Expression of Tetraspan Protein CD63 Activates Protein-tyrosine Kinase (PTK) and Enhances the PTK-induced Inhibition of ROMK Channels

Received for publication, July 6, 2007, and in revised form, January 10, 2008 Published, JBC Papers in Press, January 22, 2008, DOI 10.1074/jbc.M705574200

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In the present study, we tested the role of CD63 in regulating ROMK1 channels by protein-tyrosine kinase (PTK). Immunocytochemical staining shows that CD63 and receptor-linked tyrosine phosphatase α (RPTPα) are expressed in the cortical collecting duct and outer medulla collecting duct. Immunoprecipitation of tissue lysates from renal cortex and outer medulla or 293T cells transfected with CD63 reveals that CD63 was associated with RPTPα both in situ and in transfected cells. Expression of CD63 in 293T cells stimulated the phosphorylation of tyrosine residue 416 of c-Src but decreased the phosphorylation of tyrosine residue 527, indicating that expression of CD63 stimulates the activity of c-Src. Furthermore, c-Src was coimmunoprecipitated with RPTPα and CD63 both in 293T cells transfected with CD63 and in lysates prepared from native rat kidney. Potassium restriction had no effect on the expression of RPTPα, but it increased the association between c-Src and RPTPα in the renal cortex and outer medulla. We also used two-electrode voltage clamp to study the effect of CD63 on ROMK channels in Xenopus oocytes. Expression of CD63 had no significant effect on potassium currents in oocytes injected with ROMK1; however, it significantly enhanced the c-Src-induced inhibition of ROMK channels in oocytes injected with ROMK1+c-Src. The effect of CD63 on the c-Src-induced inhibition was not due to a decreased expression of ROMK1 channels, because blocking PTK with herbimycin A abolished the inhibitory effect of c-Src on ROMK channels in oocytes injected with ROMK1+c-Src+CD63. Furthermore, coexpression of CD63 enhanced tyrosine phosphorylation of ROMK1. We conclude that CD63 plays a role in the regulation of ROMK channels through its association with RPTPα, which in turn interacts with and activates Src family PTK, thus reducing ROMK activity.

Tetraspan proteins contain four transmembrane domains and share a common topology as well as a number of highly conserved residues. Members of this large family have been shown to be expressed in a variety of cells, including those of the immune system, epithelial cells, and glial cells (1, 2). They have been demonstrated to regulate numerous diverse cell functions through their interactions with a variety of proteins, including protein kinases, growth factor receptors, and membrane transporters (3–7). Presumably, the physiological functions of a given tetraspan protein are determined in large measure by its repertoire of interacting proteins. Tetraspan proteins such as CD53 and CD63 have been shown to associate with proteins that have tyrosine phosphatase activity (6). Because protein-tyrosine phosphatase (PTP)2 can play a key role in activation of PTK (8), these associations have the potential to regulate critical cellular signaling processes. For instance, activation of RPTPα has been reported to stimulate the activation of c-Src by increasing the phosphorylation of c-Src on tyrosine residue 416 and decreasing its phosphorylation on tyrosine residue 527 (9). Thus, it is conceivable that tetraspan proteins could modulate PTK activity through interaction with PTP. We have previously demonstrated that low potassium intake stimulates Src family PTK activity and expression (10–12). However, the molecular mechanism by which low potassium intake activates PTK was not completely understood. Therefore, the first aim of the present study is to explore the role of CD63 in activating c-Src activity, which can be taken as a representative member of non-receptor types of PTK. Also, CD63 has been shown to interact with adaptor proteins of clathrin-mediated endocytosis (13) and to stimulate the endocytosis of H-K-ATPase by a direct association with H-K-ATPase in the stomach (7). We and others have previously shown that potassium depletion increased the endocytosis of ROMK channels in the CCD (14, 15). Therefore, the second aim of the present study is to examine the role of CD63 in mediating ROMK internalization induced by stimulation of PTK.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—293T cells and HEK293 cells were obtained from American Type Culture Collection (Mannassas, VA). HEK293 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and

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‡ This work was supported by National Institutes of Health Grants DK54983 (to W.H.W.) and DK17432 (to M.C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The abbreviations used are: PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase; RPTP, receptor-linked tyrosine phosphatase; CCD, cortical collecting duct; OM, outer medulla; OMCD, OM collecting duct; GFP, green fluorescent protein; PBS, phosphate-buffered saline; KD, potassium-deficient; PKC, protein kinase C.
293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were transfected at 50–70% confluency with CD63 DNA by FuGENE HD transfection reagent (Roche Applied Science) as described by the manufacturer, and they were maintained for an additional 12 h after transfection. For harvesting, the cells were washed twice with ice-cold phosphate-buffered saline and incubated for 30 min in radioimmuno precipitation assay lysis buffer.

**Animals and Tissue Preparation**—Sprague-Dawley rats (6–8 weeks, either sex) were purchased from Taconic Farms (Germantown, NY). The rats were maintained on either a normal potassium (1.1%) or a potassium-deficient (KD) diet for 7 days. The animals (<90 g) were killed by cervical dislocation, and the kidneys were removed immediately. The animal use protocol was approved by the independent animal use committee of New York Medical College. The renal cortex and OM were dissected and suspended in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40 (pH 8.0), and protease inhibitor mixture (1%) (Sigma) was added to the lysis buffer. The tissues were then homogenized and allowed to sit on the ice for an additional 30 min. The tissue sample was subjected to centrifugation at 13,000 rpm for 8 min at 4 °C, and protein concentrations were measured in duplicate using a Bio-Rad Dc protein assay kit (Bio-Rad).

**Preparation of Xenopus Oocytes**—Xenopus laevis females were obtained from NASCO (Fort Atkinson, WI). The method for obtaining oocytes has been described previously (16). Viable oocytes were selected for injection with different cRNAs. The oocytes were incubated at 19 °C in a 66% Dulbecco's modified Eagle's medium/F-12 medium with freshly added 2.5 mM sodium pyruvate and 50 μg/ml gentamycin. The experiments were performed on days 1 and 2 after injection with two-electrode voltage clamp.

**Two-electrode Voltage Clamping**—A Warner oocyte clamp OC-725C was used to measure the whole cell potassium current. Voltage and current microelectrodes were filled with 1000 mM KCl and had resistance of less than 2 MΩ. The current was recorded on a chart recorder (Gould TA240). To exclude leak currents, 2 mM Ba2+-sensitive Ba2+ was used to determine the Ba2+-sensitive K+ current.

**Confocal Microscope**—Surface fluorescence detected by confocal microscopy at the equatorial plane of oocytes expressing GFP-tagged ROMK correlates with channel activity and has been used by us to assess channel expression in the plasma membrane (17). Briefly, GFP fluorescence was excited at 488 nm with an argon laser beam and viewed with an inverted Olympus FV300 confocal system equipped with a ×10 oil lens. Scion image software (Scion Co., Frederick, MA) was used to determine the fluorescence intensity. All of the images were acquired and processed with identical parameters.

**Immunoprecipitation and Western Blot**—For immunoprecipitation, 500 μg of protein was mixed with 4 μg of the relevant antibody and incubated for 12 h at 4 °C. Protein A affinity gel was then added and mixed at 4 °C for additional 2 h. The immunoprecipitates were washed three times with PBS. For Western blot, the proteins were separated by electrophoresis on 8–10% SDS-polyacrylamide gels and transferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with Odyssey blocking buffer and incubated with the primary antibody at 4 °C for 12 h. The membrane was washed four times for 5 min with PBS containing 0.1% Tween 20 and followed by incubation with the secondary antibody for additional 30 min. The membrane was then washed several times and scanned by an Odyssey infrared imaging system (LI-COR, Lincoln, NE) at wavelength of 700–800 nm.

**Immunostaining**—The kidneys were perfused with 50 ml of PBS containing heparin (40 unit/ml) followed by 200 ml of 4% paraformaldehyde and were fixed with 4% paraformaldehyde for 12 h. A Leica1900 cryostat was used to cut kidney slices that were dried at 42 °C for 1 h. After washing with 1× PBS, the samples were permeabilized with 0.4% Triton dissolved in 1× PBS buffer containing 1% bovine serum albumin and 0.1% lysine (pH 7.4) for 15 min. The kidney slices were blocked with 2% goat serum for 1 h at room temperature and then incubated with antibodies to ROMK, CD63, and RPTPα for 12 h at 4 °C. The slides were washed with PBS buffer followed by incubation with second antibody for 2 h at room temperature.

**Experimental Materials and Statistics**—Antibodies for RPTPα, AQP2, and CD63 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-Src (Tyr(P)416) and the phospho-Src(Tyr(P)527) antibodies were obtained from Sigma. Anti-phosphotyrosine antibody (4G10), c-Src antibody, and ROMK were obtained from Upstate and Almone (Jerusalem, Israel), respectively. As a negative control, we have also used goat anti-rabbit IgG (Molecular Probes) and goat anti-mouse IgG antibodies (Rockland, Gilbertville, PA). Protein A affinity gel was purchased from Sigma. CD63 was cloned into pcDNA3.1 vector using the BamHI and Xhol sites. RPTPα cDNA was a gift from Dr. Michael P. Sheetz at Columbia University (18). The data are presented as the means ± S.E. We used one way analysis of variance or paired Student’s t test to determine the statistical significance. If the p value is less than 0.05, the difference is considered to be significant.

**RESULTS**

We first used confocal microscopy to examine the expression of CD63 in the kidney. Fig. 1A is an immunocytochemical staining showing that CD63 is widely expressed in both cortex and medulla. To examine whether CD63 is expressed in the ROMK-positive tubules such as thick ascending lim, we conducted the double staining with ROMK and CD63 antibodies. Fig. 1B is a confocal images in OM region showing that CD63 is highly positive in the tubules where ROMK channels are expressed. To demonstrate that CD63 is expressed in the CCD and OMCD, we carried out double staining with CD63 and AQP2 antibodies. From inspection of Fig. 1 (C and D), it is apparent that CD63 is expressed in the CCD (Fig. 1C) and OMCD (Fig. 1D) as indicated by AQP2-positive staining. Fig. 1C has also shown that CD63 is expressed in some proximal tubules. CD63 has been shown to associate with proteins that have tyrosine phosphatase activity (19). Thus, we next examined whether CD63 is associated with tyrosine phosphatases such as RPTPα, which has been shown to be expressed in several nephron segments including thick ascending limb and CCD (20). We first examined whether RPTPα protein is expressed in the collecting duct. Fig. 1 demonstrates that RPTPα is expressed in
We then tested the possibility that CD63 may be associated with RPTPα by performing immunoprecipitation experiments. There are two forms of RPTPα with molecular masses of 100 and 130 kDa. The 100-kDa RPTPα represents phosphatase that is exclusively N-glycosylated, whereas the 130-kDa RPTPα contains both N- and O-linked carbohydrates. However, both forms exhibit similar enzymatic activities (21). Western blot detected only a 100-kDa form of RPTPα in the renal cortex and OM (Fig. 2A). We performed immunoprecipitations from tissue lysates of renal cortex and outer medulla using CD63 antibody. The immunoprecipitated proteins were resolved by electrophoresis and probed by Western blotting with RPTPα antibody. Fig. 2A shows a Western blot demonstrating that a 100-kDa protein band was detected by RPTPα antibody in the CD63 immunoprecipitates, suggesting that RPTPα was associated with CD63. This possibility was also supported by the experiments in which immunoprecipitation of tissue lysate with antibody directed against RPTPα was able to pull down CD63. Fig. 2B shows a Western blot demonstrating that CD63 protein was coimmunoprecipitated with RPTPα antibody. These results were not obtained in nonimmune IgG control immunoprecipitations. Therefore, our results indicate that CD63 interacts with RPTPα in the rat kidney. We then extended the study by examining the interaction between CD63 and RPTPα in 293T cells transfected with CD63. The cells were harvested and lysed 24 h after transfection, which was followed by immunoprecipitation of the cell lysate with antibody directed against RPTPα. As shown in Fig. 2C, CD63 protein was also immunoprecipitated with RPTPα antibody from the transfected 293 cells.

Because RPTPα has been shown to activate Src family PTK (22), we speculated that expression of CD63 might stimulate the activity of PTK through its association with RPTPα. This hypothesis was tested by examining the phosphorylation of c-Src on tyrosine residues 416 and 527, respectively. It is well established that increased c-Src activity is associated with an enhanced phosphorylation of tyrosine residue 416 or decreased phosphorylation of tyrosine residue 527 (23). Fig. 3A is a Western blot showing the effect of CD63 expression on the phosphorylation of tyrosine residues 416 (Fig. 3A) and 527 of c-Src (Fig. 3B). The data summarized in Fig. 3C show that the expression of CD63 significantly enhanced the phosphorylation of tyrosine residue 416 (2.5 ± 0.5-fold over the control, n = 5), whereas it decreased the phosphorylation of tyrosine residue 527 by 35 ± 5% (n = 4, p < 0.05).
After showing that expression of CD63 stimulates c-Src activity, we then explored the possibility that CD63 stimulates the activity of c-Src through its association with RPTPα, which could activate c-Src by dephosphorylating tyrosine residue 527. Thus, we immunoprecipitated the lysates of 293T cells transfected with CD63 to determine whether RPTPα is able to associate with c-Src. Fig. 4A is a Western blot showing that c-Src was coimmunoprecipitated with RPTPα from 293T cells transfected with CD63. To explore the possibility that CD63, RPTPα, and c-Src form a triplex, we conducted immunoprecipitation to determine whether c-Src is also associated with CD63 in 293T cells transfected with CD63. Fig. 4B is a Western blot demonstrating that CD63 is also coimmunoprecipitated with c-Src.

We then examined whether CD63 is also associated with c-Src and RPTPα in the rat kidney. The tissue lysates from renal cortex and outer medulla (mixture) were immunoprecipitated with RPTPα antibody, followed by electrophoresis. Fig. 4C is a Western blot showing that c-Src was clearly coimmunoprecipitated with RPTPα in the renal cortex and OM (mixture) of rats fed a potassium-deficient diet, which stimulates the tyrosine phosphorylation of ROMK channels (24). In addition, immunoprecipitation of renal tissue with CD63 antibody also reveals the association between CD63 and c-Src in rats on a KD diet (Fig. 4D). Thus, CD63, c-Src, and RPTPα also form a triplex in the rat kidney.

We then examined whether the dietary potassium intake affected the association among CD63, c-Src, and RPTPα. Fig. 5A is a Western blot showing that the association between RPTPα and c-Src is significantly decreased in renal cortex and outer medulla (mixture) in rats on a normal potassium intake diet. Potassium depletion increased the association between RPTPα and c-Src by 3.5 ± 0.6-fold. The increased association of c-Src with RPTPα was not the result of an augmented expression of RPTPα, because the expression of RPTPα was not altered by dietary potassium intake (Fig. 5B). In contrast, potassium intake did not affect the association between RPTPα and CD63 (Fig. 5C). Thus, the dietary potassium intake specifically increases the association between c-Src and RPTPα.

If CD63 stimulates c-Src activity through an association with RPTPα, it is conceivable that expression of CD63 should modulate the effect of c-Src on ROMK1 channels, which are known to be substrates of c-Src (24). Thus, we examined the effect of CD63 on potassium current in oocytes injected with ROMK1 using two-electrode voltage clamp. Oocytes were injected with water (negative control), CD63 (5 ng), ROMK1 (5 ng), ROMK1+CD63, ROMK1+c-Src (5 ng), and ROMK1+c-Src+CD63. We confirmed the previous finding (19) that injection of c-Src significantly decreased the potassium current from 11 ± 1.4 to 6 ± 0.5 μA (n = 66 from five frogs). Although injection of CD63 had no effect on the potassium current in oocytes injected with ROMK1 in the absence of c-Src (n = 36 from 4 frogs), it significantly enhanced the inhibitory effect of c-Src. The data summarized in Fig. 5A show that injection of CD63 decreased potassium current in oocytes injected with ROMK1+c-Src+CD63 from 11 ± 1.4 to 3 ± 0.3 μA, a value that was significantly lower than that in oocytes injected ROMK1+c-Src. The interpretation that CD63 enhanced the effect of c-Src on ROMK1 through stimulation of tyrosine phosphorylation was further confirmed by examining the tyrosine phosphorylation of ROMK1. We transfected 293T cells with GFP-ROMK1 (5 ng), GFP-ROMK1+c-Src (5 ng), or GFP-ROMK1+c-Src+CD63 (5 ng) and harvested ROMK protein by immunoprecipitation with GFP antibody. Fig. 5B is a Western blot showing that expression of c-Src increased tyrosine phosphorylation of ROMK1 by 350 ± 60% and that coexpression of CD63 further enhanced the tyrosine phosphorylation of ROMK by 72 ± 10% (n = 5 experiments) as determined by Western blotting using a phosphotyrosine-specific antibody. Increased tyrosine phosphorylation is expected to stimulate the internalization of ROMK1 channels. This notion is supported by experiments in which con-
The association between RPTP and c-Src, overexpression of RPTP decreased the potassium current by 40% (n = 8, p < 0.01) and that coexpression of CD63 and c-Src further decreased the potassium current to 20% (n = 8). The notion that CD63 enhances Src family PTK activity was further supported by the experiments in which the effect of herbimycin A on the potassium current was measured. Fig. 7 summarizes the results showing that application of herbimycin A (2 μM) abolished the effect of c-Src on ROMK1 channels in oocytes injected with either c-Src or CD63+c-Src. Expression of c-Src (2.5 ng) decreased potassium current from 7.1 ± 0.6 to 4.4 ± 0.5 μA in oocytes injected with 2.5 ng of ROMK1 (n = 17 from three frogs), and the potassium currents falls from 7.3 ± 0.8 to 2.7 ± 0.5 μA in oocytes injected with ROMK1 (2.5 ng) + c-Src (2.5 ng) + CD63 (2.5 ng) (n = 18 from three frogs). However, inhibition of PTK restored the potassium current to 6.5 ± 0.8 (n = 17) and 6 ± 0.7 μA (n = 24) in oocytes injected with ROMK1+c-Src and ROMK1+c-

focal microscopy was used to examine the fluorescence intensity in oocytes injected with GFP-ROMK1 (R1), R1+c-Src, and CD63+c-Src+R1. From inspection of Fig. 6C, it is apparent that expression of c-Src decreased surface fluorescence intensity by 55 ± 10% and that coexpression of CD63 causes a further reduction of surface fluorescence intensity to 20 ± 5% (n = 8) of the control value.

If the effect of CD63 on ROMK1 channels was due to facilitating the effect of c-Src on ROMK1 channels by stimulating association between RPTP and c-Src, overexpression of RPTPα should enhance the inhibitory effect of c-Src on ROMK channels. Thus, we examined the effect of c-Src on ROMK1 channels in the presence and absence of RPTPα. The data summarized in Fig. 6D show that injection of c-Src decreased ROMK1 channel activity by 60 ± 10% (n = 8, p < 0.01) and that coinjection of 10 ng of RPTPα further decreased the potassium current to 40 ± 6% of the control value (n = 8). The notion that CD63 enhances Src family PTK activity was further supported by the experiments in which the effect of herbimycin A on the potassium current in oocytes injected with ROMK1+c-Src + CD63 was measured. Fig. 7 summarizes the results showing that application of herbimycin A (2 μM) abolished the effect of c-Src on ROMK1 channels in oocytes injected with either c-Src or CD63+c-Src. Expression of c-Src (2.5 ng) decreased potassium current from 7.1 ± 0.6 to 4.4 ± 0.5 μA in oocytes injected with 2.5 ng of ROMK1 (n = 17 from three frogs), and the potassium currents falls from 7.3 ± 0.8 to 2.7 ± 0.5 μA in oocytes injected with ROMK1 (2.5 ng) + c-Src (2.5 ng) + CD63 (2.5 ng) (n = 18 from three frogs). However, inhibition of PTK restored the potassium current to 6.5 ± 0.8 (n = 17) and 6 ± 0.7 μA (n = 24) in oocytes injected with ROMK1+c-Src and ROMK1+c-

Src+Cd63, respectively. This suggests that the effect of CD63 on ROMK1 channels is the result of enhancing the PTK-induced inhibition of ROMK1 channels.

DISCUSSION

In the present study, we have demonstrated that CD63 is expressed in the CCD and can be involved in the regulation of ROMK channels. CD63 has a tyrosine-based internalization motif in the cytoplasmic C-terminal tail and has been shown to interact with adaptor protein complexes such as AP-2 and AP-3 (13). Because AP-2 and AP-3 are involved in facilitating the clathrin-mediated endocytosis, it is possible that CD63 could be directly involved in the internalization of its membrane protein partners. Indeed, CD63 has been shown to bind directly to the β-subunit of the H-K-ATPase in parietal cells of the rat stomach and to be involved in the regulation of H-K-ATPase trafficking (7). CD63 appears to stimulate the internalization of the H-K-ATPase. Although we could not completely exclude the possibility that CD63 is directly involved in the internalization of ROMK, this notion was not supported by the finding that CD63 did not affect ROMK channel activity in the absence of c-Src. Also, we did not detect a direct association between the ROMK channel and CD63 in the kidney. However, we could not completely exclude the possibility that CD63 regulates ROMK channels through direct association. But it is safe to conclude that one way by which CD63 regulates ROMK channels is through interacting with RPTPα and c-Src.

The notion that CD63 enhances the inhibitory effect of c-Src on ROMK1 is supported by three lines of evidence: 1) CD63 significantly decreased potassium currents only in oocytes injected with c-Src, but it had no effect on the activity of ROMK1 channels in the absence of c-Src; 2) c-Src-induced tyrosine phosphorylation of ROMK1 was larger in CD63-transfected cells than non-CD63-transfected cells; and 3) inhibition of PTK completely abolished the effect of c-Src on ROMK1 channels in both CD63 or non-CD63 injected oocytes. Thus, our results suggest that CD63 regulates ROMK1 channels by stimulating tyrosine phosphorylation of ROMK1 mediated by Src family PTK. We speculate that injection of c-Src stimulates the association between c-Src and unidentified endogenous PTP, which interacts also with CD63. Although we could not determine the type of PTP that interacts with c-Src and CD63 in the oocytes, the finding that c-Src, CD63, and RPTPα form a triplex in the kidney and 293T cells shed a light into the mech-

3 W.-H. Wang, unpublished observation.
The mechanism by which CD63 enhances the inhibitory effect of c-Src on ROMK1 channels is most likely through its association with RPTPα/H9251. As consequence, RPTPα activates c-Src by dephosphorylation of tyrosine residue 527 in the C terminus of c-Src. This possibility is supported by four lines of evidence: 1) CD63 and RPTPα/H9251 are expressed in the CCD, which plays an important role in the regulation of renal potassium secretion (26, 27); 2) CD63 is coimmunoprecipitated with RPTPα and CD63 in the kidney and in 293T cells transfected with CD63; 3) RPTPα is coimmunoprecipitated with c-Src in the kidney; and 4) expression of CD63 decreases the phosphorylation of tyrosine residue 527, whereas it increases the phosphorylation of tyrosine residue 416. Thus, our study suggests that CD63 plays an important role in activating Src family PTK and the phosphorylation of ROMK channels through an association directly with RPTPα.

A large body of evidence has suggested that tetraspan proteins are involved in transmembrane signal transduction. It has been reported that immunoprecipitation from cell lysates of lymph nodes and thymoma cells with antibodies directed against CD53 and CD63 reveals that immunocomplexes contain tyrosine phosphatase activity, which is able to dephosphorylate the tyrosine kinase Lck (19). CD63 has been reported to associate with Src family PTK such as Lyn in neutrophils (28).
Tetraspan proteins have also been shown to associate with activated protein kinase C (PKC) through integrins, which are constitutively associated with tetraspan proteins such as CD81 and CD53 (5). The formation of these triple complexes is essential to signal transduction from the extracellular matrix to intracellular space in fibrosarcoma cells. Therefore, we speculate that the formation of a complex among CD63, RPTPα, and Src family PTK could play an important role in activating PTK in the renal tubules such as CCD.

Our previous study has demonstrated that potassium restriction increased the activity of PTK and the expression of Src family PTK (12). However, the mechanism by which low potassium intake stimulates the activity of PTK is not understood. In the present study, we have demonstrated that association between c-Src and RPTPα was enhanced in the kidney from rats on a low potassium intake. Thus, it is possible that increased interaction between the phosphatase and PTK such as c-Src could be partially responsible for the stimulation of PTK induced by low potassium intake. Because low potassium intake did not affect the association between CD63 and RPTPα, whereas it enhanced the interaction between RPTPα and c-Src, we speculate that CD63 may constitutively associate with RPTPα in the cell membrane. However, the association between RPTPα and Src family PTK is enhanced by potassium restriction. As a consequence of an increased interaction between the RPTPα and Src family PTK, the PTK activity increased. It has been well established that RPTPα plays a major role in the activation of Src family PTK (8). For instance, RPTPα activates Src family PTK, which is essential for initiating the integrin signaling and cell migration in fibroblast cells (29). The association between RPTPα and Src family PTK has also been reported in mouse brain (22). The mechanism by which RPTPα stimulates PTK activity has been demonstrated to involve PKCδ, which phosphorylates RPTPα and increases its activity (9). In this regard, we have shown that low potassium intake stimulates the expression of PKCδ (30). Thus, it is possible that PKC may be partially involved in activation of PTK during the potassium restriction. This speculation is supported by the previous observation that inhibition of PKC attenuates the inhibitory effect of stimulating PTK on ROMK channels in the CCD (30).

**FIGURE 7.** Effect of herbimycin A (2 μM) on ROMK1 channel currents in oocytes injected with either c-Src + ROMK1 or CD63 + c-Src + ROMK1.

ROMK1 channels play an important role in renal potassium secretion in the connecting tubule and the CCD (27, 31–36). We have demonstrated in a previous study that ROMK1 channel is a substrate of Src family PTK and that tyrosine residue 337 in the C terminus of ROMK1 is one major site of tyrosine phosphorylation (24). Increased tyrosine phosphorylation has been shown to inhibit the ROMK channel activity. The inhibitory effect of PTK on ROMK channel is due to stimulation of channel endocytosis (15) because expression of a dominant-negative form of dynamin abolished the inhibitory effect of c-Src completely (37). The tyrosine phosphorylation of ROMK1 is regulated by dietary potassium intake such that potassium restriction significantly increases the tyrosine phosphorylation of ROMK channels (12). Potassium depletion-induced increase in the internalization of ROMK channels has also been reported by other investigators (14). Because CD63 plays a role in the stimulation of Src family PTK, we speculate that CD63 and other tetraspan proteins are involved in the regulation of renal potassium secretion. Fig. 8 is a model illustrating the role of CD63 in the regulation of ROMK channels. According to this scenario, potassium restriction enhances the formation of a complex among CD63, RPTPα, and c-Src and thus activates Src family PTK, which in turn inhibits ROMK channels by tyrosine phosphorylation. In this model, c-Src is associated with RPTPα. However, it is possible that c-Src could also simultaneously interact with CD63 and RPTPα. In addition, although it is not known how ROMK channels interact with the triple complex, it is conceivable that ROMK channels must be in close proximity to the CD63-cSrc-RPTPα complex. It has been reported that CD63 and c-Src are highly concentrated in cholesterol-rich lipid rafts (25). In addition, tetraspan proteins including CD63 interact with phosphatidylinositol 4-kinase to form large complexes in lipid raft-like microdomains of cells (4). Thus, it is possible that ROMK channels may also be enriched in the cholesterol-rich lipid raft, which facilitates interaction of PTK with ROMK channels. In conclusion, CD63 plays a role in the regulation of ROMK channels through the formation of a triple complex among RPTPα, Src family PTK, and tetraspan proteins.

Acknowledgments—We thank Melody Steinberg for editing the manuscript and Drs. Sheetz and Kostic for kindly providing RPTPα construct.
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JOURNAL OF BIOLOGICAL CHEMISTRY 7681