Caspr regulates the processing of contactin and inhibits its binding to neurofascin

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Introduction

Three cell adhesion molecules are present at the axoglial junctions that form between the axon and myelinating glia on either side of nodes of Ranvier. These include an axonal complex of contactin-associated protein (Caspr) and contactin, which was proposed to bind NF155, an isoform of neurofascin located on the glial paranodal loops. Here, we show that NF155 binds directly to contactin and that surprisingly, coexpression of Caspr inhibits this interaction. This inhibition reflects the association of Caspr with contactin during biosynthesis and the resulting expression of a low molecular weight (LMw), endoglycosidase H–sensitive isoform of contactin at the cell membrane, which remains associated with Caspr but is unable to bind NF155. Accordingly, deletion of Caspr in mice by gene targeting results in a shift from the LMw- to a HMw-contactin glycoform. These results demonstrate that Caspr regulates the intracellular processing and transport of contactin to the cell surface, thereby affecting its ability to interact with other cell adhesion molecules.
junction (Tait et al., 2000) and is markedly reduced at this site in contactin (Boyle et al., 2001), galactolipid (Poliak et al., 2001), or Caspr (Bhat et al., 2001)-deficient mice. It was reported recently that a soluble NF155-Fc chimera binds to cells expressing Caspr and contactin and precipitates these proteins from rat brain lysates, suggesting that NF155 serves as a receptor for the Caspr–contactin complex (Charles et al., 2002).

To determine the precise molecular mechanism of the interaction between NF155 and its neuronal partners, we have investigated whether NF155 binds to Caspr, to contactin or to a site formed by the combination of the two proteins. We found that NF155 binds directly to contactin and that, surprisingly, Caspr inhibits this interaction by regulating the intracellular processing, and cell surface expression of contactin.

Results and discussion
The glial isoform of neurofascin binds directly to contactin
To determine whether NF155 binds to contactin, we used soluble Fc-fusion proteins containing the extracellular domain of these adhesion molecules in binding experiments with cells expressing either contactin or NF155. As depicted in Fig. 1, a soluble contactin-Fc protein bound to cells expressing NF155 but not to contactin, whereas NF155-Fc bound to cells expressing contactin, as well as homophilically to cells expressing NF155. βC-Fc, a fusion protein containing the carbonic anhydrase-like domain of RPTPβ, a known high affinity ligand of contactin (Peles et al., 1995), did not bind to NF155. Nevertheless, βC-Fc pulled down NF155 from brain lysates of both wild-type and Caspr-deficient mice (unpublished data), further confirming that contactin binds NF155 even in the absence of Caspr. Because contactin is required for cell surface expression of Caspr (Faire-Sarrailh et al., 2000; Boyle et al., 2001), we could not examine the binding of NF155-Fc to cells expressing Caspr alone. Thus, we produced a soluble Caspr-Fc protein to determine whether it could bind to NF155. Although Caspr-Fc was mainly found within the cells, sufficient levels of this fusion protein were present in the medium (Fig. 1 B and not depicted). This soluble Caspr-Fc bound neither NF155, nor contactin expressing cells. The lack of Caspr-Fc binding to the latter was expected because these molecules only interact when both are present on the same membrane (i.e., in cis; Peles et al., 1997). Thus, we concluded that NF155 interacts directly with contactin but not with Caspr. The interaction between contactin and neurofascin is in agreement with previous observations using the chick homologues of these adhesion molecules (Volkmer et al., 1998).

Caspr inhibits the binding of contactin to neurofascin
Next, we determined whether the presence of Caspr affects the binding of NF155 to contactin. As a control, we used Caspr2, a homologous protein which does not interact with contactin (Poliak et al., 1999 and unpublished data). Surprisingly, binding of NF155 was substantially reduced when contactin was coexpressed with Caspr but not Caspr2 (Fig. 2, A and B). In contrast, βC-Fc bound equally well to cells expressing contactin alone or in combination with Caspr or Caspr2, indicating that contactin was available for binding other ligands at the cell surface. The expression of Caspr together with contactin on the cell surface of these cells resulted in the inhibition of NF155 binding to contactin (Fig. 2 C). It should be realized that because contactin is required for the export of Caspr from the ER (Faire-Sarrailh et al., 2000), all Caspr-positive cells detected here also express contactin on their surface. The reduction in NF155-Fc binding was proportional to the amount of Caspr used in the transfection (not depicted) and was specific to NF155-Fc, as the presence of Caspr had no effect on βC-Fc binding (Fig. 2 C, bottom). Counting the number of cells expressing Caspr (and therefore also contactin) on their surface that also bound NF155-Fc or βC-Fc (n = 500 and n = 1,000 stained cells in two different experiments), showed that βC-Fc binds to all Caspr-positive cells, whereas NF155-Fc only bound to <40% of these cells. Furthermore, clustering of NF155-Fc using a secondary antibody induced aggregation of contactin, but had no effect on Caspr (Fig. 2 D), suggesting that in Caspr–contactin expressing cells, NF155-Fc binds contactin molecules that are not associated with Caspr. The inability of NF155-Fc to cluster Caspr was in marked contrast to βC-Fc, which coclusters Caspr and contactin in
Caspr regulates the glycosylation and cell surface expression of contactin

The inhibitory effect of Caspr on the binding of contactin to neurofascin may result from direct competition for the same binding site and/or a modification of contactin by Caspr within the cells. We have shown previously that two contactin isoforms, differing in their extent of glycosylation, are found in neurons and that only the low molecular weight (LMw) form specifically associates with Caspr in the paranodes (Rios et al., 2000). Immunoprecipitation using a contactin antibody revealed that both isoforms are found in contactin-transfected HEK-293 cells (Fig. 3 A), but only the high molecular weight (HMw) isoform is expressed at the cell surface (Fig. 3 A, middle). In contrast, in the presence of Caspr, both the LMw- and HMw-contactin isoforms are detected at the cell surface. The amount of the LMw contactin present on the cell surface was directly proportional to the level of Caspr expressed in the cells (Fig. 3 B). Increasing the levels of Caspr resulted in a gradual increase of the LMw contactin and a gradual decrease in the amount of the HMw-contactin isoform present on the cell surface. The effect of Caspr on the cell surface expression of contactin was specific and was not detected when the latter was coexpressed with the closely related Caspr2 (Fig. 3 B). These results demonstrate that Caspr allows the transport of LMw contactin to the plasma membrane and reduces the amount of the HMw isoform present on the cell surface. Consistently, an increase in the expression of Caspr during the development of sciatic nerve correlates well with a detected shift between the HMw to the LMw-contactin isoform (Einheber et al., 1997).

To further examine whether this shift is directly dependent on Caspr, we have used mice lacking *Caspr* that were generated by a gene targeting approach (Fig. 3, C and D). As reported previously for another *Caspr* mutant mouse line (Bhat et al., 2001), these mice showed paranodal abnormalities (not depicted), including the absence of both Caspr and contactin from this site (Fig. 3 D). Immunoblot analysis of brain, spinal cord, and sciatic nerves from wild-type and *Caspr* mutant mice revealed that in the absence of Caspr, similar to results in transfected cells (Fig. 3, A and B), there was a clear shift to the HMw isoform (Fig. 3 E). In accordance with previous observations that noted the presence of HMw contactin in *Caspr*-deficient sciatic nerve (Bhat et al., 2001), these results demonstrate that Caspr is required for the generation of the LMw-contactin isoform normally present in the wild-type axolemma.

We have suggested previously the HMw- and LMw-contactin isoforms differed in their extent of glycosylation (Rios et al., 2000). To further characterize this difference, we treated contactin immunocomplexes with endoglycosidase H (EndoH), an enzyme that cleaves high mannose structures usually found in ER-resident proteins, but has no effect on complex oligosaccharides present on mature glycosylated proteins. As shown in Fig. 4, EndoH treatment decreases the apparent molecular weight of the LMw, but not the HMw-contactin isoform. The LMw-EndoH sensitive form was also precipitated using an antibody to Caspr, confirming that Caspr interacts with this contactin glycoform. Surface biotinylation of cells expressing Caspr and contactin showed that a complex of Caspr and the LMw contactin was present....
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on the cell surface and that both molecules were EndoH sensitive (Fig. 4 B). In contrast, the HMw-contactin glycoform found on the plasma membrane of cells expressing contactin and Caspr2 was EndoH resistant (Fig. 4 B), indicating that this contactin glycoform was further processed and carried complex oligosaccharides. These results demonstrate that contactin exists as two glycoforms, out of which only the LMw binds Caspr. Furthermore, although the HMw-contactin glycoform can reach the plasma membrane in the absence of Caspr, the latter is required for the transport of the LMw contactin to the cell surface.

In contrast to Caspr, which exclusively interacts with the LMw-contactin glycoform, NF155-Fc binds to the HMw form of contactin at the cell surface. Antibodies to NF155 or Caspr selectively immunoprecipitated the HMw- and LMw-contactin isoforms, respectively, from the cell surface of HEK-293 cells expressing all three proteins, (Fig. 4 C, left). The aim of this triple transfection was to examine the interaction of NF155 with contactin under conditions where both forms of contactin are present in the cell and that allow NF155 to interact with LMw contactin before it assembles with Caspr. Although the interaction between HMw contactin and NF155 under these conditions could thus occur in trans or cis, they clearly show that NF155 bind the HMw-contactin isoform. Similarly, NF155-Fc pulled down the HMw but not the LMw-contactin isoform from cells transfected with contactin and a moderate amount of Caspr, which allows the expression of both contactin forms on the cell surface (Fig. 4 C, right). In contrast, BC-FC interacted with both HMw and LMw. Hence, we conclude that NF155 preferentially interacts with the higher molecular weight form of contactin, which is not associated with Caspr. Whether the inability of NF155 to bind the LMw-contactin glycoform results from the immature glycosylation of the latter or steric inhibition secondary to its existence in a protein complex with Caspr on the plasma membrane will require further study.

During the biosynthesis of membrane glycoproteins, N-linked oligosaccharides are added cotranslationally in the ER and then terminal glucose and mannose residues are removed to generate a simple core-glycans (Helenius and Aebi, 2001). Properly folded proteins carrying the core-glycans then undergo further trimming and terminal glycosylation in the Golgi complex before they reach their final destination at the cell surface. The interaction between Caspr and contactin regulates each other’s transport to the cell surface. Contactin is required for the exit of Caspr from the ER (Faivre-Sarrailh et al., 2000), whereas Caspr regulates the intracellular processing and cell surface transport of contactin.

Caspr2 is presented as an inset on the left. (D) Adult-teased sciatic nerves isolated from wild-type (WT) or Caspr-deficient (KO) mice were labeled with antibodies to Na+ channels (NaCh; green) and Caspr (Caspr; red), or contactin (CNTN; red) as indicated. Note the absence of contactin from the paranodal junction in Caspr null mice. (E) Deletion of Caspr results in a shift in the molecular weight of contactin. Proteins extracts from brain (BR), spinal cord (SC), and sciatic nerve (SN) of wild-type (WT), or Caspr null mice (KO) were immunoblotted using antibodies to contactin or Caspr, as indicated at the bottom.
Caspr-dependent isoform switch of contactin

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Contactin is found as two different glycoforms, of which only the mature, EndoH-resistant HMw reaches the cell surface. The expression of Caspr in the cell chaperones the EndoH-sensitive contactin glycoform to the cell surface, where it is found in a complex with Caspr. These results have important implications on the molecular mechanisms involved in the generation of distinct domains at and around the nodes of Ranvier. First, by regulating which glycoform of contactin is present on the cell surface, Caspr controls the ability of this cell adhesion molecule to interact with additional ligands. Contactin can bind several nodal proteins, such as the β1-subunit of Na⁺ channels, NrCAM and neurofascin-186 (for review see Falk et al., 2002). The increase in Caspr expression and subsequently, the transition between HMw to LMw contactin observed during development (Einheber et al., 1997), may also regulate the ability of contactin to bind these nodal components. Second, by controlling the relative expression of contactin glycoforms at the cell surface, Caspr determines the localization of contactin in myelinated axons. Thus, in Caspr-deficient neurons, the HMw predominates (Fig. 3) and is preferentially expressed at nodes of central nervous system neurons rather than the paranodes (Bhat et al., 2001). Our results further suggest that Caspr may regulate this differential localization by directing contactin through distinct biosynthetic pathways. Finally, a major finding of this paper is the demonstration that NF155 does not bind directly to the Caspr–contactin complex. Because Caspr and contactin can be pulled down by NF155-Fc from rat brain (Charles et al., 2002), our results indicate that most likely other components are required to bridge between these proteins at the axoglial junction. Future experiments focused on identification of additional junctional components that may stabilize this tripartite complex should provide important new insights into the mechanism of paranodal junction formation.

Figure 4. Biochemical analysis of contactin isoforms. (A) Differential sensitivity of HMw and LMw contactin to endoglycosidase H (EndoH). HEK-293 cells expressing contactin with Caspr (Caspr/Con) or with neurofascin 186 as a control (NF186/Con) were subjected to immunoprecipitation with an antibody to contactin or Caspr. Immunocomplexes were incubated in the absence (–) or in the presence (+) of EndoH, following by Western blot analysis using anticontactin antibody. The Caspr-associated LMw but not the HMw-contactin isoform is EndoH sensitive. (B) Both HMw contactin and Caspr expressed on the cell surface contain high mannose structures, which are cleaved by EndoH. Intact HEK-293 cells expressing contactin with Caspr (Caspr + Con) or with Caspr2 (Caspr2 + Con) were biotinylated and subjected to immunoprecipitation with an antibody to Caspr or βC-Fc. The washed immunocomplexes were treated with EndoH as indicated (–/+), and blotted using streptavidin-HRP. (C) Preferential binding of Caspr and NF155 to the two contactin isoforms. Cells expressing Caspr, contactin and NF155 (left) or cells expressing Caspr and contactin (right) were biotinylated and subjected to immunoprecipitation (left; right, first lane), or Fc-pulldown (right) using the indicated antibodies and Fc proteins (PD). Precipitated material was blotted using streptavidin-HRP to detect cell surface proteins. In both panels, the location of the two forms of contactin, as well as Caspr and NF155, is indicated. In the left panel, only 20% of the lysates was used to precipitate contactin.

Figure 5. A schematic model describing the role of Caspr in the processing and transport of contactin. In the absence of Caspr, the high mannose residues (red dots) attached to contactin in the ER are being trimmed and replaced by complex oligosaccharide side chains (yellow dots) in the Golgi complex, resulting in the formation of an EndoH-resistant high molecular weight isoform (HMw) of contactin (only a single prototypic glycosylation site is illustrated). HMw contactin is being transported to the cell surface where it binds neurofascin and other ligands such as RPTPβ. The presence of Caspr in the cell allows the transport of an EndoH-sensitive LMw-contactin isoform to the plasma membrane. This may result from the inhibition of further processing of contactin in the Golgi complex or the transport of the Caspr–LMw-contactin complex to the cell membrane through an alternative pathway. The Caspr–LMw-contactin complex found at the cell surface binds RPTPβ but not neurofascin. The levels of Caspr determine the ratio between the HMw- and LMw-contactin isoforms present on the cell surface.

glycoforms (Fig. 5). Contactin is found as two different glycoforms, of which only the mature, EndoH-resistance HMw reaches the cell surface. The expression of Caspr in the cell chaperones the EndoH-sensitive contactin glycoform to the cell surface, where it is found in a complex with Caspr.

These results have important implications on the molecular mechanisms involved in the generation of distinct domains at and around the nodes of Ranvier. First, by regulating which glycoform of contactin is present on the cell surface, Caspr controls the ability of this cell adhesion molecule to interact with additional ligands. Contactin can bind several nodal proteins, such as the β1-subunit of Na⁺ channels, NrCAM and neurofascin-186 (for review see Falk et al., 2002). The increase in Caspr expression and subsequently, the transition between HMw to LMw contactin observed during development (Einheber et al., 1997), may also regulate the ability of contactin to bind these nodal components. Second, by controlling the relative expression of contactin glycoforms at the cell surface, Caspr determines the localization of contactin in myelinated axons. Thus, in Caspr-deficient neurons, the HMw predominates (Fig. 3) and is preferentially expressed at nodes of central nervous system neurons rather than the paranodes (Bhat et al., 2001). Our results further suggest that Caspr may regulate this differential localization by directing contactin through distinct biosynthetic pathways. Finally, a major finding of this paper is the demonstration that NF155 does not bind directly to the Caspr–contactin complex. Because Caspr and contactin can be pulled down by NF155-Fc from rat brain (Charles et al., 2002), our results indicate that most likely other components are required to bridge between these proteins at the axoglial junction. Future experiments focused on identification of additional junctional components that may stabilize this tripartite complex should provide important new insights into the mechanism of paranodal junction formation.
Materials and methods

Generation of Caspr +/- mice

A mouse genomic fragment, corresponding to the first three exons of caspr gene was isolated and used to generate a replacement-type vector, in which a neomycin-resistant gene replaced an SphI-BssHII fragment containing the first exon of Caspr including the initiator methionine and the signal sequence. ES clones were screened by Southern blot using a 2.4-kb NcoI-BglII and a 0.8-kb SpeI–SalI fragment as probes. Mice were generated as described previously for Caspr2 gene (Poliaik et al., 2003). All experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the Weizmann’s Institutional Animal Care and Use Committee.

Binding and immunofluorescence

β-C-Fc and H-con-Fc were described previously (Peles et al., 1995). For Caspr-Fc and NF155-Fc, the extracellular domain of rat Caspr (aa 1–1278) or rat NF155 (aa 1–1024) were fused to the hinge region of human IgG1-Fc. Similar results were obtained with a NF155-Fc construct containing an HA-tag after the signal sequence. ES clones were screened by Southern blot using a 2.4-kb NcoI–BglII and a 0.8-kb SpeI–SalI fragment as probes. Mice were generated as described previously for Caspr2 gene (Poliaik et al., 2003). All experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the Weizmann’s Institutional Animal Care and Use Committee.

Immunoprecipitation and immunoblot analysis

Preparation of tissue lysates, cell surface biotinylation immunoprecipitation, and Western blot analysis was performed as described previously (Poliaik et al., 2001, 2003; Goig et al., 2002). When indicated, immunocomplexes were incubated with 8 μL of EndoH (NEB) for 2 h at 37°C in 50 mM Na-citrate buffer, pH 5.7. Control samples were incubated in the same buffer without the enzyme.

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