Microfibrillar Proteins MAGP-1 and MAGP-2 Induce Notch1 Extracellular Domain Dissociation and Receptor Activation*

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Unlike most receptors, Notch serves as both the receiver and direct transducer of signaling events. Activation can be mediated by one of five membrane-bound ligands of either the Delta-like (-1, -2, -4) or Jagged/Serrate (-1, -2) families. Alternatively, dissociation of the Notch heterodimer with consequent activation can also be mediated experimentally by calcium chelators or by mutations that destabilize the Notch1 heterodimer, such as in the human disease T cell acute lymphoblastic leukemia. Here we show that MAGP-2, a protein present on microfibrils, can also interact with the EGF-like repeats of Notch1. Co-expression of MAGP-2 with Notch1 leads to both cell surface release of the Notch1 extracellular domain and subsequent activation of Notch signaling. Moreover, we demonstrate that the C-terminal domain of MAGP-2 is required for binding and activation of Notch1. Based on the high level of homology, we predicted and further showed that MAGP-1 can also bind to Notch1, cause the release of the extracellular domain, and activate signaling. Notch1 extracellular domain release induced by MAGP-2 is dependent on formation of the Notch1 heterodimer by a furin-like cleavage, but does not require the subsequent ADAM metalloprotease cleavage necessary for production of the Notch signaling fragment. Together these results demonstrate for the first time that the microfibrillar proteins MAGP-1 and MAGP-2 can function outside of their role in elastic fibers to activate a cellular signaling pathway.

Notch signaling is best known for its role in cell fate determination and is critical for regulating multiple cellular processes in many different tissues, including those of the nervous, hematopoietic, and vascular systems (1). Controlled by membrane-tethered ligands on apposing cells, ligand binding initiates canonical Notch signaling and leads to the proteolytic release of the Notch intracellular domain (NICD). The NICD fragment travels to the nucleus, interacts with a DNA-binding protein CSL (CBF1/Su(H)/LAG-1), and activates expression of target genes such as HES1.

At least three proteolytic events are required for Notch activation through CSL. The first cleavage is ligand-independent and occurs during maturation of the co-linear Notch protein. Either during trafficking or at the cell surface, Notch is cleaved by a furin-like convertase into two fragments, the extracellular domain (NICD) and the transmembrane-anchored intracellular domain (N1-NTM), that remain associated through non-covalent interactions (2–4). This “heterodimer” is the predominant form of Notch on the plasma membrane and is required for ligand-induced CSL-dependent Notch signaling (4). Interestingly, one of the mutation “hot spots” marks the area around the furin-processing site designated the heterodimerization domain (8). At least some of the heterodimerization domain mutations potentiate the dissociation of an engineered soluble form of the heterodimer, mimicking the biological effects of ligand-induced Notch signaling, and constructs encoding just the N1-NTM sequences are constitutively active (8, 9). Together, these findings imply that heterodimer dissociation leads to ligand-independent, constitutive cleavage of N1-NTM to produce active NICD. Further evidence that preservation of the heterodimer is important for maintaining Notch in an inactive state is the finding that treatment with calcium chelators, such as EDTA, disrupts the non-covalent interactions that hold the heterodimer together, leading to both receptor dissociation and activation of downstream signaling events (3).

Notch receptors and ligands of the DSL (Delta/Serrate/LAG-2) class have similarly structured extracellular domains; the bulk of which is comprised of tandem EGF-like repeats. We have recently shown that MAGP-2 interacts with the EGF-like repeats of DSL ligands Jagged1, Jagged2, and Delta1, and specifically potentiates Jagged1 shedding by ADAM sheddases (10). Therefore, given the presence of similar tandem EGF-like repeats in the N1 receptor we asked whether MAGP-2 could interact with N1 and whether this interaction had any functional consequences on N1 activity.

MAGPs are best characterized as components of microfibrils, which are important structural components of elastic tissues such as the lung, skin, and vasculature but are also present in non-elastic tissues such as the ciliary zonule of the eye (11). Biochemical dissection of microfibrils has identified the major component to be fibrillin, a large modular protein that contains 47 EGF-like repeats among other motifs (12). Under reducing conditions a number of small molecular weight proteins are also released from microfibrils, including MAGP-1 and MAGP-2 (13, 14).

The function of the small microfibrill-associated proteins has been inferred from protein-protein interactions with both extracellular matrix and cell-associated proteins. MAGP-1 and MAGP-2 share a C-terminal domain with conserved cysteine spacing that defines their gene family (15). This domain has been shown to interact with different regions of the fibrillin molecule (16, 17). MAGP-1 has also been shown...
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to interact with a number of elastic fiber components beyond fibrillin, including tropoelastin and decorin, and therefore is thought to be an integral component of the elastic fiber (18, 19). MAGP-2, on the other hand, contains a RGD sequence that can mediate interactions with integrins and has a more restricted expression pattern than MAGP-1, leading to the notion that MAGP-2 may be involved in cell signaling events (20, 21). Experimental evidence that induction of MAGP-2 expression increased collagen deposition in fibroblast culture is supportive of this notion, although in this system, no direct effect of MAGP-2 on signaling was identified (22). We now show that MAGP-2 can directly participate in a cell signaling pathway via an interaction with the Notch receptor that induces heterodimer dissociation and activation of signaling.

EXPERIMENTAL PROCEDURES

DNA Constructs—The N-terminal MAGP-2 construct was generated via PCR to delete amino acids 84–162 in the pCAGGS vector. The C-terminal MAGP-2 construct was made via standard molecular biology techniques to replace the sequences between HincII and PstI with six tandem myc epitopes. The DI3 ECDHA construct was generated via PCR to fuse the N-terminal 488 amino acids of DI3 to three tandem HA epitope tags. The N1c/s construct was generated via a PCR overlap strategy to change the two conserved cytostines at positions 1675 and 1682 in the rat N1 sequence to serines. All constructs were sequenced to verify that no PCR-induced mutations were generated.

The N1Δmyc series of constructs (23), pBosHA-N1 and pBosDI3HA (24), pBosOEDN1 (25), pBosΔEDN1 and pBosZEDN1 (26), AT-EK1 (4), mutant, and wild-type CSL reporter (JH26 and JH28, respectively) (27) constructs have been previously published. A number of constructs (24), mutant, and wild-type CSL reporter (JH26 and JH28, respectively) (27) constructs have been previously published. A number of constructs were sequenced to verify that no PCR-induced mutations were generated.

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Reporter Assays—COS7 cells were transfected via Lipofectamine (Invitrogen) using a total of 800 ng of DNA and 2 μl of Lipofectamine/ well of a 6-well dish and incubation on cells in serum-free medium for 5 h. Usually 100 ng of Notch receptor, 100 ng of CSL-reporter, and 5 ng of CMV-Not III puro luciferase (Promega) were transfected with 100 or 200 ng of MAGP-2. After serum was added back to the cultures, cells were collected at 48 h post-transfection for luciferase assays that were performed using the dual-luciferase kit (Promega) following the manufacturer’s instructions on a Turner Designs Luminometer (TD-20/20). Co-culture reporter assays using DeltaL- or Jagged1-expressing L fibroblasts to activate T3T fibroblasts transiently expressing N1 and increasing amounts of MAGP-2 were performed as described in Ref. 24.

Biotinylation of Cell Surface Proteins—Cell surface labeling and isolation of biotinylated proteins were performed as described in Ref. 24 with the following modifications. 400 ng of N1 plasmid DNA was transfected into COS7 cells with 0, 400, or 800 ng of MAGP-2 plasmid DNA, using pCAGGS as filler plasmid for MAGP-2. Cells were harvested in lysis buffer as described below for immunoprecipitations. Lysates were incubated overnight at 4 °C with streptavidin-agarose (Pierce) on a rotor and then collected and washed three times with the wash buffer described below before SDS-PAGE analysis.

Immunoprecipitations of the Soluble N1 Extracellular Domain—293T cells were transfected via standard calcium phosphate precipitation with 1 μg of HAN1 and 2 μg of MAGP2 in a total of 5 μg of DNA/ transfection into a 60-mm dish. Approximately 24 h post-transfection, Dulbecco’s modified Eagle’s medium was placed on cells and collected 2 days later. Where BB94 (British Biotech) or DAPT (Calbiochem) was used, each was added with the Dulbecco’s modified Eagle’s medium and reapplied after 1 day in culture. Conditioned medium was collected, centrifuged at 1500 × g, then the supernatant was removed and spun again at 10,000 × g. Cleared supernatants were then immunoprecipitated with a 1/100 dilution of 12CA5 myeloma supernatant, and immune complexes were collected either on Protein G-Sepharose (Amersham Biosciences) or on Protein A-agarose (Invitrogen) after an additional incubation with rabbit anti-mouse antiserum. Beads were washed twice in phosphate-buffered saline and once in wash buffer (10 mM Tris, pH 7.4, 0.5 M NaCl, 0.5% IGEPA, 1% deoxycholate, 1 mM EDTA) before elution and SDS-PAGE. Cell lysates were also collected at the same time as conditioned medium in lysis buffer (10 mM Tris, pH 8.5, 14 mM NaCl, 1 mM MgCl2, 0.5% deoxycholate, 1% IGEPA, 0.1% SDS) supplemented with protease inhibitors aprotonin, leupeptin, and phenylmethylsulfonyl fluoride.

Co-immunoprecipitations of N1 with MAGP-2 or MAGP-1—293T or COS7 cells were transfected either via calcium phosphate precipitation for 293T or via Lipofectamine for COS7. For co-immunoprecipitation studies, N1 constructs and MAGP-2 constructs were used at a 1:1 ratio, and cell lysates were collected 48 h post-transfection. Cell extracts were generated in lysis buffer as above and used for immunoprecipitation with either anti-N1 antiserum (PCR12), anti-MAGP2 antiserum, or anti-myc antibodies (9E10, from Santa Cruz Biochemicals). Beads were washed three times in wash buffer before immune complexes were eluted from the agarose beads. For NIDC immunoprecipitations, at 2 days post-transfection cells were treated for 5 h with 10 μM MG132 (BIOMOL) to block proteosomal degradation prior to collection in lysis buffer.

Western Blotting—Val1744 antiserum (Cell Signaling Technologies) and 9E10 (Santa Cruz Biochemicals) were used as per manufacturer’s instructions. Antisera to intracellular and extracellular N1 (93-4 and 93-2, respectively) and MAGP-2 have been described previously (4, 10).

Co-immunoprecipitation of Metabolically Labeled A7R5 Cells—The rat aortic smooth muscle cell line A7R5 were grown in 100-mm dishes until they reached confluence. Each dish was washed three times with phosphate-buffered saline and then incubated for 1 h with Dulbecco’s modified Eagle’s medium containing 50 μCi/ml 35S translabel (MP Biomedicals) was added to the cells and incubated overnight. The next day cell lysates were generated in a lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM CaCl2. Lysates were precleared with normal rabbit serum and then used for immunoprecipitation with anti-N1 extracellular domain antiserum (αN1-e, 93-2), preimmune serum for 93-2, anti-MAGP-2 antiserum, or anti-Fibrillin1 antiserum (exons 36–44) (28). After collection on Protein A-agarose beads, three washes were performed with the lysis buffer before the immune complexes were eluted from the agarose beads and run on SDS-PAGE. Gels were fixed in a solution of 20% methanol and 10% acetic acid, dried, and exposed to PhosphorImager screens (Amersham Biosciences). Scanning was done on the Typhoon 9410 and visualized using ImageQuant software.

RESULTS

Notch1 and MAGP-2 Interact via the Notch EGF-like Repeats—Having previously characterized Jagged1 interactions with MAGP-2 via co-immunoprecipitation (10), we first asked whether N1 and MAGP-2 would also interact in co-immunoprecipitation experiments. Given the likelihood that the EGF-like repeats of N1 would mediate this interaction, we made use of a panel of deletion constructs that encoded various amount of the extracellular domain of N1 (Fig. 1A). Either full-length Notch1 (N1) or constructs encoding 4 EGF-like repeats (ΔEDN1), 1.5 EGF-like repeats (OEDN1), or no EGF-like repeats (ZEDN1) were
expressed with MAGP-2 in COS7 cells, and immunoprecipitation with N1 antiserum was performed on cell lysates. MAGP-2 was found in immunoprecipitations from lysates containing N1 or EDN1 (Fig. 1B, lanes 2 and 3) but not OEDN1 or ZEDN1 (Fig. 1B, lanes 4 and 5). This interaction was specific, because MAGP-2 was not recognized on its own by the N1 antiserum (Fig. 1B, lane 1) and required that MAGP-2 and N1 be in the same cell, because MAGP-2 did not interact with N1 in mixed lysate controls (Fig. 1D, lower panels). Together this suggests that the EGF-like repeats of N1 mediate an interaction with MAGP-2 and that more than one N1 EGF-like repeat is required for the interaction.

Because the LIN-12/Notch repeats are also deleted in the two constructs that do not interact with MAGP-2, it remained possible that it was this domain that mediated the interaction. Therefore, we also co-expressed MAGP-2 with an Fc-tagged construct encoding only N1 EGF-like repeats 1–12 and found that the two proteins formed a complex in the conditioned medium (Fig. 1C). Together, these data indicate that the EGF-like repeats of N1 are sufficient to mediate an interaction between MAGP-2 and N1.

Endogenous MAGP-2 and Notch1 Interact—To confirm the possibility of a biological role of MAGP-2 in Notch signaling, we asked whether the endogenous proteins also interact. N1 and MAGP-2 are both expressed with MAGP-2 in COS7 cells, and immunoprecipitation with N1 antiserum was performed on cell lysates. MAGP-2 was found in immunoprecipitations from lysates containing N1 or AEDN1 (Fig. 1B, lanes 2 and 3) but not OEDN1 or ZEDN1 (Fig. 1B, lanes 4 and 5). This interaction was specific, because MAGP-2 was not recognized on its own by the N1 antiserum (Fig. 1B, lane 1) and required that MAGP-2 and N1 be in the same cell, because MAGP-2 did not interact with N1 in mixed lysate controls (Fig. 1D, lower panels). Together this suggests that the EGF-like repeats of N1 mediate an interaction with MAGP-2 and that more than one N1 EGF-like repeat is required for the interaction.

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**FIGURE 3.** MAGP-2 induces the shedding of the Notch1 extracellular domain. Using a form of N1 (HA-N1) tagged on the ECD with three HA epitopes, soluble ECD (HA-N1) is visualized by immunoprecipitation (IP) and Western blot (WB) analysis with αHA antibodies. A, 293T cells were transfected with HA-N1 and either vector (+) or MAGP-2 (+). After 2 days, conditioned medium was immunoprecipitated with αHA antibodies and then immunoblotted for either HA-N1 or MAGP-2. Whole cell lysates (WCL) show input levels of HA-N1 protein. One of three replicate experiments is shown. B, 293T cells transfected with MAGP-2 and HA-N1 were co-transfected with a competitor (AT-EK1) for furin-like convertases, and conditioned medium (CM) was analyzed for HA-N1 ECD release. The lower panels show the input levels of N1 and MAGP-2. One of three replicate experiments is shown.

**ADAM Cleavage**

We next asked what consequence MAGP-2 binding had on the N1 receptor, specifically if MAGP-2 induced cleavage of the N1 extracellular domain (ECD), much like what we reported for furin-like cleavage of the N1 ECD (HA-N1). We previously shown to prevent N1 furin-like cleavage and heterodimerization (4), along with HA-N1 and MAGP-2 and conditioned medium was assayed for HA-N1 ECD via 12CA5 immunoprecipitation. In the presence of AT-EK1, the MAGP-2-induced generation of soluble HA-N1 ECD was greatly reduced (Fig. 3A, top panel), suggesting that the furin-like cleavage of N1 into a heterodimeric receptor is necessary for MAGP-2-induced release of N1 ECD from cells.

**Notch1 ECD Release Facilitated by MAGP-2 Does Not Require ADAM Cleavage**—We initially predicted that the MAGP-2-induced shedding of HA-N1 ECD would be mediated by ADAM cleavage. This seemed likely because ADAM cleavage is a necessary step in Notch receptor activation and because MAGP-2 can induce an ADAM-like cleavage of the Notch ligand Jagged1. To test this theory we evaluated whether MAGP-2-induced production of soluble HA-N1 ECD was sensitive to ADAM inhibitors. For these studies we expressed HA-N1 and either vector or MAGP-2 in 293T cells and prepared conditioned medium in the presence of BB94, a hydroxamate-based metalloprotease inhibitor, or Me2SO control. Both in the presence and absence of BB94, HA-N1 ECD could be detected (Fig. 4A, compare lanes 2 and 4), indicating that HA-N1 ECD release by MAGP-2 is ADAM-independent.
Parallel proteolysis experiments described in the next section verified that BB94 and DAPT blocked ADAM and γ-secretase activity, respectively (Fig. 4B). Therefore, this suggests that in contrast to ADAM shedding of Jagged1, MAGP-2 induces a dissociative event of the N1 heterodimer independent of ADAM cleavage. However, it remains possible that MAGP-2 induces an alternative cleavage event that is not blocked by the pan-metalloprotease inhibitor BB94.

**MAGP-2 Leads to Increased Production of NICD and Notch-dependent Reporter Activity**—In other systems it has been shown that the presence of the extracelluar domain of N1 acts to negatively regulate receptor activation (29, 30). Furthermore, destabilizing mutations found in the heterodimerization domain in T-ALL patient samples or treatment of N1-expressing cells with calcium chelators such as EDTA can lead to N1 heterodimer dissociation and subsequent activation of signaling (3, 8). Because MAGP-2 induced dissociation of the HA-N1 heterodimer, we asked what happened to the N^TM cell-associated portion that remained after MAGP-2-induced loss of the ECD.

For these experiments, we employed a form of N1 (N1Δmyc) optimized to detect NICD in which the C terminus is replaced with six myc epitope tags (23). This truncated protein facilitates detection of N1Δmyc cleavage products that differ by only a small number of amino acids. Transfection of 293T cells with this construct and either vector or MAGP-2 was followed by immunoprecipitation with anti-myc antibodies (9E10) and Western blotting with the same antibody. As seen in Fig. 4B, MAGP-2 increased production of a NICD-like fragment from N1Δmyc (lanes 7 and 8, asterisk). Production of NICD by MAGP-2 was suppressed by both ADAM and γ-secretase inhibitors (BB94 and DAPT, respectively), indicating that the MAGP-2-induced NICD fragment requires the same cleavage events as ligand-induced Notch signaling (Fig. 4B, lanes 8, 10, and 12). Furthermore, the same precursor-product relationships described for NICD generated through Notch ligand activation (23) are also found for MAGP-2 treatment; BB94 blocks the ADAM event, preventing both S2 and S3 production (Fig. 4B, lanes 8 versus 10), whereas DAPT, which inhibits the γ-secretase cleavage of S2 to produce S3, leads to the accumulation of the S2 product (Fig. 4B, lane 12). Importantly, because we performed both experiments shown in Fig. 4 concurrently, the activity of BB94 and DAPT to block NICD production in Fig. 4B serves as a positive control for inhibitor function in the ECD experiment (Fig. 4A). This analysis indicates that increases in NICD detected for MAGP-2 are inhibited by BB94 and DAPT and allows us to conclude that ADAM cleavage is downstream of MAGP-2-induced dissociation of heterodimeric N1 and release of soluble HA-N1 ECD. To confirm that MAGP-2 could induce NICD from full-length N1, lysates from COS7 cells co-expressing MAGP-2 and full-length N1 were immunoprecipitated with anti-N1 antisera and then assayed by SDS-PAGE and Western blotting. Probing with an antisem (Val-1744) that recognizes the neo-epitope produced by the synthetic CSL reporter, the amount of biotinylated N1 detected by streptavidin-agarose did not change with increasing amounts of MAGP-2 (Fig. 5F, top two panels), suggesting that the increases in N1 activity are not because of increased levels of N1 at the cell surface.

Because MAGP-2 could activate N1 signaling we tested whether it could further potentiate signaling induced by ligand. Therefore, NIH3T3 fibroblasts co-expressing MAGP-2, N1, and the CSL reporter were co-cultured with ligand-expressing cells to activate signaling. Although co-expression of MAGP-2 with N1 increased CSL reporter activity in NIH3T3 cells in the absence of ligand (Fig. 5G, white bars), no further increase in activity was detected when both MAGP-2 and ligand cells were used to activate N1 (Fig. 5G, black bars). These data suggest that MAGP-2 may act at a point in the signaling pathway to potentiate ligand-induced CSL reporter activation.

**The C-terminal Half of MAGP-2 Is Both Necessary and Sufficient to Bind and Activate Notch1**—We next made N-terminal and C-terminal deletion mutants of MAGP-2 to map its N1-interaction domain. Because the MAGP-2 antiserum was generated to the N terminus of the full-length protein, we engineered deletion mutants of MAGP-2 to map its N1-interaction domain. deletion mutants of MAGP-2 to map its N1-interaction domain. In silico predictions and biochemical experiments indicated that it is the EGF-like repeats of N1 that interact with MAGP-2. We additionally tested whether the activity detected with the synthetic CSL reporter construct was also detected for an endogenous Notch target gene, using a Hes5 promoter reporter construct. As found for the synthetic CSL reporter, the Hes5 promoter construct was responsive to MAGP-2 (Fig. 5F).

To rule out the possibility that MAGP-2-induced potentiation of N1 signaling and ECD dissociation was because of increases in receptor levels at the cell surface, we conducted biotinylation experiments in COS7 cells transfected with N1 and increasing amounts of MAGP-2. Using the furin-cleaved ECD and TM-ICD fragments of N1 as indicators of cell surface receptor, the amount of biotinylated N1 detected by streptavidin-agarose did not change with increasing amounts of MAGP-2 (Fig. 5F, top two panels), suggesting that the increases in N1 activity are not because of increased levels of N1 at the cell surface. Because MAGP-2 could activate N1 signaling we tested whether further potentiation signaling induced by ligand. Therefore, NIH3T3 fibroblasts co-expressing MAGP-2, N1, and the CSL reporter were co-cultured with ligand-expressing cells to activate signaling. Although co-expression of MAGP-2 with N1 increased CSL reporter activity in NIH3T3 cells in the absence of ligand (Fig. 5G, white bars), no further increase in activity was detected when both MAGP-2 and ligand cells were used to activate N1 (Fig. 5G, black bars). These data suggest that MAGP-2 may act at a point in the signaling pathway to potentiate ligand-induced CSL reporter activation.

Because MAGP-2 could activate N1 signaling we tested whether it could further potentiate signaling induced by ligand. Therefore, NIH3T3 fibroblasts co-expressing MAGP-2, N1, and the CSL reporter were co-cultured with ligand-expressing cells to activate signaling. Although co-expression of MAGP-2 with N1 increased CSL reporter activity in NIH3T3 cells in the absence of ligand (Fig. 5G, white bars), no further increase in activity was detected when both MAGP-2 and ligand cells were used to activate N1 (Fig. 5G, black bars). These data suggest that MAGP-2 may act at a point in the signaling pathway to potentiate ligand-induced CSL reporter activation.

The C-terminal Half of MAGP-2 Is Both Necessary and Sufficient to Bind and Activate Notch1—We next made N-terminal and C-terminal deletion mutants of MAGP-2 to map its N1-interaction domain. Because the MAGP-2 antiserum was generated to the N terminus of MAGP-2, the construct encoding only the C-terminal half of MAGP-2 was engineered with six tandem myc epitopes replacing the N-terminal half did not co-immunoprecipitate with N1 (Fig. 6A). This analysis indicates that the C-terminal half of MAGP-2 is both necessary and sufficient for MAGP-2 effect can take place in at least two different cell lines, COS7 and NIH3T3 cells, MAGP-2 co-expression in the Notch cell does not further potentiate signaling induced by ligand co-culture with presenting cells. Because our system utilizes overexpressed ligand under a strong heterologous promoter (polyepitope chain elongation factor 1a), we note that under different conditions where the ligands are not overexpressed and may be limiting, MAGP-2 may be able to potentiate signaling.

The C-terminal Half of MAGP-2 Is Both Necessary and Sufficient to Bind and Activate Notch1—We next made N-terminal and C-terminal deletion mutants of MAGP-2 to map its N1-interaction domain. Because the MAGP-2 antiserum was generated to the N terminus of MAGP-2, the construct encoding only the C-terminal half of MAGP-2 was engineered with six tandem myc epitopes replacing the N-terminal half of MAGP-2 (Fig. 6A). Both MAGP-2 deletion constructs were detected by streptavidin-agarose did not change with increasing amounts of MAGP-2 (data not shown). Lysates from cells co-transfected with full-length N1 and full-length MAGP-2 (FL), the N-terminal half of MAGP-2 (N-MAGP-2, N), or the C-terminal half of MAGP-2 (C-myc-MAGP-2, C) were immunoprecipitated with anti-Notch1 antisem and Western blotted for MAGP-2. Both the full-length protein and the C-terminal half of MAGP-2 interacted with N1, whereas the N-terminal half did not co-immunoprecipitate with N1 (Fig. 6B). These findings are in agreement with our previous report describing a two-hybrid interaction between the C-terminal half of MAGP-2 with the EGF-like repeats of Jagged1. C-myc-MAGP-2, but not N-MAGP-2, also induced both HA-N1 ECD dissociation (Fig. 6C, top panel) and activated the CSL reporter construct in COS7 cells (Fig. 6D). Therefore we conclude that the C-terminal half of MAGP-2 is both necessary and sufficient for heterodimer dissociation and receptor activation.
To further dissect the C-terminal sequences required for N1 activation, we tested MAGP-1 for its activity on HA-N1. MAGP-1 and MAGP-2 share homology only within a 56-amino-acid domain and are otherwise not conserved. MAGP-1 can co-immunoprecipitate with Notch1 (data not shown) and both induce HA-N1 ECD dissociation from cells (Fig. 6E) and activate full-length N1 in CSL-reporter assays.

**FIGURE 5.** MAGP-2 potentiates NICD production and activation of Notch reporter constructs by full-length Notch1. A, COS7 cells were co-transfected with full-length N1 and either vector or MAGP-2 and treated with 10 μM MG132, and cell lysates were subject to N1 immunoprecipitation (IP) and then Western blot (WB) with an antibody to activated NICD (Val-1744). The lower panels show input levels of N1 and MAGP-2. One of two replicate experiments is shown. WCL, whole cell lysate. B, COS-7 cells were co-transfected with full-length N1, CSL-reporter carrying 8 CSL binding sites (pGL3PJH26) and increasing amounts of MAGP-2. Luciferase values were normalized to a CMV-Renilla luciferase construct and luciferase units shown are relative to the sample containing N1 and no MAGP-2 (0). The bar graph shows the average ± S.D. of at least 4 means from experiments done in triplicate. *, p = 0.005 (0 ng, n = 6; 100 ng, n = 4; 200 ng, n = 6). RLU, relative luciferase units. C, schematic of activated forms of N1. The two cysteine residues mutated to serine in N1c/s are noted. D, experiment done as in B, and luciferase units are shown relative to the vector only control. The bar graph shows the average ± S.D. of the means from five experiments each done in triplicate. **, p < 0.002 (p = 5). E, COS7 cells were transfected with N1, either vector or MAGP-2, and either the 8xCSL reporter (pGL3PJH26) or the Hes5 reporter. Luciferase values were normalized as in B and D, and luciferase units are shown relative to the N1 + vector control for each reporter. The bar graph shows the average ± S.D. of the means from three experiments each done in triplicate. F, MAGP-2 does not alter the amount of heterodimeric N1 receptor on the cell surface. COS7 cells co-transfected with N1 and increasing amounts of MAGP-2 were labeled with biotin and lysed, and then cell surface proteins were fractionated via streptavidin (SAV)-agarose and run on SDS-PAGE. The top two panels show streptavidin-agarose fractions immunoblotted with either antiserum to the extracellular domain of N1 (αN1E) or the intracellular domain of N1 (αN1). The two lower panels show input levels of full-length N1 and MAGP-2. One of four replicate experiments is shown. G, 3T3 cells transiently expressing N1, CSL-reporter, and increasing amounts of MAGP-2 were co-cultured with ligand-expressing L cells (L, D1HA, or J1HA) for 24 h and then assayed. Luciferase values were normalized to a CMV-Renilla luciferase construct, and luciferase units shown are relative to the sample containing N1 and no MAGP-2 that was co-cultured with parental L cells. The bar graph shows the average ± S.D. from multiple experiments each done in duplicate or triplicate (0 ng, n = 9; 25 ng, n = 9; 50 ng, n = 7; 100 ng, n = 4; 200 ng, n = 4).

**FIGURE 5.** MAGP-2 potentiates NICD production and activation of Notch reporter constructs by full-length Notch1. A, COS7 cells were co-transfected with full-length N1 and either vector or MAGP-2 and treated with 10 μM MG132, and cell lysates were subject to N1 immunoprecipitation (IP) and then Western blot (WB) with an antibody to activated NICD (Val-1744). The lower panels show input levels of N1 and MAGP-2. One of two replicate experiments is shown. WCL, whole cell lysate. B, COS-7 cells were co-transfected with full-length N1, CSL-reporter carrying 8 CSL binding sites (pGL3PJH26) and increasing amounts of MAGP-2. Luciferase values were normalized to a CMV-Renilla luciferase construct and luciferase units shown are relative to the sample containing N1 and no MAGP-2 (0). The bar graph shows the average ± S.D. of at least 4 means from experiments done in triplicate. *, p = 0.005 (0 ng, n = 6; 100 ng, n = 4; 200 ng, n = 6). RLU, relative luciferase units. C, schematic of activated forms of N1. The two cysteine residues mutated to serine in N1c/s are noted. D, experiment done as in B, and luciferase units are shown relative to the vector only control. The bar graph shows the average ± S.D. of the means from five experiments each done in triplicate. **, p < 0.002 (p = 5). E, COS7 cells were transfected with N1, either vector or MAGP-2, and either the 8xCSL reporter (pGL3PJH26) or the Hes5 reporter. Luciferase values were normalized as in B and D, and luciferase units are shown relative to the N1 + vector control for each reporter. The bar graph shows the average ± S.D. of the means from three experiments each done in triplicate. F, MAGP-2 does not alter the amount of heterodimeric N1 receptor on the cell surface. COS7 cells co-transfected with N1 and increasing amounts of MAGP-2 were labeled with biotin and lysed, and then cell surface proteins were fractionated via streptavidin (SAV)-agarose and run on SDS-PAGE. The top two panels show streptavidin-agarose fractions immunoblotted with either antiserum to the extracellular domain of N1 (αN1E) or the intracellular domain of N1 (αN1). The two lower panels show input levels of full-length N1 and MAGP-2. One of four replicate experiments is shown. G, 3T3 cells transiently expressing N1, CSL-reporter, and increasing amounts of MAGP-2 were co-cultured with ligand-expressing L cells (L, D1HA, or J1HA) for 24 h and then assayed. Luciferase values were normalized to a CMV-Renilla luciferase construct, and luciferase units shown are relative to the sample containing N1 and no MAGP-2 that was co-cultured with parental L cells. The bar graph shows the average ± S.D. from multiple experiments each done in duplicate or triplicate (0 ng, n = 9; 25 ng, n = 9; 50 ng, n = 7; 100 ng, n = 4; 200 ng, n = 4).
Together our data indicate that the homologous 56-amino-acid domain found in both MAGP-2 and MAGP-1 mediates these effects on N1. To explore the specificity of the interactions and effects detected for the cysteine-rich C-terminal domain of the MAGPs with the EGF repeats in N1, we tested whether the DSL family member, Delta-like-3 (Dll3), could also effect N1 ECD release as found for the MAGPs. Dll3 contains six EGF-like repeats and a divergent DSL domain that are both cysteine-rich, and we have previously shown that Dll3 cannot activate N1 in trans but can interact with N1 in cis similar to MAGP-2 (24). However, unlike MAGP-2, Dll3 negatively regulates Notch signaling. Because Dll3 is a single pass transmembrane-tethered protein, we also tested a soluble Dll3 mutant encoding only the extracellular domain tagged with three HA epitopes that more closely models the soluble MAGP-2 protein. Although both membrane-bound and soluble Dll3 were co-immunoprecipitated with N1 (Fig. 6G), neither induced the release of HA-N1 ECD like MAGP-2 (Fig. 6H). Therefore, the effect of MAGP-2 on N1 is not simply a consequence of fortuitous intermolecular disulfide bonding where high cysteine content drives these proteins to interact. Moreover, that Dll3 does not affect HA-N1 ECD release is consistent with our recent findings that this DSL family member is an inhibitor of Notch signaling rather than an activator as previously
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reported (24, 31). Also arguing against nonspecific alteration of N1 structure caused by interactions with MAGP-2 are the data from the CSL activation assays when the effects of MAGP-2 were tested in the presence of ligand cells. Specifically, levels of MAGP-2 that potentiated Notch activation of a CSL reporter (Fig. 5G, 100 and 200 ng), did not compromise ligand-induced activity. This implies that MAGP-2 binding to N1 does not impair ligand binding or receptor activation and that MAGP-2 may in certain conditions work in conjunction with ligand to regulate levels of Notch signaling.

DISCUSSION

A yeast two-hybrid screen first indicated that MAGP-2 could interact with EGF repeats from a number of different proteins beyond the fibrillins (17). We have now shown that MAGP-2 binding to either jagged1 (10) or Notch1 (this work) leads to the functional consequence of extracellular domain loss. However, although both jagged1 and Notch1 release their extracellular domains in response to MAGP-2, a key difference in mechanism separates the two effects. That is, the jagged1 ECD shedding in response to MAGP-2 requires metalloprotease cleavage, whereas the Notch1 ECD shedding does not. One possible reason for the different effects is that MAGP-2 binds in two distinct ways to jagged1 and Notch1 that potentiates ADAM cleavage of jagged1 but not Notch1. However, given the close resemblance between the EGF-like repeats of Notch1 and jagged1, it seems more likely that MAGP-2 binds similarly to the EGF-like repeats of these two proteins and that the mechanistic differences in extracellular domain shedding are because of intrinsic differences in the structure of the jagged1 and Notch1 proteins. Specifically, the two proteins are presented on the cell surface differently; the Notch receptors are furin-processed heterodimers, and jagged1 is not. Although the mechanism is yet to be determined, we surmise that MAGP-2 binding to the EGF repeats of either protein may induce a conformational change that is transmitted to the juxtamembrane portion within the ectodomain to potentiate ECD release. Although jagged1 is released through proteolysis, the Notch1 ECD may be released through an alternative mechanism similar to that reported for latent TGFβ activation by matricellular proteins (32).

Although Notch1 is a receptor for a signaling pathway and TGFβ is a soluble growth factor that activates intracellular signaling, there are several similarities between the maturation, latency, and activation of these two proteins. For example, the primary translation product of each gene is cleaved by furin, generating a C-terminal signaling fragment (NICD or mature TGFβ) and an N-terminal regulatory fragment (NECD or LAP, latency associated peptide). Furthermore, non-covalent interactions between the furin-produced fragments maintain the Notch1 signaling complex in an inactive state; and importantly, activation of Notch or TGFβ signaling requires disruption of these non-covalent interactions to release their respective N-terminal latency peptides (32, 33). For the small latent TGFβ complex, disruption of LAP by proteolysis, conformational change, or binding to the matricellular protein thrombospondin-1 have been shown to release active TGFβ (34, 35). Similarly, disruption of the Notch1 heterodimer and extracellular domain removal by calcium chelators (3), DSL ligand, and as we now show, binding to the matricellular proteins MAGP-1 or MAGP-2, can also lead to activation of Notch1. A final parallel is that naturally occurring activating mutations near the furin cleavage site in either protein are linked to human disease. At least some of the Notch1 mutations found in T-ALL destabilize the heterodimer and lead to receptor disso-

3 J. T. Nichols, S. L. Olsen, B. D’Souza, C. Yao, and G. Weinmaster, manuscript in preparation.

4 A. Miyamoto, unpublished data.

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