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MAG Induces Regulated Intramembrane Proteolysis of the p75 Neurotrophin Receptor to Inhibit Neurite Outgrowth

Report

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Summary

The three known inhibitors of axonal regeneration present in myelin—MAG, Nogo, and OMgp—all interact with the same receptor complex to effect inhibition via protein kinase C (PKC)-dependent activation of the small GTPase Rho. The transducing component of this receptor complex is the p75 neurotrophin receptor. Here we show that MAG binding to cerebellar neurons induces α- and then γ-secretase proteolytic cleavage of p75, in a protein kinase C-dependent manner, and that this cleavage is necessary for both activation of Rho and inhibition of neurite outgrowth.

Introduction

The p75 neurotrophin receptor, which interacts with all four known neurotrophins, initiates a plethora of signaling cascades (Gentry et al., 2004; Hempstead, 2002). Recently, p75 has also been shown to be the transducing component within the receptor complex for the three known myelin inhibitors of axonal regeneration: myelin-associated glycoprotein (MAG), a 66 amino acid sequence of Nogo-A (Nogo-66), and oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002a; Wong et al., 2002). These inhibitors all bind directly to the Nogo receptor (NgR), so named for its initial identification as a binding partner for Nogo-66 (Domeniconi et al., 2002; Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). After binding ligand, NgR interacts with p75 in a manner that most likely also involves LINGO-1, a third component of the receptor complex (Mi et al., 2004). Downstream from their receptor binding, these myelin inhibitors bring about inhibition of axonal growth through the activation of the small GTPase Rho (Lehmann et al., 1999) in a protein kinase C (PKC)-dependent manner (Sivasankaran et al., 2004). While our understanding of the signaling initiated by myelin inhibitors has grown considerably in recent years, the precise behavior of the molecular players in inhibiting axonal growth is still far from clear.

A number of years ago, "shedding" of a 50 kDa fragment of the extracellular domain (ECD) of p75 was reported to occur in culture, largely from Schwann cells, but also from some neurons (DiStefano and Johnson, 1988). This line of inquiry led more recently to the demonstration that ectodomain shedding of p75 is promoted by treatment with phorbol esters such as 12-myristate 13-acetate (PMA) (Jung et al., 2003; Kanning et al., 2003). PMA treatment results in the formation of an intermediate 30 kDa C-terminal fragment (CTF) that is further cleaved to yield a 25 kDa intracellular domain (ICD) (Figure 1A). Moreover, neurotrophin treatment does not induce cleavage of p75 (Jung et al., 2003; Kanning et al., 2003). Although the functional relevance of these cleavage events is unknown, sequential cleavage of p75 is reminiscent of regulated intramembrane proteolysis (RIP), a novel mechanism in receptor signaling typified by processing such different proteins as Notch and amyloid precursor protein (APP) (Brown et al., 2000; Ebinu and Yankner, 2002; Urban and Freeman, 2002). The initial cleavage of the ECD occurs through the action of a membrane bound metalloprotease similar to those of the ADAMS family of proteases and generally termed an α-secretase (Hooper et al., 1997). Initial cleavage by α-secretase is obligatory for the second cleavage to occur within the transmembrane domain by a γ-secretase complex such as presenilin (Kopan and Ilagan, 2004). In the case of Notch, the ICD is released after the second cleavage into the cytoplasm, where it quickly moves to the nucleus, signals differentiation events, and is then rapidly degraded (Mumm and Kopan, 2000). For the majority of receptors that undergo RIP, however, the ICD does not move to the nucleus. Instead, the ICD is typically degraded rapidly in the cytoplasm (Ebinu and Yankner, 2002).

Here we report that the p75 receptor is cleaved sequentially by α- and γ-secretase activity, respectively, in a PKC-dependent manner in order for MAG to inhibit neurite outgrowth from cerebellar neurons. Furthermore, since MAG activation of Rho is blocked if secretase activity is inhibited, it is likely that a proteolytic fragment of p75 can mediate Rho activation.

Results

MAG and PKC-Dependent Proteolytic Cleavage of p75

In cell lines transfected to express p75 in abundance as well as in immortalized neuronal cell lines, cleavage of p75 occurs following treatment with the PKC activator PMA (Jung et al., 2003; Kanning et al., 2003). The resulting fragments are the ECD and a 30 kDa CTF that is subsequently cleaved to a 25 kDa ICD fragment (Figure 1A). To assess if endogenous neuronal p75 is

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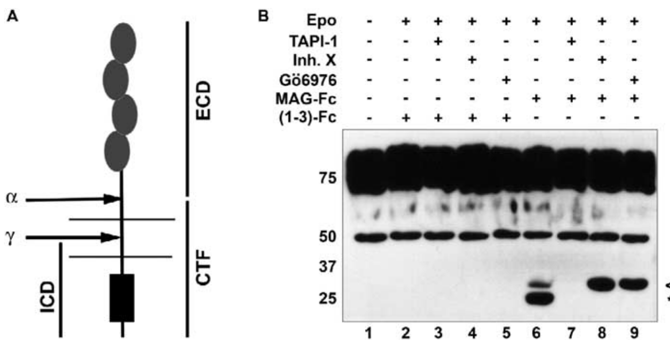

Figure 1. MAG Induces PKC-Dependent Proteolytic Cleavage of p75NTR

(A) The p75NTR ectodomain is initially shed by metalloprotease-mediated cleavage. The resulting p75NTR carboxy-terminal fragment (CTF) is subsequently cleaved by γ-secretase to release the p75NTR intracellular domain (ICD).

(B) Rat cerebellar neurons were treated with inhibitors of different proteolytic enzymes, epoxomicin (Epo, 1 μM), TAPI-1 (1 μM), inhibitor X (1 μM), and of PKC, Gö6976 (100 nM), for 60 min before addition of MAG-Fc or MAG(1-3)-Fc (both 20 μg/ml, 30 min). Western blots probed with an antibody specific to the cytoplasmic domain of p75NTR revealed a MAG-stimulated proteolytic fragment (<, ICD) produced by an inhibitor X and Gö6976-sensitive, γ-secretase-mediated cleavage. The ICD peptide is derived from a transitional product (*, CTF) that visibly accumulates in the presence of a γ-secretase or PKC inhibitor and that is lost in the presence of the metalloproteinase inhibitor TAPI-1.

cleaved in a similar manner in response to myelin-based inhibitors, we treated primary cerebellar neurons from postnatal day 5–8 rats with soluble MAG chimeras and analyzed the lysates by Western blotting with antibody to the cytoplasmic domain of p75. It has been reported before that p75 appears as a prominent 80 kDa band along with other smaller bands that represent heterogeneously glycosylated forms of the protein (Grob et al., 1985). To clarify the relationship between proteolytic processing of p75 and MAG-induced signaling, we first added inhibitors of different proteolytic enzymes or a PKC inhibitor, followed by either of two chimeric forms of MAG. MAG-Fc comprises the entire five Ig-like extracellular domains fused to the Fc portion of human IgG and has been shown to inhibit axonal growth (Tang et al., 1997). MAG (1-3)-Fc, which lacks the fourth and fifth Ig-like domains, binds to neurons in a specific, sialic acid-dependent manner, but it is not inhibitory (Tang et al., 1997). For p75 and other proteins that undergo RIP processing, it has been reported that the second cleavage leaves a cytoplasmic fragment that is degraded too rapidly for detection unless a proteasome inhibitor is included (Kanning et al., 2003; Oberg et al., 2001). Accordingly, the proteasome inhibitor epoxomicin was first added to all cells. MAG-Fc alone results in the appearance of a 25 kDa proteolytic fragment (Figure 1B, lane 6) as well as a faint band at ~30 kDa. In the absence of epoxomicin, no cleavage bands are visible (data not shown). The 30 kDa fragment corresponds to the size of the transmembrane and cytoplasmic domains after extracellular cleavage with α-secretase (Figure 1A). In the presence of compound X, a specific inhibitor of γ-secretase, the 30 kDa fragment accumulates without subsequent cleavage to 25 kDa, demonstrating the role of γ-secretase in this second cleavage reaction (Figure 1B, lane 8). Identical results are seen in the presence of Gö6976, an inhibitor of PKC (lane 9), suggesting that γ-secretase is PKC dependent. The general inhibitor of the ADAM metalloprotease family, TAPI-1, abolishes extracellular cleavage and subsequent γ-secretase cleavage (lane 7). Finally, in the presence of a control chimera, MAG(1-3)-Fc (lanes 2–5), or without addition of any ligand (data not shown), there is no cleavage of p75. These results suggest that MAG binding to primary neurons induces RIP processing of p75, in a PKC-dependent manner, to produce a final ICD p75 fragment of about 25 kDa.

Inhibition of Neurite Outgrowth by MAG Is Blocked by Secretase Inhibition

To determine if MAG-induced RIP processing of p75 is required for the inhibitory effects of MAG, we blocked γ-secretase activity by adding a specific inhibitor, compound X, to the neurite outgrowth assay. As we have reported before (Mukhopadhyay et al., 1994), when cerebellar neurons are cultured on a monolayer of MAG-expressing CHO cells, they extend processes that are about 70% shorter than when grown on control CHO cells not expressing MAG (Figures 2A and 2B). In sharp contrast, however, when the γ-secretase inhibitor is included in the cocultures, MAG has no effect on neurite outgrowth; processes are the same length on MAG-expressing cells as on control cells. Similar results were found when an α-secretase inhibitor, TAPI-1, was included in the cocultures. The PKC inhibitor Go6976 also completely blocked inhibition of neurite outgrowth by MAG (Sivasankaran et al., 2004).

As well as using MAG expressed by live cells in the neurite outgrowth assay, we also assessed if the inhibitory effects of soluble MAG-Fc could be blocked by secretase inhibitors. Figures 2C and 2D show that indeed a γ-secretase inhibitor completely blocks inhibition by MAG-Fc. These results, together with those from neurons growing on MAG-expressing CHO cells, strongly suggest that MAG binding to cerebellar neurons leads to an obligatory two-stage cleavage of p75, first by an α-secretase and then via a PKC-dependent γ-secretase, to generate MAG's inhibitory effects on axonal growth.

Inhibition by MAG Is Blocked in Neurons Expressing an Uncleavable Form of p75

To complement the pharmacological results described above, uncleavable forms of p75 were used (Zampieri et al., 2005). Chimeric proteins were created by replacing either the transmembrane (TM) or the extracellular stalk domain of p75 with equivalent domains from the Fas receptor. Both p75 and Fas are members of the tumor necrosis factor (TNF) receptor superfamily, and although they each contain similar domains, unlike p75,

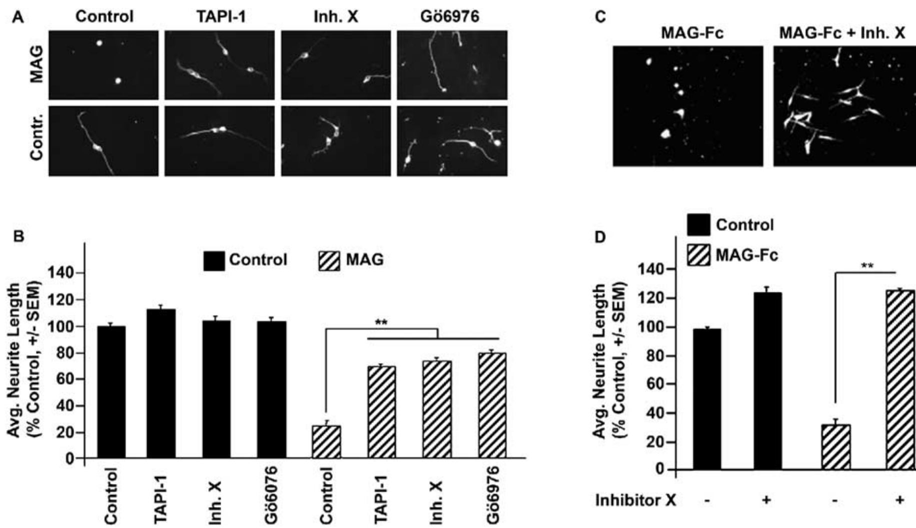

Figure 2. Inhibition of Neurite Outgrowth by MAG Is Blocked by Secretase Inhibition

(A) Neurite outgrowth of rat cerebellar neurons cultured onto confluent monolayers of control or MAG-expressing CHO cells in the presence of epoxomicin (Epo, 1 μM), TAPI-1 (1 μM), inhibitor X (1 μM), or Gö6976 (100 nM).

(B) Average neurite lengths on MAG-expressing cells were significantly longer in the presence of enzyme inhibitors (**$p < 0.001$).

(C) Neurite outgrowth of rat cerebellar neurons cultured onto an L1-Fc substrate in the presence of MAG-Fc (20 μg/ml) and inhibitor X (1 μM).

(D) Average neurite lengths from neurons cultured in the presence of inhibitor X and MAG-FC were significantly longer than those cultured with MAG-Fc alone (**$p < 0.001$).

Fas does not undergo RIP. Figure 3A is a diagram representing the chimeric proteins. The protein in which the TM domain is replaced is termed p75-FasTM, and the protein where the stalk domain is replaced is termed p75-FasS. Following transfection into NG108, both of these chimeric proteins are expressed at significantly higher levels (5- to 6-fold) than endogenous p75 (see Figure S1A in the Supplemental Data online). In addition, from surface biotinylation and precipitation studies we show that each reaches the cell surface and is able to interact with the NgR receptor (Figures S1B and S1D). When the wild-type p75 is expressed in 293 cells, both the ∼30 kDa CTF and the ∼25 kDa ICD are clearly visible after PMA treatment (Figure 3B). For the p75-FasTM chimera, only the ∼30 kDa fragment appeared after PMA treatment, while the p75-FasS chimera, under similar conditions, did not display any cleaved products. This lack of cleavage of the p75-FasTM chimera is consistent with the localization of the γ-secretase site in the transmembrane domain (Zampieri et al., 2005). In contrast, for the p75-FasS chimera, only the α-secretase site is lost from p75, and although the p75 γ-secretase site is present in the chimera, cleavage at the γ site must be preceded by cleavage at the α-secretase site. Hence, no γ cleavage occurred in the p75 FasS chimera.

To assess the role of p75 cleavage directly on inhibition of neurite outgrowth by MAG, these chimeric receptors were, first, each expressed in the neuronal cell line NG108, which had previously been shown to express wild-type p75 and to be inhibited by MAG and myelin (Domeniconi et al., 2002). When these NG108 cells expressing either of the chimeric forms of p75 were grown on MAG-expressing CHO cells, they were not inhibited from extending processes; growth was the same as on the control cells not expressing MAG. As a control, overexpression of wild-type p75 had no effect on inhibition by MAG (Figures 3C and 3D). Furthermore, when a cDNA coding for only the ICD of p75 is transfected into NG108 cells, neurite outgrowth on the control cells, not expressing MAG, is inhibited to the same extent as on MAG-expressing cells. This indicates that the ICD of p75 is, alone, sufficient to inhibit neurite outgrowth in the absence of MAG. Next, the chimeric receptors were each expressed in primary, dorsal root ganglion (DRG) neurons. As with the NG108 cells, the DRG neurons expressing either of the chimeric receptors are not inhibited by MAG; growth on MAG-expressing cells is the same as on the control cells not expressing MAG (Figure 3E). Also like the NG108 cells, overexpression of wild-type p75 has no effect on inhibition by MAG (Figure 3E). Therefore, expression of the cleavage-resistant p75 constructs in neurons blocked inhibition by MAG, confirming the results obtained by pharmacological inhibitors, namely that MAG-induced cleavage of p75 is crucial to effect inhibition.

Activation of Rho by MAG Is Blocked by Secretase Inhibition

Numerous reports have described the p75-mediated activation of Rho in response to each of the known myelin inhibitors (Mi et al., 2004; Wang et al., 2002a; Yamashita et al., 2002). To determine if cleavage of p75 is required for activation of Rho by MAG, we precipitated activated Rho with the GST-Rho binding domain (RBD) of rhotekin, a protein that binds only to GTP-Rho. Figure 4 shows a Western blot of the precipitates, immunostained for Rho. As has been reported before (Ya-

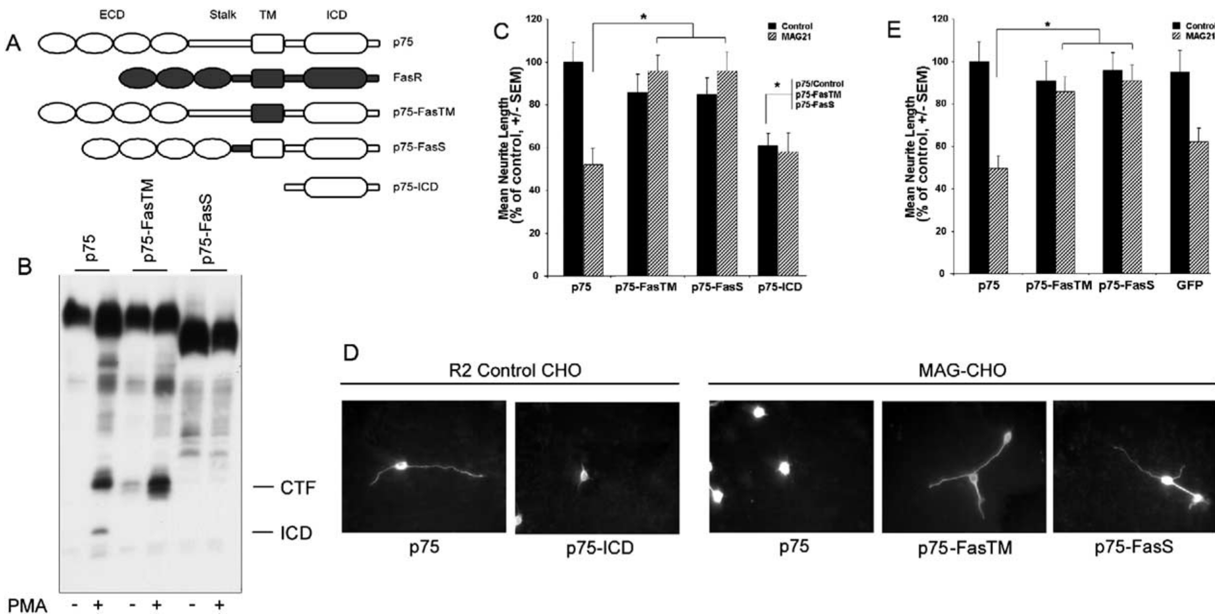

Figure 3. Inhibition of Neurite Outgrowth by MAG Is Abrogated in Neuronal Cells Expressing an Uncleavable Form of p75

(A) Schematic diagram of the p75 chimeric constructs.

(B) HEK293 cells were transfected with a chimeric construct where the transmembrane domain (p75-FasTM) or the stalk domain (p75-FasS) of p75 was replaced with the equivalent domains from FasR. Wild-type p75 was included as a control. After transfection, the cells were incubated 45 min with PMA (100 ng/ml). Lysates were analyzed by Western blot using 9992 antisera against the intracellular domain of p75.

(C) NG108 cells expressing the p75 chimeric receptors were cultured on confluent monolayers of control or MAG-expressing cells. Average neurite lengths from transfected cells were significantly longer as compared to untransfected cells (* $p < 0.05$). Expression of p75-ICD inhibited neurite outgrowth from NG108-15 cells cultured on control cells monolayers (* $p < 0.05$).

(D) Neurite outgrowth from NG108 cells cultured on confluent monolayers of control or MAG-expressing CHO cells after transfection with wild-type p75, chimeric p75 receptors, or p75-ICD.

(E) P5 DRG neurons transfected with either wild-type p75, the p75 chimeric receptors, or GFP alone grown on confluent monolayers of control or MAG-expressing CHO cells. The mean neurite lengths from neurons transfected with the chimeric receptors were significantly longer as compared to neurons transfected with wild-type p75 or GFP alone (* $p < 0.05$).

mashita and Tohyama, 2003), binding of MAG-Fc to cerebellar neurons induces the activation of Rho (Figure 4, lane 5). As expected, Rho activity in response to MAG(1-3)-Fc is comparable to that seen in the unstimulated control (Figure 4, lanes 1 and 2). The MAG-Fc-induced activation of Rho is returned to baseline levels in the presence of a γ-secretase inhibitor, an α-secretase inhibitor, or either of two inhibitors of PKC (Figure 4, lanes 6–9). The results indicate that MAG activation of Rho requires RIP processing of the p75 neurotrophin receptor.

Discussion

Based on the results reported here, we propose a mechanism of how myelin inhibitors act: MAG induces cleavage of p75, a component of the NgR complex, by activating a membrane-associated α-secretase complex. Cleavage by α-secretase results in shedding of the extracellular domain of p75 and is obligatory for a second proteolytic cleavage by a γ-secretase complex, characteristic of RIP and requiring activation of PKC. Cleavage by γ-secretase releases the ICD of p75, which is necessary for Rho activation and, in turn, initiates the downstream steps that inhibit axonal growth. Strong support for this model derives from both pharmacological and genetic data presented; inhibitors of α-secretase, γ-secretase, or PKC each block activation of Rho and inhibition by MAG. Likewise, expression of cleavage-resistant forms of p75 also block inhibition by

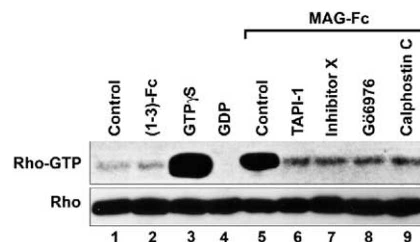

Figure 4. Activation of Rho by MAG Is Blocked by Secretase Inhibition

Rho activation detected by Rothekin-RBD pull-down assays in rat cerebellar neurons treated with MAG-Fc (20 μg/ml) in the presence of inhibitors of proteolytic enzymes, TAPI-1 (1 μM), inhibitor X (1 μM), and of PKC, Gö6976 (100 nM), calphostin C (100 nM). Baseline activation was determined in untreated or MAG(1-3)-Fc-treated neurons. Positive and negative controls were obtained by loading with GTP or GDP, respectively. Blots were probed with an antibody against Rho (-A, -B, -C).

MAG, while expression of the p75 ICD alone is sufficient to effect inhibition in the absence of MAG. Furthermore, since MAG, Nogo-66, and OMgp all serve as ligands for the same receptor complex (Domeniconi et al., 2002; Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b) and all activate Rho (Lehmann et al., 1999; Wang et al., 2002b; Yamashita et al., 2002), we also propose that this mechanism of action is very likely to be the same for all three inhibitors.

These results are important for at least two reasons. First, the identification of secretase activity in the signaling by myelin inhibitors points to an attractive target for therapeutic intervention in the treatment of CNS injury. Blocking secretase activity has the potential to halt the inhibition of axonal regeneration launched by the binding of myelin-derived ligands to the p75 receptor complex. Second, our results provide a physiological basis for cleavage of p75 by α- and γ-secretases.

Myelin inhibitors are well known to activate Rho via p75 to bring about inhibition of axonal growth (Dergham et al., 2002; Lehmann et al., 1999; Yamashita et al., 1999). In unraveling the transducing steps involving p75, it has been suggested that in the absence of ligand, p75 exists in a complex with Rho and Rho-GDI, the Rho GDP dissociation inhibitor that stabilizes the inactive Rho state (Yamashita and Tohyama, 2003). In that model, ligand binding to p75 is proposed to displace Rho-GDI and thereby permit Rho activation, in spite of the fact that MAG or Nogo binding appears to strengthen the stability of the p75:Rho:Rho-GDI complex. The implication is that Rho would remain inactive after ligand binding. However, it was also reported in the same study that a peptide fragment from the cytoplasmic domain of p75 was, on its own, capable of diminishing the inhibitory activity of Rho-GDI to permit Rho activation to its GTP bound state (Yamashita and Tohyama, 2003). The results we report here clarify the apparent discrepancy reported in this previous study. We suggest that the immediate effect of ligand binding is to stabilize the ligand:p75:Rho:Rho-GDI complex, maintaining Rho in an inactive state. Upon cleavage, however, the ICD fragment of p75 displaces Rho-GDI from Rho, and activation is induced. Thus, the previous observation that a soluble fragment of p75, in the absence of ligand, can both reduce the activity of Rho-GDI and activate Rho and the results presented here showing a similar p75 ICD fragment can inhibit neurite outgrowth are consistent with our new model.

The p75 receptor is a member of the tumor necrosis factor receptor (TNF-R) family of molecules. Recently, another member of this family of molecules, TROY (also known as TAJ), was shown to be capable of substituting for p75 to effect inhibition (Park et al., 2005; Shao et al., 2005). TROY interacts with NgR and LINGO-1: expression of TROY, NgR, and LINGO-1 in COS cells results in activation of Rho in response to myelin-associated inhibitors, and blocking TROY blocks inhibition. However, although neurons from TROY$^{-/-}$ mice extend longer processes on low concentrations of myelin inhibitors, at higher concentrations they are inhibited (Shao et al., 2005). This suggests that other molecules such as p75 are present to bring about inhibition. The identification of TROY as a functional homolog for p75 explains the apparent paradox that neurons that do not express p75 are still inhibited by myelin. We now know these neurons express TROY (Park et al., 2005; Shao et al., 2005). Because we show that inhibition of neurite outgrowth by MAG of cerebellar neurons, which express both p75 and TROY, requires secretase activity, this suggests that TROY is also likely to undergo RIP. This remains to be determined.

The number of cell surface proteins reported to undergo RIP continues to grow (Brown et al., 2000; Urban and Freeman, 2002). For the majority of these receptors, the released ICD is immediately degraded in the cytoplasm without any associated signaling. For a few other receptors, such as Notch, the ICD contains a transcriptional activator motif, and it translocates to the nucleus to activate transcription of target genes (Fryer et al., 2002; Mumm and Kopan, 2000; Nam et al., 2003). Nuclear localization of the Notch ICD is transient, and the time from release to degradation is rapid. Indeed, as we report here for the cleaved p75 ICD, the Notch ICD can be detected only after proteasome inhibition (Oberg et al., 2001). We do not know if the p75 ICD translocates after Rho activation to the nucleus to effect further signaling. The rapid rate of p75 ICD degradation does not implicate the ICD fragment in any specific metabolic fate.

In addition to its function in signaling inhibition by key myelin ligands, p75 associates with at least 15 different effectors and has been shown to be involved in both neurotrophic survival pathways and apoptotic pathways (Gentry et al., 2004; Hempstead, 2002). It also interacts with the other family of neurotrophin receptors, the Trks. Interaction between receptors increases the affinity of neurotrophin binding to result in an enhanced survival signal (Casaccia-Bonnefil et al., 1999; Chao and Hempstead, 1995). The final cleavage product of p75, the ICD, has been shown to be unable to interact with Trks, leading to the suggestion that a disruption in the Trk:p75 association diminishes the survival pathway (Jung et al., 2003). Furthermore, others have shown that apoptosis increases in transgenic mice that express a cytoplasmic domain of p75 (Majdan et al., 1997). It is not clear that this fragment is identical to the p75 fragment released by γ-secretase activity; nor is it clear that the two fragments behave identically. It is important to note, however, that we did not observe any increase in neuronal death in response to MAG or myelin, nor when we expressed the p75 ICD in NG108 cells (data not shown). What we have been able to show is that neurotrophins can overcome inhibition by MAG and myelin in general, but only if the neuron is exposed to the neurotrophin prior to being exposed to inhibitor (Cai et al., 1999). When neurons are exposed to inhibitor and neurotrophins at the same time, the neurotrophins have no effect. One possible explanation for this observation is that the inhibitory ligand disrupts the Trk:p75 association and thereby diminishes the signal required to overcome inhibition.

In summary, our current results add another layer of regulation to the mechanism of how myelin inhibitors activate Rho via the p75 receptor to cause inhibition of axonal growth. We can now add RIP processing, with the proteolytic release of the ICD of p75 by γ-secretase

activity as upstream control points for Rho activation and inhibition of regeneration.

Experimental Procedures

p75 Cleavage Assay

P6–P8 rat cerebellar neurons were cultured overnight in SATO medium. Cells were pretreated for 60 min with epoxomicin (1 μM), TAPI-1 (1 μM), inhibitor X (1 μM), Gö6976 (100 nM), calphostin (100 nM), or DMSO vehicle control and subsequently stimulated for 30 min with MAG-Fc (20 μg/ml), MAG(1-3)-Fc (20 μg/ml), or vehicle control. Cells were then washed one time with cold PBS on ice, lysed in RIPA buffer and protease inhibitors, and centrifuged 5 min at 14,000 × g; supernatants were quantified by BCA assay (Pierce) for use in SDS-PAGE.

Rho Assay

P6–P8 rat cerebellar neurons were cultured overnight in SATO medium. Cells were stimulated 30 min with individual inhibitors or appropriate controls in the presence or absence of TAPI-1 (1 μM), inhibitor X (1 μM), Gö6976 (100 nM), and calphostin (100 nM). GTP-Rho was precipitated by using beads with GST-Rho binding domain (RBD) of rhotekin following the manufacturer's instructions (Upstate Biotechnology).

Western Blotting

Cell lysate was separated by SDS-PAGE using 12% Precision gels (Pierce). For detection, proteins were transferred electrophoretically to nitrocellulose membrane. Membranes were then blocked in 5% nonfat dry milk in PBS. Incubation in a 1:2000 dilution of 9992 antiserum specific for the intracellular domain p75 or 1:1000 of anti-Rho antibody (Santa Cruz) in blocking buffer was performed overnight at 4°C. Membranes were washed three times in PBS with 0.05% Tween-20. Secondary goat anti-rabbit HRP-conjugated antibody (Sigma) was diluted 1:10,000 in blocking buffer and incubated with the membranes for 1 hr at room temperature. Membranes were then washed as before and developed using ECL (Amersham Biosciences).

Neurite Outgrowth Assay

Neurite outgrowth assays were performed as described previously (Mukhopadhyay et al., 1994). Briefly, dissociated P5–P8 rat cerebellar neurons or NG108 cells were plated at a density of 3×10^4 cells/ml onto confluent monolayers of control and MAG-expressing CHO cells. After 16–18 hr of incubation in the presence or absence of TAPI-1 (1 μM), inhibitor X (1 μM), or Gö6976 (100 nM), cultures were fixed for 30 min with 4% paraformaldehyde, permeabilized with ice-cold methanol, and immunostained with an antibody against a neuron-specific β-tubulin (Tuj-1, Covance). Neurite lengths were measured from at least 150 neurons per condition, from duplicate wells and from three independent experiments, and were quantified as described previously (Mukhopadhyay et al., 1994).

Transfection and Differentiation of NG-108 Cells and Transfection of Primary Neurons

The construction of the p75-Fas chimeric receptors and the p75-ICD receptor was described previously. The constructs were transfected into NG108 cells using lipofetamine (Life Technologies). Upon transfection, NG108 cells were differentiated with dibutyryl-cAMP (1 mM) for 24 hr. Cells were washed and cultured in unsupplemented SATO medium for an additional 24 hr prior to plating for neurite outgrowth assays as described.

DRG neurons were isolated and dissociated from P5 rat pups as described above. The single-cell suspensions were then transfected with the wild-type p75, p75-FasTM, or p75-FasS receptor chimeras via electroporation using the Amaxa Nucleofector Device (Cologne, Germany). The neurons were then plated onto 100 μg/ml poly-L-lysine in modified Sato media overnight at 37°C to allow for expression of the exogenous protein. Neurons were then removed and plated onto monolayers of MAG-expressing or control CHO cells, and the neurite outgrowth assay was performed as indicated above.

Reagents

MAG-Fc and MAG(1-3)-Fc chimeras were prepared in our lab as previously described (Tang et al., 1997). Unless otherwise stated, all reagents were purchased from Calbiochem, San Diego, CA.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/46/6/849/DC1/.

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