

# Structural Requirements for Outside-In and Inside-Out Signaling by *Drosophila* Neuroglian, a Member of the L1 Family of Cell Adhesion Molecules

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**Abstract.** Expression of the *Drosophila* cell adhesion molecule neuroglian in S2 cells leads to cell aggregation and the intracellular recruitment of ankyrin to cell contact sites. We localized the region of neuroglian that interacts with ankyrin and investigated the mechanism that limits this interaction to cell contact sites. Yeast two-hybrid analysis and expression of neuroglian deletion constructs in S2 cells identified a conserved 36-amino acid sequence that is required for ankyrin binding. Mutation of a conserved tyrosine residue within this region reduced ankyrin binding and extracellular adhesion. However, residual recruitment of ankyrin by this mutant neuroglian molecule was still limited to cell contacts, indicating that the lack of ankyrin binding at noncontact sites is not caused by tyrosine phosphoryla-

tion. A chimeric molecule, in which the extracellular domain of neuroglian was replaced with the corresponding domain from the adhesion molecule fasciclin II, also selectively recruited ankyrin to cell contacts. Thus, outside-in signaling by neuroglian in S2 cells depends on extracellular adhesion, but does not depend on any unique property of its extracellular domain. We propose that the recruitment of ankyrin to cell contact sites depends on a physical rearrangement of neuroglian in response to cell adhesion, and that ankyrin binding plays a reciprocal role in stabilizing the adhesive interaction.

Key words: ankyrins • cell aggregation • cells, cultured • cytoskeleton • *Drosophila*

MEMBERS of the L1 family of cell adhesion molecules (CAMs)<sup>1</sup> have emerged as important molecules during embryonic nervous system development and during regeneration after nerve injury (Hortsch, 1996). They are involved in processes such as myelination, long-term potentiation, neuronal migration, axonal guidance and fasciculation, and synaptogenesis. L1 family members are also thought to transduce neurite growth-promoting signals by activation of neuronal FGF receptors (Walsh and Doherty, 1997).

There is compelling evidence linking mutations in human L1-CAM to neurodevelopmental phenotypes. These phenotypes include hydrocephalus, motor neuron defects, agenesis of the corpus callosum and the corticospinal tract, and others (Wong et al., 1995b; Dahme et al., 1997). Mutations in the *Drosophila* L1 homologue, neuroglian, result

in embryonic lethality and defects in neuronal morphology and axonal pathfinding (Bieber et al., 1989; Hall and Bieber, 1997). Which of L1's many molecular functions are affected by these mutations and are therefore responsible for the observed phenotypes is currently unknown.

L1 family members with their conserved pattern of extracellular immunoglobulin (Ig) and fibronectin type III protein domains share a number of molecular functions, such as homo- and heterophilic adhesion (Hortsch, 1996). The cytoplasmic domain binds directly to ankyrin which, in turn, interacts with the spectrin cytoskeleton (Davis et al., 1993; Davis and Bennett, 1994; Dubreuil et al., 1996; Hortsch et al., 1998). Expression of the *Drosophila* L1 homologue, neuroglian, in *Drosophila* S2 tissue culture cells results in a selective recruitment of ankyrin and spectrin to sites of cell contacts (Dubreuil et al., 1996). Ankyrin recruitment is strictly limited to cell contacts, even though neuroglian is abundantly expressed over the entire cell surface. Thus, neuroglian can function as a signaling molecule that transmits the positional value of cell adhesion to the cytoplasmic assembly of ankyrin and spectrin. This outside-in signaling function appears to be conserved

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1. Abbreviation used in this paper: CAM, cell adhesion molecule.

among L1 family members, since expression of human L1 in S2 cells also results in the assembly of ankyrin at cell contact sites (Hortsch et al., 1998). The adhesion-induced rearrangement of ankyrin and spectrin can be conveyed to other membrane proteins that interact with ankyrin and spectrin and might thereby provide a mechanism for the assembly of unique plasma membrane subdomains. For example, the NaK-ATPase, which is known to interact with ankyrin in vertebrates (Nelson and Veshnock, 1987), was found to accumulate along with spectrin and ankyrin at sites of neuroglian-mediated adhesion in S2 cells (Dubreuil et al., 1997). Thus, L1-mediated adhesion events result in a reorganization and compartmentalization of the plasma membrane, which may constitute an important biological function of L1 family members.

Recent studies of the L1 family member rat neurofascin have begun to elucidate the structural requirements of the L1 family-ankyrin interaction. Deletion of a five-amino acid sequence from the conserved distal region of the neurofascin cytoplasmic domain abolished ankyrin binding (Garver et al., 1997), indicating that this sequence contributes to the ankyrin-binding site. Two tyrosine residues in this distal region (corresponding to Y1217 and Y1234 in the *Drosophila* neuroglian protein sequence) are conserved in all but two members of the L1 family. In vitro studies of neurofascin revealed that phosphorylation of one of these tyrosines (Y1234 in neuroglian) can inhibit the binding of ankyrin to neurofascin (Garver et al., 1997). Furthermore, inhibition of the ankyrin-neurofascin interaction, either by deleting or phosphorylating the critical tyrosine residue, had an inhibitory effect on neurofascin-mediated cell adhesion (Tuvia et al., 1997). Together, these observations suggest an elegant mechanism to explain the inside-out regulation of the extracellular adhesion of an L1 family member by the intracellular phosphorylation of its cytoplasmic domain.

Here we investigate the mechanisms governing outside-in signaling by neuroglian. We take advantage of the unique features of S2 cells to study not only adhesion, but also the redistribution of the spectrin cytoskeleton in response to neuroglian expression (Dubreuil et al., 1996). We used yeast two-hybrid analysis and expression of deletion constructs in S2 cells to map the region of neuroglian that is necessary and sufficient for binding to ankyrin. Similarly, we investigated the effects of cytoplasmic domain tyrosine mutations on the ability of neuroglian to interact with ankyrin in yeast and to recruit ankyrin to cell contacts in S2 cells. Finally, we tested the possibility that neuroglian outside-in signaling depends on unique features of the neuroglian extracellular domain by expressing a fasciclin II-neuroglian chimera in S2 cells. The results of these studies expand the repertoire of molecular mechanisms that are relevant to our understanding of L1 family function and the relationship between specific L1 defects and their complex phenotypes.

## Materials and Methods

### Materials and Antibodies

The 1B7 and 3F4 mAbs against *Drosophila* neuroglian, a rat antiserum specific for the neuroglian<sup>167</sup> protein (Bieber et al., 1989; Hortsch et al.,

1995) and affinity-purified rabbit anti-*Drosophila* ankyrin (Dubreuil and Yu, 1994) have been described earlier. The rat antiserum and the mouse 1D4 mAb against *Drosophila* fasciclin II, as well as the cDNA for the PEST sequence-containing, transmembrane protein form of *Drosophila* fasciclin II were kind gifts of C. Goodman (University of California, Berkeley, CA). Rabbit anti-phosphotyrosine antibody (She et al., 1997) was a gift of C. Palfrey (University of Chicago, Chicago, IL). Schneider's medium, penicillin/streptomycin solution, and fetal calf serum were from Life Technologies, Inc. (Gaithersburg, MD).

### Construction of cDNAs for S2 Cell Transfection Experiments

Cytoplasmic domain deletions were produced by PCR with neuroglian<sup>167</sup> cDNA as template and PCR products were initially subcloned in the pAS-CYH2 vector. 5' PCR primers used in these PCRs contained a NcoI site (maintaining an open reading frame with the GAL4 DNA-binding domain), followed by a NsiI site; 3' PCR primers contained a SalI site. The numbers in the designation of individual deletion constructs refer to the 85 amino acid residues of the cytoplasmic domain of the neuroglian<sup>167</sup> protein form. Carboxy-terminal deletions were created by the introduction of in-frame stop codons along the neuroglian sequence using the 3' PCR primer; deletions in the amino-terminal region of the neuroglian cDNA by using several 5' PCR primers with homology to different regions of the neuroglian cDNA. The fidelity of the PCR amplification reactions was verified by DNA sequence analysis (Sanger et al., 1977).

Constructs incorporating the cytoplasmic deletions into the complete neuroglian open reading frame were derived from the first set of verified pAS1-CYH2 plasmids. NsiI/SalI cDNA fragments were isolated from the pAS1-CYH2 DNAs and introduced into the context of the entire neuroglian open reading frame by subcloning into pRmHa3:nrg<sup>NsiI</sup> vector DNA. This vector was created by using PCR to introduce a silent C to A point mutation at position 3,480 of the neuroglian sequence at the end of the region encoding the transmembrane segment, thereby creating a unique NsiI site.

Neuroglian cDNAs with point mutations converting cytoplasmic tyrosine residues into phenylalanine residues were created using the following strategy. The unique BamHI site at position 3,710 of the neuroglian<sup>167</sup> cDNA sequence was used in a two-step PCR process to create initially single and subsequently double point mutations. This was accomplished by converting the flanking codons encoding tyrosine 1,217 and tyrosine 1,234 into phenylalanine-encoding codons. PCR primers including the BamHI site and one of the Y to F mutated codons (TAT/C to TTT/C) were used in separate PCR reactions and subsequently subcloned into BamHI and either NcoI- or SalI-digested pAS1-CYH2:nrg<sup>wt</sup> (construct no. 1, wt) vector DNA. The incorporation of only the desired point mutation(s) was verified by DNA sequence analysis. As described above for the deletion mutations, all three cDNA fragments encoding Y to F mutated, neuroglian<sup>167</sup> cytoplasmic domains were subsequently subcloned into the pRmHa3:nrg<sup>NsiI</sup> vector for transfection of S2 cells.

A chimeric cDNA was constructed by the in-frame fusion of DNA fragments encoding the extracellular domain of *Drosophila* fasciclin II and the transmembrane segment plus the cytoplasmic domain of *Drosophila* neuroglian<sup>167</sup>. A 0.45-kb KpnI/SalI PCR product encoding the neuroglian transmembrane segment and the cytoplasmic domain was derived from a *Drosophila* neuroglian<sup>167</sup> cDNA and subcloned into pRmHa3 vector DNA. Besides the KpnI cloning site, the 5' PCR primer also introduced an AatII site which was in-frame with the AatII site at position 2,581 in the fasciclin II cDNA. This interim construct was cut with KpnI and AatII and then ligated to a 2.6-kb fasciclin II cDNA fragment, resulting in the final pRmHa3-fasII<sup>EC</sup>-nrg<sup>TM+CP</sup> construct. The entire open reading frame of the transmembrane, PEST sequence-containing fasciclin II cDNA, was subcloned into the pRmHa3 vector yielding the pRmHa3-fasII<sup>PEST</sup> construct.

### Generation and Maintenance of S2 Cell Lines

All *Drosophila* S2 cell lines were transfected using Lipofectin® (Life Technologies, Inc.) and clonal lines were maintained as previously described (Bieber, 1994). After induction with 0.7 mM of CuSO<sub>4</sub>, subclones were analyzed by Western blotting for high level expression of recombinant proteins and subclones expressing comparable quantities of neuroglian were selected for further experiments.

## Cell Aggregation Experiments

The ability of mutated neuroglian to induce homophilic cell aggregation of transfected S2 cells was tested as described earlier (Hortsch et al., 1995). Cell lines were adjusted to a concentration of  $10^6$  cells/ml and neuroglian synthesis was induced by the addition of 0.7 mM of  $\text{CuSO}_4$ . Duplicate cultures ( $10^6$  cells in 1 ml) were grown in six-well tissue culture plates and induced at 25°C for 15 h. Cells were transferred to sterile 50-ml centrifuge tubes and further incubated on a shaking platform (model 62; New Brunswick Sci. Co., Edison, NJ) at 200 rpm. For a quantitative analysis, the number of nonaggregated cells was counted in a hemocytometer (Bright-Line®; Reichert, Buffalo, NY). Since cell clusters of up to five cells were occasionally observed in untransfected S2 cells, only aggregates containing more than five cells were considered to be aggregated. Eight fields, each representing 0.01  $\mu\text{l}$  of cell suspension, were counted per culture. Based on the starting number of cells, the results are presented as percent aggregated cells of total cells.

## SDS-PAGE and Western Blot Analysis

For Western blots, transfected S2 cells were pelleted and solubilized in SDS-containing buffer. Total cell proteins were separated by PAGE, transferred onto nitrocellulose filters, and then probed with specific antibodies as described (Hortsch et al., 1985; Dubreuil et al., 1987). Proteins were separated on either 7.5/0.2% or 8.0/0.08% acrylamide:bisacrylamide gels.

## Yeast Two-Hybrid Analysis

The yeast two-hybrid system was used as described by Durfee et al. (1993). The pACTII-ankyrin construct has been characterized previously (Dubreuil et al., 1996). The pACTII-control plasmid was kindly provided by J. Clemens (University of Michigan, Ann Arbor, MI). Yeast selection plates and media were made and yeast transformations were performed using established protocols (Ausubel et al., 1988). Staining for  $\beta$ -galactosidase expression in yeast cell colonies was assayed using the substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside as described (Bartel et al., 1993). Quantitative determinations of  $\beta$ -galactosidase expression were

done on 4-ml liquid yeast cultures using *O*-nitrophenyl- $\beta$ -D-galactoside as substrate (Ausubel et al., 1988).

## Immunofluorescence Staining and Microscopy

Immunofluorescence analysis of *Drosophila* ankyrin distribution in S2 cell aggregates was performed as described previously (Dubreuil et al., 1996). Briefly, cells were attached to alcian blue-coated microscope slides, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then reacted with antibodies and washed in pH 7.5 Tris-buffered saline containing 5% newborn calf serum.

## Results

### A Conserved Distal Region of the Neuroglian Cytoplasmic Domain Binds Ankyrin

To determine the cytoplasmic neuroglian subdomains that are involved in ankyrin binding, we engineered a series of neuroglian deletion constructs to test their ability to interact with *Drosophila* ankyrin (Fig. 1). Deletion breakpoints were selected to isolate several protein segments that are broadly conserved among members of the L1 gene family. It has previously been shown that these conserved regions form functionally and structurally important L1 subdomains, such as the ankyrin-binding site (Davis and Bennett, 1994; Garver et al., 1997; Tuvia et al., 1997).

Since the interaction between the full-size neuroglian cytoplasmic domain and *Drosophila* ankyrin can be readily detected using the yeast two-hybrid assay system (Dubreuil et al., 1996; Fig. 2), these neuroglian deletion constructs were initially tested for their ability to interact with a *Drosophila* ankyrin fusion protein in yeast cells. All constructs containing deletions within the amino-terminal

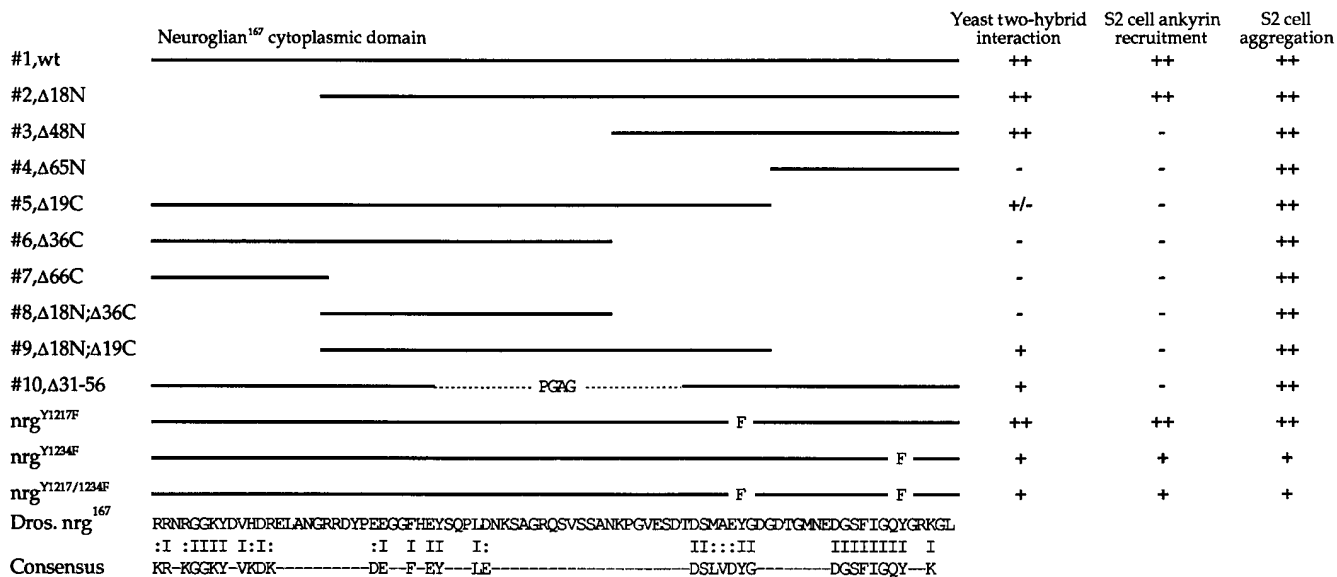
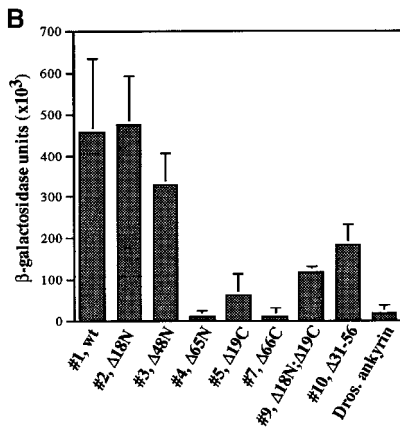
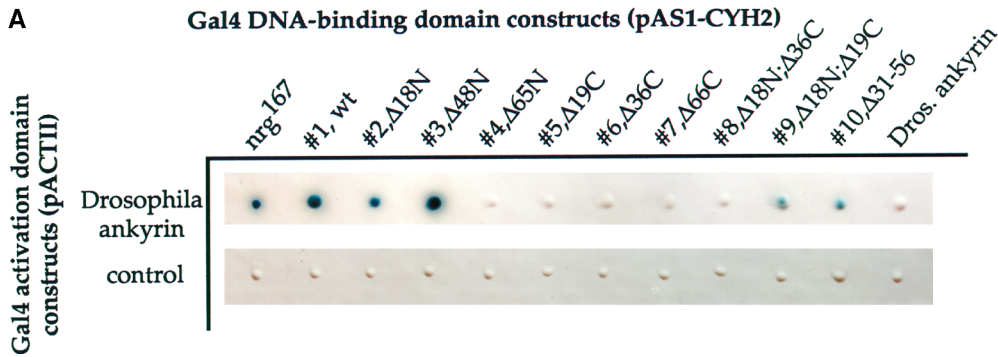


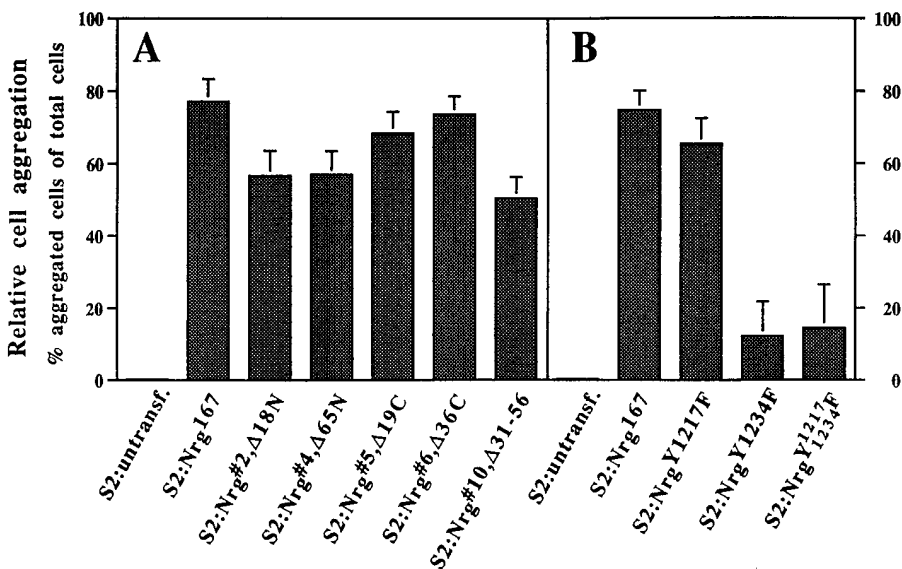
Figure 1. cDNA constructs introducing various deletions and point mutations into the cytoplasmic domain of *Drosophila* neuroglian. Truncations at the amino- and carboxyl-terminal end of the neuroglian<sup>167</sup> cytoplasmic domain, an internal deletion, and tyrosine to phenylalanine point mutations were generated by PCR as described in the Materials and Methods section. Lines represent the wild-type or mutated neuroglian cytoplasmic domains, starting at the first basic amino acid residue after the membrane-spanning segment and ending at the carboxy-terminus of the protein. The results of the yeast two-hybrid interaction between *Drosophila* ankyrin and the neuroglian cytoplasmic deletion proteins, as well as the ability of these deleted neuroglian molecules to induce S2 cell aggregation and to recruit ankyrin to S2 cell contacts are summarized on the right. Wild-type activity levels are indicated with ++. At the bottom of the figure, cytoplasmic amino acid residues which are conserved in more than 90% of all known L1 family members are shown.



**Figure 2.** A qualitative and quantitative yeast two-hybrid analysis maps the minimal ankyrin binding site to a 17-amino acid segment within the *Drosophila* neuroglial cytoplasmic domain with the next 19 carboxy-terminal amino acid residues being required for high efficiency binding. (A) Qualitative yeast two-hybrid analysis of the interaction between neuroglial cytoplasmic domain fragments and *Drosophila* ankyrin. Blue colonies result from the induction of  $\beta$ -galactosidase activity and indicate an interaction between the two GAL4 fusion proteins. *Top row*, yeast colonies containing a pACTII–*Drosophila* ankyrin construct (expressing a GAL4 activation domain–*Drosophila* ankyrin fusion protein) as well as a pAS1–CYH2 construct (expressing a fusion protein consisting of the GAL4 DNA-binding domain and a wild-type or deleted neuroglial cytoplasmic domain); *bottom row*, yeast colonies containing the same pAS1–CYH2 constructs and a pACTII vector control that did not result in an activation of  $\beta$ -galactosidase expression. (B) Quantitative analysis of the effects of neuroglial cytoplasmic deletions on the yeast two-hybrid neuroglial–ankyrin interaction. Data bars represent the mean  $\pm$  SD from three different yeast colonies performed in triplicate determinations.

48-amino acid residues of the cytoplasmic domain interacted with ankyrin at or near wild-type levels in yeast cells (Fig. 2). In all constructs missing the 19 most carboxy-terminal amino acid residues, the neuroglial–ankyrin interaction was significantly reduced, although not abolished. With the exception of construct no. 5, all constructs including residues 49–65 of the neuroglial cytoplasmic protein domain exhibited strong binding to the ankyrin fusion protein. Yeast colonies transfected with construct no. 5, which lacks the carboxy-terminal 19-amino acid residues of the neuroglial cytoplasmic domain, developed a weak signal

only after prolonged incubation. In contrast, after the same length of incubation no positive signal was ever detected in yeast colonies harboring control pAS-CYH2 plasmids or neuroglial deletion constructs nos. 4, 6, 7, or 8. The smallest region which mediated ankyrin binding at a low level consists of a well conserved 17-amino acid segment (Fig. 1; present in construct no. 3 and absent in construct no. 4). High efficiency binding between the neuroglial and the ankyrin fusion proteins, however, was only observed when the adjacent carboxy-terminal 19-amino acid residues were also present. These results indicate that

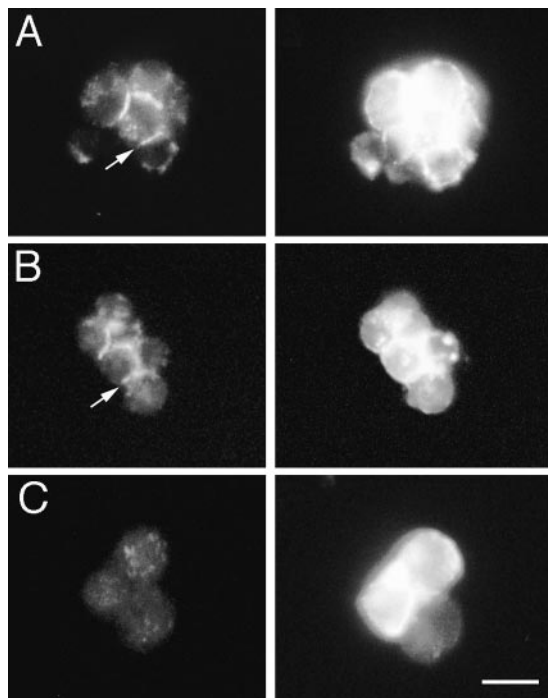


**Figure 3.** Quantitative evaluation of S2 cell aggregation induced by the expression of mutated neuroglial. For a quantification of S2 cell aggregation, duplicate cultures were prepared, induced for 15 h at low cell density, and then processed as described in the Materials and Methods section. Cells that had not aggregated were counted (minimal aggregate size >5 cells) and the number of aggregated cells was calculated as the difference to the total number of cells. Shown is the percentage of total cells which had aggregated after 2 h of shaking. Data bars represent the mean  $\pm$  SD from two independent experiments.

the conserved FIGQY motif, which was previously shown to be important for ankyrin binding (Davis and Bennett, 1994; Garver et al., 1997), is necessary but not sufficient for high efficiency ankyrin binding. A low-level interaction with ankyrin was still observed for two constructs (nos. 5 and 9) missing this motif.

The same set of cytoplasmic domain deletions was spliced to the remainder of the full-length neuroglian coding sequence for expression studies in *Drosophila* S2 cells. These recombinant neuroglian cDNA constructs yielded polypeptides with the expected apparent molecular weights in Western blots probed with anti-neuroglian antibodies (data not shown) and they induced robust S2 cell aggregation (Fig. 3 A). A double-label immunofluorescence analysis of the neuroglian and ankyrin distribution in S2 cells indicated that wild-type neuroglian was abundant over the entire cell surface, yet ankyrin was exclusively recruited to sites of cell-cell contact (Dubreuil et al., 1996; Fig. 4 A).

Expression of the  $\Delta 18N$  neuroglian deletion protein (construct no. 2, lacking the 18 amino-terminal residues of the cytoplasmic domain) in S2 cells resulted in the same pattern of ankyrin and neuroglian staining which was ob-

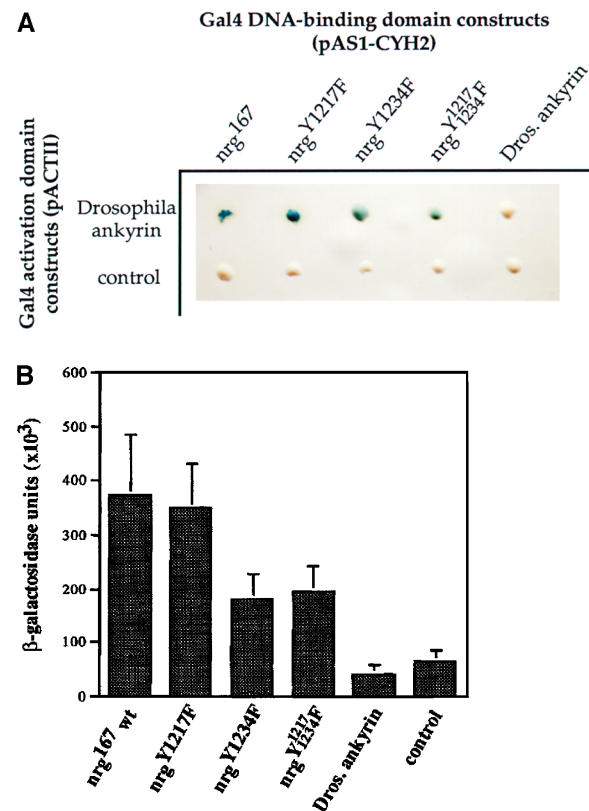


**Figure 4.** A conserved membrane-proximal segment of the neuroglian cytoplasmic domain is dispensable for recruitment of ankyrin to cell contacts in S2 cells. S2 cells expressing wild-type or cytoplasmic deletion *Drosophila* neuroglian were allowed to aggregate and were double stained with a polyclonal rabbit anti-*Drosophila* ankyrin (left column) and a mouse anti-*Drosophila* neuroglian antibody (right column) followed by fluorescently-labeled secondary antibodies. Cells were permeabilized before staining by treatment with 0.1% Triton X-100. *Drosophila* ankyrin was recruited to cell contact sites (arrows) in cell aggregates expressing either wild-type neuroglian (A) or neuroglian in which the first 18 amino acid residues of the cytoplasmic domain had been deleted (construct no. 2 shown in row B). All other deletions in the cytoplasmic neuroglian domain abolished ankyrin recruitment (as shown for construct no. 9 in panels C). Bar, 10  $\mu$ m.

served with wild-type neuroglian (Fig. 4 B). However, none of the other deletion constructs tested (e.g.,  $\Delta 18N$ ;  $\Delta 19C$  shown in Fig. 4 C) produced any detectable staining of ankyrin at cell contacts or other regions of the cell surface, even though the mutant neuroglian proteins were abundantly expressed at the plasma membrane. Thus, the ankyrin recruitment assay in S2 cells revealed a more complex requirement of the neuroglian cytoplasmic domain in ankyrin binding than was expected based on the results of the yeast two-hybrid assay.

### Functional Analysis of Conserved Tyrosine Residues in the Cytoplasmic Domain of Neuroglian

A conserved cytoplasmic tyrosine residue in rat neurofascin, corresponding to Y1234 in neuroglian, was previously



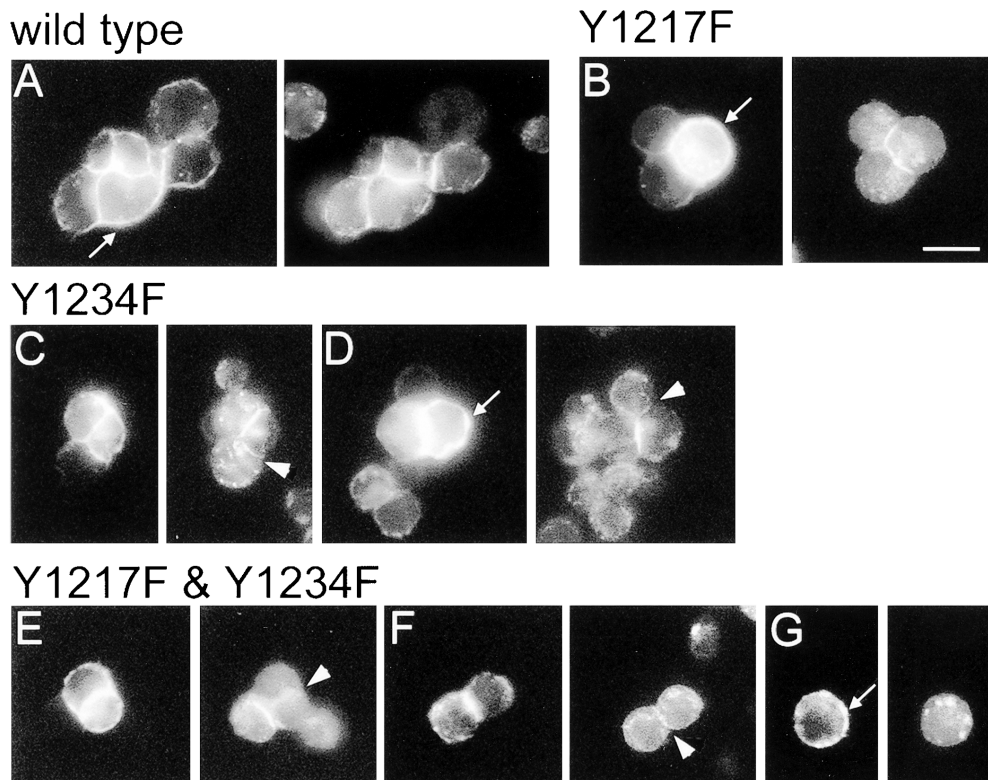
**Figure 5.** Qualitative and quantitative yeast two-hybrid analysis of the interaction between *Drosophila* ankyrin and neuroglian<sup>167</sup> cytoplasmic domains with tyrosine to phenylalanine amino acid substitutions. (A) Qualitative yeast two-hybrid analysis of the interaction between Y to F neuroglian cytoplasmic domain mutations and *Drosophila* ankyrin. Blue colonies result from the induction of  $\beta$ -galactosidase activity and indicate an interaction between the two GAL4 fusion proteins. Top row, yeast colonies containing a pACTII-*Drosophila* ankyrin construct (expressing a GAL4 activation domain-*Drosophila* ankyrin fusion protein) as well as a pAS1-CYH2 construct (expressing a fusion protein consisting of the GAL4 DNA-binding domain and a neuroglian cytoplasmic domain, which was either wild-type or contained one or two point mutations resulting in Y to F amino acid substitutions); bottom row, yeast colonies containing the same pAS1-CYH2 constructs and a pACTII vector control. (B) Quantitative evaluation of  $\beta$ -galactosidase expression in the yeast cells shown in A. Data bars represent the mean  $\pm$  SD from two different experiments performed in triplicate determinations.

shown to have a possible regulatory role, since phosphorylation of this residue inhibited the binding of ankyrin to neurofascin *in vitro* (Garver et al., 1997). Here the effects of tyrosine to phenylalanine mutations at two positions of the *Drosophila* neuroglian cytoplasmic domain on ankyrin binding were evaluated using a yeast two-hybrid assay. The residues Y1217 and Y1234 in neuroglian correspond to the tyrosine residues in rat neurofascin which were mutated and analyzed by Garver et al. (1997). Replacement of Y1217 with a phenylalanine had no detectable effect on ankyrin binding (Fig. 5). However, the Y1234F mutation had a significant effect, reducing ankyrin binding by ~50%. The double mutant Y1217F/Y1234F form reduced ankyrin binding to a similar extent. Thus, the conserved Y1234 contributes to the interaction between ankyrin and neuroglian. However, in contrast to rat neurofascin (Tuvia et al., 1997), substitution of the tyrosine residue with phenylalanine in neuroglian greatly reduced its ability to bind ankyrin.

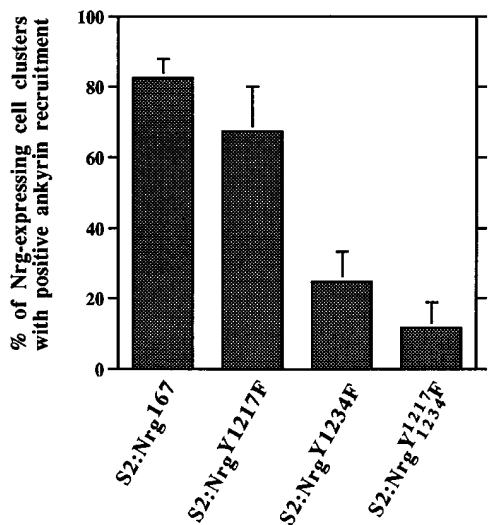
The same tyrosine mutations (Y1217F and Y1234F) were engineered into the complete neuroglian coding sequence for analysis of ankyrin-binding activity in S2 cells. Aggregates expressing wild-type neuroglian were typically large and cells often exhibited significant neuroglian staining over all regions of the plasma membrane (Fig. 6 A, *arrow*). In contrast, the most conspicuous ankyrin staining was observed at cell-cell contacts (Fig. 6 A, *right*). Additional ankyrin staining was visible as a diffuse pattern throughout the cytoplasm and, in many cases, as a punctate pattern in the peripheral cytoplasm. This fainter pattern of ankyrin staining is probably not associated with the plasma membrane since it failed to colocalize with plasma membrane markers such as neuroglian, and it was also vis-

ible in the absence of neuroglian expression (Dubreuil et al., 1996). The same patterns of neuroglian and ankyrin staining were observed in cultures expressing the Y1217F mutant form of neuroglian (Fig. 6 B), although the efficiency of ankyrin recruitment was slightly lower than with wild-type neuroglian (Fig. 7). There was a dramatic drop in the efficiency of ankyrin recruitment in cells expressing the Y1234F neuroglian mutation or the double mutant (Fig. 6, C-F, *arrowheads*). Most contact sites in which neuroglian was prominently stained showed little or no recruitment of ankyrin in cells expressing the Y1234F mutation (e.g., Fig. 6 F). Nevertheless, ankyrin was consistently detected at sites of cell-cell contact in a fraction of cell aggregates expressing these mutations (Fig. 6, C-F and Fig. 7).

Although there was a significant effect of the Y1234F mutation on the efficiency of ankyrin recruitment, there was no apparent effect on the selectivity of recruitment. As with wild type, the only significant colocalization of ankyrin with Y1234F neuroglian occurred at sites of cell contact (Fig. 6, C-E), even though neuroglian was often abundant at noncontact regions of the plasma membrane (e.g., Fig. 6 D, *arrow*). It should be noted that although the panels shown in the figure were chosen to demonstrate the selectivity of ankyrin recruitment, most of the mutant cell clusters analyzed in the experiment did not exhibit detectable ankyrin staining at cell contacts (Fig. 7). Ankyrin also failed to colocalize with neuroglian in single cells that had not yet joined an aggregate (Fig. 6 G). These results are consistent with the previous observation that ankyrin colocalizes with neuroglian only at sites of cell contact, and not with neuroglian found at noncontact regions of the plasma membrane (Dubreuil et al., 1996). Since cytoplasmic tyrosine mutations did not lead to colocalization of ankyrin



**Figure 6.** A cytoplasmic tyrosine residue is required for efficient recruitment of ankyrin to cell contacts in neuroglian-expressing S2 cells. S2 cells were induced to express wild-type neuroglian (A),  $\text{nr}g^{\text{Y1217F}}$  (B),  $\text{nr}g^{\text{Y1234F}}$  (C and D), or double mutant neuroglian (E-G) for 24 h before processing for antibody labeling of neuroglian (left panels) and ankyrin (right panels) as in Fig. 4. *Arrows*, abundance of neuroglian at noncontact regions of the plasma membrane; *arrowheads*, cell contact sites in mutant cells with little or no recruitment of ankyrin. Bar, 10  $\mu\text{M}$ .



**Figure 7.** Quantification of ankyrin recruitment in S2 cells expressing neuroglian proteins mutated at cytoplasmic tyrosines. Populations of transfected S2 cells expressing wild-type neuroglian (*Nrg*<sup>167</sup>) or the indicated tyrosine mutations of neuroglian were double labeled with mouse anti-neuroglian and rabbit anti-ankyrin antibodies as in Fig. 3. In each experiment, 100 S2 cell clusters were identified by virtue of their neuroglian expression and then scored for the presence or absence of detectable ankyrin staining at cell contacts. Data bars represent the mean  $\pm$  SD from two independent experiments.

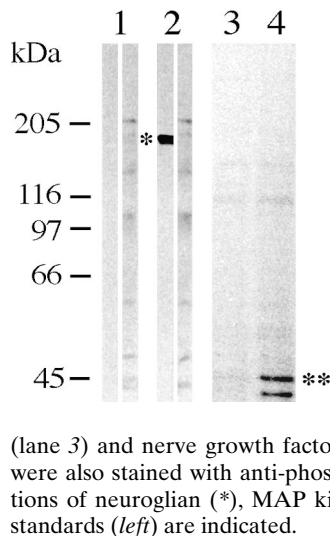
with neuroglian at noncontact regions of the plasma membrane, a mechanism other than the phosphorylation of these residues must be responsible for the observed selectivity of ankyrin recruitment.

#### *Drosophila Neuroglian Is Not Detectably Phosphorylated at Tyrosine Residues in S2 Cells*

Further evidence that tyrosine phosphorylation is not responsible for neuroglian regulation in S2 cells was obtained by staining Western blots of neuroglian-expressing S2 cell lysates with a phosphotyrosine-specific antibody (Fig. 8). After induction, neuroglian was readily detected with a neuroglian-specific antibody (Fig. 8, lane 2, *left column*), but not with the anti-phosphotyrosine antibody (Fig. 8, lane 2, *right column*). Faint bands in the region of neuroglian were also detected by the anti-phosphotyrosine antibody in uninduced cells (Fig. 8, lane 1, *right column*) where neuroglian was undetectable (Fig. 8, lane 1, *left column*). PC12 cells treated with nerve growth factor were used as a positive control. Tyrosine-phosphorylated mitogen-activated protein (MAP) kinase was readily detected in growth factor-induced PC12 cells (Fig. 8, lane 4, \*\*) but not in control untreated cells (Fig. 8, lane 3) (Boulton et al., 1991).

#### *Neuroglian-mediated Adhesion Is Subject to Inside-Out Regulation*

The effect of cytoplasmic mutations on neuroglian-mediated cell adhesion was analyzed in qualitative and quantitative S2 cell aggregation assays. It was previously shown that a glycosyl phosphatidylinositol-linked form of neuro-

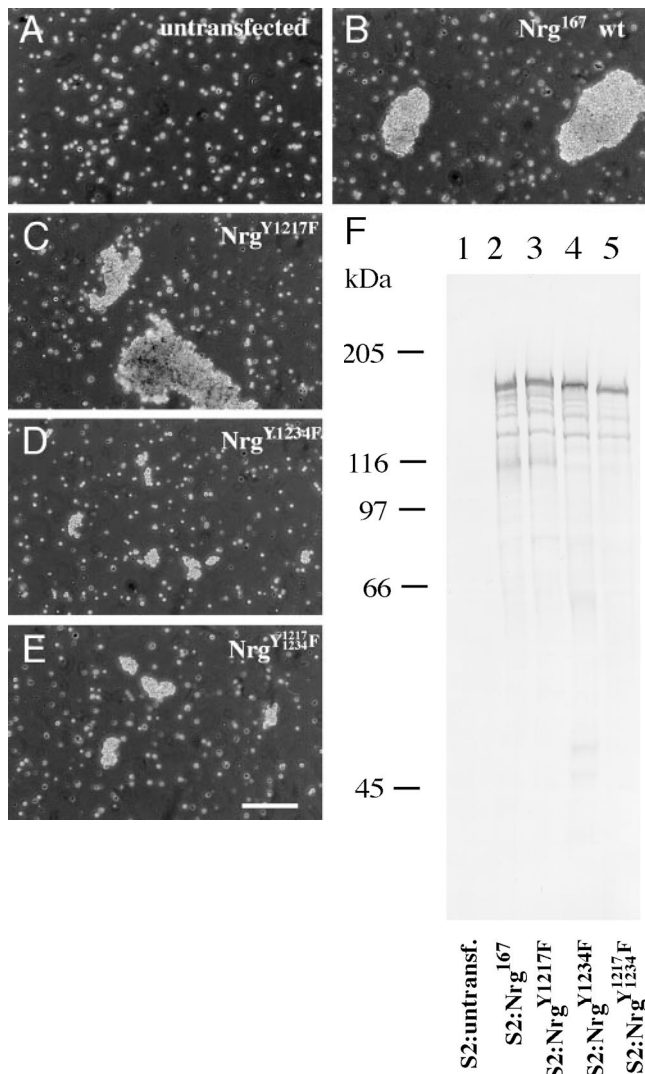


**Figure 8.** Neuroglian is not phosphorylated on tyrosine residues in S2 cells. Western blots of total S2 cell proteins from uninduced cells (lane 1) or cells induced to express neuroglian (lane 2) were stained with mouse anti-neuroglian (lanes 1 and 2, *left columns*) or rabbit anti-phosphotyrosine antibodies (*right columns*). Antibody binding was detected with alkaline phosphatase-conjugated secondary antibodies. Total proteins from control PC12 cells (lane 3) and nerve growth factor-stimulated PC12 cells (lane 4) were also stained with anti-phosphotyrosine antibody. The positions of neuroglian (\*), MAP kinase (\*\*) and molecular weight standards (*left*) are indicated.

glian lacking the entire cytoplasmic domain nevertheless exhibited robust cell adhesion (Hortsch et al., 1995). All of the neuroglian cytoplasmic domain deletion mutants analyzed here also exhibited strong cell aggregation comparable to cells expressing wild-type neuroglian (refer to Fig. 3 A). In contrast, when analyzing the Y to F mutant neuroglian forms for their competence to recruit ankyrin in S2 cells, we observed that neuroglian molecules harboring a Y1234F mutation consistently induced smaller cell aggregates when compared with wild-type or Y1217F-mutant forms (Fig. 9, A–E). Although a majority of cells expressing wild-type or Y1217F-mutant neuroglian had joined large cell aggregates after 2 h of incubation, cell aggregation was significantly reduced in S2 cell cultures expressing Y1234F-mutant neuroglian (Fig. 9 D). After prolonged incubation times (4–24 h) the mutant neuroglian-expressing cell lines did form somewhat larger aggregates, but never reached wild-type levels (data not shown). This effect was not due to a reduced level of neuroglian protein produced by these cell lines, since Western blot analysis demonstrated similar quantities of neuroglian in induced cultures (Fig. 9 F).

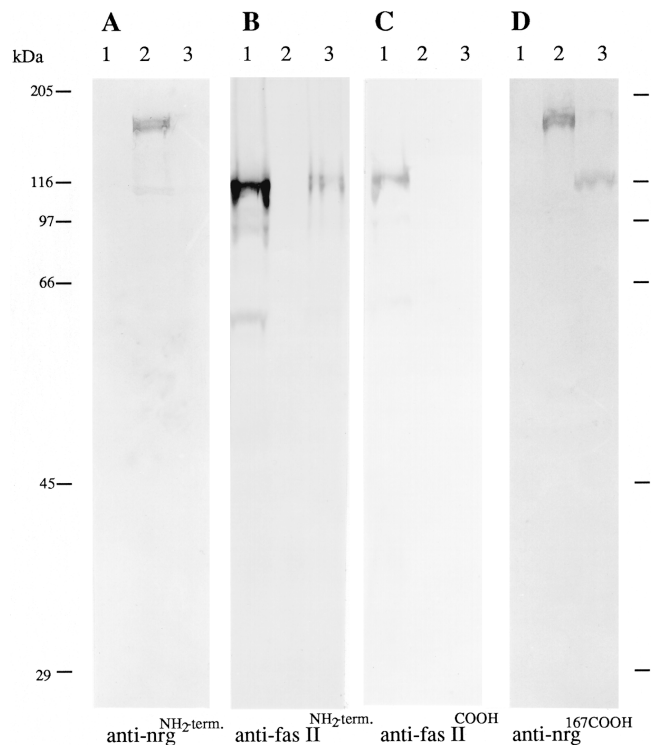
#### *Outside-In Signaling by a Fasciclin II–Neuroglian Chimera*

The mechanism behind the adhesion-based neuroglian signal was further examined with a chimeric cDNA composed of the extracellular domain of the *Drosophila* CAM fasciclin II linked to the cytoplasmic and transmembrane domains of neuroglian. Fasciclin II is also a member of the Ig superfamily, but it is distantly related in sequence and distinct from neuroglian in domain organization (Grenningloh et al., 1991). If the neuroglian signal is simply a consequence of cell adhesion, then the chimera should mimic authentic neuroglian in its ability to induce the recruitment of ankyrin to sites of cell contact. However, if the signal depends on allosteric communication between the extracellular and intracellular domains of neuroglian, then the signal should be uncoupled in the chimeric molecule. The fasciclin II cDNA used here encodes a 115-kD transmembrane protein with a 104-amino acid cytoplasmic do-



**Figure 9.** S2 cells expressing neuroglian with a cytoplasmic Y1234F mutation exhibit a reduced level of cell aggregation. S2 cell lines that had been transfected with either wild-type (B) or mutated neuroglian cDNA constructs (C, *Nrg*<sup>Y1217F</sup>; D, *Nrg*<sup>Y1234F</sup>; and E, *Nrg*<sup>Y1217/1234F</sup>) were induced for 15 h with Cu<sup>2+</sup> ions and subsequently incubated at room temperature on a shaking platform at 200 rpm for 4 h. (A) Control cells not expressing neuroglian. S2 cells cultures were processed in parallel for Western blot analysis and probed with the neuroglian-specific 3F4 mAb (F). The equivalent of 5 × 10<sup>5</sup> cells was loaded in each lane. Bar, 200 μm.

main that is unrelated to neuroglian (Fig. 10). Expression of authentic fasciclin II in S2 cells caused extensive cell aggregation (Fig. 11, A and B), but did not direct ankyrin recruitment to cell contacts (Fig. 11 A). In contrast, expression of the fasciclin II-neuroglian chimera resulted in cell aggregation and recruitment of ankyrin to sites of cell-cell contact (Fig. 11, C and E). Ankyrin was not recruited to nonadherent regions of the plasma membrane in cell aggregates or to the plasma membrane in single cells expressing the chimera (data not shown). The fasciclin II staining pattern appeared concentrated at sites of cell-cell contact in detergent-permeabilized cells (Fig. 11, B and D). However, control experiments with nonpermeabilized



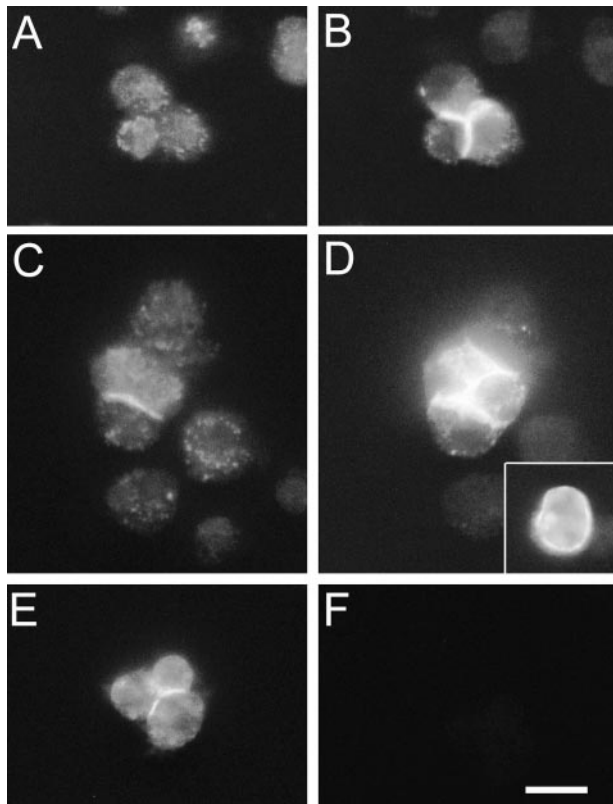
**Figure 10.** Western blots of fasciclin II, neuroglian, and fasciclin II<sup>EC</sup>-neuroglian<sup>TM+CP</sup> hybrid proteins expressed in S2 cells. S2 cells, which had been transfected with a fasciclin II (PEST form) (lanes 1), a neuroglian<sup>167</sup> (lanes 2), or a fasciclin II<sup>EC</sup>-neuroglian<sup>TM+CP</sup> (lanes 3) cDNA construct, were induced overnight and processed for Western blot analysis. Blot A was incubated with the neuroglian-specific mAb 3F4 which recognizes an epitope on the neuroglian extracellular domain, blot B with a rat polyclonal antiserum which was raised against the entire fasciclin II protein, blot C with the mAb 1D4 which binds to a cytoplasmic epitope of the fasciclin II (PEST) protein form, and blot D with a polyclonal rat antiserum which was raised against a small peptide representing the eight carboxy-terminal amino acid residues of the neuroglian<sup>167</sup> form.

cells revealed that the chimera was also abundant at non-adherent regions of the plasma membrane (Fig. 11 D, inset). Thus, ankyrin recruitment coincided with sites of cell adhesion and not with the total distribution of the chimeric molecule. These results indicate that cell adhesion is a sufficient signal to activate recruitment of ankyrin by the cytoplasmic domain of neuroglian.

## Discussion

In addition to their primary roles in cell adhesion, adhesion molecules from the integrin and cadherin families transduce signals across the plasma membrane (Clark and Brugge, 1995; Gumbiner, 1995). Here we show that neuroglian, a CAM from the Ig superfamily, also has the ability to transduce signals in both directions across the plasma membrane. First, we have investigated the structural requirements for outside-in signaling which leads to selective binding of ankyrin to neuroglian at sites of cell contact. Studies of a fasciclin II-neuroglian chimera revealed that adhesion, rather than any unique property of neuro-





**Figure 11.** Selective recruitment of ankyrin to cell contacts in S2 cells expressing a chimeric neuroglian molecule. S2 cells were induced to express the adhesion molecule fasciclin II (A and B) or a chimeric molecule (fasciclin II<sup>EC</sup>-neuroglian<sup>TM+CP</sup>) in which the extracellular neuroglian domain was replaced with the corresponding domain from fasciclin II (C–F). Cells were induced for 48 h and then processed for immunofluorescent staining with antibodies against ankyrin (A, C, and E) and against the extracellular domain of either fasciclin II (B and D) or neuroglian (F). Control experiments (D, inset) revealed that fasciclin II was abundantly detected at all regions of the plasma membrane when the detergent permeabilization step was omitted. Bar, 10  $\mu$ M.

glian's extracellular domain, activates ankyrin binding to the intracellular domain. Phosphorylation of a conserved cytoplasmic tyrosine residue, which was recently shown to inhibit ankyrin binding to a vertebrate neuroglian homologue, neurofascin (Garver et al., 1997; Tuvia et al., 1997), did not regulate ankyrin binding to neuroglian in S2 cells. Second, the extracellular adhesive activity of neuroglian was found to be regulated by the state of the cytoplasmic domain. Although the biological significance of this level of regulation is not yet known, it provides a simple mechanism for the spatial and temporal regulation of L1 family members. These observations broaden our understanding of how extracellular adhesion directs rearrangement of the spectrin/ankyrin membrane skeleton within the cell and how protein interactions inside the cell can regulate extracellular adhesion.

Whereas the inside-out regulation of neuroglian's adhesive function is one finding of the present study, our approach has largely been directed toward the mechanism of outside-in signaling. *Drosophila* S2 cells provide a unique

model system in which to study the recruitment of ankyrin to the plasma membrane, since ankyrin is not ordinarily associated with the plasma membrane of these cells. We previously demonstrated that both *Drosophila* neuroglian (Dubreuil et al., 1996) and human L1-CAM (Vallejo et al., 1997; Hortsch et al., 1998) induce the selective recruitment of ankyrin to sites of cell contact, even though the adhesion molecules themselves are broadly expressed over the entire cell surface. Once neuroglian has interacted with ankyrin at cell–cell contact sites, it becomes resistant to extraction by nonionic detergents (Dubreuil et al., 1996). This change in the solubility of neuroglian precludes an analysis of the neuroglian–ankyrin interaction by immunoprecipitation techniques and, when using immunofluorescence microscopy, results in an underrepresentation of neuroglian staining at noncontact areas of the S2 plasma membrane.

It was previously shown that the FIGQY sequence, which is highly conserved throughout the L1 family, provides an essential part of the ankyrin-binding domain in rat neurofascin (Davis and Bennett, 1994; Garver et al., 1997). Through a combination of yeast two-hybrid analysis and expression of mutated neuroglian molecules in S2 cells, we have mapped a larger, 36-amino acid segment in the neuroglian cytoplasmic domain, which includes this conserved motif, that is both necessary and sufficient to allow the interaction between neuroglian and ankyrin.

The demonstration that ankyrin binding to rat neurofascin can be regulated by phosphorylation of the tyrosine residue in the FIGQY motif (Garver et al., 1997; Tuvia et al., 1997) suggested a possible mechanism to explain outside-in regulation of neuroglian. Phosphorylation of this conserved tyrosine in the cytoplasmic domain of neurofascin caused an  $\sim$ 40% reduction in ankyrin binding and a comparable decrease in cell aggregation. Based on these properties of neurofascin, we hypothesized that the association of ankyrin with neuroglian in S2 cells could also be regulated by tyrosine phosphorylation, assuming that the phosphorylation of the tyrosine residue in the FIGQY motif is regulated by cell adhesion.

If neuroglian were phosphorylated on tyrosine residues in S2 cells, and phosphorylation was responsible for the lack of ankyrin binding to neuroglian at noncontact sites of aggregated S2 cells, then the mutation of tyrosine residue 1234 in *Drosophila* neuroglian would be expected to cause ankyrin binding indiscriminately at all regions of the plasma membrane. Although the neuroglian Y1234F mutation greatly reduced the extent of ankyrin binding to neuroglian, it did not affect the selective association of ankyrin at sites of cell–cell contact or the lack of ankyrin at noncontact regions of the plasma membrane. Thus, another mechanism must be responsible for the outside-in regulation of ankyrin recruitment in S2 cells. We therefore suggest that the previously observed effects of tyrosine phosphorylation on rat neurofascin constitutes a mechanism of regulation that might be specific for the neurofascin subfamily. Alternatively, tyrosine phosphorylation may operate in regulating ankyrin binding to L1 molecules in some cell types, such as the B104 neuroblastoma cell line, but not in other cells. S2 cells may simply lack a tyrosine kinase of the appropriate specificity for the FIGQY motif to produce an effect on ankyrin binding. It is impor-

tant to note that these two mechanisms for regulating the interaction between ankyrin and L1 molecules are not mutually exclusive, and the phosphorylation of the conserved tyrosine residue in the FIGQY motif might provide an additional way to inhibit L1-mediated cell adhesion and L1 binding to the membrane skeleton.

Other types of protein phosphorylation might also be involved in regulating the association of ankyrin with neuroglial in vivo or in S2 cells. Several cytoplasmic serine residues are also highly conserved throughout the L1 family and some have been shown to be the target of protein kinases (Wong et al., 1996a,b). The phosphorylation of these residues might have an effect on ankyrin binding to L1 family members and play an important role in the regulation of this interaction in S2 cells.

The regulation of ankyrin binding to L1-type molecules might also involve the adhesion-induced clustering of several L1 molecules within the plane of the plasma membrane, similar to the mechanism of growth factor receptor activation (Schlessinger and Ullrich, 1992). Alternatively, the homophilic adhesion process could induce an allosteric change in the neuroglial molecule. However, the finding that a chimeric molecule, composed of the extracellular domain of a distantly related adhesion molecule coupled to the cytoplasmic and transmembrane domains of neuroglial, exhibits the same regulated association with ankyrin as wild-type neuroglial argues against the allosteric model.

Our results indicate that the tyrosine residue in the conserved FIGQY motif plays an important role in L1's interaction with the membrane skeleton and its adhesive function. One of the identified L1-CAM mutations, which causes a range of neurological phenotypes in humans, results in the exchange of tyrosine 1229 in the human L1 protein for a histidine residue (Van Camp et al., 1996). Residue Y1229 in human L1-CAM is homologous to Y1234 in the *Drosophila* neuroglial sequence. Considering that the cytoplasmic domain of human L1 CAM is dispensable for its adhesive function (Wong et al., 1995a), it has been an enigma why mutations in the cytoplasmic domain of human L1-CAM can cause the same range of phenotypes as mutations in the extracellular domain. It now appears that extracellular adhesive and intracellular cytoskeletal interactions of L1 molecules are functionally connected and therefore influence each other. Members of the L1 family are expressed on advancing growth cones during development and during regeneration after nerve injury and by other motile neuronal cells, such as migrating granule cells (Lindner et al., 1983; Kamiguchi and Lemmon, 1997). A precise regulation of neuronal adhesiveness must be very important in these cells to allow dynamic interactions with their cellular environment. Any mutation interrupting this delicate adhesive balance might therefore cause the observed wide range of L1 mutant phenotypes (Wong et al., 1995b; Dahme et al., 1997; Hall and Bieber, 1997).

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