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Abstract

The neural cell adhesion molecule axonin-1/TAG-1 mediates cell-cell interactions via homophilic and heterophilic contacts. It consists of six Ig and four fibronectin type III domains anchored to the membrane by glycosylphosphatidylinositol. The recently solved crystal structure indicates a module composed of the four N-terminal Ig domains as the contact site between trans-interacting axonin-1 molecules from apposed membranes. Here, we have tested domain-specific monoclonal antibodies for their capacity to interfere with homophilic binding in a cell aggregation assay. The results confirmed the existence of a binding region within the N-terminal Ig domains and identified a second region contributing to homophilic binding on the third and fourth fibronectin domains near the C terminus. The perturbation of each region alone resulted in a complete loss of cell aggregation, suggesting that axonin-1-mediated cell-cell contact results from a cooperative action of two homophilic binding regions. The data support that axonin-1-mediated cell-cell contact is formed by cis-assisted trans-binding. The N-terminal binding regions of axonin-1 establish a linear zipper-like string of trans-interacting axonin-1 molecules alternately provided by the two apposed membranes. The C-terminal binding regions strengthen the cell-cell contact by enhancing the expansion of the linear string into a two-dimensional array via cis-interactions. Cis-assisted trans-binding may be a basic binding mechanism common to many cell adhesion molecules.
Axonin-1/TAG-1 Mediates Cell-Cell Adhesion by a Cis-assisted Trans-interaction*

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The neural cell adhesion molecule axonin-1/TAG-1 mediates cell-cell interactions via homophilic and heterophilic contacts. It consists of six Ig and four fibronectin type III domains anchored to the membrane by glycosylphosphatidylinositol. The recently solved crystal structure indicates a module composed of the four N-terminal Ig domains as the contact site between trans-interacting axonin-1 molecules from opposed membranes. Here, we have tested domain-specific monoclonal antibodies for their capacity to interfere with homophilic binding in a cell aggregation assay. The results confirmed the existence of a binding region within the N-terminal Ig domains and identified a second region contributing to homophilic binding on the third and fourth fibronectin domains near the C terminus. The perturbation of each region alone resulted in a complete loss of cell aggregation, suggesting that axonin-1-mediated cell-cell contact results from a cooperative action of two homophilic binding regions. The data support that axonin-1-mediated cell-cell contact is formed by cis-assisted trans-binding. The N-terminal binding regions of axonin-1 establish a linear zipper-like string of trans-interacting axonin-1 molecules alternately provided by the two apposed membranes. The C-terminal binding regions strengthen the cell-cell contact by enhancing the expansion of the linear string into a two-dimensional array via cis-interactions. Cis-assisted trans-binding may be a basic binding mechanism common to many cell adhesion molecules.

Interactions between cell adhesion molecules on the surface of neural cells play a key role in several stages of neural development, including cell migration, axon growth and guidance, establishment of synaptic connections, and myelinization. A well characterized representative of a neural cell adhesion molecule is axonin-1 (1), the avian homolog of TAG-1 (2). Axonin-1 is a member of the immunoglobulin superfamily. It consists of six Ig and four fibronectin type III (Fn) domains and is anchored to the cell membrane by a glycosylphosphatidylinositol anchor. Axonin-1 has been found to engage in different interactions with other cell-surface or extracellular matrix components. Axonin-1 on one cell binds homophilically to axonin-1 on another cell (3). Heterophilic interactions of axonin-1 have been reported with the cell adhesion molecules NgCAM (4), NrCAM (5), and NCAM (6); the proteoglycans neurocan and phosphacan; and the extracellular matrix component tenascin C (6).

As a first step toward a detailed molecular description of the homophilic and heterophilic interactions of axonin-1, we have determined the crystal structure of the first four N-terminal domains of axonin-1 (7). We found a U-shaped arrangement of the Ig domains, resulting in the formation of a compact module. In the crystals, these modules are aligned in a string. Adjacent modules exhibit a large edge-to-face contact and are oriented in an antiparallel fashion, with their C termini pointing perpendicularly to the string. This arrangement suggests that cell adhesion by homophilic axonin-1 interactions occurs by the formation of a linear zipper-like array in which the axonin-1 molecules are alternately provided by the two apposed membranes (Fig. 1). In confirmation of this model, mutations in a loop critical for the formation of the zipper result in the loss of the homophilic binding capacity of axonin-1 (7).

Here, we provide evidence that monoclonal antibodies binding either to the first Ig domain or to the third and fourth Fn domains perturb the formation of aggregates between axonin-1-expressing myeloma cells. Our results indicate that two distinct binding sites, one at the N terminus and one at the C terminus of axonin-1, are involved in axonin-1-mediated cell-cell contact. Perturbing the binding in each region alone resulted in a complete loss of axonin-1-mediated cell-cell contact. Therefore, we propose a model for axonin-1-mediated cell-cell binding based on a cooperative action of the N- and C-terminal binding sites.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies—**Axonin-1 was purified from the ocular vitreous fluid of embryonic day 14 chicken embryos as described previously (8). For the production of monoclonal antibodies, four 6-week-old BALB/c mice were immunized by subcutaneous injection of 20 or 25 μg of axonin-1 in complete Freund’s adjuvant, followed by three to four booster injections of 10–20 μg of axonin-1 in incomplete Freund’s adjuvant. 3.5 days before spleens were removed, the mice received a last booster injection of 15 μg of axonin-1 in phosphate-buffered saline. One-half was injected into the tail vein, and the other half into the peritoneum. Monoclonal antibodies were produced according to the protocol given by Fazekas de St. Groth and Scheidegger (9). For fusion with spleen cells, the myeloma cell line X63-Ag8.653 was used. All clones resulting from a fusion were tested 11 days after the fusion, and the test was repeated for slowly growing and negative clones at days 13, 15, and 19. Supernatants were tested for their capacity to recognize native purified axonin-1 in a dot immunobinding assay on nitrocellulose. Binding mAbs were visualized with peroxidase-conjugated anti-mouse IgG antibody (Kirkegaard & Perry Laboratories). Positive clones were subcloned at least three times.
to the domain that was deleted. For example, in Ig2, the second Ig domain of axonin-1 was excised. The domain deletion mutants used in this study and the corresponding peptide fragments that were deleted are listed below, giving the first and last amino acids of the excised peptide fragment in reference to the amino acid sequence position of wild-type axonin-1 (1): Ig1: (Met27–Phe38); Ig2: (Thr706–Pro807); Ig3: (Pro807–Pro908); Ig4: (Pro908–Pro1009); Ig5: (Pro1009–Gly1110); Ig1234: (Met27–Phe124/Ser131–Ala147); Ig1234: (Met27–Phe124/Ser131–Leu147), and Ig1234: (Ala492–Ala569–Ala661–Arg679–Pro705–Pro735). The hybrids between axonin-1 and the related cell adhesion molecule F11 (contactin) were previously described by Lierheimer et al. (11). The axonin-1/F11 hybrid consisted of Met1–Leu666 of axonin-1 and Pro566–Phe1009 of F11. The F11/axonin-1 hybrid was composed of Met1–Pro566 of F11 and Ser666–Leu1006 of axonin-1.

Transfection of Myeloma Cells—Stably transfected myeloma cells (J558L) were obtained by protoplast fusion (12) with the constructs specified above. To obtain independent transfected clones, the cells were diluted in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and cultivated in microtiter plates at 37 °C in 10% CO2. After 2 days, transfected clones were selected by addition of 5 mM l-histidinol (Sigma, Buchs, Switzerland). Transfection efficiencies were ~10^-4. After 10 days, the cells were screened for recombinant expression by immunofluorescence staining using rabbit antiserum against axonin-1 or F11 (1:500 dilution) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (1:100 dilution; Zymed Laboratories Inc., South San Francisco, CA). Colonies with high level expression were subcloned. Expression of domain deletion mutants was tested by Western blot analysis. Cell-surface exposition of the expressed domain deletion mutants was visualized by indirect immunofluorescence using goat anti-axonin-1 serum and FITC-conjugated rabbit anti-goat IgG antibody (Zymed Laboratories Inc.).

Epitope Mapping of mAbs with Axonin-1 Domain Deletion Mutants Expressed by J558L Cells—To test binding of mAbs, the J558L myeloma cells expressing wild-type or mutant axonin-1 were preincubated for 2 h at 37 °C with hybridoma supernatant or monoclonal anti-IgG antibody at a concentration of 10 μg/ml in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells that had bound the antibodies were detected by incubation with FITC-conjugated goat anti-mouse IgG antibody (1:200 dilution; Cappel Organon Teknika nv, Turnhout, Belgium). The preparation was examined with a Leitz DMR fluorescence microscope (Leica AG, Glattbrugg, Switzerland) and analyzed with an automated microplate fluorescence reader (CytoFluor 2000, Millipore). Expression of mutant axonin-1 was confirmed using goat polyclonal anti-axonin-1 antibody at a concentration of 500 μg/ml and FITC-conjugated rabbit anti-goat IgG antibody at a dilution of 1:100.

Microscopy—Clustering of axonin-1 molecules was recorded by a Zeiss laser scanning microscope (Axioplan LSM 310) using identical conditions for all images. Contrast was set to 418, and brightness to 9999.

Adenoviral Expression in CV-1 Cells—An adenovirus vector containing an expression cassette for the deletion mutant Ig5, termed AdAxIg5, was constructed as previously reported for the adenovirus vector coding for the full-length axonin-1 sequence (AdAx) (13). In both constructs, expression of the transgene is controlled by the cytomegalovirus promoter. For viral infection, CV-1 cells were cultured in 60-mm culture dishes until they reached ~30% confluency. The cells were then trypsinized, counted, and allowed to settle down in culture medium. adenovirus (multiplicity of infection = 20) was added; and 3 h later, the medium was replaced with new culture medium. The experiments were started 48 h after transfection.

Quantification of Immunostaining in Axonin-1-expressing CV-1 Cells—To quantify the accumulation of axonin-1 and Ig5 at the cell contact area of two expressing cells, we determined the distribution coefficient (q) using a laser scanning microscope and NIH Image software. q was equal to the ratio between the fluorescence intensity in the contact area of the two cells (Icontact) per area (a) and the average of the fluorescence intensity of the two cell surfaces (Icell1 and Icell2 respectively) per area: q = (Icontact/a)/(Icell1 + Icell2)/a.

RESULTS

Perturbation Studies Identify Monoclonal Antibodies That Inhibit Axonin-1-mediated Cell-Cell Aggregates—To localize the surface areas of axonin-1 that are involved in homophilic

Fig. 1. Zipper model for axonin-1-mediated cell-cell adhesion as deduced from the crystal structure of the first four Ig domains of axonin-1. The crystal structure reveals that the first four Ig domains of axonin-1 are arranged in a U-shape and form a composite module (7). The contacts of the axonin-1 molecules in the crystals prompted a zipper model for axonin-1-mediated cell-cell adhesion in which trans-interacting axonin-1 molecules are alternately provided by the two apposed membranes. With an extended structure of the trans-binding axonin-1 molecules are alternately provided by the two apposed membranes. With an extended structure of the trans-binding axonin-1 molecules, apposed membranes would assume a maximum distance of 58 nm. For clarity, axonin-1 molecules of the upper and lower membrane are shown in distinct colors. Ig domains are green (upper membrane) or red (lower membrane), and Fn domains are turquoise (upper membrane) or orange (lower membrane).
Fig. 2. Antibody perturbation of the aggregation of axonin-1-expressing myeloma cells. a–h, phase-contrast images of axonin-1-expressing myeloma cells under selected conditions. Axonin-1-expressing myeloma cells formed aggregates (a). Cell aggregation was not perturbed by non-binding antibody 187.1 (b) and anti-axonin-1 mAb X9H8 (c). Polyclonal anti-axonin IgG antibody prevented cell aggregation (d). Anti-axonin-1 mAbs U77F8 (e), V3B3 (f), X9C3 (g), and X9F6 (h) prevented cell aggregation. Bar = 50 μm. i, quantification of myeloma cell aggregation. Axonin-1-expressing myeloma cells were incubated with 50 μg/ml monoclonal anti-axonin-1 Fab antibodies or 1 μg/ml polyclonal anti-IgG antibodies. The percentage of cell aggregates containing >4 cells and >10 cells, respectively, was determined by counting aggregates and forming the ratio to the aggregates in the negative control (no antibody (Ab)). Two classes are presented: aggregates containing >4 cells and aggregates containing >10 cells. Error bars indicate the S.E. (n = 3). With mAbs V10C7 and X9C3 and with polyclonal goat anti-axonin-1 antibodies (Gax-A), no aggregates with >10 cells were found. With mAb U77F8, we found no aggregates at all.

binding, we tested the capacity of monoclonal antibodies to perturb axonin-1-mediated cell-cell binding and mapped their epitopes on the surface of native axonin-1. To identify such antibodies, we used a cell aggregation assay with myeloma cells stably expressing wild-type axonin-1. The axonin-1-expressing myeloma cells form large cell aggregates mediated by homophilic interactions between axonin-1 molecules (3). We tested 27 mAbs for their capacity to interfere with axonin-1-dependent myeloma cell aggregation. In a pilot experiment, purified mAbs were tested in their IgG form. We found seven mAbs that clearly prevented the formation of aggregates. Together with a random selection of non-perturbing mAbs, these aggregation-perturbing mAbs were subsequently tested as Fab for their capacity to dissociate axonin-1-expressing myeloma cell aggregates (Fig. 2). All seven aggregation-perturbing mAbs reduced the percentage of aggregates composed of >10 cells to 10% or less (Fig. 2i). Smaller aggregates composed of at least five cells were still observed, but their number was <45% of the control values. The most potent inhibitors of homophilic binding (U77F8, V10C7, and X9C3) perturbed myeloma cell aggregation completely. In the presence of these mAbs, as with polyclonal anti-axonin-1 Fab antibodies, virtually no cell aggregates were formed. Indirect immunofluorescence staining was used to show that the mAbs that had no effect on the formation of myeloma cell aggregates were capable of binding to cell-surface exposed axonin-1, indicating that their inability to perturb axonin-1-mediated cell aggregation was not due to an inaccessibility of their corresponding epitopes in the membrane-bound form of axonin-1.

Anti-axonin-1 Antibodies Perturbing Cell-Cell Aggregation Are Directed against Two Distinct Regions: Either the N or C Terminus—To localize the epitopes of the mAbs, we used domain deletion mutants of axonin-1 and axonin-1/F11 chimeras stably expressed on the surface of J558L myeloma cells. Entire domains of axonin-1, as defined by their homology to the Ig variable domains or the fibronectin type III domains (1) and by exon/intron borders of the axonin-1 gene (14), were deleted by oligonucleotide-directed mutagenesis. Mutant forms of axonin-1 were cloned into the axonin-1 expression vector pMAX (3). Vectors carrying these constructs were introduced into myeloma cells by protoplast fusion (12). Immunocytochemical staining revealed that all mutants, except ΔIg1, were expressed on the surface of J558L cells. No axonin-1 immunoreactivity was detectable on parental myeloma cells. Myeloma cells stably expressing axonin-1 and axonin-1 deletion mutants on their surface were used for epitope mapping. The presence of an epitope recognized by a particular mAb was assessed by indirect immunofluorescence staining using FITC-conjugated anti-mouse IgG antibody and fluorescence measurement in the Cytofluor 2000 automated microplate fluorescence reader. Based on the absence of binding to myeloma cells expressing a particular deletion mutant, the epitope of a mAb was allocated to a specific domain of axonin-1 (Fig. 3). In our series of 27 mAbs, three were mapped to Ig domain 1 (Ig1), one to Ig3, four to Ig4, one to Ig5, one to Ig6, one to Fn domain 1 (Fn1), one to Fn2, two to Fn3, and five to Fn4. Five mAbs failed to bind to mutants lacking one of the first three Ig domains. Of these five mAbs, four were also unable to bind to mutants lacking the fourth Ig domain (10). Three mAbs did not bind to mutants lacking either the third or fourth Fn domain.

Three of the seven mAbs that interfered with myeloma cell aggregation (U77F8, X7F6, and V3B3) had their cognate epitopes on the first Ig domain. The other four mAbs (X9E9, X9F6, V10C7, and X9C3) were directed to epitopes located on the two C-terminal Fn domains. The epitopes of V10C7 and X9C3 were unequivocally on Fn4, whereas the antibodies of X9E9 and X9F6 required the presence of both Fn3 and Fn4 for binding. The epitopes of the 20 axonin-1-binding antibodies that did not affect axonin-1-mediated myeloma cell aggregation were distributed over the entire axonin-1 molecule. In particular, we found non-perturbing antibodies with epitopes on the same domains as the epitopes of the aggregation-perturbing antibodies, i.e. on the Ig1234 domain conglomerate and on the third and fourth Fn domains. One of the Ig1234-binding inef-
Cis-assisted Trans-binding of Axonin-1/TAG-1

**Fig. 3. Epitope mapping of monoclonal anti-axonin-1 antibodies.** a, domain structure of axonin-1, hybrids composed of axonin-1 and F11, and axonin-1 deletion mutants used for epitope mapping. The Ig and Fn domains of axonin-1 are represented as red and orange ellipsoids, respectively; the corresponding domains of F11 are indicated in dark blue and light blue, respectively. The axonin-1/F11 hybrid (Ax-F11) is composed of all Ig domains and the first Fn domain of axonin-1 and the last three Fn domains of F11; the complementary hybrid F11/axonin-1 (F11-Ax) is composed of all Ig domain and the first Fn domain of F11 and the last three Fn domains of axonin-1. Deleted domains are indicated by lines, and the deleted domain(s) are indicated on the left. b, profiles of the binding of 27 mAbs raised against native axonin-1 to axonin-1/F11 hybrids and deletion mutants of axonin-1 (Ax-1). The hybrids and deletion mutants were expressed on the surface of myeloma cells, and binding was analyzed by indirect immunofluorescence using an automated microplate fluorescence reader. +, binding; −, no binding. No sign indicates “not tested.” The second line from the bottom indicates the domain(s) bearing the epitope. The last line indicates whether the antibody perturbed the aggregation of myeloma cells expressing wild-type axonin-1. The colored areas indicate the aggregation-perturbing mAbs.

Cells were infected with a recombinant adenovirus containing an expression cassette of either wild-type axonin-1 (AdAx) or the ΔIg5 mutant (AdAxΔIg5). 2 days after viral infection, the cells were stained for surface axonin-1 by indirect immunofluorescence (Fig. 4). As demonstrated in Fig. 4c, the cell contact zones between axonin-1-expressing cells showed no or at best weak accumulation of axonin-1 immunoreactivity. In contrast, cells expressing the ΔIg5 mutant of axonin-1 exhibited a pronounced accumulation of axonin-1 immunoreactivity along the contact sites with other cells expressing the axonin-1 mutant (Fig. 4b). Measurements of the fluorescence intensities over free cell-surface membranes and over cell contact lines confirmed a significant accumulation of mutant axonin-1 compared with wild-type axonin-1 at cell contact lines (Fig. 4e). These results indicate an enhanced propensity of the extended form of axonin-1 to associate with areas of cell-cell contact.

**DISCUSSION**

Cis-assisted Trans-binding: A Model for Axonin-1-mediated Cell-Cell Adhesion—We found that the epitopes of function-blocking monoclonal antibodies against axonin-1 were located in two distant regions, one close to the N terminus (Ig1) and the other close to the C terminus (Fn3 and Fn4). Perturbing the binding of each region alone resulted in a complete loss of axonin-1-mediated cell-cell adhesion. Theoretically, homophilic axonin-1 binding involving two distant binding sites could be formed by (a) an interaction of the N-terminal site of axonin-1A (N1) with the C-terminal site of axonin-1B (C1) and (b) two distinct trans-interactions between axonin-1A and axonin-1B (NC-N2 and C2-C3), or (c) a cooperation of a trans-interaction between the N termini (NC-N2) and a cis-interaction between the C termini of the trans-interacting axonin-1 molecules with the C termini of adjacent axonin-1 molecules.
(C\textsubscript{A}-C\textsubscript{A} and C\textsubscript{B}-C\textsubscript{B}). The reverse of the interactions of possibility \(c\) is also possible (C\textsubscript{A}-C\textsubscript{N} with N\textsubscript{A}-N\textsubscript{A} and N\textsubscript{B}-N\textsubscript{B}), although, based on steric reasons, less likely.

The finding that mAbs against either region alone are capable of abolishing axonin-1-mediated cell-cell contacts is obvious for possibilities \(a\) and \(c\). For possibility \(b\), the explanation would be that only N-N or C-C trans-interactions would not be sufficient to support cell-cell binding.

Alternatively, for possibility \(d\), one could speculate that the binding of mAbs to the membrane-proximal epitopes of axonin-1 might perturb a role of the Fn region in controlling the conformation or dynamic accessibility of the Ig1–4 region. This possibility is plausibly supported by the observation that monomeric axonin-1 has a horseshoe form that is thought to be stabilized by an intramolecular contact between the N-terminal Ig region and the C-terminal Fn region (10).

From these possibilities, we favor a cooperation of two interactions, one among N-terminal binding sites and another among C-terminal binding sites of adjacent axonin-1 molecules anchored to the same membrane because previous studies have clearly demonstrated that both isolated N- and C-terminal fragments exhibit the capacity of self-binding (7, 16). In the crystals of the module formed by the first four Ig domains of axonin-1, Freigang \textit{et al.} (7) have identified an extended edge-to-face contact between adjacent molecules, consistent with the formation of a linear string of molecules. Adjacent molecules in the string are in antiparallel orientation, and their C termini point perpendicularly to the axis of the string. This arrangement prompted a model for axonin-1-mediated cell-cell adhesion in which the N-terminal Ig1–4 modules of axonin-1 molecules of apposed membranes become engaged in a homophilic trans-contact by interlacing in a zipper-like manner. In support of the model, the mutation of two amino acids located within the edge-to-face contact region (H186A and F189A) resulted in a complete loss of axonin-1-mediated cell aggregation. Likewise, Tsiotra \textit{et al.} (16) have reported an aggregation of Schneider line 2 cells transfected with the Fn moiety of TAX-1, the human ortholog of axonin-1. These results unequivocally reflect the existence of a homophilic binding site within the Fn moiety. However, although the evidence for this binding site has been obtained in an assay reporting for trans-interactions, it is possible that the biologically relevant \textit{in vivo} interaction reflected in this assay occurs in \textit{cis}. As previously observed with axonin-1 and NgCAM, topological information may be lost in binding assays due to orientation, conformational, or steric deviations from the normal situation (for a well documented example, compare Refs. 4 and 15). In the case of truncated TAX-1 molecules overexpressed on the surface of Schneider cells, a cis-binding site between Fn segments arranged in parallel (as expected for a cis-interaction) could readily cause cell-cell aggregation via trans-binding if enough orientation freedom is provided to allow a tilt of the binding segment from the perpendicular to the membrane plane. Because of their proximity to the membrane, these binding sites in the Fn region are more likely to interact in \textit{cis} than in \textit{trans}. Distance measurements between a silicon substrate coated with covalently coupled full-length axonin-1 and growth cones of cultivated sensory ganglial neurons (17) indeed revealed values that exclude a trans-interaction between the Fn regions (see below for quantitative aspects). Therefore, we propose that the binding site in the Fn region mediates cis-interactions between the membrane-proximal Fn regions of axonin-1 molecules of the same membrane.

Based on the results presented here and published observations, we propose a cis-assisted trans-binding mechanism for the axonin-1-mediated cell-cell aggregation. As depicted in Fig. 5, the N-terminal binding site (located on a domain module comprising Ig1–4) binds in \textit{trans}, forming a linear zipper-like array with molecules alternately provided by the two apposed membranes (7). The binding is strengthened by the cis-contacts of the C-terminal binding sites (located on Fn3 and Fn4), which mediate a side-by-side arrangement of the linear strings into a two-dimensional array. This model reconciles the currently available observations on direct interactions between recombinant fragments of axonin-1 (7, 16) and the results of our antibody perturbation studies on cell-cell adhesion mediated by full-length axonin-1. The model is also in agreement with recent distance measurements. Using fluorescence interference contrast microscopy, a minimal distance of 37 nm between growth cones of cultivated sensory ganglial neurons and a silicon substrate coated with covalently coupled axonin-1 has been measured (17). Based on the distance of the C termini of adjacent axonin-1 Ig1–4 modules in the crystals (10 nm) (7), the length of a tandem array of two Ig domains (9 nm) (7), the length of a tandem array of two Fn domains (6 nm) (18), and the length of the proline- and glycine-rich decapptide between the Ig and Fn moieties (3 nm), the membranes connected by trans-bound axonin-1 in a completely extended conformation would be at a maximum distance of 58 nm. Assuming that cis-interactions between Fn domains would result in a tilt of at least part of the domains from the perpendicular to the membrane plane or that tandems of Ig and Fn domains are partially juxtaposed, such as in CD4 (19), a considerably shorter intermembrane distance than that calculated for an extended structure of axonin-1 would result. Based on the measured minimal distance of 37 nm, a homophilic trans-contact between Fn domains (and thus, a binding model with two trans-contacts) is excluded. With a homophilic trans-contact between the two membrane-proximal Fn domains, membranes would be re-
Therefore, an associated monomeric axonin-1 with trans-bound axo-
masked in monomeric axonin-1 by the back-folded Ig1 
horseshoe-like form. It is possible that the cis-site in the Fn region is 
Membrane-bound monomeric axonin-1 is thought to be folded in a 
front were trimmed down to the three membrane-proximal Fn domains. 

better insight into the organization of axonin-1 molecules interacting in 
the two apposed membranes are shown in distinct colors. To allow 
of linear strings into a two-dimensional array and thus enlarge and 
contacts, the membrane-proximal Fn domains promote the association 
ing of axonin-1.

large edge-to-face contact between adjacent Ig1 
naturally provided by the two apposed membranes and trans-bound by a 
the installment of a linear string of axonin-1 molecules that are alter-
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required to approach each other to 12 nm or less; with a hom-
ophilic trans-contact between the first Fn domains, an inter-
membrane distance of 24 nm or less would be required.

Establishment of an Axonin-1-mediated Cell-Cell Contact Re-
quires a Conformational Transition from the Horseshoe-like Structure to an Extended Structure of Axonin-1—In the structure 
found by negative staining electron microscopy of purified soluble axonin-1, the axonin-1 molecule is bent in a horseshoe-
like form so that its two ends become located in close proximity (10). Thus, in the glycosylphosphatidylinositol-anchored form 
of axonin-1, the four N-terminal Ig domains bearing the Ng-
CAM-binding site (20) are turned back to the cell membrane. 
Based on this structure, the absence of heterophilic trans-
binding between axonin-1 and NgCAM exposed on the surface 
of different myeloma cells (15) is explained. Interestingly, the 
ΔIg5 mutant of axonin-1, in which the fifth Ig domain was 
deleted, exhibits a strikingly enhanced trans-binding of Ng-
CAM (10). It is plausible for sterical reasons that the deletion of 
an Ig domain in the interior of the molecule restrains the back-folding and thus favors the extended structure in which the N-terminal Ig1–4 module is better accessible for a trans-

In this study, we have found that the ΔIg5 mutant of axo-

The hypothesis that a major conformational change is re-
quired for the establishment of homophilic axonin-1/axonin-1 
binding is further supported by the observed discrepancy be-
tween cell-cell and Covaspheres-Covaspheres binding studies. Axonin-1 expressed on the surface of myeloma cells in the 
glycosylphosphatidylinositol-anchored form mediates cell ag-
gregation by homophilic binding (for details, see Ref. 3), 
whereas no homophilic binding is observed when axonin-1 is 
covalently coupled to the surface of Covaspheres via a reaction 
with amino group-reactive sites on the Covaspheres. Such a 
discrepancy between the function of the cell surface-anchored 
form and the Covaspheres-conjugated form has not been ob-
erved for NgCAM (4, 15) and NrCAM (5, 21). As a possible 
 speculation, one may speculate that binding to the Covas-
phers, possibly via multiple amino groups on the surface of 
each axonin-1 molecule, restricts the conformational freedom of 
axonin-1 that much that it may no longer be capable of assum-
ing the extended conformation that is required for an engage-
ment in homophilic binding.

In conclusion, these results indicate that the transition from 
a horseshoe to an extended form of axonin-1 is required for the 
establishment of homophilic trans-binding between axonin-1 
molecules of different cells. The Fn region, by binding to Ig1–4, 
stabilizes the horseshoe conformation and thus reduces the 
accessibility of the Ig1–4 module for trans-binding. Therefore, 
the most plausible conformational consequence of a mAb bind-
ing to Fn would be an enhanced trans-interaction. Thus, both 
restraining the back-bending into the horseshoe structure and 
interfering with the conformational function of the Fn region 
by antibodies should enhance trans-binding. The loss of cell-
cell binding in the presence of mAbs against Fn can thus not be 
explained by a conformational effect. Therefore, we speculate 
that the transition from the horseshoe to the extended form not 
only increases the accessibility of the Ig1–4 modules for ho-
ophilic trans-binding, but in addition interferes with another 
function. In view of the published homophilic binding site in Fn 
(16), an interference with the side-by-side association of adja-
cent axonin-1 molecules seems to us the most plausible explana-
tion for the binding-perturbing activity of the mAbs against 
Fn.

The Homophilic Cis-binding Site in the Fn Region May En-

The Homophilic Cis-binding Site in the Fn Region May En-
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Fig. 5. Model for cell-cell adhesion by cis-assisted trans-bind-
ing of axonin-1. An axonin-1-mediated cell-cell contact is initiated by the installment of a linear string of axonin-1 molecules that are alter-
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Figure 5: Model for cell-cell adhesion by cis-assisted trans-bind-
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ately provided by the two apposed membranes and trans-bound by a 

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the absence of the Fn moiety. Thus, the cis-binding among Fn regions of axonin-1 rather plays a role after the trans-binding by the Ig1–4 modules has been installed. It serves the expansion and enhancement of the cell-cell contact area initiated by the formation of linear strings of trans-bound axonin-1. After the first cis-contact of Fn domains of axonin-1 of two linear strings has become established, adjacent axonin-1 molecules of the one string would preferably become engaged in cis-contacts with adjacent axonin-1 molecules of the other string, resulting in the lateral alignment of the strings. Weak affinity between Fn domains provided, imperfect alignments could be improved, as single contacts could be broken and new ones established repeatedly during an ongoing optimization period until a parallel arrangement of multiple strings is reached. A further optimization of the package of trans-bound axonin-1 molecules would be realized by the exclusion of monomeric axonin-1 molecules from the contact area.

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REFERENCES