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Brief report

Identification of a functional proprotein convertase cleavage site in microfibril-associated glycoprotein 2

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ABSTRACT

Microfibril-associated glycoprotein 2 (MAGP2) is a secreted protein associated with multiple cellular activities including the organization of elastic fibers in the extracellular matrix (ECM), angiogenesis, as well as regulating Notch and integrin signaling. Importantly, increases in MAGP2 positively correlate with poor prognosis for some ovarian cancers. It has been assumed that full-length MAGP2 is responsible for all reported effects; however, here we show MAGP2 is a substrate for the proprotein convertase (PC) family of endoproteases. Proteolytic processing of MAGP2 by PC cleavage could serve to regulate secretion and thus, activity and function as reported for other extracellular and cell-surface proteins. In support of this idea, MAGP2 contains an evolutionarily conserved PC consensus cleavage site, and amino acid sequencing of a newly identified MAGP2 C-terminal cleavage product confirmed functional PC cleavage. Additionally, mutagenesis of the MAGP2 PC consensus cleavage site or treatment with PC inhibitors prevented MAGP2 proteolytic processing. Finally, both cleaved and uncleaved MAGP2 were detected extracellularly and MAGP2 secretion appeared independent of PC cleavage, suggesting that PC processing occurs mainly outside the cell. Our characterization of alternative forms of MAGP2 present in the extracellular space not only enhances diversity of this ECM protein but also provides a previously unrecognized molecular mechanism for regulation of MAGP2 biological activity.

1. Introduction

Microfibril-associated glycoprotein 2 (MAGP2) is a secreted protein that was first identified based on its association with extracellular elastic microfibrils (Gibson et al., 1996). MAGP2 can also induce changes in cellular function, such as the induction of blood vessel formation in cell culture and in tumor models in live mice. MAGP2 modulation of Notch and/or integrin activation is thought to underlie these pro-angiogenic effects of MAGP2 (Miyamoto et al., 2006; Albig et al., 2007, 2008; Mok et al., 2009). MAGP2 has also been implicated as an independent prognostic factor in ovarian cancer, as elevated MAGP2 levels in ovarian cancer patient samples are correlated with poor prognosis (Mok et al., 2009). Whether the different functions of MAGP2 are regulated by biochemical modifications such as proteolytic cleavage or other post-translational modifications beyond glycosylation has not been addressed previously.

Many secreted and plasma membrane proteins, such as Notch and Fibrillin1, are cleaved by one or more members of the proprotein convertase (PC) family of proteins (Logeat et al., 1998; Lonnqvist et al., 1998; Raghunath et al., 1999; Bush et al., 2001; Wallis et al., 2003). Endoproteolytic cleavage by PC proteases often has a regulatory function, such as altering secretion, extracellular matrix deposition, or enzyme activity (Seidah, 2011). Most PC substrates contain a consensus site, RX(K/R)R, where X is any amino acid except cysteine, which allows for cleavage by the PC family of endoproteases (Seidah, 2011). PC cleavage can occur either within the biosynthetic pathway, at the cell surface, or in the extracellular space consistent with the location and functional range of the PC family members (Mesnard et al., 2011).

In this study, we have identified an evolutionarily conserved PC consensus site found in MAGP2 sequences from fish to humans, and provide evidence for PC-mediated proteolytic processing of MAGP2. Specifically, we show that MAGP2 can serve as a substrate for the PC family of endoproteases, and can exist extracellularly in both a cleaved and uncleaved form. To our knowledge, this is the first report for MAGP2 proteolytic processing during its normal maturation, which extends both the diversity and potentially, the function, of this important ECM protein.

2. Results

2.1. MAGP2 is cleaved near its C-terminal end at a conserved proprotein convertase consensus site

A candidate PC cleavage site in mouse MAGP2 that matched the PC consensus site, RX(K/R)R (amino acids 140–143) was identified using ProP 1.0 analysis (Duckert et al., 2004). Amino acid alignment of

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MAGP2 among multiple species indicated that the RLRR sequence is identical in sequences from placental mammals, birds, and reptiles. There is also conservation, albeit more limited, in marsupial and fish sequences (Fig. 1A) (Segade, 2009). The predicted cleavage would occur between arginine 143 and serine 144 and produce a large N-terminal cleavage fragment and a small 20 amino acid C-terminal cleavage fragment (Fig. 1B).

To determine if MAGP2 was cleaved at this putative PC cleavage site, a panel of anti-MAGP2 antibodies was used in immunoblotting assays to detect the predicted cleavage fragments. Detection of mouse MAGP2 secreted from transiently transfected HEK 293T using an antibody against the C-terminus revealed a full-length MAGP2 band at 23 kDa and a 3–4 kDa band, whose mass is consistent with cleavage near the C-terminal end of MAGP2 (Fig. 2A, WT).

Further evidence for the predicted C-terminal cleavage was generated from a fusion protein construct, MAGP2-Fc, in which MAGP2 is joined at its C-terminal end to the Fc region of human IgG. When this fusion construct was transiently overexpressed in HEK 293T cells and analyzed on immunoblots probed with anti-Fc antiserum, two immunoreactive species were detected that were consistent in size with full-length fusion protein (54 kDa) and the C-terminal cleavage fragment (36 kDa) (Fig. 2B, WT). The 36 kDa fragment is within the range of the apparent molecular mass of recombinant human IgG Fc, suggesting that only a small portion of MAGP2 remained attached to Fc. Furthermore, when duplicate samples of secreted MAGP2-Fc were probed for the corresponding N-terminal cleavage fragment, the detected immunoreactive species were in agreement with the expected sizes of the full-length fusion protein (54 kDa) and an N-terminal cleavage fragment (21 kDa) that is predicted to be only 20 amino acids shorter than wild-type MAGP2 (Fig. 2C, WT, arrowheads). Based on its mass, the minor 36 kDa band (Fig. 2C, WT, asterisk) is probably the C-terminal cleavage fragment of MAGP2-Fc, whose human Fc domain is recognized weakly by the secondary goat anti-rabbit immunoglobulin antiserum (Fig. 2C, asterisk). Taken together, the protein masses identified by the different antibodies indicate that MAGP2 is cleaved near the C-terminal end of the protein by an endogenous protease found in HEK 293T cells.

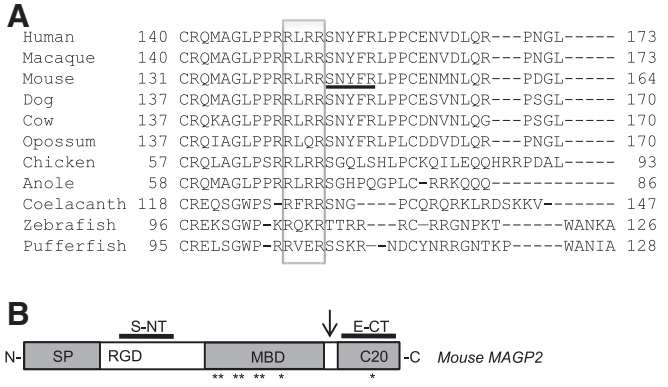

Fig. 1. MAGP2 contains a conserved PC consensus site. A. Amino acid alignment of C-terminal MAGP2 sequences from species ranging from fish to humans, starting from the seventh conserved cysteine. The predicted PC consensus site (RXRR) is boxed. The amino acids underlined in the mouse sequence were identified in Edman degradation analysis of the C-terminal cleavage fragment. The following accession numbers were used to generate the alignment on the UniProt Sequence Alignment website (UniProt Consortium, 2012)—human: Q13361, macaque: F7FYE8, mouse: Q9QZJ6, dog: E2RTJ1, cow: Q28022, opossum: F7B7R8, chicken: F1NIR2, anole: G1KTW6, coelacanth: H3ATW4, zebrafish: E7FA84, and pufferfish: H3CRQ7. B. Schematic of the domain structure of MAGP2. The PC cleavage site is indicated by the arrow. SP, signal peptide; RGD, integrin-binding site; MBD, matrix binding domain; C20, C-terminal 20 amino acids; *, conserved cysteine residues. Black bars indicate antibody binding sites (approximate). S-NT, Santa Cruz Biotechnology N-terminal MAGP2 antibody, E-CT, Epitomics C-terminal MAGP2 antiserum.

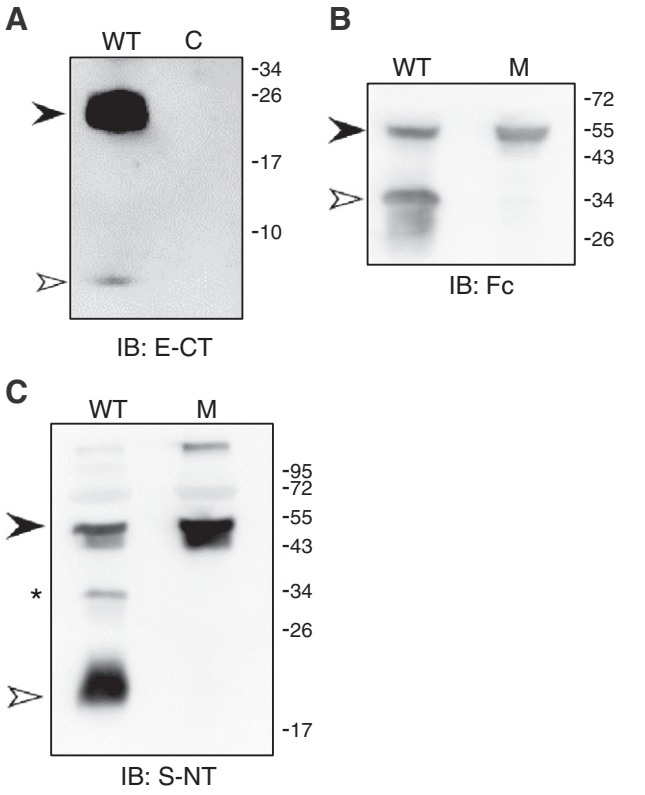

Fig. 2. MAGP2 cleavage by endogenous proteases requires the PC consensus site. A. HEK 293T cells were transfected with wild-type (WT) mouse MAGP2 or a control (C) plasmid and conditioned medium was collected after 3 days. Samples were analyzed on 16% Tris–Tricine SDS-PAGE followed by immunoblotting (IB) with the E-CT polyclonal antiserum. B and C. HEK 293T cells were transfected with either a wild-type (WT) mouse MAGP2-Fc fusion construct or a mutant (M) MAGP2-Fc-RRAA construct, and conditioned medium was collected after 3 days. Samples were analyzed by immunoblotting with either (B) an anti-Fc-HRP antibody or (C) a MAGP2 N-terminal antibody (S-NT). Filled arrowheads represent full length MAGP2-Fc, open arrowheads indicate the PC-cleavage fragments of MAGP2-Fc, and the asterisk indicates a minor species that is most likely due to cross-reactivity of secondary anti-rabbit IgG antibodies to the human IgG Fc portion of MAGP2-Fc.

2.2. The PC consensus site in MAGP2 is functional and necessary for cleavage

To determine the specific site of the proteolytic cleavage in MAGP2, direct amino acid sequencing of the C-terminal cleavage fragment of MAGP2-Fc was conducted. Edman degradation analysis of the 36 kDa MAGP2-Fc cleavage fragment revealed an N-terminal sequence of SNYFR (underlined, Fig. 1A). This sequence matches the predicted PC cleavage fragment of MAGP2, supporting the notion that MAGP2 is cleaved by a PC family endoprotease.

Since evolutionary conservation, immunoblotting, and amino acid sequencing indicated that MAGP2 is cleaved by PC endoprotease(s), a mutant form of MAGP2-Fc was made to formally prove that the predicted PC consensus sequence, RLRR, was necessary for cleavage of MAGP2. To this end, the sequence was changed from RLRR to RLAA, replacing the back-to-back arginines to alanines (Sasanami et al., 2003) in the MAGP2-Fc construct to generate MAGP2-Fc-RRAA. When this mutant construct was expressed in HEK 293T cells and evaluated for cleavage, the RRAA mutation effectively blocked cleavage of the fusion protein as seen by the loss of the faster migrating MAGP2-Fc species on immunoblots (Fig. 2C, panels B and C, lane M), indicating that the RLRR sequence is required for cleavage of MAGP2.

We then wanted to positively identify the corresponding N-terminal cleavage fragment in wild-type MAGP2. To facilitate these studies, a mutant construct of MAGP2 was designed to mimic the N-terminal