire axonal terminals are labeled to some degree, it seems likely that the synapses are labeled as well.

We do not yet know the precise functional role of TERM-1 at the labeled terminals. TERM-1 might be involved in the recognition of specific synaptic partner neurons or in stabilizing synaptic contacts, once these have been formed. The accumulation of TERM-1 in the cleft that separates the labeled terminal endings from other neighboring small-diameter terminal profiles could indicate a role in the formation of adhesive contacts or in the organization of subcellular synaptic specializations. Molecules that become associated with the nascent synaptic extracellular matrix, fill out the synaptic cleft, and affect postsynaptic differentiation have been described at the developing neuromuscular junction in vertebrates (McMahan and Wallace, 1989). TERM-1 could conceivably be a neuron-specific form of this more general type of secreted, terminal-associated molecules. Alternatively, TERM-1 might be involved in the synaptic or secretory functions of the labeled interneurons. Given the fact that neurosecretory and transmitter molecules in insects can show a very high degree of cellular specialization (Truman and Copenhaver, 1989; Nässel et al., 1989; Lundquist and Nässel, 1990), TERM-1 might be a specialized, synapse-associated protein that is initially expressed very early in neurogenesis but whose functional role only becomes apparent after the onset of synaptic transmission.

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