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Human membrane metallo-endopeptidase-like protein (MMEL) degrades both A β ₄₂ and A β ₄₀

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Abstract

Beta-amyloid (A β) degrading endopeptidases are thought to protect against Alzheimer disease (AD) and are potentially therapeutic. Of particular interest are endopeptidases that are blocked by thiorphan and phosphoramidon (T/P), as these inhibitors rapidly induce A β deposition in rodents. Neprilysin is the best known target of T/P; however neprilysin knockout results in only modest A β increases insufficient to induce deposition. Therefore, other endopeptidases targeted by T/P must be critical for A β catabolism. Another candidate is the T/P sensitive membrane metallo-endopeptidase-like protein (MMEL), a close homolog of neprilysin.

The endopeptidase properties of β and γ splice forms of human MMEL were determined in HEK293T cells transduced with the human cDNAs for the two splice forms; this showed degradation of both A β ₄₂ and A β ₄₀ by hMMEL- β but not hMMEL- γ . hMMEL- β activity was found at the extracellular surface with no significant secreted activity. hMMEL- γ was not expressed at the extracellular surface. Finally, it was found that hMMEL cleaves A β near the α -secretase site (producing A β ₁₋₁₇ >> A β ₁₋₁₆). These data establish hMMEL as a mediator of A β catabolism and raises the possibility of its involvement in the etiology of Alzheimer's disease and as a target for intervention.

Keywords

endopeptidase; neprilysin; beta-amyloid; Alzheimer's disease; membrane metallo-endopeptidase like protein

Introduction

Previous studies have identified metalloendopeptidases as mediators of A β catabolism (Hersh, 2003). One key enzyme is neprilysin, a cell-surface associated endopeptidase (Howell et al., 1995, Iwata et al., 2000). It has been reported that NEP localization in the brain is reduced in AD and in aging, particularly in brain regions vulnerable to plaque formation in humans and rodents (Akiyama et al., 2001, Reilly, 2001, Yasojima et al., 2001a, Yasojima et al., 2001b, Iwata et al., 2002, Apelt et al., 2003, Caccamo et al., 2005, Maruyama et al., 2005, Wang et

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al., 2005). Infusion of the NEP inhibitor, thiorphan, has been shown to induce a dramatic increase in A β levels (~30-fold) sufficient to produce plaque deposition after only 1 month in wild-type rats and mice (Iwata et al., 2000, Dolev and Michaelson, 2004). Another NEP inhibitor, phosphoramidon, produced similar results in mice (personal communication from D. M. Michaelson). This implicates that enzymes targeted by these inhibitors are critical for maintaining normal A β levels *in vivo*.

The murine NEP homolog termed membrane metallo-endopeptidase like protein -1/2 (MMEL) has a membrane bound (mMMEL- α), and a secreted form (mMMEL- β) as a result of alternative splicing (Fig. 1) (Ikeda et al., 1999, Ghaddar et al., 2000). MMEL (also known as SEP, NL1, NEPLP, and NEP2) was shown to be able to degrade vasoactive peptides, and was determined to be sensitive to inhibition by thiorphan and phosphoramidon (Ikeda et al., 1999). The α splice-form of MMEL was shown to degrade A β (Shirotani et al., 2001). The human ortholog of MMEL has been identified, and was found to be expressed in the brain (Bonvouloir et al., 2001). Figure 1 compares alternate splice forms of human MMEL (β , γ , and δ) to known splice forms of the murine gene (α and β). The murine β form retains an alternate exon that contains a furin-like cleavage site (arrow) resulting in its secretion, and loss of activity (Ikeda et al., 1999, Shirotani et al., 2001). There is 77% identity between the mouse and human β homologs (Bonvouloir et al., 2001) and all three hMMEL isoforms retain the homologous furin-site containing exon. However, the human β (accession # BC101027) and δ (accession # BC101029) forms contain a large alternate exon excluded from the human γ (accession # BC101028) form, and the δ form utilizes an alternate splice acceptor site resulting in a frameshift mutation and truncation of the protein, eliminating the zinc binding motif critical for activity. Very little is known regarding the properties of hMMEL. Therefore, we set out to test the A β degrading activity and molecular properties of these splice forms in a HEK293T cell culture system.

Results and Discussion

Plasmids containing the human MMEL cDNAs were purchased from Open Biosystems (Huntsville, AL). To express the human β and γ isoforms of hMMEL, we constructed lentiviral gene transfer vectors expressing these cDNAs. A lentiviral vector expressing murine MMEL- β was also constructed for testing. All vectors expressed the desired transgene driven by the human cytomegalovirus promoter in a 'third generation' lentiviral vector (Tiscornia et al., 2006). Control vectors included: lenti-NEP (positive control) and lenti-GFP (negative control) (Marr et al., 2003). To achieve the production of A β in our HEK293T cell cultures, a lentiviral vector (lenti-APP) directing the expression of mutant human APP (Swedish and London mutations-K670M/N671L/V717I) and PS-1 (Δ 9) was used (Singer et al., 2005).

Two sets of experiments were performed aimed at transducing HEK293T cells with lentiviral vectors expressing our endopeptidases of interest and APP. In the first set of experiments (co-infection), HEK293T cells were transduced with the lenti-APP vector and then these same cells were expanded and then co-infected with a lentiviral vector expressing the endopeptidases or GFP control. In the second set of experiments (co-culture), the lenti-APP transduced HEK293T cells were expanded and co-cultured with HEK293T cells transduced separately with the lentiviral vectors expressing the endopeptidases or GFP control. In both experimental sets, the same lenti-APP transduced cells were used across the test and control groups ensuring consistent levels of A β production. Our results indicate that hMMEL- β was able to degrade cell-produced A β ₄₂ and A β ₄₀ in both co-infection (Fig. 2A, B) and co-culture (Fig. 2C, D) experiments (hMMEL- γ was inactive). The catabolic activity of hMMEL- β was found to be sensitive to thiorphan and phosphoramidon similar to reports for the murine α homolog (data not shown). Murine MMEL- α has previously been shown to degrade A β (Shirotani et al., 2001). However, the reported activity was highly selective for A β ₄₀ rather than A β ₄₂. This has

relevance to AD research as it is thought that A β ₄₂ is more amyloidogenic and has been implicated as the primary mediator of the disease. Our findings clearly suggest the human homolog of MMEL has similar activities against both forms of A β (Fig. 2).

MMEL endopeptidases have preferred substrate cleavage sites. To further characterize hMMEL, synthetic A β ₁₋₄₀ or A β ₁₋₄₂ was digested with purified recombinant hMMEL- β and the products analyzed by mass spectrometry (Fig. 3). The primary substrate cleavage for both forms of A β was A β ₁₋₁₇ (hydrolyzing between Leu17 and Val18) and to a lesser extent A β ₁₋₁₆ (hydrolysis at the α -secretase cut site between Lys16 and Leu17). This preference is within a subset of sites also cut by ECE-1, a more distantly related homologue of MMEL (Eckman et al., 2001).

We next set out to determine if hMMEL- β is a secreted or cell surface associated enzyme. Western blot analysis of transfected HEK293T cells indicated that both hMMEL- β and hMMEL- γ are detected in the cell lysate (Fig. 4A). The γ isoform showed the predicted shift in electrophoretic mobility due to the absence of an alternate exon. hMMEL- β and not γ was detected in the cell supernatant. hMMEL- β was immunodetected on the surface of transfected and unpermeabilized HEK293T cells by flow cytometry (Fig. 4B). The majority of the cell population transfected with hMMEL- β showed more intense green fluorescence producing a shift to the right in the population histogram. However, very little hMMEL- γ was detected at the cell surface as there was not a substantial shift in the transfected cell population. A β degrading activity was assayed in the cell culture supernatants of these transfected cells. Supernatants expressing hMMEL- β , hNEP (also cell surface associated), and GFP were mixed with A β -containing medium and later assayed for remaining A β levels. An expression vector producing a secreted-active ectodomain of hNEP (secNEP) (Marr et al., 2004) was used as a positive control in this experiment. As expected, only cell culture supernatant from secNEP transfected cells showed reduced A β . This indicated a lack of soluble enzyme activity for hMMEL- β and NEP (Fig. 4C). Combined with the data above (Fig. 2) these results show that hMMEL- β , in its active form, is a cell-surface associated protein with an extracellular active site, similar to NEP. However, it was also detected as a secreted higher molecular weight form that did not possess significant A β degrading activity (Fig. 4A). This observation is similar to what was reported for murine MMEL, as the β form (SEP- β) was shown to be secreted as an inactive higher molecular weight protein (Ikeda et al., 1999). The increased mass of the processed / secreted form was shown to be due to glycosylation. Therefore, our results differ from the reported murine data in that the human β splice form was also found to be active on the cell surface. The hMMEL- γ splice variant was not detected at the cell surface in significant amounts but was found in the cell lysate. This suggests this isoform has altered protein trafficking patterns.

MMEL expression studies have been performed in mice and rats (Carpentier et al., 2003, Facchinetti et al., 2003). It has been suggested that MMEL and NEP exhibit a complementary expression pattern in the brain. For example, both enzymes were found to be expressed in the hippocampal formation in rats. However, NEP was found primarily in the granule cell layer of the dentate gyrus while MMEL was found throughout the pyramidal cell layers (Facchinetti et al., 2003). MMEL and NEP have been reported to degrade many of the same smaller neuropeptide substrates including substance P, enkephalin, and bradykinin (Ghaddar et al., 2000, Carpentier et al., 2003). This suggests a redundancy in the functions performed by these enzymes in the brain, similar to the related enzymes thimet oligopeptidase and neurolysin (Massarelli et al., 1999, Tullai et al., 2000). Also, both enzymes have been reported to be expressed in the human brain (Akiyama et al., 2001, Bonvouloir et al., 2001) and our results demonstrate degradation of A β ₄₂ and A β ₄₀ by both. Therefore, the shared functions between MMEL and NEP could include the control of A β peptide levels in humans. The potential use of NEP augmentation to treat AD has been extensively explored in rodent models (Leissring

et al., 2003, Marr et al., 2003, Hong et al., 2006, Hemming et al., 2007). Our data support the potential use of MMEL augmentation for the treatment of Alzheimer's disease and indicate the possibility that MMEL dysfunction could be involved in progression of neurodegeneration.

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Figure 1. Representations of the murine and human forms of MMEL

Alternate exons are shown in grey boxes. These are predicted type-II integral membrane proteins with short N-terminal cytoplasmic tails and a single transmembrane (TM) spanning region. The arrow indicates a consensus furin-like cleavage sequence. Murine MMEL-α has been shown to be a membrane bound protein which can degrade Aβ₄₀. Murine MMEL-β has been shown to be a secreted protein which lacks Aβ degrading activity. Human MMEL-β is the corresponding homolog of mMMEL-β. Human MMEL-γ does not contain a single alternate exon while hMMEL-δ utilizes an alternate splice acceptor site near its C-terminus resulting in a frameshift mutation / truncation lacking the critical zinc binding motif [HEITH].

Figure 2. The β splice form of MMEL can degrade A β

A β ₄₂ (A, C) and A β ₄₀ (B, D) were degraded by hMMEL- β but not hMMEL- γ in co-infection (A, B) and co-culture assays (C, D). Data are presented as the % A β remaining compared to a control condition (i.e. cells expressing GFP and APP). **Methods:** Lentiviral vectors were produced as previously described (Marr et al. 2003, Singer et al. 2005). Briefly, HEK293T cells were transfected (Ca-phosphate method) (Graham and van der Eb, 1973, Tiscornia et al., 2006) with vector and packaging plasmids and the cell culture supernatants containing virus collected. For co-infection, HEK293T cells were transduced with the lenti-APP vector (100 μ l) and then split into wells of a 24 well plate 1 day later. After this the APP-infected wells were co-infected with 100 μ l of a lenti-endopeptidase or control vector. 2 days later the culture medium was replaced with serum free medium (OptiMEM + 1% antibiotic/antimycotic, Gibco, Grand Island, NY) and the medium collected 24 hrs later for A β quantitation using specific ELISA (BioSource, Nivelles, Belgium). For co-culture experiments, cells were infected with the lenti-APP vector preparation (100 μ l). Also separate wells of cells were infected with a lenti-endopeptidase or control vector (100 μ l). The next day the APP-infected cells were split and co-cultured with endopeptidase or control infected cells. These cells were cultured for 2 days and A β -containing medium collected and assayed as done for the co-infection assay (above). Experiments using recombinant A β ₄₂ and A β ₄₀ established that the cross reactivity between A β isoforms on the specific A β ELISA kits were < 0.1% as reported by the manufacturer (not shown). The data are expressed as mean $\pm$ S.E.M. (n = 5, *p<0.05, **p<0.01).

Figure 3. $A\beta_{1-17}$ and $A\beta_{1-16}$ are preferred cleavage products produced by hMMEL- β
 Incubation of synthetic $A\beta$ with recombinant hMMEL- β produced major products corresponding to $A\beta_{1-17}$ and $A\beta_{1-16}$ (A). A representative MS profile is shown below (10 min incubation) (B). **Methods:** $A\beta_{40}$ or $A\beta_{42}$ (Quality Controlled Biochemicals, Hopkinton, MA) was dissolved in dimethyl sulfoxide at 20 mg/ml as a stock solution. 7 μ g of $A\beta$ was incubated with 1.7 μ g of recombinant hNEP2 (MMEL) ~100:1 [substrate:enzyme] (R&D Systems, Minneapolis, MN) in 50 μ l of 150 mM NaCl, 50 mM Tris, pH 7.5 at 37° C. The reactions were stopped with 0.2% formate. Mass spectrometry analysis was performed on a Voyager DE-STR Biospectrometry workstation (Applied Biosystems, Foster City, CA; as described in Tullai et al., 2000) with α -cyano-4-hydroxycinnamic acid as matrix in delayed extraction and reflector mode with 200 shots after calibration.

Figure 4. hMMELβ is present at the cell surface

hMMEL-β and γ was associated with the cell lysate and hMMEL-β was also detected in the cell culture supernatant by immunoblot. Recombinant human MMEL-β (rhMMEL-β, R&D Systems) was included as a positive control (5ng) (A). hMMEL-β but not γ was found at the cell surface as immunodetected by flow cytometry. The histogram shows the number of cells (y-axis) that were counted at the specific intensity of green fluorescence (x-axis) (B). Aβ degrading activity was not transferable in the medium for hMMEL-β and NEP; however a secreted mutant of NEP (secNEP) degraded Aβ (C). **Methods:** *Western blot:* Transfected cells were homogenized in RIPA buffer with protease inhibitors (Complete mini™, Roche, Indianapolis, IN). Cell lysates and cell culture supernatants were then analyzed by SDS-PAGE /

immunoblot (1° antibody - polyclonal goat anti-human MMEL2 (AF2340, R&D Systems) (1:5000)) and visualized by HRP-conjugated 2°- antibody and chemiluminescence (Lumi-Light^{PLUS}, Roche). *Flow cytometry*: Transfected cells were blocked in PBS + 5% donor goat serum (Gemini Bio-Products, West Sacramento, CA) for 30 minutes (4°C). Cells were then immunostained with a monoclonal mouse anti-human MMEL2 1° antibody (1:100) (MAB2340, R&D Systems) (1 hr, 4°C), and goat anti-mouse-IgG Alexa Fluor 488 2° antibody (1:20) (Invitrogen) (30 min, 4°C) before analysis by flow cytometry. *Secreted A β degrading activity*: Transfected cell culture medium was collected and then mixed (1:1) with A β ₄₀ containing medium and incubated (6hrs) / analyzed by specific ELISA (as above). The data are averages $\pm$ S.E.M. (n=3, **p<0.01).