occur before cleavage but after sulfinic or sulfonic acid modification, as we were able to observe these derivatives in our peptide analysis of proMMP-9. To confirm the pathophysiological relevance of these findings, we performed the same ischemia and reperfusion experiments after nNOS inhibition with 3br7NI, which is known to be neuroprotective and decrease stroke size. Under these conditions with NO formation blocked, the sulfinic and sulfonic acid oxidation products of activated MMP-9 were not observed in our MALDI-TOF analysis (Fig. 4C). One caveat with these findings is that nNOS deletion or NOS inhibition diminishes stroke damage, and hence one could argue that other stroke-related processes responsible for MMP activation would be reduced. Nonetheless, taken together with the data demonstrating S-nitrosylation of MMPs and our finding that MMPs activated in this manner cause neuronal apoptosis in vitro, it is likely that NO activation of MMPs participates in neuronal injury in vivo.

S-Nitrosylation and subsequent oxidation of protein thiol in the prodomain of MMP-9 can thus lead to enzyme activation, and homologous MMPs may be activated in a similar manner. This series of reactions confers responsiveness of the extracellular matrix to nitrosative and oxidative stress. Such insults are relevant to a number of pathophysiological conditions, including cerebral ischemia and neurodegenerative diseases. Extracellular proteolytic cascades triggered by MMPs can disrupt the extracellular matrix, contribute to cell detachment, and lead to a form of apoptotic cell death known as anoikis, similar to that observed in our neuronal cultures (*27*). The elucidation of an extracellular signaling pathway to neuronal apoptosis involving NO-activated MMPs may contribute to the development of new therapies for stroke and other disorders associated with nitrosative and oxidative stress.

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Methods
Figs. S1 to S5

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Myelin-Associated Glycoprotein as a Functional Ligand for the Nogo-66 Receptor

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Axonal regeneration in the adult central nervous system (CNS) is limited by two proteins in myelin, Nogo and myelin-associated glycoprotein (MAG). The receptor for Nogo (NgR) has been identified as an axonal glycosyl-phosphatidylinositol (GPI)–anchored protein, whereas the MAG receptor has remained elusive. Here, we show that MAG binds directly, with high affinity, to NgR. Cleavage of GPI-linked proteins from axons protects growth cones from MAG-induced collapse, and dominant-negative NgR eliminates MAG inhibition of neurite outgrowth. MAG-resistant embryonic neurons are rendered MAG-sensitive by expression of NgR. MAG and Nogo-66 activate NgR independently and serve as redundant NgR ligands that may limit axonal regeneration after CNS injury.

Axons of the adult mammalian CNS possess an extremely limited ability to regenerate after injury, largely because of environmental factors preventing axon growth (*1*, *2*). Characterization of the molecular mechanisms limiting axonal regeneration holds promise for the development of therapeutics to promote recovery after human CNS injury (*3*, *4*). Two factors limiting CNS regeneration, Nogo and MAG, are produced by oligodendrocytes and are distributed in the myelin that ensheathes CNS axons (*5*, *6*). Successful axon regeneration in the peripheral nervous system can be attributed to the absence of Nogo in myelinating Schwann cells, the rapid clearance of MAG by macrophages, and the induction of regeneration-associated genes (*1*–*6*). The recently identified Nogo receptor (NgR) is a leucine-rich repeat (LRR) protein that is GPI-anchored to the outer leaflet of the plasma membrane and binds a discrete cell-surface Nogo domain, Nogo-66 (*7*). MAG is recognized as a sialic acid–binding SIGLEC (sialic acid–dependent immunoglobulin-like family member lectin) protein with an affinity for gangliosides GD1a and GT1b (*8*–*14*). It has been suggested that these gangliosides might serve as axonal MAG receptors (*11*). No axonal proteins with high affinity for MAG have been identified. Here, we find that NgR serves as a receptor for MAG as well as Nogo-66, indicating that blockade of this one receptor may reverse the action of two inhibitors of CNS axonal regeneration.

NgR lacks an intracellular component, suggesting that it serves as the primary Nogo-66 binding site while an associated transmembrane coreceptor subunit mediates intracellular signal transduction (*7*). We sought to identify a coreceptor component by using an expression-cloning strategy based on the binding of a soluble fusion protein composed

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of alkaline phosphatase (AP) and the entire NgR ectodomain. In initial studies, the soluble AP-NgR fusion protein bound to neurons in a saturable manner and with high affinity (*15*), indicating a potential coreceptor binding site for NgR. The AP-NgR fusion protein was used to screen pools of an adult mouse brain cDNA expression library transfected into COS-7 cells. This screen demonstrated that the NgR can self-associate, with 6 out of 10 positive pools containing a cDNA encoding NgR itself (see supporting online material) and mediating binding of AP-Nogo-66 as effectively as AP-NgR. NgR self-association is clear in assays of AP-NgR binding to NgR-expressing cells (Fig. 1A). The 4 remaining positive pools from the screen do not exhibit AP-Nogo-66 binding. A single cDNA species, Clone 91, was isolated by sib selection from one of these positive pools. Transfection of Clone 91 cDNA into COS-7 cells resulted in the expression of a binding site with high affinity for AP-NgR; the binding affinity (K_D) for this is 15 nM (Fig. 1B). Neither AP (*15*) nor AP-Nogo-66 (Fig. 1A) binds to Clone 91–expressing cells.

To our surprise, DNA sequence analysis indicated that Clone 91 does not encode a neuronal Nogo coreceptor but rather a myelin-derived inhibitor of axon regeneration, MAG. Sib selection from all of the three other non–NgR-positive pools also yielded MAG. This suggests that NgR is a ligand-binding receptor for both Nogo-66 and MAG. Indeed, using soluble AP-MAG-ectodomain, MAG binding to cell-surface NgR exhibits a K_D of about 20 nM (Fig. 1C). This binding affinity is consistent with the potency of soluble MAG as an axonal growth cone–collapsing agent (*16*). Because MAG is known to bind sialic acid, we considered whether the binding event might be dependent on sialic acid residues on NgR. Neuroaminidase treatment strips sialic acid from COS-7–expressing exogenous NgR and neurons that express endogenous NgR but does not alter AP-MAG binding (Fig. 1, D and E). Thus, neither sialic acid on NgR nor sialic acid on cell-surface gangliosides appears essential for AP-MAG binding to NgR-expressing cells. Alternatively, phosphatidyl-inositol–specific phospholipase C (PI-PLC) treatment to remove NgR and other GPI-anchored proteins (but not gangliosides) from the cell surface completely abolishes AP-MAG binding to NgR-expressing COS-7 cells and neurons (Fig. 1, D and E). These data are consistent with NgR being the primary high-affinity binding site for MAG on neurons. The partial sialidase sensitivity of neuronal MAG binding sites observed in some previous reports (*8–10*, *13*) may reflect the fact that solid-phase assays favor multivalent, low-affinity interactions of MAG with sialic acid residues.

One explanation for AP-MAG binding to NgR-expressing cells and AP-NgR binding to MAG-expressing cells is that the two proteins associate directly. This mechanism was tested with purified soluble proteins. The extracellular domain of MAG fused to the Fc portion of human immunoglobulin G (IgG) (Fc-MAG) was immobilized on a protein A resin. The Fc-MAG affinity matrix retains purified NgR, as determined by anti-NgR immunoblot, whereas protein A alone does not (Fig. 1F). The MAG-NgR interaction is not modulated by the presence of 2 μg/ml of ganglioside GT1b; bound NgR in the presence of GT1b is 91 ± 9% (mean ± SEM) of that without GT1b. Fc-MAG was also immobilized in anti-human IgG–coated microtiter wells and exposed to AP-NgR or AP-Nogo-66. Selective binding of NgR to MAG is detectable as bound AP activity (Fig. 1G).

If NgR is indeed a functional receptor for MAG in neurons, removal of NgR from the axonal surface should prevent MAG-induced inhibition of outgrowth. PI-PLC pretreatment of embryonic day 14 (E14) chick DRG neurons removes GPI-anchored proteins, including NgR, from the neuronal cell surface (*7*). This pretreatment does not alter basal growth cone morphology but renders neurons insensitive to Fc-MAG–induced growth cone collapse (Fig. 2, A and B). To selectively interfere with NgR action, we tested a dominant-negative interfering form of NgR. The truncated soluble NgR fragment containing amino acids 27 to 310 (NgR-Ecto) selectively antagonizes Nogo-66 and myelin inhibition of neurite outgrowth (*17*). The presence of NgR-Ecto protein also reverses MAG inhi-

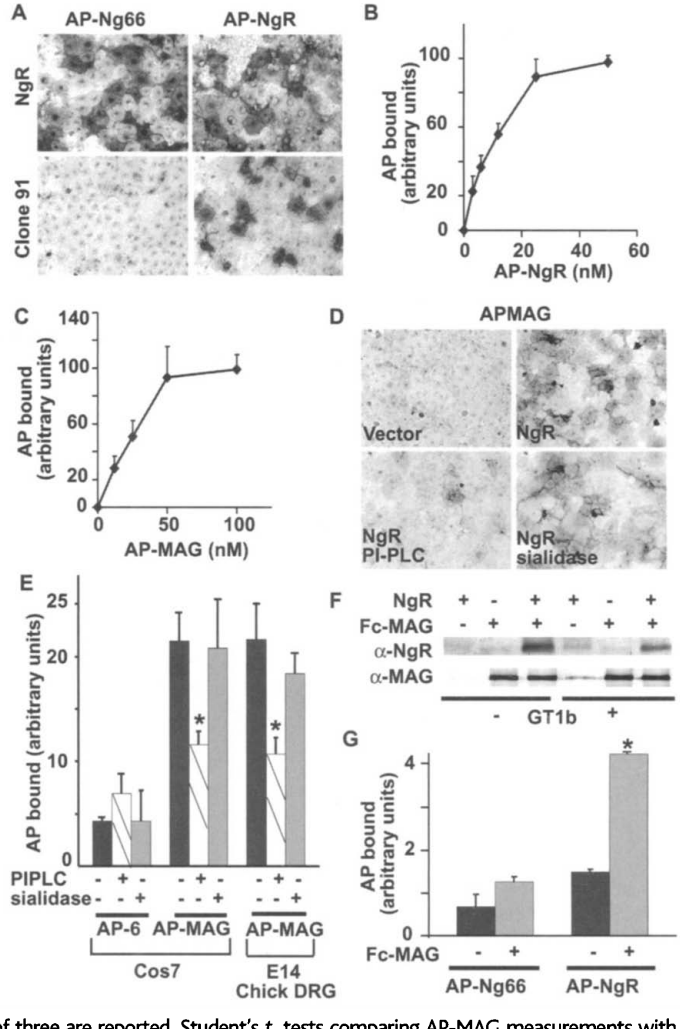

Fig. 1. MAG binds to the Nogo-66 receptor. (**A**) Identification of a cDNA clone that encodes a cell-surface protein binding AP-NgR but not AP-Nogo-66. Binding of AP-Nogo-66 (10 nM) and AP-NgR (50 nM) to COS-7 cells expressing either NgR or Clone 91 (MAG) was visualized by AP reaction. (**B**) AP-NgR binding to Clone 91–expressing COS-7 cells measured as a function of AP-NgR concentration. Mean ± SEM for three experiments. (**C**) AP-MAG binds to NgR-expressing cells in a dose-dependent manner. Mean ± SEM for three experiments. (**D**) Binding of MAG to the cellular NgR does not depend on the presence of sialic acids. COS-7 cells transfected with either NgR expression vector or control vector were treated with either 1 U PI-PLC, 50 mU Vibrio cholera sialidase, or no addition for 1 hour at 37°C. AP-MAG (25 nM) binding was assessed. (**E**) Quantification of AP-MAG bound to COS-7 cells was performed as in (D). Means ± SEM from one representative experiment of three are reported. Student's *t* tests comparing AP-MAG measurements with and without PI-PLC on NgR-expressing E13 chick DRGs and COS-7 cells are $^*P \leq 0.05$. (**F**) NgR directly associates with MAG. Purified soluble NgR-Ecto was precipitated with either Fc-MAG bound to protein A or protein A resin alone in the presence or absence of ganglioside GT1b and analyzed by anti-NgR immunoblot. (**G**) AP-Nogo-66 or AP-NgR bound to Fc-MAG. Fc-MAG was absorbed to microtiter plates precoated with anti-human IgG. AP-tagged proteins were applied for 1 hour at room temperature. After being washed, bound AP-proteins were measured. Average values for six wells from one experiment out of three with similar results are reported. Student's *t* test comparing AP-NgR bound to Fc-MAG or control is $^*P \leq 0.05$.

bition of neurite outgrowth (Fig. 2, C and D). The specificity of NgR-Ecto blockade is apparent in its lack of effect on outgrowth inhibition by a reagent that does not bind to NgR, Amino-Nogo (Fig. 2D) (*17*). Together, these data indicate that NgR is essential for MAG inhibition of axon outgrowth.

The sensitivity of neurons to MAG is regulated developmentally in rodents (*18*), with MAG-dependent inhibition beginning only in the early postnatal period. If NgR mediates MAG action, then NgR expression may follow a similar pattern. Indeed, NgR immunohistology reveals strong expression in adult (P64) mouse DRG neurons, but no detectable expression in E17 mouse DRG (Fig. 3A). We confirm that NgR-expressing adult DRG neurons are inhibited by MAG and that this inhibition is abolished in the presence of NgR-Ecto or by treatment with PI-PLC (Fig. 3B). By contrast, embryonic DRGs lack NgR and are not sensitive to MAG in the neurite outgrowth assay (Fig. 3, B). For these neurons, MAG sensitivity is correlated with NgR expression. This correlation is likely to be widespread, because a variety of postnatal neurons are responsive to MAG (*19*), and all adult CNS neurons appear to express NgR (*20*).

To investigate whether NgR expression is sufficient to convert MAG-insensitive neurons to a state in which MAG is inhibitory, we expressed NgR in early embryonic DRG neurons that are not inhibited by MAG. A recombinant herpes simplex virus (HSV) preparation drives expression of NgR in chick E7 DRG neurons and supports both Nogo-66 and MAG action, as determined in growth cone–collapse assays (Fig. 3, C and D). Thus, NgR can mediate both Nogo-66 and MAG signaling.

To understand the mechanism by which NgR is stimulated by either Nogo or MAG, we sought to characterize the domains on the NgR responsible for receptor-MAG interaction by deletion analysis. Binding of Nogo-66 is known to require all eight LRRs, but not the 140–amino acid domain connecting the LRRs to the GPI anchorage site (*17*). MAG binds to the same region of NgR as does Nogo-66, requiring all of the LRR domains but not the 140–amino acid domain (Fig. 4A). Deletion of any two sequential LRRs in NgR abolishes AP-MAG binding (*15*). This raises the possibility that Nogo-66 and MAG might compete for the same binding site in the NgR LRRs. However, excess Nogo-66 does not prevent MAG-NgR binding (*15*). Thus, the two myelin-derived outgrowth inhibitors appear to possess separate binding sites within the same domain of NgR. To verify the distinctness of these sites in axons, the selective Nogo-66 antagonist peptide NEP1-40 was included in neurite outgrowth assays on MAG or Nogo-66 substrate. We have shown that peptide NEP1-40, derived from the amino-terminal fragment of the Nogo-66 domain, antagonizes Nogo-66 activity (*21*). Here, we show that, although NEP1-40 blocks Nogo-66 action, it does not reduce MAG inhibition of neurite outgrowth (Fig. 4B).

This study identifies NgR as a receptor

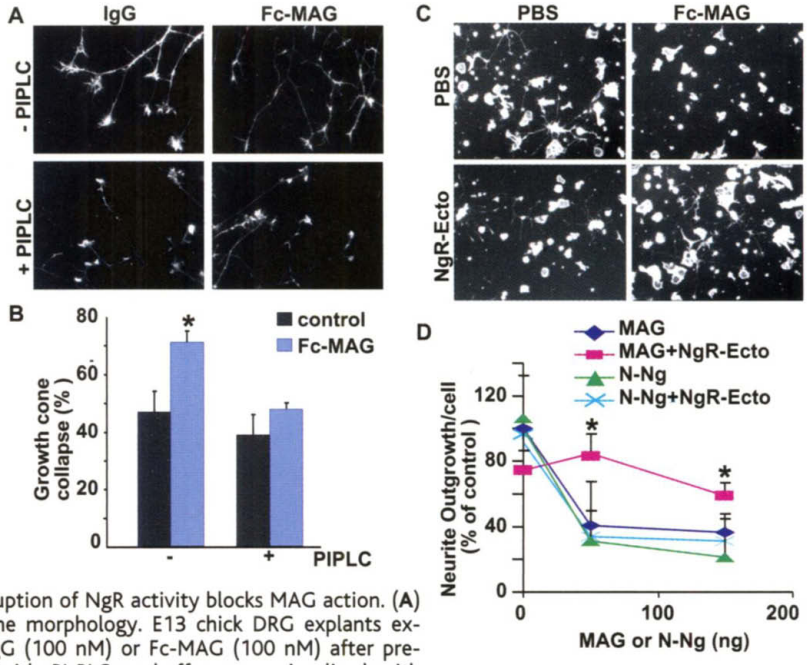

Fig. 2. Disruption of NgR activity blocks MAG action. (**A**) Growth cone morphology. E13 chick DRG explants exposed to IgG (100 nM) or Fc-MAG (100 nM) after pretreatment with PI-PLC or buffer were visualized with rhodamine phalloidin staining. (**B**) Quantification of growth cone collapse from (A). Mean ± SEM of three experiments. (**C**) Dissociated E13 DRGs were plated on control phosphate-buffered saline (PBS) spots or MAG spots mixed with 500 ng of glutathione S-transferase (GST) or NgR-Ecto. MAG inhibition is completely reversed by the addition of NgR-Ecto. (**D**) Measurements of DRG outgrowth on spots of MAG or Amino-Nogo alone or mixed with NgR-Ecto. Neurite outgrowth is expressed as μm of growth per cell. Means ± SEM for three to four experiments are reported.

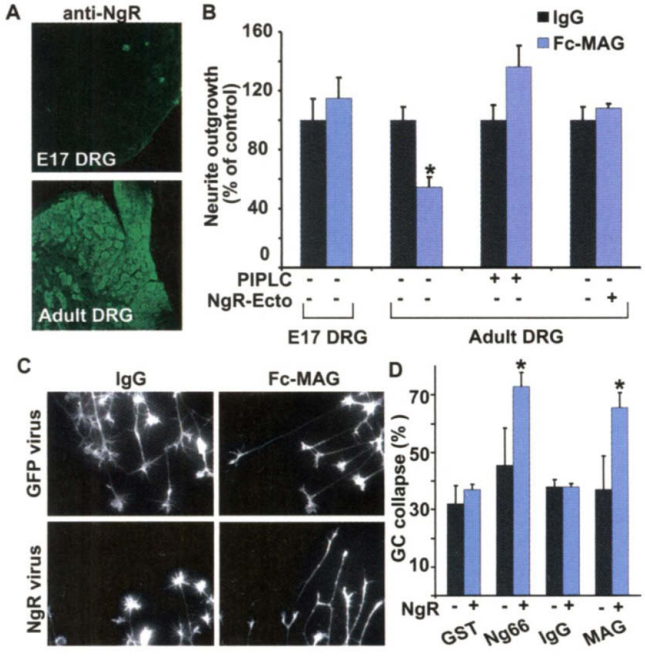

Fig. 3. NgR mediates the inhibitory responses induced by MAG. (**A**) NgR expression in mouse E17 and adult DRG. E17 and postnatal day 64 DRGs were fixed, sectioned, and stained with antibody to NgR followed by FITC-conjugated secondary antibody. Composite Z sections of FITC-labeled cells were obtained with a confocal microscope. (**B**) Quantitation of neurite length of E17 and adult DRGs plated on IgG (50 ng) control or Fc-MAG (50 ng) in the presence of PBS, PI-PLC (1 U/ml), or NgR-Ecto (50 ng). Mean ± SEM of four to six determinations. Student's *t* test comparing adult IgG neurite outgrowth measurements with Fc-MAG in the presence of PBS is $^*P \leq 0.05$. (**C**) Chick E7 DRG explants infected with HSV-GFP (green fluorescent protein) or HSV-Nogo receptor and treated for 30 min with either 100 nM of GST, GST-Nogo-66, IgG, or Fc-MAG. (**D**) Quantitation of growth cone collapse in (C). Means ± SEM for three experiments are reported. Student's *t* tests comparing NgR measurements to control GFP are $^*P \leq 0.003$.

for MAG. MAG binds directly to NgR, and NgR is necessary and sufficient for MAG inhibition of neurite outgrowth. Indeed, NgR expression in vivo is correlated with neuronal sensitivity to MAG, and the NgR protein is juxtaposed to compact myelin–containing MAG and Nogo (*20*). Thus, NgR must be considered a general receptor for restrictive effects of CNS myelin on axon growth in the adult mammalian CNS (Fig. 4C). Although MAG and Nogo-66 both bind to the LRRs of NgR, they appear to bind independently. This provides an explanation for similar but additive effects of Nogo and MAG on inhibition of axon growth. Evidence indicates that the NgR ligands, Nogo and MAG, are the two primary inhibitors in CNS myelin. Myelin prepared from mice lacking Nogo-A exhibits reduced inhibition of axon outgrowth, and the residual inhibitory activity is abolished by antibodies to MAG (*22*). Because one receptor mediates the action of both known myelin-derived inhibitors, interference with NgR function after CNS axonal injury may significantly alleviate myelin-dependent limitation of axonal regeneration.

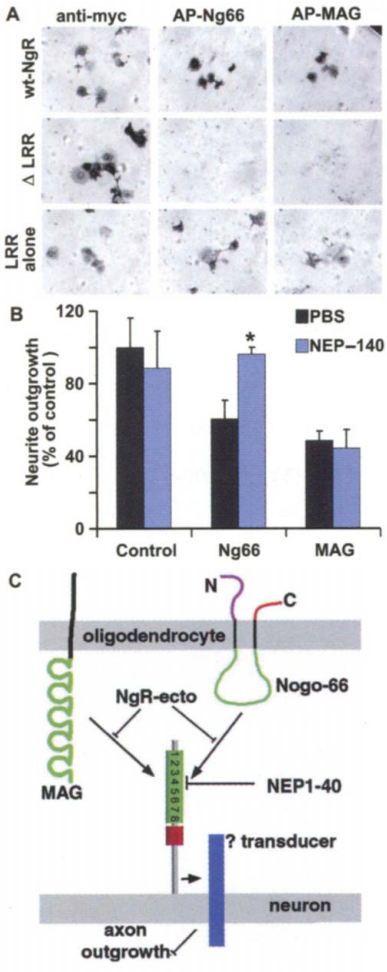

Fig. 4. Characterization of MAG binding site on the Nogo receptor. (**A**) COS-7 cells expressing full-length NgR (wtNgR), a NgR mutant lacking LRR 1-8 (ΔLRR), or a mutant containing LRR1-8 fused to the GPI linkage site (LRR alone) were stained for Myc immunoreactivity or tested for AP-Nogo-66 and AP-MAG binding. MAG and Nogo bind only to wtNgR and LRR alone transfected COS-7 cells. (**B**) NEP1-40 blocks Nogo-66 inhibitory activity but not that of MAG. Quantification of neurite outgrowth from dissociated E13 chick DRG cultures grown for 5 to 7 hours on PBS or MAG spots in the presence or absence of 1 μM NEP1-40. Means ± SEM of three experiments are reported. All $^*P$ values ≤ 0.002 (student's t test). (**C**) Model of NgR-mediated signaling. Either MAG or Nogo-66 can activate NgR. These interactions are blocked by the presence of a dominant-negative NgR protein, NgR-Ecto. The peptide antagonist, NEP1-40, specifically inhibits Nogo-66 activity but not that of MAG. Interaction of the axonal NgR with either one of its ligands on oligodendrocytes is predicted to activate a transmembrane signal transducer to inhibit axon outgrowth.

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Amphiphysin 2 (Bin1) and T-Tubule Biogenesis in Muscle

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In striated muscle, the plasma membrane forms tubular invaginations (transverse tubules or T-tubules) that function in depolarization-contraction coupling. Caveolin-3 and amphiphysin were implicated in their biogenesis. Amphiphysin isoforms have a putative role in membrane deformation at endocytic sites. An isoform of amphiphysin 2 concentrated at T-tubules induced tubular plasma membrane invaginations when expressed in non-muscle cells. This property required exon 10, a phosphoinositide-binding module. In developing myotubes, amphiphysin 2 and caveolin-3 segregated in tubular and vesicular portions of the T-tubule system, respectively. These findings support a role of the bilayer-deforming properties of amphiphysin at T-tubules and, more generally, a physiological role of amphiphysin in membrane deformation.

Ultrastructural observations have suggested that T-tubules of striated muscle develop from beaded tubular invaginations of the plasma membrane that resemble strings of caveolae (*1*, *2*). Accordingly, recent studies have demonstrated a critical role for caveolin-3 in T-tubule biogenesis (*3*–*5*) and have implicated caveolin-3 in a form of human muscular dystrophy (*6*). However, the smooth tubular profile of the T-tubule system of mature muscles indicates that the function of caveolin is, at least in part, replaced by other proteins during muscle differentiation. In addition, T-tubules, albeit with an abnormal morphology, are present in mice lacking caveolin-3 (*5*), indicating that other proteins participate in tubulogenesis.

It was reported that a splice variant of amphiphysin 2 is expressed at very high levels in adult striated muscle [muscle or M-amphiphysin 2, also referred to as Bin1 (*7*, *8*)] and is localized at T-tubules (*7*). Amphiphysin proteins function