Neuroglian-mediated Cell Adhesion Induces Assembly of the Membrane Skeleton at Cell Contact Sites

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Abstract. The protein ankyrin links integral membrane proteins to the spectrin-based membrane skeleton. Ankyrin is often concentrated within restricted membrane domains of polarized epithelia and neurons, but the mechanisms responsible for membrane targeting and its segregation within a continuous lipid bilayer remain unexplained. We provide evidence that neuroglian, a cell adhesion molecule related to L1 and neurofascin, can transmit positional information directly to ankyrin and thereby polarize its distribution in *Drosophila* S2 tissue culture cells. Ankyrin was not normally associated with the plasma membrane of these cells. Upon expression of an inducible neuroglian minigene, however, cells aggregated into large clusters and ankyrin became concentrated at sites of cell–cell contact. Spectrin was also recruited to sites of cell contact in response to neuroglian expression. The accumulation of ankyrin at cell contacts required the presence of the cytoplasmic domain of neuroglian since a glycosyl phosphatidylinositol–linked form of neuroglian failed to recruit ankyrin to sites of cell–cell contact. Double-labeling experiments revealed that, whereas ankyrin was strictly associated with sites of cell–cell contact, neuroglian was more broadly distributed over the cell surface. A direct interaction between neuroglian and ankyrin was demonstrated using yeast two-hybrid analysis. Thus, neuroglian appears to be activated by extracellular adhesion so that ankyrin and the membrane skeleton selectively associate with sites of cell contact and not with other regions of the plasma membrane.

MANY cell types (e.g., epithelia, neurons) exhibit cell-surface polarity that results from the segregation of membrane components within restricted membrane domains. Several mechanisms have been proposed to explain the establishment and maintenance of cell-surface polarity. Sorting events in the secretory pathway are thought to contribute to the delivery of membrane components to specific cell surface domains (Matter and Mellman, 1994; Simons et al., 1992), but additional mechanisms are required to explain the initial establishment of a polarized secretory apparatus and maintenance of the polarized state (Nelson, 1992; Shiel and Caplan, 1995). One proposed mechanism is the selective retention of membrane components within specialized regions by a polarized membrane skeleton (Hammerton et al., 1991).

To evaluate the contribution of the membrane skeleton to cell polarity, it will be necessary to increase our understanding of its assembly. The membrane skeleton consists of a spectrin–actin network that is attached to the plasma membrane through the protein ankyrin (reviewed by Bennett and Gilligan, 1993). Ankyrin, in turn, interacts with the cytoplasmic domains of several integral membrane proteins, including ion channels, ion pumps, and certain cell adhesion molecules. Little is yet known about how these multiple interactions affect the membrane skeleton. In addition to the multiplicity of potential membrane binding sites, there are at least three ankyrin genes in mammals (Peters et al., 1995; Kordeli et al., 1995), and more than one may be expressed in a single cell. Because of the number of different binding partners involved, the control of ankyrin's interaction with the plasma membrane is likely to be quite complex.

The complexity of ankyrin–membrane interactions might be simplified somewhat if different membrane proteins interact with ankyrin in different ways. Some membrane proteins may simply act as ankyrin receptors that recruit ankyrin from the cytoplasm to the plasma membrane, potentially in an indiscriminate manner. To produce the po-

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1. *Abbreviations used in this paper*: GPI, glycosyl phosphatidylinositol; nrg^{GPI}, GPI-linked form of neuroglian; nrg^{167}, 167-kD isoform of neuroglian; nrg^{180}, 180-kD isoform of neuroglian.

larized distribution of ankyrin observed in some cell types (Nelson and Lazarides, 1984; Drenckhahn and Bennett, 1987; Morrow et al., 1989; Nelson et al., 1990; McNeill et al., 1990), such receptor molecules must somehow be targeted to the appropriate membrane domain independently of their interaction with ankyrin. Other membrane proteins may respond to ankyrin, rather than directing its distribution in the cell. Ankyrin may act as the receptor for these interacting proteins as they arrive at the cell surface. In polarized cells, binding sites on ankyrin are thought to capture and retain diffusible membrane proteins (e.g., the sodium pump; Hammerton et al., 1991) within a domain defined by the membrane skeleton. An individual ankyrin molecule may play both roles by simultaneously binding to a receptor protein and to a responding membrane protein. Alternatively, the membrane skeleton may serve as a multivalent scaffold in which individual ankyrin molecules only interact with a single class of ankyrin-binding membrane proteins.

So far, it has been difficult to establish the order of events that lead to polarized assembly of the membrane skeleton. In the human erythrocyte, interactions of ankyrin with the major integral membrane protein band 3 and with spectrin have been extensively characterized (Bennett, 1990). Yet, even band 3 (which is the best-characterized ankyrin-associated membrane protein) exhibits puzzling properties. It was proposed to be a limiting factor in the assembly of ankyrin during erythrocyte development (Moon and Lazarides, 1984). The stable accumulation of band 3, however, was found to occur later in development than the membrane association of other membrane skeleton proteins (Cox et al., 1987). There must be other protein interactions that direct ankyrin to the plasma membrane before the availability of band 3.

Recent studies of mammalian tissue culture cells have pointed to cell adhesion as a cue that targets ankyrin to specific plasma membrane domains in epithelial cells. Polarization of ankyrin in MDCK cells and E-cadherin–transfected fibroblasts coincides with the formation of cell contacts and the accumulation of E-cadherin at these sites (McNeill et al., 1990; Nelson and Veshnock, 1987; Morrow et al., 1989). While there is obviously a connection between cell adhesion and membrane skeleton assembly in these cells, its molecular basis (direct or indirect) remains to be established (Nelson et al., 1990).

Here we describe a molecular link between neuroglian (a cell adhesion molecule belonging to the Ig superfamily; Bieber et al., 1989) and ankyrin. Using *Drosophila* S2 tissue culture cells as a simple model system, we show that the interaction between these molecules occurs by a mechanism that couples the positional information of cell adhesion to the polar assembly of ankyrin.

Materials and Methods

Origin and Maintenance of Cell Lines

Cells were cultured under standard conditions (Schneider, 1972) using Schneider's medium and 10% FCS both purchased from GIBCO BRL (Gaithersburg, MD). Three stably transfected cell lines were used to produce the two natural isoforms of neuroglian (nrg^{167} and nrg^{180}) and an artificial glycosyl phosphatidylinositol (GPI)-linked form of neuroglian (nrg^{GPI}) under control of an inducible metallothionein promoter (Hortsch et al., 1995). Neuroglian expression was induced by the addition of 0.7 mM $CuSO_4$ to the growth medium for 24 h. Cell aggregates formed during 2 h of gentle rocking at room temperature.

Immunofluorescent Staining and Microscopy

S2 cells and neuroglian-expressing cell aggregates were immobilized on Alcian blue–coated microscope slides and stained as previously described (Lee et al., 1993). Briefly, cells were fixed for 10 min in 2% fresh formaldehyde in PBS, pH 7.2. Reactive aldehydes were quenched in a Tris-containing buffer. Cells were permeabilized with 0.5% Triton X-100 in Tris-buffered saline for 10 min, then equilibrated in Tris-buffered saline/0.1% Tween 20 containing 5% newborn calf serum (GIBCO BRL). Primary and secondary antibodies were each diluted in Tris/Tween buffer with serum and were allowed to react with cells for 2 h at room temperature. Cells were rinsed four times with Tris/Tween buffer after each antibody incubation, mounted in FITC-Guard (Testog, Inc., Chicago, IL), and viewed and photographed with an ausJena microscope (Jena, Germany) using a 50× 0.95 NA Plan Apo objective. Confocal images were obtained using a Noran Odyssey XL laser scanning microscope and an Axiovert 135 HDTV (Carl Zeiss, Inc., Thornwood, NY) in the Al Robin Confocal Microscopy Unit of the University of Chicago. Cells were imaged using a Zeiss 63× 1.4 NA Plan Apo objective at 488 and 568 nm.

Primary antibodies were used as follows: ankyrin, 4 μg/ml affinity-purified rabbit antibody (Dubreuil and Yu, 1994); neuroglian, 1:500 culture supernatant of hybridoma clone 1B7 (Bieber et al., 1989); myc epitope tag, 1:1,000 ascites fluid of hybridoma clone 9E10 (Evan et al., 1985, a gift from Eric Branden, Harvard University, Boston, MA); and β-spectrin, 1:250 dilution of antibody 89 (Byers et al., 1989). Affinity-purified FITC-conjugated goat anti–rabbit Ig (Zymed Laboratories, South San Francisco, CA) and Texas red–conjugated goat anti–mouse Ig (Jackson Immuno Research Laboratories, West Grove, PA) secondary antibodies were used at 1:500 dilutions. The epitope(s) recognized by the ankyrin antibody are lost upon exposure to methanol or glutaraldehyde. Therefore, all of the ankyrin localizations reported here were carried out in formaldehyde-fixed preparations.

Construction of a Recombinant Ankyrin Minigene

A myc-tagged ankyrin minigene was built using a previously described strategy (Deng et al., 1995). An ankyrin cDNA insert corresponding to codons 6–1,550 of the published sequence (Dubreuil and Yu, 1994) was spliced downstream of a 10–amino acid myc epitope tag, and expression of the tagged product was driven by a HindIII–NcoI fragment of the *Drosophila* ubiquitin promoter (Lee et al., 1988). The NcoI site was added during PCR amplification of the promoter region at the position of the ubiquitin start codon (Lee et al., 1993). The recombinant ankyrin product is predicted to be several amino acids longer than authentic *Drosophila* ankyrin, but for unknown reasons, it migrates slightly ahead of ankyrin on SDS-polyacrylamide gels. The construct was transiently expressed in cells that were stably transfected with the pRmHa2-nrg^{180} neuroglian minigene (Hortsch et al., 1995). Transient transfections were carried out essentially as described by Chen and Okayama (1987). 3 d after ankyrin transfection, neuroglian synthesis was induced with 0.7 mM $CuSO_4$, and cell aggregates were processed for immunofluorescence on day 4.

Yeast Two-hybrid Analysis

The two-hybrid system developed by Elledge and co-workers was used as described by Durfee et al. (1993). For constructs pAS1-CYH2-$nrg^{167-cyto}$ and pAS1-CYH2-$nrg^{180-cyto}$, 350- and 550-bp EcoRI/SalI fragments were obtained by PCR from cDNA clones encoding the shorter and longer neuroglian cytoplasmic domain isoforms, respectively. These fragments were ligated into SfiI/SalI cut pAS1-CYH2 vector DNA. The SfiI end of the vector and the EcoRI end of the insert were joined using the heptanucleotide (5′ AATTCCA 3′). When transformed into Y190 yeast cells and tested for β-galactosidase expression, most transformants did not express detectable β-galactosidase. Some yeast colonies, however, were positive for β-galactosidase, possible because of a rare recombination event between the linker regions of the GAL4 cDNA and the neuroglian cDNAs. Therefore, the original neuroglian constructs were modified by excision of the linker region (between NdeI and KpnI sites) followed by religation in the presence of hexanucleotide (5′ TAGTAC 3′). The resulting constructs encode fusions between the GAL4 DNA–binding domain and the 85 or 147 carboxy-terminal amino acid residues of the two neuroglian isoforms.

When transformed into yeast Y190 cells, these constructs did not lead to the inappropriate expression of β-galactosidase.

For the construction of neuroglian fusions in the pACTII yeast vector, PCR was used to introduce an in-frame NcoI site (underlined) immediately upstream of the cytoplasmic domain coding region of the two neuroglian isoforms (5′ oligonucleotide: 5′ GAGCCATGGTCCGACGCAA-TCGGGGC 3′). A 3′ EcoRI site was derived from the parent pBluescript vector. The constructs pACTII-nrg$^{167-cyto}$ and pACTII-nrg$^{180-cyto}$ were assembled using the NcoI and EcoRI sites of the vector and PCR products.

A 2.8-kb EcoRI/BamHI fragment of *Drosophila* ankyrin cDNA (Dubreuil and Yu, 1994) was used to produce clone pAS1-CYH2-ankyrin. Using the heptanucleotide bridge described above, the ankyrin insert was inserted into the SfiI/BamHI–digested vector DNA. A 2.8-kb NcoI/BamHI fragment from the pAS1-CYH2 construct was used to produce clone pACTII-ankyrin. Both ankyrin constructs fuse 941 amino acids from the amino terminal region of *Drosophila* ankyrin to the GAL4 DNA–binding or GAL4-activation domain. This region of ankyrin includes all of the putative membrane-binding domain and much of the spectrin-binding domain of ankyrin (Bennett and Gilligan, 1993).

Yeast selection plates and media were made, and transformation and screening procedures were performed by established methods (Ausubel et al., 1988). Staining for β-galactosidase expression in yeast cells was done using the substrate X-gal as described by Bartel et al. (1993).

Cell Extractions, Fractionations, and Electrophoresis

The amounts of ankyrin in induced and uninduced cells were compared in Western blots of total cellular proteins probed with antiankyrin antibody. Cultures were seeded at 10^6 cells/ml in the presence or absence of copper and incubated 24 h at 25°C. Cells were rocked for 2 h to facilitate contact formation then harvested by centrifugation. Cells were rinsed once in *Drosophila* Ringer's solution (Ashburner, 1989), and cell pellets were dissolved in SDS sample buffer, boiled, and prepared for gel electrophoresis and Western blotting. The solubility of ankyrin and neuroglian in S2 cells was determined by sonicating cells in the presence or absence of detergent. 2×10^7 cells were harvested and resuspended in 2 ml lysis buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5 mM EDTA, 2 μg/ml leupeptin, 60 μg/ml pepstatin A) either with or without 1% Triton X-100 (Sigma Immunochemicals, St. Louis, MO). Cells were lysed by 3 × 10–s bursts with a Branson sonifier microtip at 20 W, and then immediately centrifuged at 75,000 rpm for 20 min at 4°C in a TLA 100.3 rotor (Beckman Instruments, Palo Alto, CA). Gel samples were prepared from the homogenate and supernatant by the addition of 5× SDS sample buffer. The pellet was resuspended in 2 ml of lysis buffer before the addition of sample buffer. Samples were proportionately loaded on SDS gels with material from 10^5 cells/lane, and they were subsequently processed for Western blotting.

The fate of cellular proteins during permeabilization for immunofluorescent staining was also monitored on Western blots. Neuroglian-expressing S2 cells were collected in a microfuge tube by centrifugation at setting 5 in an Eppendorf 2415 microfuge (Brinkmann Instruments, Inc., Westbury, NY) for 1 min. The cell pellet was sequentially resuspended in formaldehyde fixative, quenching, and permeabilization solutions described above. Between steps, cells were collected by centrifugation. Equal proportions of the total fixed cell sample, detergent extract, and detergent insoluble residue were dissolved in SDS gel sample buffer.

Gel electrophoresis, electrophoretic transfer to nitrocellulose, and reaction with antibodies were carried out as previously described (Dubreuil et al., 1987). Rabbit antiankyrin was used at 4 μg/ml, and antineuroglian monoclonal antibody 3F4 was used at 1:1,000 (the 1B7 antineuroglian antibody used in immunolocalizaion does not work efficiently in Western blots).

Results

We previously found that *Drosophila* Schneider's 2 (S2) tissue culture cells (Schneider, 1972) abundantly express components of the membrane skeleton, including spectrin and ankyrin (Dubreuil and Yu, 1994). The distribution of ankyrin in S2 cells was examined by indirect immunofluorescence with a polyclonal ankyrin antibody. A diffuse staining pattern was detected in the cytoplasm of control S2 cells, and in some cells, a brighter punctate pattern was detected in the cytoplasm (Fig. 1 *A*, *inset*). But there was no detectable plasma membrane staining, even though ankyrin was readily detected in Western blots of these cells (see below).

One possible explanation for the lack of detectable ankyrin staining at the plasma membrane is that S2 cells do not express an appropriate ankyrin-binding plasma membrane protein. Previous biochemical studies established that neural cell adhesion molecules of the L1 family, of which *Drosophila* neuroglian is a member, interact with ankyrin through their cytoplasmic domain (Davis et al., 1993; Davis and Bennett, 1994). S2 cells are ordinarily nonadherent, but they form large, stable aggregates upon induction of a transfected neuroglian minigene (Hortsch et al., 1995). Ankyrin specifically associated with zones of cell–cell contact in these aggregates (Fig. 1 *A*, *arrow*, nrg^{180}-expressing cells), and was not detectable on surfaces facing away from cell contacts. Identical results were obtained with cells expressing an alternately spliced form of neuroglian (nrg^{167}; not shown), suggesting that the ankyrin targeting activity must occur within a 69–amino acid cyto-

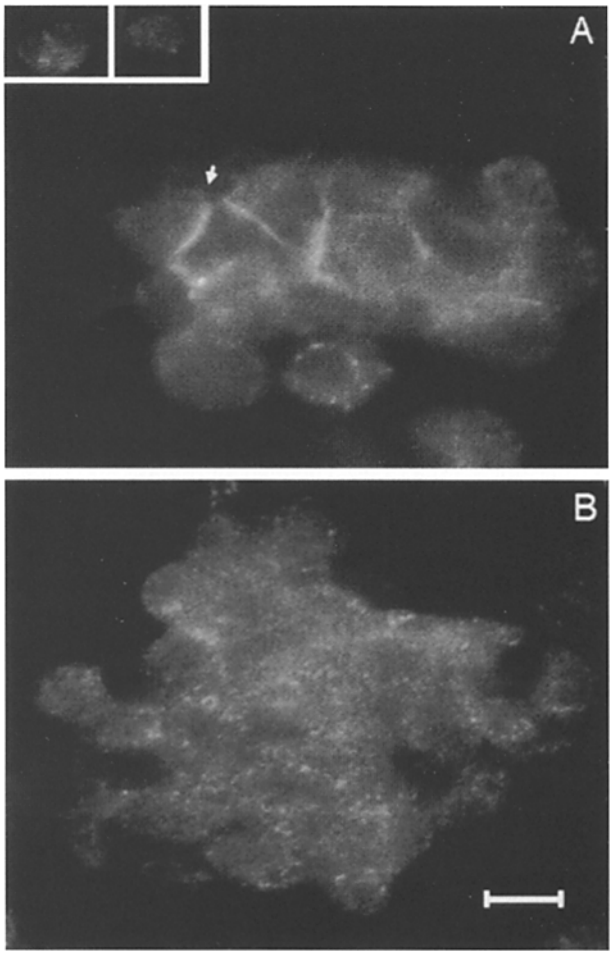
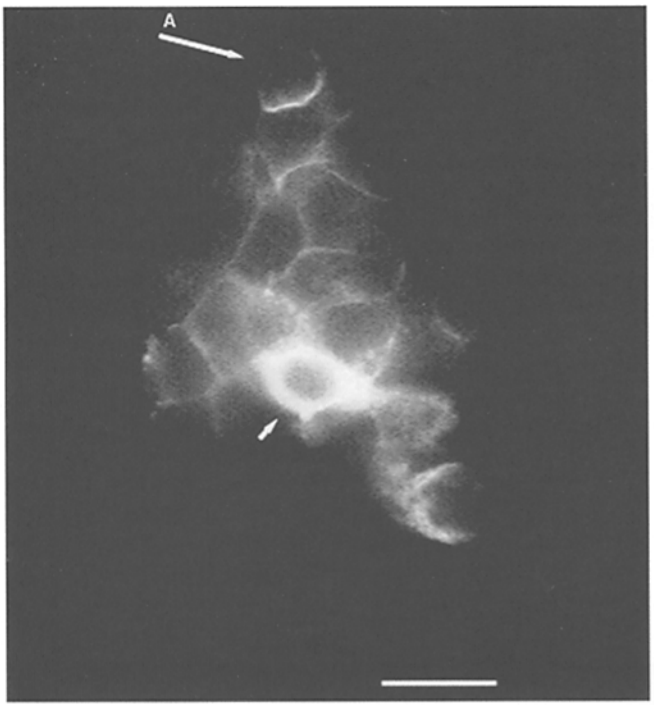


Figure 1. Redistribution of ankyrin in response to cell adhesion. Control S2 cells (*A*, *inset*), neuroglian-expressing cell aggregates (*A*, *nrg*^{180}), and control aggregates expressing a GPI-linked form of neuroglian (*B*, *nrg*^{GPI}) were stained with a polyclonal antiankyrin antibody and fluorescent secondary antibody. Ankyrin was recruited to cell contacts (*arrow*) only in cells that expressed the cytoplasmic domain of neuroglian (*A*). Bar, 10 μM.



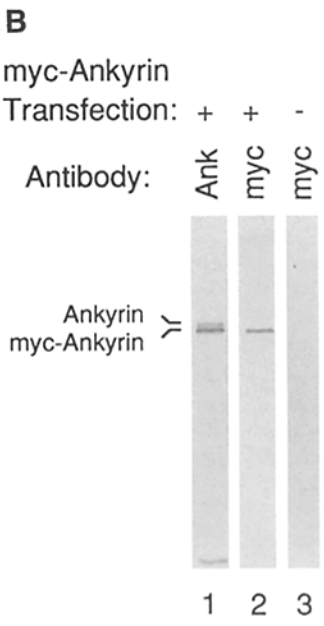


Figure 2. (*A*) Detection of recombinant epitope-tagged ankyrin at cell contacts. Doubly transfected S2 cells expressing neuroglian (nrg^{180}) and myc-tagged ankyrin were stained with the tag-specific antibody 9E10 and fluorescent secondary antibody. Staining intensity varied with the level of recombinant ankyrin expression in the transiently transfected population. Moderate to high levels of ankyrin expression resulted in intense staining at sites of cell contact (*long arrow*) or staining throughout the cytoplasm (*short arrow*), respectively. Bar, 10 μM. (*B*) Specificity of the ankyrin and 9E10 antibodies in Western blots of total S2 cell proteins. Antibody reactions were detected with alkaline phosphatase–conjugated secondary antibodies. Endogenous ankyrin (*upper band*) and *myc*-tagged recombinant ankyrin (*lower band*) reacted with affinity-purified rabbit antiankyrin (lane *1*), but only the myc-tagged ankyrin was detected by antibody 9E10 (lane *2*). The 9E10 antibody did not react with proteins from nontransfected cells (lane *3*).

plasmic sequence that is shared between the two neuroglian isoforms (Hortsch et al., 1990).

The distribution of ankyrin was also examined in cell aggregates expressing a recombinant form of neuroglian in which the transmembrane domain and cytoplasmic tail were replaced with a signal that specifies attachment of a GPI membrane anchor (nrg^{GPI}; Hortsch et al., 1995). The nrg^{GPI}-expressing cells formed large aggregates, indicating that the adhesive activity of neuroglian was not dependent on cytoplasmic interactions. The distribution of ankyrin in these aggregates (Fig. 1 *B*), however, was indistinguishable from control cells that did not express neuroglian (Fig. 1 *A*, *inset*), implying that ankyrin accumulation at cell contacts was dependent on the cytoplasmic domain of neuroglian.

A selective association of ankyrin with cell contacts was also detected in cells that overexpressed ankyrin. Stably transfected S2 cells harboring a nrg^{180} minigene were further transfected with a recombinant myc-tagged ankyrin minigene. The ankyrin minigene product was localized with a tag-specific antibody after formation of cell aggregates via neuroglian expression (Fig. 2 *A*). This experiment addressed two important issues. First, it provided a useful control for the specificity of the ankyrin antibody in immunofluorescence. It was formally possible that the pattern observed in Fig. 1 was the result of an antibody cross-reaction with a component that was not easily detected on Western blots. Second, it provided a way to test the relation between the level of ankyrin and its distribution within cells. The doubly transfected cells exhibited a range of tagged ankyrin expression levels. Low level expression resulted in faint ankyrin staining at sites of cell contact. Moderate expression resulted in intense staining that was still limited to sites of cell contact (*long arrow*). High levels of ankyrin expression appeared to saturate available membrane-binding sites, resulting in bright staining throughout the cytoplasm (*short arrow*). The myc epitope antibody reacted specifically with recombinant tagged ankyrin in transfected S2 cells (Fig. 2 *B*, lane *2*), and not with control S2 cells expressing only endogenous ankyrin (lane *3*). The antiankyrin antibody reacted strongly with endogenous S2 cell ankyrin and recombinant ankyrin, and also with unidentified lower molecular weight components (lane *1*).

The above experiments demonstrated a striking change in the immunofluorescent ankyrin staining pattern after the induction of neuroglian. There are two likely explanations for this change. First, there could be an upregulation of ankyrin levels through either new synthesis or stabilization of the protein. Second, a change in ankyrin staining could simply result from a redistribution of ankyrin within the cell or altered targeting, without a significant change in its amount. The amount of ankyrin present in cultures induced to express neuroglian was compared to control uninduced cultures on Western blots (Fig. 3 *A*). Induced and uninduced cells were loaded at either 10^5 cells per lane (lanes *1* and *2*) or at 3×10^5 cells per lane (lanes *3* and *4*). Similar amounts of ankyrin were detected in induced (lane *1*) and uninduced cells (lane *2*) under conditions where a threefold difference in concentration was clearly visible (compare lanes *1* and *3*). The same fractions were compared on a Coomassie blue–stained gel where there was also no significant difference in protein composition (not shown).

Since there did not appear to be a substantial change in ankyrin levels in the same time frame in which there was a substantial change in the immunofluorescent staining pat-

tern, fractionation experiments were carried out to examine the state of ankyrin before and after neuroglian induction. Ankyrin was present primarily in the insoluble residue of S2 cells lysed in the absence of detergent (Fig. 3 *B*, *P*), and not in the soluble fraction (*S*). Other proteins were efficiently extracted into the soluble fraction, indicating that the lack of ankyrin in the supernatant was a result of its insolubility rather than poor efficiency of cell breakage (not shown). When 1% Triton X-100 was included during cell lysis (Fig. 3 *C*), most of the ankyrin present in the homogenate (*H*) appeared in the high speed supernatant (*S*) rather than the pellet (*P*). Thus, ankyrin appeared to be present in a particulate fraction of S2 cells that was dissociated in the presence of neutral detergent. The solubility of ankyrin was also examined in neuroglian-expressing cells. Whereas most of the ankyrin was detergent soluble in the absence of neuroglian, a large proportion of the ankyrin was detergent insoluble after the formation of cell aggregates (Fig. 3 *D*). Interestingly, most of the neuroglian was present in the detergent soluble supernatant of the same preparation (Fig. 3 *E*). Subsequent experiments were directed toward the puzzling observation that, while induction of cell adhesion caused ankyrin to shift from a detergent soluble fraction to a detergent insoluble fraction, the adhesion molecule itself remained largely detergent soluble.

Double-labeling experiments were carried out to compare the distribution of neuroglian and ankyrin within individual cells. Single neuroglian-expressing cells were present in neuroglian-expressing cultures, although their frequency decreased as large cell masses formed over time. These single cells exhibited no detectable ankyrin staining at the plasma membrane (Fig. 4 *B*, *arrowhead*), despite the abundance of neuroglian at the cell surface (Fig. 4 *A*). Other cells in the same field that had lost the ability to express neuroglian exhibited the same distribution of ankyrin (Fig. 4, *A* and *B*, *arrows*). It is important to note that within the same time frame, other cells from the same culture formed ankyrin-associated cell–cell contacts (e.g., Fig. 1 *A*). Thus, it appeared that the redistribution of ankyrin observed in cell aggregates was not simply caused by the presence of neuroglian at the cell surface.

Ankyrin became enriched at sites of cell–cell contact in aggregates of neuroglian-expressing cells (Fig. 5 *B*). Neuroglian, in contrast to ankyrin, was detectable at sites of cell contact, as well as noncontact regions of the plasma membrane (Fig 5 *A*, *arrow*). A merged image of the two labeling patterns revealed that ankyrin and neuroglian overlap significantly only at sites of cell–cell contact (Fig. 5 *C*).

Neuroglian often appeared to be enriched at sites of cell–cell interaction, with significantly less staining detected at sites facing away from cell contacts when cells were fixed with formaldehyde (e.g., Fig. 5 *A*). In contrast, neuroglian was not enriched at sites of cell contact in glutaraldehyde-fixed preparations, but instead was uniformly distributed over cell surfaces (Fig. 6 *A*). The neuroglian staining pattern was also more intense than observed with paraformaldehyde-fixed samples so that numerous filopodia, which are visible in living cells, were highlighted by staining with antineuroglian antibody. We tested the possibility that some of the neuroglian was somehow lost from cells when formaldehyde was used, but not when glutaral-

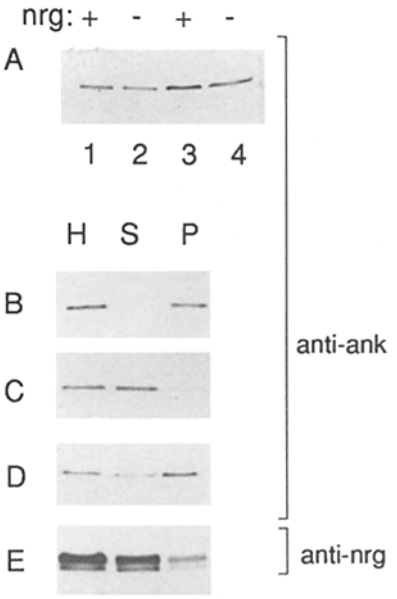


Figure 3. Effects of neuroglian expression on the level and solubility of ankyrin in S2 cells. (*A*) SDS-solubilized proteins from 10^5 (lanes *1* and *2*) or 3×10^5 cells (lanes *3* and *4*). Cells were plated for 24 h either with (lanes *1* and *3*) or without (lanes *2* and *4*) induction of neuroglian synthesis. Western blots of total proteins were reacted with antiankyrin antibody and alkaline phosphatase–conjugated secondary antibody. (*B* and *C*) S2 cells were lysed by sonication in the presence (*C*) or absence (*B*) of 1% Triton X-100. Total proteins (*H*, homogenate), the high speed supernatant (*S*), and the high speed pellet (*P*) were proportionately loaded for Western blotting and reacted with antiankyrin antibody as in *A*. (*D*) Transfected S2 cells were lysed in the presence of detergent (as in *C*) after induction of neuroglian synthesis, and the resulting fractions were probed in a Western blot with antiankyrin antibody. (*E*) Neuroglian-expressing cells were fractionated as described above in the presence of detergent and stained with antineuroglian antibody 3F4.

dehyde was used as a fixative. Specifically, the amount of neuroglian present before and after detergent permeabilization was compared on Western blots of SDS-solubilized proteins from paraformaldehyde-fixed cells. Equal proportions of total fixed cell protein, a detergent soluble supernatant, and detergent insoluble pellet (corresponding to the cells observed by immunofluorescence) were compared. Surprisingly, most of the neuroglian was extracted with detergent during cell permeabilization (Fig. 6 *B*), as with unfixed cells (Fig. 3 *E*). Ankyrin was not detectable extracted from the same preparations. Thus, the apparent concentration of neuroglian at sites of cell contact (Fig. 5) is an artifact that occurs by extraction of neuroglian from nonadherent regions of the plasma membrane during fixation and permeabilization.

The intracellular distribution of spectrin was examined after induction of neuroglian, as a marker for the behavior of other membrane skeleton proteins during the formation of cell–cell contacts. Spectrin was detected with a previously-characterized β-spectrin–specific antibody (Byers et al, 1989). Control nonadherent cells exhibited a diffuse staining pattern throughout the cytoplasm, similar to the pattern observed after ankyrin antibody staining. Identical results were obtained with uninduced cells (not shown)

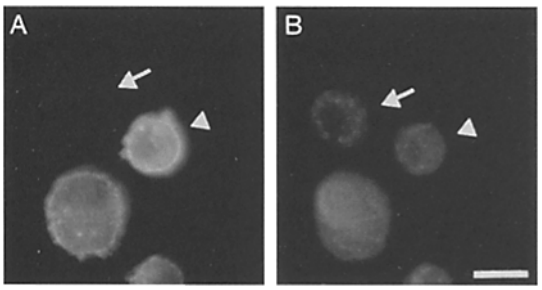


Figure 4. Distributions of ankyrin and neuroglian before formation of cell–cell contacts. Cells were double labeled with mouse antineuroglian and rabbit antiankyrin antibodies followed by Texas red– and fluorescein-labeled secondary antibodies, respectively. Single cells exhibited a cytoplasmic distribution of ankyrin whether or not they exhibited neuroglian staining of the plasma membrane (*arrowhead*, neuroglian-positive cell; *arrow*, neuroglian-negative cell). Bar, 10 μM.

and with induced cells that had not yet formed cell–cell contacts (Fig. 7 *A*). After the induction of neuroglian synthesis and cell contact formation, β-spectrin became concentrated at cell contacts in the same manner as ankyrin (Fig. 7 *B*).

The yeast two-hybrid system was used to demonstrate that the interaction between *Drosophila* ankyrin and neuroglian was direct. Previous studies had demonstrated a biochemical interaction between ankyrin and neuroglian homologues from mammals (Davis et al., 1993; Davis and Bennett, 1994). Coding sequences corresponding to the two cytoplasmic domain isoforms of neuroglian and to the amino-terminal repeat region of ankyrin were subcloned into the appropriate yeast GAL4 vectors and tested alone or in pairwise combinations for their ability to promote the expression of β-galactosidase in response to GAL4 activation (Durfee et al., 1993). Yeast colonies expressing either neuroglian isoform in combination with ankyrin produced positive reactions (Fig. 8), whereas all control combinations of plasmids (single vectors or redundant pairs) were negative. Thus the interaction between ankyrin and L1 cellular adhesion molecule family members is conserved in evolution and can be detected in vitro and in living cells.

Discussion

The above results establish that neuroglian transmits the positional information of cell adhesion to ankyrin in living cells. The results further suggest that neuroglian is a receptor for ankyrin and that ankyrin is sensitive to the adhesive state of neuroglian. Ankyrin is not detectably associated with the plasma membrane of individual control cells or control cell aggregates, but it becomes associated with the site of interaction between cells that express either one of the naturally occurring neuroglian isoforms. The association of ankyrin with the plasma membrane is strictly dependent on the cytoplasmic domain of neuroglian. Overexpression of ankyrin in cells increases the level of ankyrin detected at cell contacts, yet does not lead to staining at other surface sites. Thus, the lack of detectable ankyrin staining at the plasma membrane of control cells and at nonadherent regions of neuroglian expressing cells cannot be attributed to inadequate antibody sensitivity. Double-labeling experiments show that the distribution of ankyrin in cells follows the pattern of cell adhesion and not the distribution of neuroglian. Finally, yeast two-hybrid experiments confirm that there is a direct interaction between ankyrin and neuroglian, as expected on the basis of biochemical studies of the corresponding mammalian proteins (Davis et al., 1993; Davis and Bennett, 1994).

We initially failed to detect an interaction between neuroglian and ankyrin by coimmunoprecipitation from detergent extracts of *Drosophila* embryos (Dubreuil and Yu, 1994), or by several other binding assays, cross-linking experiments, and affinity purification attempts (Hortsch, M., unpublished observations). One possible explanation is that the detergent solubilization conditions used in those previous experiments somehow interfered with the interaction between ankyrin and neuroglian. Alternatively, detergent extraction may have preferentially released inactive ankyrin and neuroglian molecules from cells. Indeed,

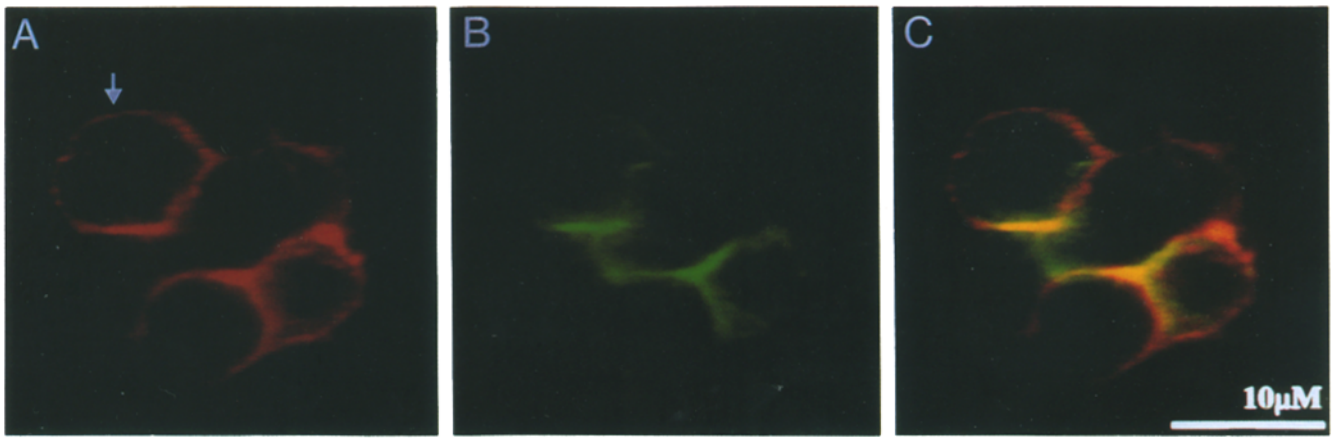


Figure 5. Distributions of ankyrin and neuroglian after formation of cell–cell contacts. Cells were prepared and labeled as described in Fig. 3. Neuroglian was detectable over adherent and nonadherent regions (*arrow*) of the S2 cell surface (*A*). Ankyrin, in contrast, was found at sites of cell–cell contact and in the cytoplasm, but not at nonadherent regions of the plasma membrane (*B*). The merged image reveals that the distributions of ankyrin and neuroglian overlap significantly at sites of cell contact (*C*). Bar, 10 μM.

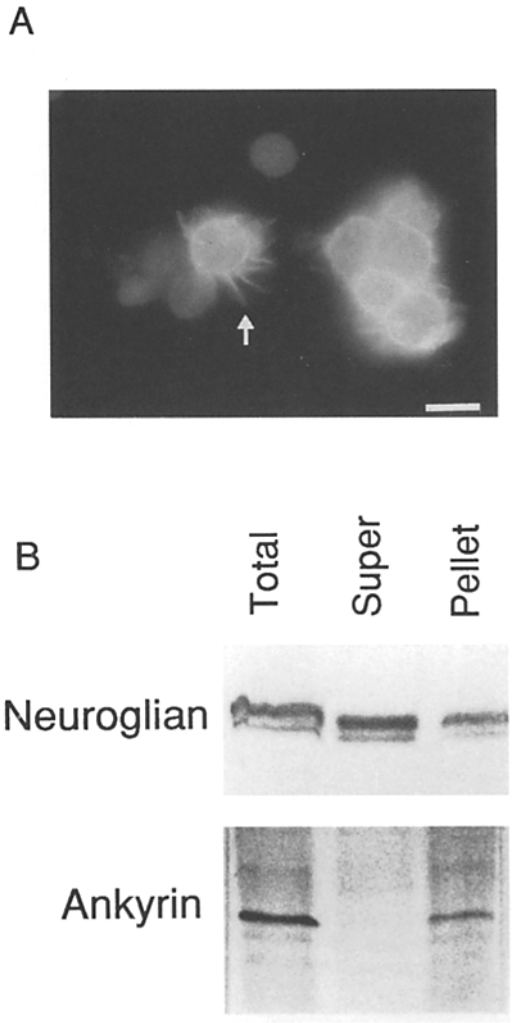


Figure 6. (*A*) Localization of neuroglian in glutaraldehyde-fixed cells. Cells were prepared and stained with antineuroglian antibody as described in Fig. 4, except that 0.1% glutaraldehyde was included in the fixative. Bright staining of the entire cell surface, including numerous filopodia (*arrow*), was observed. Bar, 10 μm. (*B*) Extraction of neuroglian from fixed cells. Cells were processed in suspension using the same formaldehyde fixation and permeabilization conditions used for immunolocalization. Equal proportions (corresponding to $\sim 5 \times 10^5$ cells/lane) of total fixed cell proteins, the detergent-soluble supernatant from cells, and the pellet of insoluble material were probed with antineuroglian (*top*) and antiankyrin (*bottom*) antibodies, as described in Fig. 3.

ankyrin was detergent soluble in S2 cells before the expression of neuroglian. Likewise, much of the neuroglian found at nonadherent regions of the cell surface was detergent soluble, giving the false impression that most of the neuroglian was present at sites of cell–cell contact. It was unexpected (and initially puzzling) that so much of the neuroglian in S2 cells could be extracted with detergent, even after formaldehyde fixation. In contrast, ankyrin was enriched in the detergent-resistant fraction of neuroglian-expressing cells. The detergent-resistant population of neuroglian was found to represent those molecules enriched at sites of cell–cell contact. Thus, the ankyrin–neuroglian complex appeared to be detergent-resistant upon

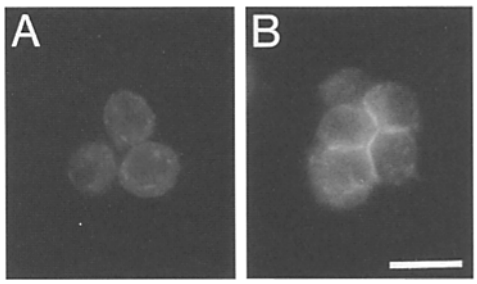


Figure 7. Distribution of spectrin in transfected S2 cells in response to neuroglian synthesis and cell adhesion. Uninduced (*A*) and induced cells (*B*) were stained with a rabbit anti–β-spectrin antibody and FITC-conjugated secondary antibody. Bar, 10 μm.

formation of cell–cell contacts because of intracellular associations with the membrane skeleton, extracellular interactions between adhesive domains, or both.

The present results reveal that there is a marked change in ankyrin behavior in response to neuroglian-mediated adhesion in S2 cells. Studies of mammalian cells have shown that cell adhesion can promote stabilization of the membrane skeleton (Nelson and Veshnock, 1987) and the formation of new ankyrin transcripts (Marrs et al., 1995). In these systems, however, a dramatic change in membrane skeleton protein levels occurs over a period of days. The changes reported here occur within ~1 d and do not appear to involve dramatic changes in membrane skeleton protein levels. A redistribution of ankyrin in response to the availability of neuroglian binding sites at the plasma membrane seems more likely. Studies of lymphocytes provide a precedent for the recruitment of ankyrin from a cytoplasmic store to the plasma membrane (Gregorio et al., 1994). The increased staining intensity of ankyrin (and spectrin) at cell contacts most likely results from its increased concentration relative to the rest of the cytoplasm.

Since there are multiple interactions between the membrane skeleton and the plasma membrane, we would like to know which ones initiate assembly of the membrane skeleton. *Drosophila* S2 cells provide a unique model in which to address the order of events in membrane skeleton assembly, since ankyrin and spectrin are not detectably associated with the plasma membrane before neuroglian expression. Integral membrane proteins that interact with ankyrin are obvious candidates to direct ankyrin to the plasma membrane after its synthesis in the cytoplasm. Yet, ankyrin-independent binding sites can also attach spectrin (and indirectly ankyrin) to the plasma membrane (Steiner and Bennett, 1988). These sites, rather than ankyrin, could potentially promote and target assembly of the membrane skeleton (Lombardo et al., 1994). The present results support the former mechanism in which an integral membrane receptor initiates a cascade of protein interactions, as originally proposed for the erythrocyte membrane (Moon and Lazarides, 1984). The integral membrane protein acts first to attract ankyrin to the plasma membrane. Ankyrin then provides a membrane attachment site for spectrin and other proteins of the membrane skeleton. In this case, the receptor (neuroglian) appears to require activation via homophilic cell adhesion before it is capable of recruiting ankyrin.

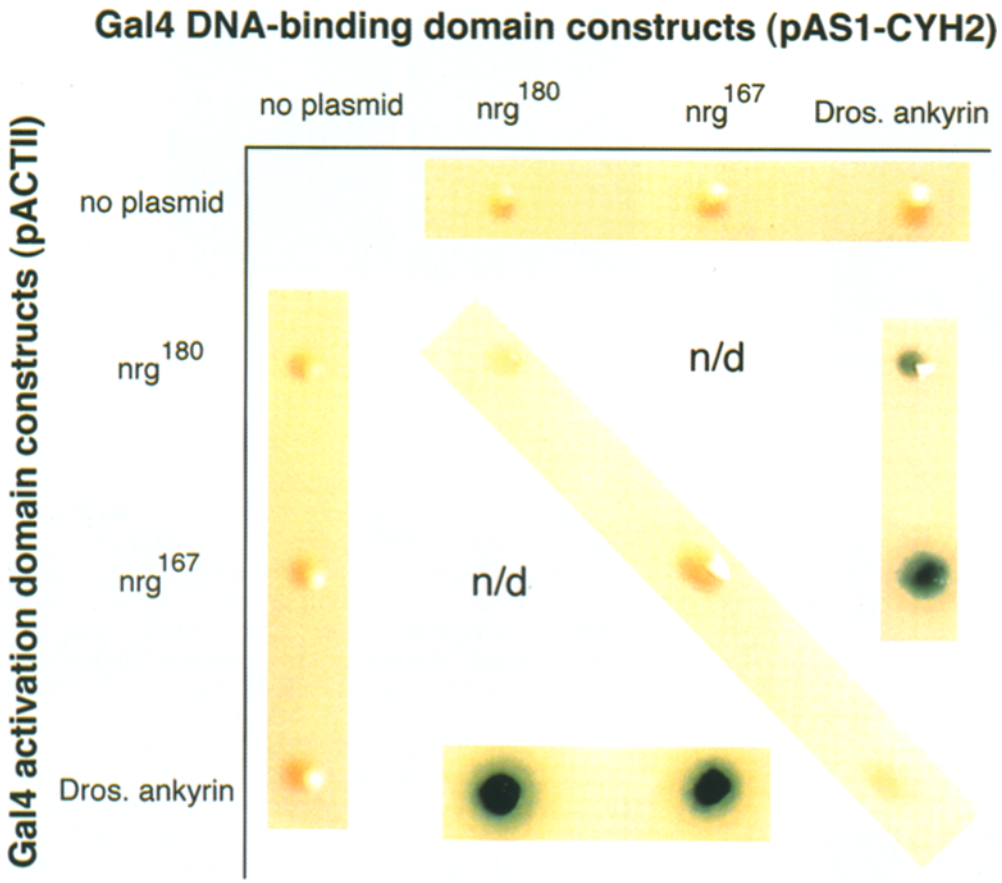


Figure 8. Demonstration of a direct interaction between ankyrin and neuroglian by yeast two-hybrid analysis. The cytoplasmic domains of both neuroglian isoforms and the amino terminal repeat–containing region of ankyrin were cloned and expressed as GAL4 fusions using the yeast plasmids pAS1-CYH2 and pACTII in the indicated combinations. Blue colonies resulted from the induction of β-galactosidase activity and hydrolysis of X-gal substrate, which in turn required an interaction between domains of neuroglian and ankyrin. Yeast colonies expressing only the ankyrin fusion or only the neuroglian fusions (*top row*, Y190 cells, pAS-CYH2 vector, grown on Trp^-SD medium; *left column*, Y190 cells, pACTII vector, grown on Leu^- SD medium) did not activate β-galactosidase expression. Colonies expressing combinations of ankyrin and neuroglian fusions were originally triple selected on $Trp^-Leu^-His^-$ medium. They were subsequently assayed for β-galactosidase activity on Trp^-Leu^- plates (to facilitate the growth of colonies expressing noninteracting GAL4 fusions). Colonies were transferred to nitrocellulose and tested for β-galactosidase activity using the substrate X-gal.

Although the mechanism of neuroglian activation is not yet known, the data so far suggest a few possibilities. Neuroglian and ankyrin did not appear to interact at nonadhesive regions of S2 cells; however, an interaction between recombinant protein fragments was detected in yeast. There may be a factor present in S2 cells, but not in yeast, that blocks the interaction in the absence of cell adhesion. Alternatively, regulation may occur through a change in neuroglian's affinity for ankyrin. A low affinity interaction may be detectable by the sensitive yeast two-hybrid method, but may not be sufficient to recruit ankyrin in S2 cells before activation through cell adhesion. Another possibility is that the neuroglian fragment tested in yeast was constitutively activated. Finally, there are potential phosphorylation sites in the cytoplasmic domain of neuroglian and in ankyrin that may regulate their interaction.

Neuroglian differs from many other cell adhesion molecules in that the cytoplasmic domain is dispensable for adhesive activity (since a GPI-linked form is fully active in cell adhesion; Hortsch et al., 1995). In contrast, integrins and classical cadherins lose their adhesive capability when their cytoplasmic domain is deleted and their interaction with the cytoskeleton is disrupted (inside-out signaling; Ginsberg et al., 1992; Kemler, 1993). At the same time, extracellular adhesion triggers, among other physiological responses, the assembly of cytoskeletal complexes at sites of cell adhesion (outside-in signaling, e.g., Podack and Kupfer, 1991; McNeill et al., 1990; Miyamoto et al., 1995). So far, it appears that ankyrin and neuroglian exhibit only the latter outside-in signaling mechanism. We postulate that the ability of neuroglian to communicate the positional information of homophilic cell adhesion is equally important to its role in maintaining cell–cell interactions.

Neuroglian is abundantly expressed in neurons of *Drosophila*, and can be found in a variety of other *Drosophila* cell types (Bieber et al., 1989; Hortsch et al., 1990). Questions of cell polarity that have been studied extensively in epithelial cells are also relevant to our understanding of polarity in neurons (Simons et al., 1992). Based on its behavior in S2 cells, we predict that neuroglian recruits membrane skeleton assembly within specialized domains of neurons in response to cell adhesion. Interestingly, immunolocalization experiments have shown that the distribution of neuroglian in embryonic neurons is not compartmentalized along the usual boundaries of soma, axon, and dendrites. Instead, it appears to be distributed over the entire surface of neurons (Hortsch et al., 1990). An important future goal will be to examine the fate and distribution of other membrane components (such as ankyrin and spectrin) as they respond to neuroglian during neural growth and differentiation.

Cell adhesion molecules have been shown to be important signal transducing molecules. Vertebrate L1 is a signal transducer that promotes neurite outgrowth in response to

extracellular adhesion (Doherty and Walsh, 1994). Our results show that neuroglian is also a signal-transducing molecule that recruits ankyrin to the plasma membrane in response to extracellular adhesion. Given the similarity between L1 and neuroglian, it will be interesting to determine if ankyrin binding to members of this gene family actively contributes to signal transduction, perhaps by recruitment of additional signaling molecules to sites of cell–cell adhesion.

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References

Ashburner, M. 1989. *Drosophila*: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 378.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1988. *Saccharomyces cerevisiae. In* Current Protocols in Molecular Biology. J. Wiley & Sons, NY.

Bartel, P.L., C.-T. Chien, R. Sternglanz, and S. Fields. 1993. Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach. D.A. Hartley, editor. 153–179.

Bennett, V. 1990. Spectrin based membrane skeleton. A multipotential adaptor between membrane and cytoplasm. *Physiol. Rev.* 70:1029–1065.

Bennett, V., and D.M. Gilligan. 1993. The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. *Annu. Rev. Cell Biol.* 9:27–66.

Bieber, A.J., P.M. Snow, M. Hortsch, N.H. Patel, J.R. Jacobs, Z.R. Traquina, J. Schilling, and C.S. Goodman. 1989. *Drosophila* neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell.* 59:447–460.

Byers, T.J., A. Husain-Chishti, R.R. Dubreuil, D. Branton, and L.S.B. Goldstein. 1989. Drosophila beta-spectrin: sequence similarity to the amino-terminal domain of α-actinin and dystrophin. *J. Cell Biol.* 109:1633–1641.

Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745–2752.

Cox, J.V., J.H. Stack, and E. Lazarides. 1987. Erythroid anion transporter assembly is mediated by a developmentally regulated recruitment onto a preassembled membrane skeleton. *J. Cell Biol.* 105:1405–1416.

Davis, J.Q., and V. Bennett. 1994. Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *J. Biol. Chem.* 269:27163–27166.

Davis, J.Q., T. McLaughlin, and V. Bennett. 1993. Ankyrin-binding proteins related to nervous system cell adhesion molecules: candidates to provide transmembrane and intercellular connections in adult brain. *J. Cell Biol.* 121:121–133.

Deng, H., J.K. Lee, L.S.B. Goldstein, and D. Branton. 1995. Drosophila development requires spectrin network formation. *J. Cell Biol.* 128:71–79.

Doherty, P., and F.S. Walsh. 1994. Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. *Curr. Opin. Neurobiol.* 4:49–55.

Drenckhahn, D., and V. Bennett. 1987. Polarized distribution of M_r 210,000 and 190,000 analogs of erythrocyte ankyrin along the plasma membrane of transporting epithelia, neurons and photoreceptors. *Eur. J. Cell Biol.* 43:479–486.

Dubreuil, R., T.J. Byers, D. Branton, L.S.B. Goldstein, and D.P. Kiehart. 1987. Drosophila spectrin. I. Characterization of the purified protein. *J. Cell Biol.* 105:2095–2102.

Dubreuil, R.R., and J. Yu. 1994. Ankyrin and beta spectrin accumulate independently of alpha spectrin in Drosophila. *Proc. Natl. Acad. Sci. USA.* 91: 10285–10289.

Durfee, T., K. Becherer, P.L. Chen, S.H. Yeh, Y. Yang, A.E. Kilburn, W.H. Lee, and S.J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7:555–569.

Evan, G.I., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.

Ginsberg, M.H., X. Du, and E.F. Plow. 1992. Inside-out integrin signalling. *Curr. Opin. Cell Biol.* 4:766–771.

Gregorio, C.C., E.A. Repasky, V.M. Fowler, and J.D. Black. 1994. Dynamic properties of ankyrin in lymphocytes: colocalization with spectrin and protein kinase Cβ. *J. Cell Biol.* 125:345–358.

Hammerton, R.W., K.A. Krzeminski, R.W. Mays, T.A. Ryan, D.A. Wollner, and W.J. Nelson. 1991. Mechanism for regulating cell surface distribution of Na^+, K^+-ATPase in polarized epithelial cells. *Science (Wash. DC).* 254:847–850.

Hortsch, M., A.J. Bieber, N.H. Patel, and C.S. Goodman. 1990. Differential splicing generates a nervous system-specific form of *Drosophila* neuroglian. *Neuron.* 4:697–709.

Hortsch, M., Y.-M.E. Wang, Y. Marikar and A.J. Bieber. 1995. The cytoplasmic domain of the *Drosophila* cell adhesion molecule neuroglian is not essential for its homophilic adhesive properties. *J. Biol. Chem.* 270:18809–18817.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.

Kordeli, E., S. Lambert, and V. Bennett. 1995. Ankyrin-G. *J. Biol. Chem.* 270: 2352–2359.

Lee, H., J.A. Simon, and J.T. Lis. 1988. Structure and expression of ubiquitin genes of *Drosophila melanogaster. Mol. Cell. Biol.* 8:4727–4735.

Lee, J., R. Coyne, R.R. Dubreuil, L.S.B. Goldstein, and D. Branton. 1993. Cell shape and interaction defects in α-spectrin mutants of *Drosophila melanogaster. J. Cell Biol.* 123:1797–1809.

Lombardo, C.R., S.A. Weed, S.P. Kennedy, B.G. Forget, and J.S. Morrow. 1994. BetaII-spectrin (fodrin) and beta IE2-Spectrin (muscle) contain NH_2- and COOH-terminal membrane association domains (MAD-1 and MAD2). *J. Biol. Chem.* 269:29212–29219.

Marrs, J.A., C. Andersson-Fisone, M.C. Jeong, L. Cohen-Gould, C. Zurzolo, I.R. Nabi, E. Rodriguez-Boulan, and W.J. Nelson. 1995. Plasticity in epithelial cell phenotype: modulation by expression of different cadherin cell adhesion molecules. *J. Cell Biol.* 129:507–519.

Matter, K., and I. Mellman. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* 6:545–554.

McNeill, H., M. Ozawa, R. Kemler, and W.J. Nelson. 1990. Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell.* 62:309–316.

Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.

Moon, R.T., and E. Lazarides. 1984. Biogenesis of the avian erythroid membrane skeleton: receptor-mediated assembly and stabilization of ankyrin (goblin) and spectrin. *J. Cell Biol.* 98:1899–1904.

Morrow, J.S., C.D. Cianci, T. Ardito, A.S. Mann, and M. Kashgarian. 1989. Ankyrin links fodrin to the α subunit of Na,K ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–466.

Nelson, W.J., and E. Lazarides. 1984. The patterns of expression of two ankyrin isoforms demonstrate distinct steps in the assembly of the membrane skeleton in neuronal morphogenesis. *Cell.* 39:309–320.

Nelson, W.J., and P.J. Veshnock. 1987. Modulation of fodrin (membrane skeleton) stability by cell–cell contact in Madin-Darby Canine kidney epithelial cells. *J. Cell Biol.* 104:1527–1537.

Nelson, W.J., E.M. Shore, A.Z. Wang, and R.W. Hammerton. 1990. Identification of a membrane-cytoskeleton complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 110:349–357.

Nelson, W.J. 1992. Regulation of cell surface polarity from bacteria to mammals. *Science (Wash. DC).* 258:948–955.

Peters, L.L., K.M. John, F.M. Lu, E.M. Eicher, A. Higgins, M. Yialamas, L.C. Turtzo, A.J. Otsuka and S.E. Lux. 1995. Ank3 (epithelial ankyrin), a widely distributed new member of the ankyrin gene family and the major ankyrin in kidney, is expressed in alternatively spliced forms, including forms that lack the repeat domain. *J. Cell Biol.* 130:313–330.

Podack, E.R., and A. Kupfer. 1991. T-cell effector functions: mechanisms for delivery of cytotoxicity and help. *Annu. Rev. Cell Biol.* 7:479–504.

Schneider, I. 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster. J. Embryol. Exp. Morphol.* 27:353–365.

Shiel, M.J., and M.J. Caplan. 1995. The generation of epithelial polarity in mammalian and *Drosophila* embryos. *Sem. Dev. Biol.* 6:39–46.

Simons, K., P. Dupree, K. Fiedler, L.A. Huber, T. Kobayashi, T. Kurzchalia, V. Olkkonen, S. Pimplikar, R. Parton, and C. Dotti. 1992. Biogenesis of cell-surface polarity in epithelial cells and neurons. *Cold Spring Harbor Symp. Quant. Biol.* 57:611–619.

Steiner, J.P., and V. Bennett. 1988. Ankyrin-independent membrane protein-binding sites for brain and erythrocyte spectrin. *J. Biol. Chem.* 263:14417–14425.