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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

細胞膜を構成するリン脂質は非対称的に分布している。この非対称性はアポトーシス細胞や活性化したリンパ球などで崩壊し、通常は細胞膜の内側に存在するフォスファチジルセリン(PS)が細胞膜の外側に露出される。この過程にはリン脂質を区別なく双方向に移層する、スクランブラーゼと呼ばれる分子が働く。Xkr8はアポトーシス時にカスパーゼ依存的に活性化するスクランブラーゼとして同定されたが、マウスプロB細胞株においては生きた状態でも活性化することが知られていた。

申請者らは、生きた細胞においてXkr8がリン酸化依存的に活性化されることを示した。また、質量分析によりXkr8のリン酸化部位を同定し、変異体の解析によってカスパーゼによる活性化とリン酸化による活性化が独立した機序であることを示した。

以上の研究は、リンパ球や腫瘍細胞が生きた状態でPSを露出する現象の理解につながる可能性を持っており、医学の発展に貢献するものである。

したがって、本論文は学位に値するものと認める。

論　文　内　容　の　要　旨
Synopsis of Thesis

氏　名 Name	櫻木 崇晴
論文題名 Title	Phosphorylation-mediated activation of mouse Xkr8 scramblase for phosphatidylserine exposure （リン酸化依存的マウスXkr8スクランブラーゼ活性化によるフォスファチジルセリン露出）

論文内容の要旨

〔目　的(Purpose)〕

Phospholipids are asymmetrically distributed between the outer and inner leaflets of the plasma membrane, and phosphatidylserine (PtdSer) is exclusively localized in the inner leaflet. PtdSer is exposed to the cell surface in various biological processes, such as apoptosis, blood clotting and activation of lymphocytes. The exposure of PtdSer is regulated by the down-regulation of flippases and the activation of scramblases, which nonspecifically and bidirectionally transport phospholipids between the leaflets. Xkr8 has been identified as a scramblase that is activated during apoptosis, but its exogenous expression in the mouse Ba/F3 pro B cell line induces constitutive PtdSer exposure. The purpose of this study is to elucidate how Xkr8 is activated independently from cleavage by caspase.

〔方法ならびに成績(Methods/Results)〕

TMEM16F$^{-/-}$Ba/F3 expressing mouse (m) Xkr8 (*16F*$^{-/-}$BaF-Xkr8) were incubated with Cy5-Annexin V or NBD-PC at 4°C or 20°C and analyzed by flow cytometry. The transformants exposed PtdSer at 4°C but not at 20°C, although they incorporated NBD-PC at 20°C, suggesting that mXkr8 in Ba/F3 constitutively scrambles phospholipids, but the PtdSer exposure is blocked by flippases at 20°C. When *16F*$^{-/-}$BaF-Xkr8 were pretreated with staurosporine (STS), a broad kinase inhibitor, PtdSer exposure was blocked. In contrast, PtdSer exposure was observed even at 20°C after treatment with phosphatase inhibitors such as pervanadate (PV) or calyculin A. In line with this, pre-treatment with STS significantly inhibited the NBD-PC incorporation, while PV and calyculin A enhanced it, indicating that Xkr8-mediated phospholipid scrambling is regulated by phosphorylation.

To identify the phosphorylation sites, mXkr8 protein was purified from *16F*$^{-/-}$BaF-Xkr8 cells that had been treated with PV, digested with AspN or chymotrypsin, and analyzed by LC-MS/MS. These analyses revealed three phosphorylation residues (Thr-356, Ser-361, and Thr-375) downstream of the caspase recognition site of mXkr8. To confirm these residues were the targets for phosphorylation, they were mutated to alanine, and the mutant protein (S/T-3A) was introduced into *Xkr8*$^{-/-}$*16F*$^{-/-}$Ba/F3 cells. Transformants expressing S/T-3A mutant mXkr8 did not expose PtdSer, while mXkr8 in which two aspartate residues in the caspase recognition site were mutated still supported constitutive PtdSer exposure, indicating that the phosphorylation-induced PtdSer exposure did not require caspase. On the other hand, *Xkr8*$^{-/-}$*16F*$^{-/-}$Ba/F3 expressing phosphomimic mutant mXkr8, in which the three phosphorylation sites were mutated to aspartate (S/T-3D), exposed PtdSer at 4°C after treatment with STS.

Unlike phosphatase inhibitors, however, S/T-3D mutant did not support PtdSer exposure at 20°C. To examine the possibility that flippases were inhibited by phosphorylation, the flippase activity was assayed by measuring the incorporation of NBD-PS. Pre-treatment with STS had little effect on the flippase activity, but PV or calyculin A strongly inhibited the incorporation of NBD-PS. To confirm the effect of the flippases on Xkr8-mediated PtdSer exposure, mXkr8 were introduced into wild-type W3 cells or *ATP11A*$^{-/-}$*ATP11C*$^{-/-}$*TMEM16F*$^{-/-}$W3 (TKO-W3) cells, in which two flippases expressed in W3 cells (ATP11A and 11C) were knocked out. W3 cells expressing mXkr8 did not support PtdSer exposure at 15°C, but TKO-W3 cells expressing mXkr8 exposed PtdSer, indicating that the flippase activity normally counteracted Xkr8's ability to expose PtdSer.

〔総　括(Conclusion)〕

mXkr8 scramblase can be activated by phosphorylation independently from the caspase-mediated cleavage. PtdSer exposure to the cell surface can occur by the phosphorylation-mediated activation of Xkr8 and flippase down-regulation.