Matrix Biology 28 (2009) 463–469

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

The C-terminal domains of ADAMTS-4 and ADAMTS-5 promote association with N-TIMP-3

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ARTICLE INFO

Article history:
Received 8 April 2009
Received in revised 20 July 2009
Accepted 20 July 2009

Keywords:
Aggrecanase
Inhibition kinetics
MMPs

ABSTRACT

We investigated whether the affinity of tissue inhibitor of metalloproteinases (TIMP)-3 for adamalysins with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5 is affected by the non-catalytic ancillary domains of the enzymes. For this purpose, we first established a novel method of purifying recombinant FLAG-tagged TIMP-3 and its inhibitory N-terminal domain (N-TIMP-3) by treating transfected HEK293 cells with sodium chlorate to prevent heparan sulfate proteoglycan-mediated TIMP-3 internalization. TIMP-3 and N-TIMP-3 affinity for selected matrix metalloproteinases and forms of ADAMTS-4 and -5 lacking sequential C-terminal domains was determined. TIMP-3 and N-TIMP-3 displayed similar affinity for various matrix metalloproteinases as has been previously reported for *E. coli*-expressed N-TIMP-3. ADAMTS-4 and -5 were inhibited more strongly by N-TIMP-3 than by full-length TIMP-3. The C-terminal domains of the enzymes enhanced interaction with N-TIMP-3 and to a lesser extent with the full-length inhibitor. For example, N-TIMP-3 had 7.5-fold better K_i value for full-length ADAMTS-5 than for the catalytic and disintegrin domain alone. We propose that the C-terminal domains of the enzymes affect the structure around the active site, favouring interaction with TIMP-3.

1. Introduction

In addition to their catalytic domains, proteolytic enzymes often have non-catalytic ancillary domains that modulate interaction of the enzyme with substrates or inhibitors. Indeed, almost all members of the metzincin family of metalloproteinases have such ancillary domains, and they have been shown to mediate recognition and cleavage of numerous substrates. For example, the hemopexin domain of matrix metalloproteinase 1 (MMP-1) is required for cleavage of collagen (Clark and Cawston, 1989). Among the related adamalysins with thrombospondin motifs (ADAMTSs), the C-terminal domains of ADAMTS-4 and ADAMTS-5 have been shown to promote aggrecan cleavage (Kashiwagi et al., 2004; Gendron et al., 2007; Fushimi et al., 2008), the C-terminal spacer domain of ADAMTS13 promotes cleavage of von Willebrand factor (Gao et al., 2008) and C-terminal domains of ADAMTS-2 modulate processing of the N-terminal propeptide of procollagen (Colige et al., 2005). These ancillary domains have also been shown to modulate interaction of some metzincins with the endogenous tissue inhibitors of metalloproteinases (TIMPs). For example, the MMP-2 hemopexin domain interacts strongly with TIMP-2 (Murphy et al., 1992; Butler et al., 1999; Morgunova et al., 2002). While the MMPs are inhibited by all four of the mammalian TIMPs, most adamalysins and ADAMTSs are inhibited only by TIMP-3. Compared to the MMPs, the adamalysins and ADAMTSs have a greater number and diversity of C-terminal ancillary domains and the role of these in modulating interactions with TIMP-3 is largely unknown, as a lack of sensitive substrates has hampered in-depth kinetic analysis. Good substrates are available for ADAM17, or tumour necrosis factor-α converting enzyme (TACE) and the C-terminal domains of this enzyme have been shown to reduce the affinity of the enzyme for both full-length TIMP-3 and N-TIMP-3 by 10-fold (Lee et al., 2002).

ADAMTS-4 and -5 cleave the cartilage matrix proteoglycan aggrecan at various sites, releasing the chondroitin and keratan sulfate-bearing region of the molecule from the tissue. This is an early and crucial step in the development of osteoarthritis as it reduces the ability of the tissue to resist compressive loads. Both enzymes are readily proteolyzed to smaller isoforms, which have altered proteolytic activity (Gao et al., 2004). Here we investigate TIMP-3 inhibition of the isoforms, with an

Abbreviations: ADAM, adamalysin; ADAMTS, adamalysin with thrombospondin motifs; cat, catalytic domain; CysR, cysteine-rich; Dis, disintegrin; LRP, low-density lipoprotein receptor-related protein; MMP, matrix metalloproteinase; N-TIMP, N-terminal domain of TIMP; RAP, receptor-associated protein; Sp, spacer; TACE, tumour necrosis factor-α converting enzyme; TIMP, tissue inhibitor of metalloproteinase; TS, thrombospondin; VAP, vascular apoptosis-inducing protein.

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doi:10.1016/j.matbio.2009.07.005

aim to understanding whether, as in the case of ADAM17, the C-terminal ancillary domains of the enzymes regulate TIMP-3 binding to the active site. To do this, we developed a novel method of purifying sufficient soluble full-length TIMP-3 and its inhibitory N-terminal domain (N-TIMP-3) for kinetic analysis.

N-TIMP-3 can be successfully expressed in *Escherichia coli* and refolded *in vitro* (Kashiwagi et al., 2001), but we have not been able to refold full-length TIMP-3 using this system. Unlike the other TIMPs, TIMP-3 binds tightly to the extracellular matrix and is not readily soluble (Lee et al., 2007; Yu et al., 2000). It is thus difficult to recombinantly express TIMP-3 in mammalian cells, and to date full-length TIMP-3 has only been recombinantly produced in NSO mouse myeloma cells, which do not produce an extracellular matrix (Apte et al., 1995). Here we describe successful purification of full-length TIMP-3 and N-TIMP-3 recombinantly expressed in human embryonic kidney HEK293 cells. Recently developed synthetic fluorescent quenched substrates allowed us to determine the inhibition constants of both TIMP-3 and N-TIMP-3 for various forms of ADAMTS-4 and ADAMTS-5.

2. Results

2.1. Purification of recombinant TIMP-3 and N-TIMP-3

This study describes a novel method of purifying soluble full-length TIMP-3. No TIMP-3 is observed in the conditioned medium of transfected HEK293 cells, and we have previously shown that TIMP-3 is rapidly endocytosed after secretion from the cell by a scavenger endocytic receptor of the low-density lipoprotein receptor-related protein (LRP) family (Troeberg et al., 2008). TIMP-3 accumulates in the conditioned medium if this endocytic pathway is blocked, for example by the addition of receptor-associated protein (RAP), an inhibitor of LRP-mediated endocytosis (Troeberg et al., 2008). Heparin also causes an accumulation of TIMP-3, indicating that a heparan sulfate proteoglycan co-receptor is possibly required for LRP-mediated endocytosis as has been reported for other LRP ligands (Godyna et al., 1995; Sarafanov et al., 2001). However, heparin binds to TIMP-3 tightly and purification of TIMP-3 from heparin-treated cells proved difficult. To keep recombinantly expressed TIMP-3 in the medium, we treated transfected cells with sodium chlorate ($NaClO_3$), which blocks sulfation of cell surface glycosaminoglycans (Baeuerle and Huttner, 1986; Safaiyan et al., 1999). This resulted in the accumulation of FLAG-tagged TIMP-3 and N-TIMP-3 in the conditioned medium of transfected cells. Both proteins were purified from the conditioned medium by anti-FLAG affinity chromatography (Fig. 1A). As previously reported for full-length ADAMTS-4 (Kashiwagi et al., 2004), substantial processing of the C-terminal FLAG-tag occurred, reducing the yield to approximately 50 μg of purified protein per litre of conditioned medium. Both TIMP-3 and N-TIMP-3 expressed in HEK293 were confirmed to have the expected N-terminal sequence ^{1}CTCSPSHPQD and to react with a polyclonal anti-TIMP-3 antibody (Fig 1B). Titration against a known concentration of MMP-1 showed all preparations of TIMP-3 to be 100% active.

TIMP-3 contains a single N-glycosylation site in its C-terminal domain (Apte et al., 1995) and various possible O-glycosylation sites. Various cell types have been shown to express both a 27 kDa glycosylated and a 24 kDa non-glycosylated form (Apte et al., 1995; Fabunmi et al., 1996; Langton et al., 1998). Transfected HEK293 cells also synthesized both glycosylated and non-glycosylated forms, but only the glycosylated form remained after purification, with the non-glycosylated form possibly lost due to increased "stickiness". Indeed, we obtained lower yields when we treated cells with tunicamycin to obtain only the non-N-glycosylated form. N-TIMP-3 was expressed primarily as a non-glycosylated form, with a lower amount of a higher molecular mass glycosylated form present. Since N-TIMP-3 contains no N-glycosylation sites, this possibly reflects O-glycosylation.

2.2. $K_{i(app)}$ of TIMP-3 and N-TIMP-3 for selected MMPs

We compared the inhibitory activity of our mammalian-expressed TIMP-3 and N-TIMP-3 with the previously characterized *E. coli*-expressed N-TIMP-3 with a C-terminal His-tag (Kashiwagi et al., 2001). $K_{i(app)}$ values determined in the current study for *E. coli*-expressed N-TIMP-3 agreed well with previously published values for this protein (Kashiwagi et al., 2001). Although we determined a 6-fold lower $K_{i(app)}$ value for N-TIMP-3 inhibition of the catalytic domain of MMP-3 (MMP-3_{cat}), our results agree with that found in the previous study that N-TIMP-3 is a weaker inhibitor of MMP-3_{cat} than of the catalytic domain of MMP-1 (MMP-1_{cat}) or MMP-2 (Table 1).

We found that mammalian-expressed N-TIMP-3 had essentially identical inhibitory properties to the *E. coli*-expressed N-TIMP-3, being a strong inhibitor of MMP-1_{cat} and MMP-2, and a 10-fold weaker inhibitor of MMP-3_{cat}.

MMP-1_{cat} was equally well inhibited by N-TIMP-3 and full-length TIMP-3. MMP-3_{cat}, however, was more strongly inhibited by full-length TIMP-3 than by N-TIMP-3, implying that the C-terminal domain of the inhibitor contributes to binding. The MMP-2 catalytic domain (MMP-2_{cat}) was equally well inhibited by N-TIMP-3 and full-length TIMP-3, but the full-length enzyme was more strongly inhibited by the full-length TIMP-3 than by N-TIMP-3. This indicates that the C-terminal domain of TIMP-3 interacts with the C-terminal hemopexin domain of MMP-2.

2.3. K_i of TIMP-3 and N-TIMP-3 for ADAMTS-4 and ADAMTS-5

We analyzed TIMP-3 and N-TIMP-3 inhibition of various isoforms (Fig. 2) of ADAMTS-4 and -5 lacking C-terminal ancillary domains. Fig. 3 shows representative data for four enzyme isoforms fitted to the tight-binding equation (Bieth, 1995). All isoforms of ADAMTS-4 and -5 were effectively inhibited by TIMP-3 and N-TIMP-3, with K_i values in the sub-nanomolar range. All ADAMTS-5 isoforms were inhibited more strongly by N-TIMP-3 than by full-length TIMP-3 (Table 2). The C-terminal domains of ADAMTS-5 enhanced inhibition by N-TIMP-3, with full-length ADAMTS-5 (ADAMTS5-1) having a 7.5-fold better K_i value than ADAMTS5-5, which consists of the catalytic and disintegrin domain. The C-terminal domains of the enzyme had a similar, although less marked, effect on association with the full-length inhibitor. The C-terminal domains of ADAMTS-4 also had a similar effect on association with full-length and N-TIMP-3 (Table 2).

Glycosylation had little effect on TIMP-3 or N-TIMP-3 inhibition of ADAMT5-2 or ADAMTS4-2 (Table 3).

The isolated catalytic domains of ADAMTS-4 and -5 (ADAMTS4-5 and ADAMTS5-6) had only minimal activity on natural substrates and the synthetic substrates used, so their inhibition by TIMP-3 could not be analyzed by enzyme inhibition kinetics.

3. Discussion

Here we report a novel method to generate recombinant TIMP-3 protein by treating transfected HEK293 cells with sodium chlorate. Our initial attempts to express TIMP-3 in HEK293 or HTB94 chondrosarcoma cells were hampered by the observation that, although these cells transcribe a considerable amount of TIMP-3 mRNA, no TIMP-3 accumulates in the conditioned medium. We have previously reported that TIMP-3 is normally internalized but accumulates in medium in the presence of an antagonist of the LRP endocytic receptor or heparin (Troeberg et al., 2008). We thus treated cells with sodium chlorate, which blocks sulfation of cell surface glycosaminoglycans (Baeuerle and Huttner, 1986; Safaiyan et al., 1999) and observed accumulation of soluble TIMP-3 in the medium. TIMP-3 has previously been purified from the conditioned medium of transfected NSO mouse myeloma cells (Apte et al., 1995), which appear to lack this endocytic pathway. Our transfected cells showed no signs of TIMP-3-induced apoptosis (Bond et al., 2000) in the absence of sodium chlorate, but exhibited