（ 続紙 1 ）

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論文題目	EDEM2 stably disulfide-bonded to TXNDC11 catalyzes the first mannose trimming step in mammalian glycoprotein ERAD （哺乳動物の構造異常糖タンパク質分解におけるマンノーストリミングの第一ステップはTXNDC11と安定なジスルフィド結合を形成したEDEM2により触媒される）		

（論文内容の要旨）

In the endoplasmic reticulum (ER), misfolded proteins that cannot be brought back to their native state are recognized, ubiquitylated and targeted to the cytoplasm for proteasomal degradation. These processes are collectively termed ER-associated degradation (ERAD). The ERAD pathway deals with different substrates; hence, depending on the type of misfolded lesion, ERAD is classified into three pathways, ERAD-L (ERAD of substrates with misfolded lesions within the ER lumen), ERAD-C (cytoplasm), or ERAD-M (membrane). ERAD-L deals with the clearance of both glycosylated (gpERAD-L) and non-glycosylated (non-gpERAD-L) misfolded proteins from the lumen. The $Glc_3Man_9GlcNAc_2$ sugar moiety is attached to the majority of nascent polypeptides that are translocated into the ER lumen at an Asn residue, which is not only important for ensuring the proper topology, but is also essential for their ERAD if they are unable to gain their native conformation. During the folding process of glycoproteins, the terminal three glucose residues are trimmed by glucosidase I and II sequentially to produce $Man_9GlcNAc_2$. In higher eukaryotes, the UDP-glucose glucosyltransferase (UGGT) repeatedly adds a glucose residue to $Man_9GlcNAc_2$. The mono-glucosylated glycoprotein is specifically recognized by two lectin chaperones, calnexin and calreticulin, and retained in the ER until it gets fully folded. In gpERAD, the recognition of misfolded glycoproteins requires this N-linked glycan ($Man_9GlcNAc_2$) and protein determinant. The recognition of misfolded glycoproteins in yeast gpERAD has been extensively studied. In this process, sequential mannose trimming from $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ to $Man_7GlcNAc_2$ plays a crucial role. Briefly, Mns1p, an α1,2-mannosidase, catalyzes the first step, conversion of $Man_9GlcNAc_2$ (M9) to $Man_8GlcNAc_2$ isomer B (M8B); and α1,2-mannosidase Htm1p catalyzes the second step, conversion of M8B to $Man_7GlcNAc_2$ isomer A (M7A) with the α1,6-mannose exposed. M7A oligosaccharides are recognized by lectin Yos9p for subsequent retro-translocation to the cytoplasm and degradation. In mammalian ER, ER mannosidase I (ERmanI) is considered to be the sole homolog of Mns1. In contrast, Htm1p has 3 homologues,

namely EDEM1, EDEM2, and EDEM3. According to the previous model, the mammalian gpERAD is initiated by ERmanI, which converts M9 to M8B. EDEM1 or EDEM3 then carry out the second step, M8B to M7 conversion. However, gene knockout studies of the mammalian homologues in chicken DT40 cells and human HCT116 cells indicated that EDEM2 is required for the trimming of M9 to M8B, and EDEM3 (primarily) and EDEM1 (partly) are required for the trimming of this M8B to M7A. M7A is further recognized by lectin proteins OS9 and XTP3B for retro-translocation to the cytoplasm. However, the confirmation that these homologues as true mannosidases can be achieved by checking their mannosidase activity in vitro. It was previously reported that recombinant EDEM2 purified from HEK293 cells showed no mannosidase activity towards pyridylamine (PA)-tagged M9 or PA-tagged M8 in vitro. Hence, the mannosidase activity of EDEM2 remains in debate. Interestingly, Htm1p is stably disulfide-bonded to Pdi1p, a protein disulfide isomerase, via the C-terminal domain. The association with Pdi1p leads to introduction of a disulfide bond between C65 and C445 in the mannosidase homology domain to function as a mannosidase. The Htm1p-Pdi1p complex is able to convert M8B to M7 in vitro.

My present study showed the vital role of the three cysteines (C65, C408 and C558) in EDEM2 for its mannosidase activity. Among these cysteines, C65 and C408 in the mannosidase homology domain are strictly conserved in EDEM1, EDEM3 and yeast Htm1p. Both mutations of C65 and C408 to alanine lead to the defects in EDEM2 mannosidase activity and degradation of ERAD substrate CD3-d-TM-HA, suggesting that C65 and C408 are likely to be linked by intramolecular disulfide bonding. I found that EDEM2 was stably disulfide-bonded to TXNDC11, an endoplasmic reticulum protein containing five thioredoxin (Trx)-like domains. C558 present outside of the mannosidase homology domain of EDEM2 was linked to C692 in Trx5, which solely contains the CXXC motif in TXNDC11. The C558A mutant of EDEM2 and the C692S mutant of TXNDC11 did not rescued the defects in mannose trimming and degradation of CD3-d-TM-HA in EDEM2-KO and TXNDC11-KO cells, respectively. TXNDC11 was consistently detected as a doublet band after SDS-PAGE. By site directed mutagenesis, I found that this doublet band is due to alternative translation initiation from M58 residue. Centrifugal fractionation after repeated freezing and thawing of cells indicated that the M58A mutant was a membrane protein like calnexin, whereas a majority of the M1A mutant was a calreticulin-like soluble protein. Gene knockout studies of TXNDC11 clearly indicated the importance of a stable heterodimer association between TXNDC11 and EDEM2; disruption of TXNDC11 gene lead to the defect in EDEM2 mannosidase activity, and degradation of gpERAD substrates such as ATF6α and CD3-d-TM-HA is significantly delayed in TXNDC11-KO cells. EDEM2 showed altered protein conformation in the absence of its stable association with TXNDC11. Most

importantly, I successfully purified the EDEM2-TXNDC11 complex and the complex extensively converted PA-M9 to PA-M8 in vitro. The further mannose isomer analysis showed that the M8 produced by EDEM2 was M8B.

The present study clearly demonstrates the mannosidase activity of EDEM2 towards free oligosaccharides in vitro, and this effect is even clearer than the case of yeast Htm1p. Hence, the present study represents the first clear demonstration of in vitro activity amongst EDEMs family proteins. The notion that EDEM2 catalyzes the first mannose trimming step and thereby initiates gpERAD is now firmly supported by both genetic and biochemical analyses.

（続紙　２　）

（論文審査の結果の要旨）

構造異常糖タンパク質の小胞体関連分解（構造異常糖タンパク質を小胞体から細胞質に引きだし、ユビキチン化してプロテアソームで分解するシステム）において、Ｎ型糖鎖上のマンノースのトリミング（刈り込み，マンノース９個→８個→７個）が鍵となる。マンノース７個になると隠れていたα1,6 結合が露出し、これを認識するレクチン分子によって分解へと運命決定されるからである。

遺伝学的解析（遺伝子ノックアウト細胞の解析）によって、９個→８個の刈り込みにはEDEM2が、８個→７個の刈り込みには主としてEDEM3が、部分的にEDEM1が必要であることが示されていた。しかし、精製したEDEM2はマンノシダーゼ活性を示さないという報告と矛盾が生じていた。

本研究によって、EDEM2はTXNDC11という小胞体局在性のチオレドキシン・タンパク質と安定なジスルフィド結合を形成していること、この複合体形成が９個→８個の刈り込みおよび構造異常糖タンパク質の小胞体関連分解に必要であること、TXNDC11が９個→８個の刈り込みおよび構造異常糖タンパク質の小胞体関連分解に必要であること、精製したEDEM2・TXNDC11複合体が試験管内で９個→８個の刈り込み活性を示すことが明らかになった。

以上のように本論文は、構造異常糖タンパク質の小胞体関連分解において、EDEM2がマンノース刈り込みの第一ステップを触媒するマンノシダーゼとして機能することを明瞭に示した。令和２年３月３１日、論文内容とそれに関連した事項について試問を行った上で、４月１０日に調査委員の間で博士論文および公聴会における発表と質疑応答に関して審議・議論し、博士（理学）に十分に値するものと判断されたので合格と認めた。

要旨公表可能日：　　年　　月　　日以降