Receptor Clustering Drives Polarized Assembly of Ankyrin

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Summary

Expression of the L1 family cell adhesion molecule neuroglian in Drosophila S2 cells leads to cell aggregation and polarized ankyrin accumulation at sites of cell-cell contact. Thus neuroglian adhesion generates a spatial cue for polarized assembly of ankyrin and the spectrin cytoskeleton. Here we characterized a chimera of the extracellular and transmembrane domains of rat CD2 fused to the cytoplasmic domain of neuroglian. The chimera was used to test the hypothesis that clustering of neuroglian at sites of adhesion generates the signal that activates ankyrin binding. Abundant expression of the chimera at the plasma membrane was not a sufficient cue to drive ankyrin assembly, since ankyrin remained diffusely distributed throughout the cytoplasm of CD2-neuroglian expressing cells. However, ankyrin became highly enriched at sites of antibody-induced capping of CD2-neuroglian. Spectrin codistributed with ankyrin at capped sites. A green fluorescent protein-tagged ankyrin was used to monitor ankyrin distribution in living cells. EGFP-ankyrin behaved identically to antibody-stained endogenous ankyrin, proving that the polarized accumulation of ankyrin was not an artefact of fixing and staining cells. We propose a model in which clustering of neuroglian induces a conformational change in the cytoplasmic domain that drives polarized assembly of the spectrin cytoskeleton.
**Introduction**

Spectrin forms a submembrane protein scaffold in a variety of eukaryotic cell types. The scaffold is thought to consist primarily of $\alpha_2\beta_2$ spectrin tetramers crosslinked into a hexagonal lattice by their interaction with actin filaments. The major attachment between spectrin and the plasma membrane occurs through the protein ankyrin, which functions as an adapter linking the $\beta$ subunit of spectrin to the cytoplasmic domains of integral membrane proteins (1).

Recent studies have established a role for the spectrin cytoskeleton in the formation of specialized plasma membrane domains of polarized cells. A number of integral membrane proteins have been found to interact directly with ankyrin (2,3) and their behavior is altered by mutations in ankyrin or spectrin. Voltage-gated sodium channels failed to accumulate at axon initial segments of ankyrin$\text{C}$ knockout mice, leading to impaired action potential firing (4). Similarly, a reduced level of L1 was observed in axons of ankyrin$\text{B}$ knockout mice (5). The basolateral accumulation of Na,K ATPase was severely perturbed in epithelial cells of *Drosophila* $\beta$ spectrin mutants (6). These results support a model in which the spectrin cytoskeleton acts to capture and stabilize interacting proteins, leading to their accumulation within specialized functional domains of the plasma membrane.

While the downstream effects of spectrin and ankyrin mutations on other interacting proteins are being elucidated, relatively little is known about the upstream cues that polarize the distributions of ankyrin and spectrin. The same is true of other cytoskeletal proteins that are thought to modulate the accumulation of interacting membrane activities. For example, dystrophin is known to stabilize the expression of a complex of dystrophin-associated glycoproteins at the sarcolemma (7,8). The PDZ domain protein *disks large* is required for the accumulation of fasciclin III and coracle at septate junctions of *Drosophila* epithelial cells (9) and for K$^+$ channel clustering at synapses in the nervous system (10). In *C. elegans*, mutations of the PDZ protein *lin7* result in mislocalization of the *let23* tyrosine kinase (11). The PDZ protein *InaD* is responsible for the formation of signaling protein complexes in the rhabdomeres.
of *Drosophila* photoreceptor cells (12). However, the upstream cues responsible for targeting of each of these cytoskeletal proteins are poorly defined. The identification of such cues and their mechanisms is therefore of broad relevance.

One of the cues that leads to polarized assembly of the spectrin cytoskeleton is cell-cell adhesion. Expression of neuroglian, which contains an ankyrin-binding site in its cytoplasmic domain, causes a dramatic redistribution of ankyrin and spectrin to sites of cell-cell contact in *Drosophila* S2 cells (13). Human L1 expressed in S2 cells has the same effect, indicating that the polarizing activity of L1 family members is conserved between *Drosophila* and mammals (14). In both cases the accumulation of ankyrin is observed exclusively at cell contacts, even though the adhesion molecule itself is abundantly distributed over the remainder of the cell surface. Thus cell adhesion appears to generate a signal that specifies the recruitment of ankyrin exclusively by those neuroglian molecules present at cell contacts. Several possible signaling mechanisms were tested and ruled out in previous studies of wild type and mutant neuroglian molecules (13,15).

Here we tested the hypothesis that the aggregation and/or immobilization of neuroglian at cell contacts triggers ankyrin recruitment. A transgene composed of the extracellular domain of rat CD2 fused to the cytoplasmic domain of neuroglian (CD2/nrg) was expressed in S2 cells and the conditions necessary to activate accumulation of ankyrin were identified. An important advantage of this approach is that it allowed high level expression of the cytoplasmic domain of neuroglian without the formation of large, unwieldy cell aggregates which are induced by authentic neuroglian. We studied the behavior of ankyrin in response to expression of the CD2/nrg chimera by antibody staining and by monitoring the distribution of EGFP-tagged ankyrin in living cells. The results support a model in which clustering of neuroglian activates the polarized accumulation of ankyrin at sites of cell-cell contact.
Experimental Procedures

Origin and Maintenance of Cell Lines

*Drosophila* S2 cells were cultured under standard conditions (16) using Schneider’s medium and 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD). A stably transfected cell line was used to produce a natural isoform of neuroglian (nrg\(^{167}\)) under the control of an inducible metallothionein promoter (17). Neuroglian expression was induced by the addition of 0.7 mM CuCl\(_2\) to the growth medium for 24 h. Transient expression of recombinant ankyrin and neuroglian transgenes in S2 cells was carried out by transfection essentially as described by Chen & Okayama (18).

Construction of CD2/nrg and CD2\(\Delta\) Genes

A cDNA fragment encoding rat CD2 was a generous gift from Dr. Nick Brown (19). An NcoI - HindIII fragment of the CD2 coding region, extending from the start codon to codon 18 of the cytoplasmic domain, was spliced into the pUip2 vector (20) at the start site of the ubiquitin promoter. A HindIII site was introduced at codon 16 of the cytoplasmic domain of a neuroglian cDNA (17) by PCR. The cytoplasmic domain of the resulting CD2/neuroglian chimera, confirmed by DNA sequencing, includes the first 18 amino acids of CD2 followed by amino acids 15 - 85 the neuroglian cytoplasmic domain. A control construct encoding truncated CD2 (CD2\(\Delta\)) was identical except that it lacked the cytoplasmic fragment of neuroglian and had an in-frame stop codon immediately after codon 18 of the cytoplasmic domain.

Construction of an ankyrin-EGFP fusion

A fusion of the ankyrin coding sequence with the enhanced green fluorescent protein (EGFP) coding sequence was produced from a previously described myc-tagged *Drosophila* ankyrin transgene (13). An NcoI site was engineered at the position of the ankyrin stop codon by PCR and confirmed by DNA sequencing of the PCR product. A NcoI-NotI fragment of the EGFP coding region (Clontech Laboratories) was spliced to the ankyrin coding sequence.
Antibody Staining and Microscopy

Drosophila S2 cells were processed for antibody staining and microscopy as previously described (13) either before or after treatment of live cells with anti-CD2 antibody. Briefly, cells were immobilized on Alcian blue-coated microscope slides, fixed in 2% fresh formaldehyde in PBS, pH 7.2, permeabilized with 0.1% Triton X-100 in Tris-buffered saline, then equilibrated in Tris-buffered saline/0.1% Tween 20 containing 5% newborn calf serum (GIBCO BRL) for antibody staining. Primary antibodies were used as follows: ankyrin, 1:250 affinity-purified rabbit antibody (21); neuroglian, 1:1000 culture supernatant of mouse hybridoma clone 1B7 (22); β-spectrin 1:500 (23); CD2, 1:88 mouse anti-rat CD2 (Serotec). Affinity-purified FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA) and Texas red-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA) were used at 1:1000 dilutions.

In capping experiments, S2 cells and transiently-transfected CD2/nrg and CD2Δ S2 cells were incubated live with anti-rat CD2 antibody (1:88) in fly Ringer's solution for two hours in microfuge tubes, rinsed in fly Ringer's and microcentrifuged for 30 seconds at setting 3 (Eppendorf 5415C) three times. Cells were either transferred to alcian blue-coated slides (primary capping alone) or were further treated with goat anti-mouse Texas red antibody (1:1000 in fly Ringer’s; secondary capping) before transfer to slides. Once transferred to slides, cells were stained as above for CD2 and either ankyrin or β spectrin.

Capping experiments were also carried out with cells expressing ankyrin-EGFP. Stably transfected cells carrying the metallothionein promoter-neuroglian transgene (13) were transiently transfected with the ubiquitin promoter-ankyrin-EGFP transgene then induced to express neuroglian by addition of CuCl₂ to the medium 2 days later. Live cells were transferred to viewing chambers for confocal microscopy of cell aggregates. Digital images were captured using an Olympus IX70 microscope with Fluoview software.

In parallel experiments S2 cells were transiently co-transfected with ankyrin-EGFP and CD2.
transgenes and processed for live cell capping with primary and secondary antibodies two days later, as described above. Live cells were viewed by epifluorescence, photographed using an ausJena Jenalumar photomicroscope. Images were obtained on photographic film, digitized using a Polaroid SprintScan scanner and edited using Adobe Photoshop 5.0.

Western blotting was carried out as previously described (24). Total S2 cell proteins (5 x 10^5 cells/lane) were dissolved in SDS sample buffer, resolved by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Nitrocellulose strips were stained with rabbit anti-ankyrin (1:250), mouse anti myc-epitope tag (1:500; ref. 25), or mouse anti rat CD2 (1:200) and alkaline phosphatase conjugated secondary antibodies (1:1000; Zymed Laboratories).

Quantification of capping experiments

Capped S2 cells expressing CD2/nrg or CD2Δ were scored for the expression of fluorescent antibody stained caps using the rhodamine channel. To score primary antibody-treated cells, cells were fixed prior to addition of fluorescent secondary antibody. Fifty cells were scored for ankyrin or β spectrin colocalization at caps in each experiment and results were expressed as a percentage of total capped cells. Control uncapped cells were scored for the presence of conspicuous CD2 staining, then scored for ankyrin colocalization.
**Results**

Expression of the adhesion molecule neuroglian in *Drosophila* S2 tissue culture cells leads to the formation of large aggregates of cells as well as the recruitment of ankyrin specifically to sites of cell-cell contact (13). To study the ankyrin recruitment process independently of cell-cell adhesion, we engineered a chimeric protein composed of the extracellular and transmembrane domains of rat CD2 fused to the cytoplasmic sequence of the nrg\(^{167}\) isoform of neuroglian (Fig. 1). A truncated form of CD2 (CD2\(\Delta\)), lacking most of the cytoplasmic domain, was used as a control. The Drosophila ubiquitin promoter which directs high-level constitutive expression of transfected genes in S2 cells drove expression of both constructs. Expression of the CD2/nrg chimera (Fig. 1, lane 3) and CD2\(\Delta\) (lane 2) was demonstrated in Western blots of transiently transfected S2 cultures probed with mouse anti-rat CD2 antibody. No reaction of the antibody was observed in control non-transfected S2 cells (lane 1). The CD2\(\Delta\) product migrated with a relative mobility of 30 kD (lane 2) and the CD2/nrg chimera migrated at 42 kD. These mobilities are consistent with glycosylated products having predicted molecular masses of 28 kD and 38 kD, respectively.

Expression of recombinant CD2 molecules and their effects on assembly of ankyrin in S2 cells were examined by immunofluorescent staining of transiently transfected populations. Typically less than 50% of the cells in the population expressed recombinant CD2, and among these there was a broad range of expression level (data not shown). Cells were fixed and permeabilized for antibody staining. Conspicuous uniform plasma membrane staining and variable intracellular staining were observed in cells expressing either CD2/nrg or CD2\(\Delta\) (Fig. 2, column 1). There was no detectable effect of CD2 expression on the intracellular distribution of ankyrin (column 2). Ankyrin staining was found in a variable speckled pattern throughout the cytoplasm of S2 cells, whether or not the cells were transfected. Thus the presence of the neuroglian cytoplasmic domain in S2 cells was not sufficient to bring about a detectable redistribution of ankyrin to the plasma membrane.
The distribution of ankyrin was also examined after capping CD2 by addition of anti-CD2 antibody to living cells. Capping was induced by the sequential addition of monoclonal anti-CD2 antibody and fluorescent goat anti-mouse secondary antibody. Addition of antibody to living cells resulted in extensive capping of both CD2/nrg and CD2Δ (Fig. 2, column 3). Cap morphology varied from numerous small islands of staining (arrows) to large polar caps (arrowhead). After capping, cells were fixed and permeabilized for staining with rabbit anti-ankyrin antibody (Fig. 2, column 4). CD2/nrg expressing cells exhibited a dramatic redistribution of ankyrin to capped sites (Fig. 2, E-F), although not all caps were associated with detectable ankyrin staining (e.g. Fig. 2F, *). In contrast, capped cells expressing CD2Δ never exhibited a detectable redistribution of ankyrin (Fig. 2, G-H). Thus colocalization of ankyrin with the CD2/nrg chimera was dependent on the presence of the neuroglian cytoplasmic domain as well as capping of the receptor.

The effects of CD2/nrg expression on ankyrin distribution were quantified in large populations of transfected cells (Fig. 3). The fraction of cells with caps that exhibited colocalization of ankyrin was expressed as a percentage of total capped cells (n = 50). Forty percent of cells expressing the CD2/nrg chimera exhibited prominent ankyrin colocalization. About 20% of cells treated with primary antibody alone also exhibited recruitment of ankyrin to cap sites. Treatment of cells with monoclonal primary antibody alone was expected to induce formation of CD2 dimers rather than caps. However, antibody binding appears to induce a conformational change in CD2 that favors lateral clustering of molecules in the plane of the membrane (26). Thus, while caps formed by primary antibody alone were not as prominent or compact as those induced with primary and secondary antibodies (data not shown), there was a conspicuous redistribution of CD2/nrg as well as ankyrin in primary antibody-treated cells that was distinct from non-antibody-treated control cells.

CD2/nrg capping was also expected to induce a redistribution of β spectrin, since ankyrin is the adapter protein that links spectrin to the plasma membrane. There was a dramatic redistribution of β
spectrin in capped CD2/nrg cells (Fig. 4A-B). The β spectrin in control cells (not shown) and capped CD2Δ cells (Fig. 4C-D) was found in a diffuse distribution in the cytoplasm and in numerous perinuclear puncta, as previously described (27). Quantification of the β spectrin redistribution revealed that it was not as efficient as ankyrin redistribution (Fig. 3, right), but it was equally dependent on the cytoplasmic domain of neuroglian and capping. Thus antibody capping of CD2/nrg induced assembly of a membrane cytoskeleton complex of ankyrin and spectrin comparable to what was previously observed in S2 cells expressing authentic neuroglian (13).

The response of ankyrin to neuroglian expression was also monitored in cells expressing a recombinant ankyrin-green fluorescent protein (EGFP) construct (Fig. 5). It was important to establish that the patterns of ankyrin staining in S2 cells were not an artefact of fixing and/or permeabilizing the cells. The NcoI start site of EGFP was fused to a modified ankyrin coding sequence in which the stop codon was replaced with an NcoI site. The ankyrin template used also included an amino-terminal myc epitope tag (13). Expression of the desired product was confirmed in Western blots of transfected cells (Fig. 5). A unique 197 kD product was detected in transfected cells by rabbit anti-ankyrin antibody (lane 2) and by an antibody against the myc epitope (lane 4), but not in control untransfected cells (lanes 1 & 3). Thus the ankyrin-EGFP product was abundantly expressed and stable in S2 cells.

The ankyrin-EGFP marker was monitored in living, neuroglian-expressing S2 cells by confocal microscopy. Ankyrin-EGFP was excluded from the nucleus but was otherwise diffusely distributed throughout the cytoplasm of non-aggregated cells (not shown). A confocal through-focus series revealed that ankyrin-EGFP was dramatically redistributed to sites of cell-cell contact in neuroglian expressing cells (Fig. 6A, arrow). No ankyrin-EGFP above the background of cytoplasmic staining was detected at non-contact regions of the plasma membrane (arrowhead). Thus the distribution of ankyrin-EGFP in living cells precisely matched the previously described distribution of ankyrin in antibody-stained cells (13).
Ankyrin-EGFP was also used to monitor the effects of expression and capping of CD2/nrg in living S2 cells (Fig. 6B). Cells were treated with primary and secondary antibody, as described above, then directly viewed and photographed while still alive. Antibody treatment caused a dramatic redistribution of CD2/nrg and CD2Δ into caps (Fig. 6B, arrows). Ankyrin-EGFP codistributed with caps in cells expressing the chimera, but not in cells expressing truncated CD2 (Fig. 6C, arrows). Ankyrin-EGFP was not detectably associated with the plasma membrane prior to antibody-induced capping (data not shown). Thus the dramatic enrichment of ankyrin at sites of antibody-induced capping of CD2/nrg and at sites of neuroglian-mediated cell-cell adhesion was not an artefact of the processing steps used in antibody labeling experiments.
Discussion

Previous studies established that the cytoplasmic domain of neuroglian recruits ankyrin to sites of cell-cell adhesion in neuroglian-expressing S2 cells (13,15). Several possible explanations for the polarizing effect of neuroglian on ankyrin were proposed, including 1) mobilization of neuroglian to cell contacts, thereby sequestering the ankyrin binding site; 2) transmission of an allosteric signal that selectively activates ankyrin binding by neuroglian molecules engaged in extracellular adhesion; and 3) inhibition of ankyrin binding to neuroglian at non-contact sites by tyrosine phosphorylation within the ankyrin-binding site of neuroglian. However, each of these mechanisms was ruled out in previous studies of wild type and mutant neuroglian molecules (13,15). A fourth mechanism, local activation of ankyrin recruitment by clustering of neuroglian molecules in the plane of the membrane, had not been tested. The finding here that ankyrin codistributes with a CD2-neuroglian chimera only after antibody-induced capping provides compelling evidence that ankyrin binding to neuroglian is modulated by receptor clustering.

It was important to establish that the apparent concentration of ankyrin at sites of neuroglian clustering was not an artefact of either cell aggregation or the processing steps used in antibody staining experiments. The CD2/nrg chimera used here made it possible to uncouple ankyrin recruitment from cell adhesion and to exert precise control over the timecourse of ankyrin recruitment. The chimera could be expressed at high levels without recruiting ankyrin to the plasma membrane. It was not possible to express authentic neuroglian without inducing aggregation and ankyrin recruitment once neuroglian accumulated to a sufficient level. The fixation and permeabilization steps before antibody staining were a source of further concern. Most of the neuroglian in cell aggregates was present at non-contact regions of the plasma membrane, but was extracted during permeabilization with detergent (13). Consequently, ankyrin and neuroglian both appeared to be concentrated at cell contacts in antibody-stained cells. Use of the ankyrin-EGFP fusion described here made it possible to monitor the distribution of ankyrin without cell processing artefacts. Ankyrin-EGFP strictly codistributed with sites of neuroglian-mediated adhesion.
and not with the distribution of total neuroglian.

The behavior of ankyrin-EGFP in CD2/nrg-expressing S2 cells was remarkably different from the behavior of EGFP-tagged ankyrin in mammalian cells. When ankyrinG-EGFP was expressed in human kidney 293 cells it was diffusely distributed throughout the cytoplasm and was not detectably associated with the plasma membrane. When co-expressed with intact neurofascin, ankyrinG-EGFP was efficiently recruited to the plasma membrane, whether or not the cells formed aggregates (28). Thus the recruitment of ankyrin to the plasma membrane by rat neurofascin was independent of cell adhesion. This is in marked contrast to Drosophila ankyrin-EGFP which only associated with the plasma membrane at cell contacts in neuroglian-expressing cells or with antibody-induced caps in CD2/nrg-expressing cells (this study). The difference in the ankyrin recruiting activities of the two proteins was unexpected given the sequence conservation of their ankyrin binding sites (70% identity over 27 residues; Fig. 6).

Further comparison of the properties of neurofascin and neuroglian provided the rationale for a model to explain the regulated recruitment of ankyrin in response to neuroglian adhesion. Biochemical studies revealed that the cytoplasmic domain of neurofascin behaves as a dimer in solution (28). In contrast, the cytoplasmic domain of neuroglian did not detectably dimerize in yeast two-hybrid experiments (13). Since the ankyrin binding regions of neuroglian and neurofascin are conserved, it is likely that the dimerization site of neurofascin resides in the more divergent upstream region of the cytoplasmic domain. Further inspection of the ankyrin-binding regions of neurofascin and neuroglian revealed a high density of acidic amino acids (8/27 residues). We integrated these observations (acidic character, dimeric vs. monomeric states, and constitutive vs. regulated association with ankyrin) into a simple model in which repulsion of negative charges in the cytoplasmic domain drives a conformational change which activates the ankyrin-binding activity of L1 family members (Fig. 6). In the model, neurofascin is constitutively active because the native molecule exists as a dimer in which the acidic cytoplasmic domains are juxtaposed. The CD2/nrg chimera is activated by antibody crosslinking of the
extracellular domain, which mimics the activated state of the neurofascin molecule. While this working model is speculative, it illustrates many of the questions that will be important to address in future experiments.

One question concerns the valency of the ankyrin interaction with L1 family members. Molecular and biochemical studies indicate that there are two binding sites for neurofascin in mammalian ankyrins and that a single ankyrin can simultaneously interact with two neurofascin molecules, as well as band 3 (29). However, there is no direct evidence that both sites are utilized or required in situ. The model posits that a single ankyrin molecule associates with the cytoplasmic domains of two neuroglian or neurofascin molecules, although 1:1 binding of ankyrin to neuroglian would also serve to mask charges. We attempted to address the valency of ankyrin in the present study without success. CD2/nrg was co-expressed with authentic neuroglian in S2 cells to ask if recruitment of ankyrin to cell contacts by neuroglian would lead to a simultaneous recruitment of CD2/nrg. CD2 staining was observed at cell contacts (data not shown), but the same result was obtained with CD2Δ, indicating that recruitment to cell contacts occurred via the extracellular domain of CD2, rather than the ankyrin-binding site of neuroglian. Thus other experimental approaches, perhaps using different neuroglian chimeras, will be required to determine the number of neuroglian molecules that associate with ankyrin in situ.

Neuroglian molecules are likely to be clustered at cell contact sites by diffusion-mediated trapping. Clustering of adhesion molecules at adhesive sites by this mechanism is thought to enhance the strength and stability of cell contacts (30-32). The model described here suggests that clustering of neuroglian is also responsible for the selective recruitment of ankyrin to cell contacts. In addition, the model provides a rationale to explain inside-out regulation of neuroglian mediated adhesion (15,33). Mutations in the cytoplasmic domains of neuroglian and neurofascin that interfere with ankyrin binding dramatically reduce the efficiency of extracellular adhesion. It was difficult to reconcile this finding with the earlier observation that a lipid-linked form of neuroglian altogether lacking a cytoplasmic domain
mediated robust cell-cell adhesion (17). In one case the activity of the cytoplasmic domain was required for efficient cell adhesion, in the other case it was not. These observations can be reconciled by assuming that the cytoplasmic domain of neuroglian has an inhibitory effect on adhesion caused by charge repulsion. The inhibitory effect can be relieved either by removing the cytoplasmic domain, as in the case of lipid-linked neuroglian, or by masking the charges with ankyrin in the case of wild type neuroglian. This hypothesis can be tested in future studies by manipulating the density and distribution of charges in the cytoplasmic domain of neuroglian and assaying effects on ankyrin recruitment and adhesion.

The model proposed here suggests that ankyrin binding to neuroglian is activated by receptor clustering. An alternative interpretation of the data is that ankyrin is constitutively bound to neuroglian in the absence of clustering, but the concentration of ankyrin at unclustered sites is below its threshold of detection. Clustering of neuroglian could conceivably elevate the ankyrin concentration above that threshold. This alternate explanation seems unlikely for a number of reasons. First, threshold of detection was not an issue with mammalian ankyrin-EGFP which was dramatically redistributed to the plasma membrane in response to neurofascin expression (28). Drosophila ankyrin labeled with the same EGFP tag did not codistribute with neuroglian at non-contact regions of the plasma membrane in neuroglian-expressing cells or with uncapped CD2/nrg. Second, not all of the caps formed by antibody treatment of CD2/nrg expressing cells were positive for ankyrin. This result is more consistent with an induced association that is moderately efficient as opposed to accretion of ankyrin that is constitutively associated with neuroglian. In the latter case, ankyrin staining should always be observed when its receptor is clustered. Third, ankyrin did not colocalize with neuroglian at non-contact regions of the S2 cell plasma membrane under conditions where detection of ankyrin relative to neuroglian was not limiting (13). Antibody staining experiments suggest that the enrichment of neuroglian staining at cell contacts is relatively small compared to the dramatic enrichment of ankyrin at contacts. We suggest that the enrichment of ankyrin is caused by an increase in the affinity of the ankyrin-neuroglian interaction that is
brought about by receptor clustering.

There are likely to be additional activating mechanisms that operate during assembly of the cytoskeleton, even within the L1 family of proteins. In the nervous system, spectrin, ankyrin, sodium channels and neurofascin all codistribute at the node of Ranvier (34). However, targeting to the node does not require cell-cell adhesion since these molecules also form node-like clusters in cultured neurons in response to a protein factor secreted by oligodendrocytes (35). In light of this result, it is not surprising that the recruitment of ankyrin to the plasma membrane by neurofascin in 293 cells was independent of cell-cell adhesion. One possibility is that recruitment of ankyrin by neurofascin at the node is regulated by tyrosine phosphorylation, which is known to have a potent modulatory effect on ankyrin binding in vitro (36).

Direct coupling of cytoskeleton assembly to cell adhesion provides a way to propagate positional information during the development of polarized cells. Neuroglian is expressed in polarized epithelial cells in vivo where the clustering mechanism described here is likely to have a role in polarized assembly of the spectrin cytoskeleton (27). The model provides a conceptual framework for further studies aimed at elucidating the structural basis for ankyrin recruitment by L1 family members. The model may also be relevant to understanding the interactions of other plasma membrane-associated cytoskeletal complexes, such as dystrophin and PDZ domain proteins, with their respective membrane receptors.
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**Figure legends**

**Figure 1:** Expression of CD2 transgenes in *Drosophila* S2 cells. A HindIII restriction site was engineered into the cytoplasmic domain of *Drosophila* neuroglian by PCR then spliced to the endogenous HindIII site of rat CD2. A truncated CD2 molecule (CD2Δ), with a stop codon immediately downstream of the HindIII site, was used as a control construct. Chimeric CD2/nrg (lane 3) and CD2Δ (lane 2) were expressed transiently in S2 cells under control of the *Drosophila* ubiquitin promoter. Total proteins from the transfected populations and from non-transfected control cells (lane 1) were analyzed on Western blots probed with mouse anti-CD2 and alkaline phosphatase conjugated secondary antibody. Molecular weight standards are shown to the left in kD.

**Figure 2:** Localization of ankyrin before and after capping of CD2 products in S2 cells. Chimeric CD2/nrg and CD2Δ were transiently expressed in S2 cells, cells were fixed in 2% formaldehyde, then double labeled with mouse anti-CD2 and rabbit anti-ankyrin antibodies, followed by rhodamine conjugated anti-mouse and fluorescein conjugated anti-rabbit antibodies. Abundant expression of CD2 was detected at the plasma membrane of transfected cells (A-D), but there was no detectable colocalization of ankyrin. However, when cells were treated for 3 hours with anti-CD2 primary and secondary antibodies before fixation, extensive capping of CD2 into compact islands of staining (arrows) or polar caps (arrowhead) was observed (E-H). Some CD2/nrg caps did not exhibit detectable ankyrin staining (*). Bar = 10 uM.

**Figure 3:** Quantitative analysis of ankyrin recruitment in response to CD2 capping. S2 cells expressing either the CD2/nrg chimera or truncated CD2Δ were scored for ankyrin colocalization before capping (-), after treatment with primary antibody alone (1°), or after capping with both primary and secondary antibodies (1°+2°), as shown in Figure 2. Each bar represents analysis of 50 capped cells that were scored
for the presence or absence of ankyrin at caps. Uncapped cells were scored for colocalization of ankyrin with sites of abundant CD2 expression. Colocalization of β spectrin with CD2 caps was scored after staining with rabbit anti-β spectrin antibody. Results are tabulated as the mean percentage from 3 independent experiments ± standard deviation.

**Figure 4:** Localization of β spectrin in capped cells expressing CD2/nrg or CD2Δ. Cells were capped and stained with β spectrin antibody, as described for ankyrin in Figure 2. β spectrin was redistributed to capped sites in CD2/nrg-expressing cells (A-B, arrows), but not in CD2Δ-expressing cells (C-D, arrows). Bar = 10 uM.

**Figure 5:** Expression of an epitope-tagged ankyrin-EGFP fusion protein. A previously-described myc epitope-tagged ankyrin transgene, in which ankyrin expression was driven by the *Drosophila* ubiquitin promoter (13), was engineered by PCR to include an NcoI restriction site in place of the endogenous stop codon from the ankyrin cDNA sequence. The NcoI site was used to splice the coding sequence for EGFP in frame with the ankyrin coding sequence. Total proteins from control S2 cells (lanes 1 &3) or from transiently-transfected S2 cells expressing ankyrin-EGFP (lanes 2 & 4) were analyzed on Western blots stained with either rabbit anti-ankyrin antibody (lanes 1 & 2) or mouse anti myc epitope tag antibody (lanes 3 & 4) followed by alkaline phosphatase conjugated secondary antibody. The 197 kD ankyrin-EGFP protein was detected with both antibodies (arrowhead), indicating that the fusion product was stable. Molecular weight standards are shown to the left in kD.

**Figure 6:** Localization of ankyrin-EGFP in living S2 cells. A. The ankyrin-EGFP gene was transiently transfected into stably-transfected S2 cells carrying an inducible transgene encoding the 167 kD isoform
of Drosophila neuroglian. Three days after transfection the population was induced to express neuroglian which led to formation of cell aggregates. Panels shown represent a through-focus confocal series of an ankyrin-EGFP expressing cell and its contacts (arrow) with neighboring neuroglian-expressing cells. Ankyrin-EGFP was not detectably recruited by neuroglian to non-contact regions of the plasma membrane (arrowhead). B & C. Ankyrin-EGFP and CD2/nrg or CD2Δ were transiently cotransfected into S2 cells. Three days after transfection, CD2 was capped by addition of primary and secondary antibody to living cells. The rhodamine conjugated secondary antibody and EGFP fluorescence were viewed and photographed in living cells. Ankyrin-EGFP codistributed with CD2 caps in chimera expressing cells (B, arrows) but not in cells expressing truncated CD2Δ (C, arrows). Bar = 10 μM.

Figure 7: Recruitment of ankyrin in response to receptor aggregation. The minimal ankyrin binding sites of neuroglian and the related protein neurofascin include 8 acidic residues (*) in a span of 27 residues. These acidic residues may trigger a conformational change that activates ankyrin binding in response to dimerization or capping. The cytoplasmic domain of neuroglian appears to exist as a monomer in yeast 2-hybrid studies (13) and ankyrin does not detectably associate with CD2/nrg until molecules are crosslinked by antibody capping. The cytoplasmic domain of neurofascin exists as a dimer in solution (28) and recruitment of ankyrin to the plasma membrane by neurofascin is constitutive.
Jefford Figure 5

ubiquitin promoter

myc

EcoRI

Ncol

GFP

ankyrin

1 2 3 4

205

116

97

anti ankyrin

anti myc
ANKYRIN BINDING SITE

neuroglian: ESDTDSMAEYGDGDGMNEDGSFIGQY
* * * * * * *

neurofascin: ESDDSLVDYGELEGQFNEDEGSFIGQY
* * * * * * *

CD2-neuroglian

adhesion/capping → + ankyrin

Neurofascin

dimerization site
