Identification of KRAP-expressing cells and the functional relevance of KRAP to the subcellular localization of IP$_3$R in the stomach and kidney

TAKAHIRO FUJIMOTO$^{1,2}$ and SENJI SHIRASAWA$^{1,2}$

$^1$Department of Cell Biology, Faculty of Medicine and $^2$Central Research Institute for Advanced Molecular Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

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Abstract. KRAS-induced actin-interacting protein (KRAP), originally identified as one of the deregulated genes expressed in colorectal cancer, participates under physiological conditions in the regulation of systemic energy homeostasis and of the exocrine system. We have recently found that KRAP is a molecule associated with inositol 1,4,5-trisphosphate receptor (IP$_R$) and is critical for the proper subcellular localization of IP$_R$ in the liver and the pancreas. However, the expression of KRAP and its precise function in other tissues remain elusive. In this study, we aimed to identify the KRAP-expressing cells in mouse stomach and kidneys and to examine the relevance of KRAP expression in the regulation of IP$_R$ localization in these tissues. In the stomach, double immunohistochemical staining for KRAP and IP$_R$ demonstrated that KRAP was expressed along with the apical regions in the mucous cells and the chief cells, and IP$_R$3 was dominantly co-localized with KRAP in these cells. Furthermore, IP$_R$2 was also co-localized with IP$_R$3 in the chief cells. It is of note that the proper localization of IP$_R$3 and IP$_R$2 in the chief cells and of IP$_R$3 in the mucous cells were significantly abrogated in KRAP-deficient mice. In the kidneys, KRAP was expressed in both the apical and the basal regions of the proximal tubular cells. Intriguingly, KRAP deficiency abrogated the localization of IP$_R$1 in the proximal tubular cells. Finally, co-immunoprecipitation study in the stomachs and the kidneys validated the physical association of KRAP with IP$_R$s. These findings demonstrate that KRAP physically associates with IP$_R$s and regulates the proper localization of IP$_R$s in the mucous cells and the chief cells of the stomach and in the proximal tubular cells of the kidneys.

Introduction

Three inositol 1,4,5-trisphosphate receptor (IP$_R$) subtypes, IP$_R$1, IP$_R$2, and IP$_R$3, are differentially expressed among tissues (1-5) and function as the Ca$^{2+}$ release channel on endoplasmic reticulum membranes (6-10). IP$_R$3 is regulated by many intracellular modulators, phosphorylation by kinases, and associated proteins (11-15).

KRAS-induced actin-interacting protein (KRAP) was originally identified as one of the deregulated expression gene in the colorectal cancer cell line, HCT116 (16). The previous studies using KRAP-knockout (KRAP-KO) mice demonstrate that KRAP participates in the regulation of systemic energy homeostasis (17) and of exocrine system (18). Among the adult mouse tissues, KRAP is ubiquitously expressed, with high levels in the pancreas, liver, and brown adipose tissues, and KRAP localizes in the restricted apical regions of the liver parenchymal cells and of the pancreatic exocrine acinar cells (19). Our recent findings indicate that KRAP associates with IP$_R$ to regulate its proper subcellular localization in the mouse liver and the pancreas (20) as well as in immortalized cultured cell lines (21). Despite these advances, it remains largely unknown which cell types express KRAP among the other tissues including stomach and kidneys.

Herein, we performed immunohistological analysis and identified the exact KRAP-expressing cells in the stomach and the kidneys, and demonstrated that KRAP plays critical role in the regulation of the precise subcellular localization of IP$_R$ in the mucous and the chief cells of the stomach and in the proximal tubular cells of the kidneys.

Materials and methods

Animals. All animals used in this study were treated in accordance with the guidelines of Fukuoka University. KRAP-knockout mice were generated as described previously (17).

Immunohistochemical staining. Immunohistochemical staining was performed as described previously (19,20). Specific signals were detected by using rabbit polyclonal anti-KRAP antibody (19), mouse monoclonal anti-ZO-1 antibody (ZYMED), mouse monoclonal anti-IP$_R$3 antibody (610313) from BD Transduction...
Laboratories, rabbit polyclonal anti-IP,R2 antibody (AB3000) from Millipore, and rabbit polyclonal anti-IP,R1 antibody (ab5840) from Abcam.

**Immunoprecipitations and western blotting.** Immunoprecipitations and western blotting were performed as described previously (19,20).

**Results**

**Localization of KRAP protein in the adult mouse stomach.** To examine the cellular distribution of KRAP protein in the adult mouse tissues, we performed immunohistochemical staining by using anti-KRAP antibody. In the stomach, strong KRAP immunoreactivity was restricted to the pit regions of gastric glands (Fig. 1A), whereas significant expression of KRAP was not detected in the muscularis mucosae beneath the gastric glands (Fig. 1A, arrows). The specificity of KRAP expression in the stomach was confirmed by using KRAP-KO tissue as a control (Fig. 1B). In the pit region of the gastric gland, where columnar surface mucous cells mainly exist (22), KRAP was localized beneath the apical membranes of the mucous cells (Fig. 1C). In the base region of the gastric glands, where zymogenic chief cells mainly exist, coronal plane of deeper gastric...
glands showed that KRAP was restricted to the apical regions of the chief cells (Fig. 1D, arrowheads), whereas KRAP was not detected in the parietal cells (Fig. 1D, asterisks). The distinction between the chief and the parietal cells was validated by ZO-1 staining as described (23), indicating that KRAP was expressed in the ZO-1-positive chief cells but not in the ZO-1-negative parietal cells (Fig. 1E).

KRAP co-localized with IP3R in the stomach. Since we previously reported that KRAP associates with particular subtypes of IP3R in the liver and the pancreas (20), we examined whether KRAP in the stomach is also co-localized with IP3R. Double-immunostaining of the stomach for KRAP and IP3R revealed that KRAP was co-localized with IP3R3 in the apical regions of both the chief cells (Fig. 2A, arrows) and the mucous cells (Fig. 2B, arrows). Of note, IP3R2 co-existed with IP3R3 in the chief cells (Fig. 2C, arrow) but not in the parietal cells (Fig. 2C, asterisks). Furthermore, IP3R2 was not detected in the mucous cells (Fig. 2D, arrows). These results indicated that KRAP was co-localized with IP3R2 and IP3R3 in the chief cells and with IP3R3 in the mucous cells.

Impaired localization of IP3R in the KRAP-deficient chief cells and the mucous cells. We addressed the functional relevance of KRAP to the proper localization of IP3R by using KRAP-KO mice. IP3R3 was located in the apical region of the chief cells (Fig. 3A, arrow) and of the mucous cells (Fig. 3C, arrows) in the wild-type (WT) mouse stomach, whereas the restricted localization of IP3R3 appeared to be diminished in the KRAP-KO stomach (Fig. 3B, arrow; 3D, arrows). Furthermore, IP3R2 was detected in both the chief cells (Fig. 3E, arrows) and the parietal cells (Fig. 3E, asterisks) in the WT stomach, whereas the localization of IP3R2 in the KRAP-KO stomach was impaired in the chief cells (Fig. 3F, arrows) but not in the parietal cells (Fig. 3F, asterisks). Thus, KRAP plays critical role in the regulation of the proper localization of IP3R2 and IP3R3 in the chief cells and of IP3R3 in the mucous cells.

KRAP expression and its contribution to the localization of IP3R1 in the proximal tubules of the mouse kidney. To examine the cellular distribution of KRAP protein in the adult mouse kidneys, we performed immunohistochemical staining by

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**Figure 2. Colocalization of KRAP with IP3Rs in the chief cells and the mucous cells of the mouse stomach.** (A) Fluorescent confocal images of the base region of gastric glands for KRAP (red), IP3R3 (green), and the merged photo. Arrows indicate the apical membranes of the chief cells. (B) Fluorescent confocal images of the pit region of gastric glands for KRAP (red), IP3R3 (green), and the merged photo. Arrows indicate the apical membranes of the chief cells. (C) Fluorescent confocal images of the base region of gastric glands for IP3R2 (red), IP3R3 (green), and the merged photo. Asterisks and arrow indicate the parietal cells and the apical membranes of the chief cells, respectively. (D) Fluorescent confocal images of the pit region of gastric glands for IP3R2 (red), IP3R3 (green), and the merged photo. Arrows indicate the apical membranes of the mucous cells. Blue, 4',6-diamidino-2-phenylindole (DAPI) staining; scale bar, 25 µm.
using anti-KRAP antibody. The specificities of the signals were validated by comparing the immunoreactivities of WT and KRAP-KO mouse tissues. In the WT kidneys, intense immunoreactivities were observed in the renal proximal tubules (Fig. 4A) but not in the renal distal tubules (data not shown). On the other hand, significant immunoreactive signal was

Figure 3. Impaired localization of IP$_3$Rs in the KRAP-deficient chief cells and the mucous cells. (A and B) Fluorescent confocal images of the base region of gastric glands for IP$_3$R3 (red), F-actin with phalloidin (green), and the merged photo from wild-type (WT) (A) or KRAP-deficient (KO) (B) mice. Asterisks and arrows indicate the parietal cells and the apical membranes of the chief cells, respectively. (C and D) Fluorescent confocal images of the pit region of gastric glands for IP$_3$R3 (red), F-actin (green), and the merged photo from WT (C) or KO (D) mice. Arrows indicate the apical membranes of the mucous cells. (E and F) Fluorescent confocal images of the base region of gastric glands for IP$_3$R2 (red), F-actin (green), and the merged photo from WT (E) or KO (F) mice. Asterisks and arrows indicate the parietal cells and the apical membranes of the chief cells, respectively. Blue, 4',6-diamidino-2-phenylindole (DAPI) staining; scale bar, 25 µm.
not detected in the proximal tubules in the KRAP-KO mice (Fig. 4B). Taken together, these results indicate that KRAP was exactly expressed in the proximal tubules. The proximal tubules were identified by the presence of the brush-border stained with phalloidin (Fig. 4A and B). Immunostaining in the proximal region showed that KRAP was accumulated beneath the brush-border (Fig. 4A, arrowheads) and KRAP was also detected in the basolateral actin bundles (Fig. 4A, arrows). We next examined which subtypes of IP₃R, IP₃R₁, IP₃R₂, and IP₃R₃, expressed in the proximal tubular cells, revealing that IP₃R₁ (Fig. 4C) but not IP₃R₂ or IP₃R₃ (data not shown) was detected in the beneath the brush-border and in the basolateral actin bundles. Finally, we addressed the functional relevance of KRAP expression in the proximal tubular cells to the regulation of IP₃R localization. It is of note that the restricted localization of IP₃R₁ detected in the WT mouse kidney (Fig. 4C) was disturbed in the KRAP-KO mouse kidney (Fig. 4D). Thus, KRAP plays critical role in the regulation of the proper localization of IP₃R₁ in the proximal tubular cells.

**KRAP interacts with IP₃R₁ in the kidneys and with IP₃R₃ in the stomach.** As described above, immunohistochemical signals for particular IP₃R subtypes in the KRAP-KO mouse kidneys or the stomach were abrogated, leading us to check the expression levels of IP₃R between the WT and KRAP-KO mouse tissues. Normal expression levels of IP₃R₁ and IP₃R₃ were detected in the KRAP-KO mouse kidney and the stomach, respectively, compared with the WT mouse tissues (Fig. 5A), suggesting that mislocalizations but not deregulated expressions of IP₃R occur in the KRAP-KO mouse kidneys and the stomach. Next, to examine the physical association of KRAP with IP₃R, we performed co-immunoprecipitations by anti-KRAP antibody in the kidneys or the stomach, in which we could not evaluate the specific association of IP₃R₂ with KRAP due to lack of IP₃R₂-specific antibody available for western blotting. In the preparations from the WT mouse tissues, KRAP precipitates IP₃R₁ and IP₃R₃ in the kidney and the stomach, respectively (Fig. 5B). The specificity of co-immunoprecipitations of IP₃R was confirmed by using KRAP-KO mouse tissue as a control (Fig. 5B). Thus, KRAP
physically interacts with IP$_3$R1 in the kidneys and with IP$_3$R3 in the stomach.

**Discussion**

In this study, we demonstrated that KRAP protein expression and the subcellular localization was restricted beneath the apical and/or basolateral membranes in specific cell types of the stomach and the kidneys, in which KRAP physically associated with particular IP$_3$R subtype(s). In the KRAP-KO mouse stomach and the kidneys, the polarized localization of IP$_3$R was impaired, indicating that KRAP plays critical roles in the regulation of the proper subcellular localization of IP$_3$R in the stomach and the kidneys.

Notably, KRAP as well as IP$_3$R3 proteins were polarized beneath the apical membranes facing the gastric gland lumen and were absent in the parietal cells (Fig. 1), suggesting an association of these proteins with chief cell functions including pepsinogen secretion (22-24). From this view point, KRAP expression and the localization beneath the apical membranes of the pancreatic acinar cells (19), another type of zymogen cells, may suggest a similar role for KRAP in the stomach and the pancreas. Considering the fact that KRAP physically interacts with IP$_3$R to regulate its proper subcellular localization in vivo, further research on the exact relevance of the association between KRAP and IP$_3$R to the biological phenomena will lead to a better understanding of physiological metabolic processes.

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