TMCC3 localizes at the three-way junctions for the proper tubular network of the endoplasmic reticulum

Wisesa, Sindhu

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学 位 論 文 の 内 容 要 旨

TMCC3 localizes at the three-way junctions for the proper tubular network of the endoplasmic reticulum

TMCC3 は小胞体の適切なチューブネットワークのために three-way junction に局在する

神戸大学大学院医学研究科医科学専攻
膜動態学
(指導教員：匂坂 敏朗 教授)

SINDHU WISESA

Introduction

The tubular network of the endoplasmic reticulum (ER) is formed by connecting ER tubules through three-way junctions. Atlastins and lunapark, two classes of the ER membrane proteins conserved from yeasts to mammals, have been shown to be involved in the generation and stabilization of the three-way junctions. Atlastins are dynamin-like GTPases anchored on the ER tubules. GTP-bound atlastins dimerize *in trans* through their GTPase domains and form a bridge between the tip of an ER tubule and the side of another one. Upon GTP hydrolysis, atlastins catalyze homotypic membrane fusion between the two ER tubules, thereby generating a new three-way junction. The nascent three-way junctions are stabilized by lunapark. Upon oligomerization, lunapark localizes to the nascent three-way junctions where atlastins reside and preferentially sits on their concave edges, leading to stabilization of the negative curvature of the three-way junctions. However, it remains unknown whether mammalian cells employ an additional ER membrane protein(s) specific to higher eukaryote to shape the three-way junctions.

The testis-expressed 28 (TEX28) family is a higher eukaryote-specific membrane protein family that composed of the four family members, transmembrane and coiled-coil domain family (TMCC) 1, TMCC2, TMCC3, and TEX28. Several studies have suggested that TMCC1 and TMCC2 localize at the ER and are associated with some cellular processes. TMCC3 also localizes at the ER and has been suggested to bind to 14-3-3 proteins, the multifunctional cytosolic proteins involved in the regulation of various cellular processes. In this study, we report TMCC3 as another ER membrane protein that shapes the three-way junctions in mammalian cells.

Results

1. TMCC3 localizes at the three-way junctions.

In this study, we set out to compare the ER localization between the TEX28 family members. HA-tagged TEX28 family members were transfected into human osteosarcoma U2OS cells along with GFP-tagged Sec61β, an ER membrane marker protein, followed by immunostaining. While HA-TMCC1, HA-TMCC2, and HA-TEX28 were uniformly distributed throughout the reticular network of the ER, HA-TMCC3 was concentrated at the three-way junctions. These results indicate that among TEX28 family members, TMCC3 specifically localizes at the three-way junctions in the peripheral ER.

2. TMCC3 binds to atlastins.

We examined whether TMCC3 colocalized with atlastins and lunapark. HA-TMCC3 was transfected into U2OS cells along with FLAG-tagged atlastin-1, Myc-tagged atlastin-2, FLAG-tagged lunapark, and GFP-Sec61β, followed by immunostaining. HA-TMCC3 colocalized with FLAG-atlastin-1, Myc-atlastin-2, and lunapark-FLAG at the three-way junctions. We next examined whether TMCC3 bound to atlastins and lunapark by coimmunoprecipitation analysis. Endogenous TMCC3 was immunoprecipitated with the anti-TMCC3 mAb from the U2OS cells extract. Endogenous atlastin-1 and atlastin-2 but not endogenous lunapark were coimmunoprecipitated with endogenous TMCC3. These results indicate that TMCC3 binds to atlastins at the three-way junctions *in vivo*.

3. The C-terminal transmembrane domains of TMCC3 are responsible for binding to atlastins.

We sought to determine which region of TMCC3 was responsible for binding to atlastins. The primary structure of TMCC3 is characterized by the long N-terminal cytoplasmic region encompassing two coiled-coil domains and the C-terminal transmembrane domains. HA-tagged N-terminal cytoplasmic region of TMCC3 (HA-TMCC3-N), HA-tagged C-terminal transmembrane domain of TMCC3 (HA-TMCC3-C), or HA-TMCC3 was transfected into HEK293 cells along with FLAG-atlastin-1 or Myc-atlastin-2, followed by the coimmunoprecipitation analysis. FLAG-atlastin-1 was coimmunoprecipitated with HA-TMCC3-C and HA-TMCC3 but not HA-TMCC3-N. Myc-atlastin-2 was coimmunoprecipitated with HA-TMCC3-C and HA-TMCC3, and to a lesser extent with HA-TMCC3-N. These results indicate that TMCC3 binds to atlastins through the C-terminal transmembrane domains.

We examined whether TMCC3 was localized to the three-way junctions by binding to atlastins. HA-TMCC3-N or HA-TMCC3-C was transfected into U2OS cells along with GFP-Sec61β, followed by immunostaining. HA-TMCC3-C but not HA-TMCC3-N localized at the ER. HA-TMCC3-C was distributed throughout the tubular ER and not concentrated at the three-way junctions, although TMCC3-C has the potency to bind to atlastins as described above. These results indicate that TMCC3 localizes independently of binding to atlastins.

4. The first coiled-coil domain of TMCC3 is required for its localization to the three-way junctions.

We examined whether the coiled-coil domains in the N-terminal cytoplasmic region were required for localization to the three-way junctions. HA-tagged TMCC3 mutant lacking the first coiled-coil domain (HA-TMCC3-ΔCC1) or that lacking the second coiled-coil domain (HA-TMCC3-ΔCC2) was transfected into U2OS cells along with GFP-Sec61β, followed by immunostaining. While HA-TMCC3-ΔCC2 localized at a subpopulation of the three-way junctions, HA-TMCC3-ΔCC1 was distributed throughout the tubular ER and not concentrated at the three-way junctions. These results indicate that the first coiled-coil domain of TMCC3 is required for its localization to the three-way junctions.

5. TMCC3 is involved in the regulation of the tubular ER network.

We examined whether TMCC3 was involved in the regulation of the tubular ER network. Three independent siRNAs targeting *TMCC3 or control siRNA* were transfected into U2OS cells, followed by the transfection with GFP-Sec61β. In the control siRNA-transfected cells, GFP-Sec61β showed reticular signal indicative of the tubular ER network in the peripheral area and a dense signal indicative of the ER sheets in the perinuclear area. *TMCC3* knockdown cells reduced the peripheral reticular signal and showed the dense signal in the peripheral area. When we counted the number of three-way junctions of the peripheral ER, *TMCC3* knockdown cells significantly decreased the number of three-way junctions relative to the control siRNA-transfected cells. We next examined the effect of *TMCC3* knockdown on the abundance of ER sheets. The siRNAs targeting *TMCC3 or control siRNA* were transfected into U2OS cells, followed by immunostaining with anti-CLIMP-63 pAb, an ER sheet marker. In the control siRNA-transfected cells, CLIMP-63 localization was restricted predominantly to the perinuclear area where the ER sheets were enriched. On the other hand, CLIMP-63 localization was aberrantly extended to the peripheral area in the *TMCC3* knockdown cells. These results indicate that *TMCC3* knockdown causes decrease in the number of three-way junctions and expansion of ER sheets, leading to reduction of the tubular ER network.

6. Overexpression of atlastin-2 partially rescues the phenotype caused by *TMCC3* knockdown.

Binding of TMCC3 to atlastins prompted us to examine whether overexpression of atlastins rescued the phenotype caused by *TMCC3* knockdown. Myc-atlastin-2 or the control vector was transfected into the *TMCC3* knockdown cells along with GFP-Sec61β, followed by immunostaining. *TMCC3* knockdown cells transfected with Myc-atlastin-2 exhibited more extensive peripheral reticular ER network as judged from GFP-Sec61β than the *TMCC3* knockdown cells transfected with the control vector. When we counted the number of three-way junctions of the peripheral ER, *TMCC3* knockdown cells transfected with Myc-atlastin-2 significantly increased the number of three-way junctions relative to the *TMCC3* knockdown cells transfected with the control vector. These results indicate overexpression of atlastin-2 restored the tubular ER network impaired by *TMCC3* knockdown to some extent. We next assessed how much overexpression of atlastin-2 restored the tubular ER network. The tubular ER network in the *TMCC3* knockdown cells transfected with Myc-atlastin-2 was less extensive than that in the control siRNA-transfected cells. The number of three-way junctions *in TMCC3* knockdown cells transfected with Myc-atlastin-2 was lower than in the control siRNA-transfected cells. Overall, these results indicate that *TMCC3* knockdown phenotype is partially rescued by the overexpression of atlastin-2.

Conclusion

This study demonstrates that mammalian cells localize TMCC3 to the three-way junctions for the proper tubular ER network. TMCC3 acts upstream of atlastins for three-way junction formation, suggesting that mammalian cells will have a special mechanism to regulate actions of atlastins by TMCC3.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

受付番号	甲 第2983号	氏名	SINDHU WISESA
論文題目 Title of Dissertation	TMCC3 localizes at the three-way junctions for the proper tubular network of the endoplasmic reticulum TMCC3 は小胞体の適切なチューブネットワークのために three-way junction に局在する		
審査委員 Examiner	主査 Chief Examiner 南 康博 副査 Vice-examiner 中村俊一 副査 Vice-examiner 勝二郁夫		

（要旨は１，０００字～２，０００字程度）

（目的）
小胞体はチューブ状の脂質膜とシート状の脂質膜が組み合わさった細胞内小器官であり、タンパク質合成、脂質合成、小胞輸送、カルシウム貯蔵等の生命の恒常性維持に必須の役割を担う。チューブ状の小胞体は three-way junction と呼ばれる部位で連結し、複雑な網目状のネットワークを細胞質全体に構築している。Three-way junction の形成には膜融合タンパク質 atlastin と膜変形タンパク質 lunapark が必須の働きをしていることが明らかにされているが、その制御機構については依然として不明な点が多い。本研究では、機能未知の小胞体膜タンパク質ファミリーである TEX28 ファミリーに着目し、ファミリーメンバーの１つである TMCC3 の性状解析を通じて、three-way junction の形成を制御する分子機構の解明をおこなった。

（方法）
・ TEX28 ファミリーの細胞内局在の解析
U2OS 細胞に HA タグをつけた TEX28 ファミリーメンバーと GFP-Sec61β（小胞体マーカー膜タンパク質）をトランスフェクションし、抗 HA 抗体で免疫染色することで、細胞内局在を解析した。内在性の TMCC3 は抗 TMCC3 抗体で免疫染色することで解析した。

・ 免疫沈降実験
U2OS 細胞の TritonX-100 抽出液に抗 TMCC3 抗体を加えて免疫沈降した。免疫沈降サンプルを抗 TMCC3 抗体、抗 atlastin 抗体、抗 lunapark 抗体で Western blot し、TMCC3 と atlastin 及び lunapark の共沈降を検討した。

・ TMCC3 ノックダウン細胞の解析
U2OS 細胞に TMCC3 siRNA をトランスフェクションして TMCC3 ノックダウン細胞を調製した。TMCC3 ノックダウン細胞に GFP-Sec61β をトランスフェクションし、GFP 蛍光を観察することで、小胞体のチューブネットワークと three-way junction 数に与える効果を解析した。TMCC3 ノックダウン細胞を抗 CLIMP-63（シート状小胞体マーカータンパク質）抗体で免疫染色し、シート状小胞体に与える効果を解析した。TMCC3 ノックダウン細胞に GFP-Sec61β と Myc-atlastin-2 または lunapark-HA をトランスフェクションし、GFP 蛍光を観察することで、atlastin-2 または lunapark が TMCC3 ノックダウン細胞をレスキューできるかを検討した。

（結果）
1. TMCC3 の three-way junction への局在
TEX28 ファミリーメンバーである TMCC1, TMCC2, TMCC3, TEX28 を U2OS 細胞にトランスフェクションしたところ、TMCC3 のみが three-way junction に濃縮して局在し、その他のメンバーは小胞体全体に一様に局在した。内在性の TMCC3 も同様に three-way junction に濃縮して局在した。

2. TMCC3 と atlastin の結合
U2OS 細胞から TMCC3 を免疫沈降したところ、atlastin-1 と atlastin-2 が共沈降した。他方、lunapark は共沈降しなかった。

3. TMCC3 ノックダウンによる three-way junction の減少
TMCC3 ノックダウン細胞では小胞体のチューブネットワークが障害され、three-way junction の数が減少した。

4. TMCC3 ノックダウンによるシート状小胞体の増大
シート状小胞体の局在は核周辺に限局するが、TMCC3 ノックダウン細胞ではシート状小胞体が細胞辺縁部にまで広がり、シート状小胞体の量が増加した。

5. Atlastin-2 の過剰発現による TMCC3 ノックダウン細胞のレスキュー
TMCC3 ノックダウン細胞に atlastin-2 を過剰発現すると、小胞体のチューブネットワーク障害が部分的に回復し、three-way junction の数が増加した。他方、lunapark の過剰発現にはこのような回復効果はなかった。

（結論）
TMCC3 は three-way junction に局在し、atlastin と物理的かつ機能的に相互作用することで three-way junction の形成ひいては小胞体のチューブネットワーク形成を制御していることが明らかとなった。

本研究は、three-way junction に局在する新しい小胞体膜タンパク質として TMCC3 を解析したものである。その結果、TMCC3 が膜融合タンパク質 atlastin と協調して three-way junction の形成ひいては小胞体のチューブネットワーク形成を制御していることを証明した点において、価値ある業績であると認める。よって、本研究者は博士（医学）の学位を得る資格があると認める。