The G protein α subunit variant XLαs promotes inositol 1,4,5-trisphosphate signaling and mediates the renal actions of parathyroid hormone in vivo

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GNAS, which encodes the stimulatory G protein (heterotrimeric guanine nucleotide–binding protein) α subunit (Gαs), also encodes a large variant of Gαs termed extra-large α subunit (XLαs), and alterations in XLαs abundance or activity are implicated in various human disorders. Although XLαs, like Gαs, stimulates generation of the second messenger cyclic adenosine monophosphate (cAMP), evidence suggests that XLαs and Gαs have opposing effects in vivo. We investigated the role of XLαs in mediating signaling by parathyroid hormone (PTH), which activates a G protein–coupled receptor (GPCR) that stimulates both Gαs and Gαq/11 in renal proximal tubules to maintain phosphate and vitamin D homeostasis. At postnatal day 2 (P2), XLαs knockout (XLKO) mice exhibited hyperphosphatemia, hypocalcemia, and increased serum concentrations of PTH and 1,25-dihydroxyvitamin D. The ability of PTH to reduce serum phosphate concentrations was impaired, and the abundance of the sodium phosphate cotransporter Npt2a in renal brush border membranes was reduced in XLKO mice, whereas PTH-induced cAMP excretion in the urine was modestly increased. Basal and PTH-stimulated production of inositol 1,4,5-trisphosphate (IP3), which is the second messenger produced by Gαq/11 signaling, was repressed in renal proximal tubules from XLKO mice. Crossing of XLKO mice with mice overexpressing XLαs specifically in renal proximal tubules rescued the phenotype of the XLKO mice. Overexpression of XLαs in HEK 293 cells enhanced IP3 generation in unstimulated cells and in cells stimulated with PTH or thrombin, which acts through a Gαq/11-coupled receptor. Together, our findings suggest that XLαs enhances Gαq/11 signaling to mediate the renal actions of PTH during early postnatal development.

INTRODUCTION

The α subunit of the stimulatory heterotrimeric guanine nucleotide–binding protein (Gαs) mediates the actions of many hormones, neurotransmitters, and autocrine or paracrine factors by stimulating the generation of the second messenger cyclic adenosine monophosphate (cAMP) (1–3). Gαs is encoded by the imprinted GNAS complex locus, mutations in which cause multiple genetic diseases with complex parent-of-origin–specific phenotypes. Furthermore, somatic mutations in GNAS that lead to constitutive Gαs activity or increased GNAS copy number are found in multiple benign and malignant tumors. In addition to Gαs, GNAS gives rise to several different gene products, including a variant of Gαs termed extra-large α subunit (XLαs) (4). XLαs has a distinct N-terminal domain but is otherwise identical to Gαs. Gαs is expressed biallelically in most tissues; however, paternal Gαs expression is silenced in some tissues, such as certain parts of the brain, thyroid, pituitary gland, and renal proximal tubules (5). In contrast, the XLαs promoter is silenced on the maternal allele and activates transcription exclusively from the paternal allele (6, 7). Thus, most inactive and all activating GNAS mutations affect the expression and activity of XLαs when located on the paternal allele.

In mice and humans, loss of function of XLαs is implicated in intrauterine and perinatal growth retardation, as well as poor adaptation to feeding, hypoglycemia, and disrupted glucose counterregulation (8–11). Epigenetic silencing of XLαs is found in patients with a platelet abnormality characterized by the poor effectiveness of agonists that stimulate the Gαs-cAMP signaling pathway, whereas its overexpression is postulated to contribute to the development of chromosome 20q–amplified breast cancers (12, 13). Loss of imprinting at the XLαs promoter in mice, which leads to increased abundance of XLαs mRNA, also leads to early postnatal hypoglycemia and lethality (14). Despite the clear importance of this protein in multiple systems, the cellular functions of XLαs itself and relative to the functions of Gαs remain currently unclear. At the biochemical level, XLαs behaves similarly to Gαs, and it stimulates G protein–coupled receptor (GPCR)–activated generation of cAMP when it is increased in abundance (15, 16). On the other hand, comparison of the phenotypes of XLαs knockout (XLKO) mice with those of Gαs knockout mice suggests that the two proteins play opposing roles in vivo and that XLαs has actions that are distinct from those of Gαs (9, 17). Moreover, XLαs has several splice variants and alternative translation products (6, 18–21), which are also ablated or overexpressed upon genetic or epigenetic defects that alter XLαs expression. The relative contributions of XLαs and these other variants to physiology and disease pathogenesis have also remained largely unclear.

To examine the cellular roles of XLαs, we focused on the actions of parathyroid hormone (PTH), which acts through Gαs in the renal proximal tubule to inhibit phosphate reabsorption and to stimulate the synthesis of the bioactive form of vitamin D (22). PTH also uses the Gαq/11 signaling pathway to inhibit phosphate reabsorption in the proximal tubule, thus stimulating phospholipase Cβ (PLC-β) and thereby generating the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which is followed by the activation of certain protein kinase C (PKC) isozymes (23). Overexpression studies have also shown that PTH acts through XLαs to stimulate cAMP generation (24). Because XLαs is found in the kidney (16, 24, 25), we investigated whether this imprinted GNAS product mediated the actions of PTH in vivo and whether its effects occurred through cAMP signaling. Our investigations showed that the loss of XLαs in the renal proximal tubule disrupted PTH-mediated phosphate handling...
XL\(_{\alpha}\) protein is found in whole mouse kidneys in readily detectable amounts at postnatal day 2 (P2) but not P6 (25). Through immunostaining with an XL\(_{\alpha}\)-specific antiserum, we showed that XL\(_{\alpha}\) protein was located in the renal proximal tubules of P2 wild-type mice and, albeit less abundantly, in distal tubules, whereas no immunostaining was detected in other parts of the kidney (Fig. 1, A and B and fig. S1A). XL\(_{\alpha}\) protein was undetectable in samples from mice in which the paternal XL\(_{\alpha}\) allele was disrupted (XLKO mice) (Fig. 1, A and B, and fig. S1A). Loss of detectable XL\(_{\alpha}\) protein upon ablation of the paternal allele alone was consistent with the exclusively paternal expression of this GNAS product, as described previously (6). Because XL\(_{\alpha}\) mRNA abundance was greater at P2 than at later times (fig. S1B), we focused our efforts on determining the role of XL\(_{\alpha}\) at P2. Histological analysis detected no obvious defects in tubule structures in P2 XLKO mice, although the kidneys from these mice were smaller compared to those from their wild-type littermates, consistent with the overall smaller size of the XLKO pups (fig. S1C) (9). Nevertheless, the abundance of mRNA for Kid1, which encodes a renal transcription factor whose mRNA accumulates in the course of postnatal renal development, was decreased by about 50% in whole kidneys from P2 XLKO mice compared to that in kidneys from wild-type mice, which suggests that there was a delay in proximal tubule development (fig. S1D).

**RESULTS**

**XL\(_{\alpha}\) is located in renal proximal tubule cells during the early postnatal development of mice**

XL\(_{\alpha}\) protein is found in whole mouse kidneys in readily detectable amounts at postnatal day 2 (P2) but not P6 (25). Through immunostaining with an XL\(_{\alpha}\)-specific antiserum, we showed that XL\(_{\alpha}\) protein was located in the renal proximal tubules of P2 wild-type mice and, albeit less abundantly, in distal tubules, whereas no immunostaining was detected in other parts of the kidney (Fig. 1, A and B and fig. S1A). XL\(_{\alpha}\) protein was undetectable in samples from mice in which the paternal XL\(_{\alpha}\) allele was disrupted (XLKO mice) (Fig. 1, A and B, and fig. S1A). Loss of detectable XL\(_{\alpha}\) protein upon ablation of the paternal allele alone was consistent with the exclusively paternal expression of this GNAS product, as described previously (6). Because XL\(_{\alpha}\) mRNA abundance was greater at P2 than at later times (fig. S1B), we focused our efforts on determining the role of XL\(_{\alpha}\) at P2. Histological analysis detected no obvious defects in tubule structures in P2 XLKO mice, although the kidneys from these mice were smaller compared to those from their wild-type littermates, consistent with the overall smaller size of the XLKO pups (fig. S1C) (9). Nevertheless, the abundance of mRNA for Kid1, which encodes a renal transcription factor whose mRNA accumulates in the course of postnatal renal development, was decreased by about 50% in whole kidneys from P2 XLKO mice compared to that in kidneys from wild-type mice, which suggests that there was a delay in proximal tubule development (fig. S1D).

**XLKO mice exhibit hyperphosphatemia and hypocalcemia together with increased serum PTH**

At the renal proximal tubule, PTH inhibits the reabsorption of inorganic phosphate from the glomerular filtrate and stimulates the synthesis of the active vitamin D metabolite 1,25 dihydroxyvitamin D \([1,25(OH)_{2}D]\), which increases calcium absorption in the small intestine. P2 XLKO pups showed substantially increased concentrations of serum phosphate (hyperphosphatemia), decreased concentrations of serum calcium (hypocalcemia), as well as increased concentrations of serum PTH compared to wild-type mice (Table 1). However, the concentration of 1,25(OH)_{2}D in the serum was also markedly increased, whereas the serum concentration of fibroblast growth factor 23 (FGF23), another phosphaturic hormone that suppresses 1,25(OH)_{2}D synthesis in the proximal tubule (26), was substantially reduced in abundance in P2 XLKO pups (Table 1).

We then examined the abundance of mRNA for renal 25-hydroxyvitamin D 1α-hydroxylase (Cyp27b1), which synthesizes 1,25(OH)_{2}D, as well as that of 25-hydroxyvitamin D 24-hydroxylase (Cyp24a1), which metabolizes 1,25(OH)_{2}D. The abundance of Cyp27b1 mRNA was increased more than fivefold in P2 XLKO kidneys compared to that in P2 wild-type kidneys (Fig. 2A), whereas that of Cyp24a1 mRNA was moderately increased in P2 XLKO kidneys (Fig. 2B), suggesting that the increased 1,25(OH)_{2}D abundance resulted from induced expression of Cyp27b1. We then examined the type II sodium-dependent phosphate cotransporter Npt2a in the renal brush border membrane because of its critical role in the reabsorption of phosphate, and we found that the abundance of its protein in P2 XLKO mice was markedly increased compared to those in P2 wild-type mice (Fig. 2, C

![Table 1. Comparison of serum biochemistries.](http://stke.sciencemag.org/)

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<th>WT</th>
<th>XLKO</th>
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<tr>
<td>Phosphate (mg/dl)</td>
<td>10.32 ± 0.24</td>
<td>11.42 ± 0.41*</td>
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<tr>
<td>Ca(^{2+}) (mM)</td>
<td>1.48 ± 0.01</td>
<td>1.42 ± 0.02*</td>
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<tr>
<td>PTH (pg/ml)</td>
<td>65.25 ± 1.34</td>
<td>138.10 ± 4.99*</td>
</tr>
<tr>
<td>1.25(OH)(_{2})D (pM)</td>
<td>183.42 ± 4.64</td>
<td>378.02 ± 6.86***</td>
</tr>
<tr>
<td>FGF23 (pg/ml)</td>
<td>524.11 ± 11.64</td>
<td>158.86 ± 9.29***</td>
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and D). In contrast, the abundances of Npt2c protein and Slc20A2 (Pit2) mRNA, which encodes another sodium-dependent phosphate transporter, were similar in renal brush border membranes from XLKO and wild-type mice, as determined by Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, respectively (fig. S2, A to C).

**XLKO mice are resistant to PTH despite exhibiting increased excretion of urinary cAMP**

PTH stimulates the expression of Cyp27b1 by acting on its promoter region (27). As expected, 2 hours after they were injected subcutaneously with PTH (50 nmol/kg), wild-type mice exhibited a 10-fold increase in the abundance of Cyp27b1 mRNA in their kidneys compared to that in the kidneys of wild-type mice injected with vehicle (Fig. 3A). In contrast, the abundance of Cyp27b1 mRNA was increased by only 1.42-fold in XLKO mice injected with PTH compared to that in vehicle-treated XLKO mice (Fig. 3A). Note that the XLKO pups had much higher basal Cyp27b1 expression than wild-type pups (Fig. 3A). PTH stimulates renal phosphate excretion by decreasing the abundance of Npt2a protein in renal brush border membranes and thus leads to hypophosphatemia (28). After they were injected with PTH, P2 wild-type mice had statistically significantly reduced serum phosphate concentrations compared to those in vehicle-treated wild-type mice, whereas the serum phosphate concentration of PTH-treated XLKO mice was not substantially reduced compared to that in vehicle-treated wild-type mice (Fig. 3B). Similarly, divergent effects of PTH on renal Npt2a abundance in wild-type and XLKO mice were also observed (Fig. 3, C and D). Fifteen minutes after they were injected with PTH, wild-type mice showed an 80% decrease in the abundance of Npt2a in the renal brush border membrane than did vehicle-treated wild-type mice, whereas the effect of PTH on reducing Npt2a abundance in the renal brush border membranes of XLKO mice was less substantial (Fig. 3, C to E). These results suggest that the proximal tubular actions of PTH in XLKO mice were blunted compared to those in wild-type mice.

To characterize the role of XLO α in mediating the actions of PTH, we tested whether XLKO exhibited a reduction in the PTH-induced secretion of cAMP from the kidney into the urine, a test that is used to assess the proximal tubular action of PTH and to establish a diagnosis of PTH resistance in patients (29). The basal concentrations of urinary cAMP in P2 wild-type and XLKO mice were similar 15 min after they received subcutaneous injections of vehicle (Fig. 4A). Injection with PTH led to increased concentrations of urinary cAMP in wild-type and XLKO pups; however, the concentrations were statistically significantly greater in the
XLKO mice than in their wild-type littermates (Fig. 4A). We then measured Gαs mRNA abundance in whole kidneys and found a moderate, but statistically significant, increase in those of P2 XLKO mice compared to those of wild-type mice (Fig. 4B). Western blotting analysis revealed a threefold increase in the abundance of Gαs protein in the brush border membranes of XLKO mice compared to that in the brush border membranes of wild-type mice (Fig. 4, C and D). Immunostaining of kidney sections with antiserum directed against the unique N-terminal portion of Gαs (and that cannot recognize XLαs) was also suggestive of increased amounts of Gαs protein in the kidneys of XLKO mice (fig. S3).

**XLKO pups show repressed Gq/11 signaling**

Because the PTH-stimulated generation of cAMP was not impaired in P2 XLKO mice, we examined the Gq/11 signaling pathway, which can be activated by PTH and contributes to the regulation of serum phosphate concentrations (23, 30, 31). In proximal tubule–enriched renal cortices isolated from P2 XLKO kidneys, we found that the intracellular amount of the IP3 metabolite inositol monophosphate (IP1), which reflects IP3 generation, was substantially reduced under basal conditions, as well as upon stimulation with PTH (Fig. 5A). Western blotting analysis showed that the abundances of PKCδ, PKCa, and PKCζ, but not PKCβII or PKCθ, were decreased in the cytosolic fractions of XLKO samples compared to those in cytosolic fractions of samples from wild-type mice (Fig. 5B and fig. S4, A and B). PKCδ abundance was also markedly decreased in the membrane fractions of XLKO samples, whereas the abundances of PKCa and PKCζ were only slightly reduced (Fig. 5, B to E). Moreover, the abundances of PKCδ, PKCa, and PKCζ after PTH treatment were substantially decreased in both the cytosolic and membrane fractions of XLKO samples compared to those of wild-type samples (Fig. 5, B to E). Western blotting analysis indicated no reduction in the abundance of phosphorylated extracellular signal–regulated kinase 1 (ERK1) and ERK2 in the proximal tubules from XLKO mice either under basal conditions or after stimulation with PTH (fig. S4, C and D), whereas the amounts of phosphorylated PKA targets were slightly increased in membrane fractions after stimulation with PTH (Fig. 5B and fig. S4E).

**Hyperphosphatemia and reduced Gq/11 signaling in XLKO mice are rescued by overexpression of XLαs specifically in proximal tubules**

Knockout of the gene encoding XLαs in XLKO mice ablates not only XLαs but also its variants in all tissues (9). To determine whether the proximal tubule–specific overexpression of XLαs could rescue the hyperphosphatemia phenotype of P2 XLKO mice, we crossed XLKO mice to transgenic mice in which XLαs expression was targeted to the renal proximal tubule with the...
promoter of the gene encoding the type I rat γ-glutamyltranspeptidase (rptXLα mice) (25). We found that this promoter was active at the P2 stage and that the abundance of XLαs mRNA in rptXLα mice was 12-fold higher than that in wild-type littermates (Fig. 6A). Whereas the XLKO offspring from these matings showed hyperphosphatemia, rptXLα offspring tended to have reduced concentrations of serum phosphate (Fig. 6B). Serum phosphate concentrations were statistically significantly lower in the XLKO:rptXLα offspring than in the XLKO offspring, and these concentrations were comparable to those in wild-type littermates (Fig. 6B). In addition, the increase in Cyp27b1 expression observed in XLKO mice was effectively reversed by transient overexpression of XLαs (Fig. 6C), although the expression of Cyp24a1 was still increased in the XLKO:rptXLα mice (Fig. 6D). The reduced abundance of Kid1 mRNA in XLKO mice was also fully rescued by overexpression of XLαs in proximal tubules (Fig. 6E).

Western blotting analysis revealed that the reduction in the abundances of PKCδ, PKCa, and PKCζ in the proximal tubules of XLKO mice was also rescued by the proximal tubule–specific overexpression of XLαs (Fig. 6, F to I). In particular, overexpression of XLαs in the proximal tubule substantially increased the membrane abundance of PKCδ, which was evident in both rptXLαs and XLKO:rptXLαpups (Fig. 6, F and G).

**XLαs expression enhances the basal and agonist-stimulated amounts of IP1**

Basal and PTH-stimulated concentrations of IP1 were statistically significantly increased in proximal tubule–enriched cortices isolated from P2 pups (Fig. 6A). Concordant with these results, transient transfection of human embryonic kidney (HEK) 293 cells stably expressing the PTH receptor with increasing amounts of plasmid encoding XLαs resulted in a dose-dependent increase in basal IP1 concentrations (Fig. 7B). Moreover, cells transfected with either control plasmid or plasmid encoding XLαs responded to PTH, but the overexpression of XLαs substantially enhanced the generation of IP1 in response to different concentrations of PTH (Fig. 7C). Furthermore, overexpression of XLαs in these cells also enhanced the amount of IP1 generated in response to thrombin, another agonist that acts through its own endogenous Gs-coupled receptor (Fig. 7D). In addition, we examined a guanosine triphosphatase (GTPase)–deficient mutant of XLαs (XLαs-R543H), which shows constitutive activity with respect to cAMP generation (24). As expected, HEK 293 cells transiently expressing this mutant XLαs displayed increased basal cAMP generation compared to those expressing wild-type XLαs (Fig. 7E). Similarly, basal IP1 concentrations were substantially greater in HEK 293 cells expressing XLαs-R543H than in those expressing wild-type XLαs (Fig. 7F).

To determine whether XLαs could mimic the actions of Goq11 proteins, we expressed XLαs in HEK 293 cells in which Goq and Go11 were both ablated through the use of the CRISPR (clustered regularly interspersed short palindromic repeats)/Cas9 system (CRISPR-associated). Thrombin stimulated a 2.55-fold increase in IP1 generation in native HEK 293 cells compared to that in unstimulated cells (Fig. 7G), whereas it failed to stimulate IP1 generation in Goq11-deficient HEK 293 cells that were transfected with control plasmid (Fig. 7H). Transient transfection of the Goq11-deficient HEK 293 cells with plasmid encoding either Goq or Go11 led to an increase in basal IP1 concentrations and rescued the thrombin-stimulated generation of IP1 (Fig. 7H). In Goq11-deficient HEK 293 cells transfected with plasmid encoding XLαs, the basal IP1 concentration was 3.39-fold greater than that in cells transfected with the control plasmid, and thrombin stimulated a 1.81-fold increase in IP1 abundance compared to that in unstimulated Goq11-deficient cells expressing XLαs (Fig. 7H).

**DISCUSSION**

We investigated the role of XLαs, a variant of Goq, by focusing on the action of PTH, and our data suggest that XLαs is essential for the PTH-mediated activation of the CYP27B1/CYP24A1 pathway.
regulation of phosphate handling in the renal proximal tubule during early postnatal development. Whereas this role of XLα₃ appears to be similar to that of Gₛₐ in the same context, we found that the ablation of XLα₃ in mice repressed Gₛ₁₁₁-mediated signaling rather than Gₛ-mediated signaling. Our additional results suggest that XLα₃ promotes basal and agonist-stimulated signaling that is typically downstream of Gₛ₁₁₁ proteins.

By stimulating at least two signaling pathways, which are mediated by Gₛ and Gₛ₁₁₁ proteins, PTH inhibits renal phosphate reabsorption by decreasing the abundance of Npt2a protein. The hyperphosphatemia in XLKO mice is thus consistent with an increase in the amount of Npt2a at steady state and the blunted PTH-induced reduction in Npt2a abundance. FGFR23 also decreases the abundance of Npt2a (32), and therefore, the increased Npt2a abundance would be consistent with the reduction in the amount of FGFR23 in XLKO mice. However, a role for reduced FGFR23 in P2 XLKO mice is unlikely because complete ablation of FGFR23 in mice does not lead to alterations in serum phosphate and calcium concentrations until after P6 (33, 34). PTH also increases the synthesis of 1,25(OH)₂D by inducing the expression of Cyp27b1, but this effect depends largely on Gₛ-dependent signaling (27, 35). Because the serum PTH concentration was increased in XLKO pups compared to that in wild-type pups, and because this was concurrent with markedly increased amounts of renal Cyp27b1 mRNA and 1,25(OH)₂D, it is possible that the action of PTH on Cyp27b1 expression is somewhat preserved in the absence of XLα₃.

In support of this possibility was the tendency of renal Cyp27b1 mRNA abundance to increase even more than the already enhanced amounts in response to PTH in XLKO pups (Fig. 4A). A reduction in the amount of serum FGFR23 is unlikely to account for the increased amount of 1,25(OH)₂D in P2 XLKO pups because FGFR23-deficient mice do not display increased amounts of 1,25(OH)₂D until after P10 (33). 1,25(OH)₂D stimulates the expression of Cyp24a1, which would thus explain the increased renal abundance of Cyp24a1 mRNA in XLKO pups.

The hypocalcemia in XLKO pups is more difficult to explain, given their increased amounts of 1,25(OH)₂D, which normally enhances Ca²⁺ absorption in the gut. In newborns, however, deficiency in 1,25(OH)₂D or the loss of its receptor does not lead to hypocalcemia, which suggests that this hormone is not essential for regulating serum Ca²⁺ concentrations during early postnatal life (36). It is possible that the reduced serum concentration of Ca²⁺ in XLKO pups is secondary to hyperphosphatemia or reflects the poor...
feeding of the XLKO pups (9). In addition, PTH enhances the reabsorption of Ca\textsuperscript{2+} in the renal distal tubule (22). Because we detected XL\(\alpha\) protein in this part of the nephron, PTH-induced reabsorption of Ca\textsuperscript{2+} may be impaired in P2 XLKO mice, thus contributing to the hypocalcemia despite the presence of increased amounts of PTH in the serum of these mice.

The PTH resistance in XLKO pups was observed in the presence of intact, and modestly increased, G\(\alpha\)t abundance and an enhanced PTH-stimulated cAMP response. In contrast, basal and PTH-stimulated IP\(_3\) signaling and the abundances of certain PKC isoforms were repressed. In addition, overexpression of XL\(\alpha\), in the proximal tubules of XLKO pups rescued both the hyperphosphatemia and the diminished generation of IP\(_3\). It thus appears that the impaired responsiveness of Npt2a to PTH in the proximal tubules of XLKO mice, and the resultant changes in serum biochemistries of XLKO pups, is at least partly a result of the impaired activation of IP\(_3\)-DAG signaling. Together with the results of our cell culture experiments, these findings suggest that XL\(\alpha\), serves as a G\(_{q/11}\)-like signaling protein and that it mediates the PTH-induced inhibition of phosphate reabsorption from the glomerular filtrate.

Previous studies showed that the G\(_{q/11}\) signaling pathway and the activation of PKC play crucial roles in the PTH-stimulated internalization of Npt2a and regulation of phosphate homeostasis (37–39). Our findings confirm the importance of this signaling pathway to the renal actions of PTH, and they identify XL\(\alpha\), as an indispensable mediator of those actions during early postnatal development.

Given that XL\(\alpha\), mice that survive to weaning show normal concentrations of an indispensable mediator of those actions during early postnatal development, patients have mutations in exon 1 of their paternal G\(_{q/11}\) subunit. In this tissue, note that the XLKO:rptXL\(\alpha\), variant scould be involved in the XL\(\alpha\),-mediated generation of IP\(_3\), particularly under basal conditions; however, thrombin receptors do not couple to G\(_\beta\)\(\gamma\) proteins (49), and therefore, thrombin-induced IP\(_3\) generation in XL\(\alpha\),-expressing, G\(_{q/11}\)-deficient cells is unlikely to be secondary to cAMP generation. Our findings from in vivo experiments also argue against the involvement of a cAMP-dependent mechanism, because XLKO pups showed increased PTH-stimulated generation of cAMP in the proximal tubule despite having diminished G\(_{q/11}\)-mediated signaling.

PKCs are divided into three subfamilies: conventional, novel, and atypical. Conventional PKCs, such as PKC\(\alpha\), and novel PKCs, such as PKC\(\delta\), require DAG for their activation and are thus downstream of G\(_{q/11}\) signaling. The inhibited generation of IP\(_3\), as well as the decreased abundances of PKCS and PKC\(\alpha\), in the proximal tubules of P2 XLKO mice suggests that XL\(\alpha\), plays a stimulatory role in G\(_{q/11}\) signaling. On the other hand, PKC\(\gamma\), which is an atypical PKC that does not require DAG or Ca\textsuperscript{2+} for activation (51), was also reduced in abundance in samples from XLKO mice, suggesting that XL\(\alpha\), might also regulate the abundance of atypical PKCs through an as-yet-undefined mechanism. PTH stimulates PKC activation in a PLC-independent manner (52), which might possibly be mediated by XL\(\alpha\),.

That loss of XL\(\alpha\), increased the PTH-stimulated generation of cAMP is consistent with data from experiments with brown adipose tissue (BAT) from XLKO mice, in which basal and isoproterenol-stimulated cAMP generation were enhanced compared to those in BAT from wild-type mice (9). Our results suggest that the increased production of cAMP in the proximal tubules of XLKO mice is at least partly a result of increased G\(\alpha\)t abundance. It is tempting to speculate that the changes in G\(\alpha\)t abundance reflect a physiological response to help counteract the hyperphosphatemia. The change in G\(\alpha\)t abundance in proximal tubules from XLKO mice may also result from the loss of a potential regulatory mechanism involving an interaction in cis between the paternal XL\(\alpha\), transcript and the downstream G\(\alpha\)t promoter, because a similar mechanism involving another paternally expressed upstream GNAS transcript (A/B) and the G\(\alpha\)t promoter has been proposed (53).

\(\text{GNAS}\) is a highly complex locus, and XL\(\alpha\), itself has multiple variants and alternative translational products (6, 18–21). The XLKO mice have a deletion in extra-large exon 1, thus abating all of these variants (9). Thus, the PTH-resistant phenotype of XLKO pups could reflect a deficiency in one or more of these proteins. However, the phenotype was rescued by overexpressing XL\(\alpha\), in the proximal tubule, and it is therefore likely that the phenotype reflects a specific deficiency in XL\(\alpha\), rather than its other variants, in this tissue. Note that the XLKO:rptXL\(\alpha\), pups appeared grossly indistinguishable from their XLKO littermates and showed a similar degree of early postnatal lethality, indicating that other XL\(\alpha\), variants could be responsible for these other defects.
In summary, our study revealed a hitherto unknown function of the large G<sub>a</sub> variant XL<sub>a</sub><sub>s</sub>, demonstrating that this imprinted protein can enhance the G<sub>q/11</sub> signaling pathway. Our studies with XL<sub>a</sub><sub>s</sub>-deficient mice also suggest that XL<sub>a</sub><sub>s</sub> is required for the action of PTH in the proximal tubules of early postnatal mice with respect to phosphate handling. These findings have broad implications regarding the pathogenesis of human diseases caused by GNAS mutations or copy number variations.

**MATERIALS AND METHODS**

**Mice**

XLKO mice and rptXL<sub>a</sub> mice (9, 25) were maintained in a CD1 genetic background. All the animal experiments were conducted in accordance with the accepted standards of the Institutional Animal Care and Use Committee, and the studies were approved by the Massachusetts General Hospital Sub-committee on Research Animal Care.

**Expression plasmids**

Construction of plasmids encoding XL<sub>a</sub><sub>s</sub> and the GTPase-deficient XL<sub>a</sub><sub>s</sub> mutant XL<sub>a</sub><sub>s</sub>-R543H was described previously (20, 24). Plasmids encoding G<sub>a</sub><sub>q</sub> and G<sub>a</sub><sub>11</sub> were obtained from www.cDNA.org.

**cDNA synthesis and qRT-PCR analysis**

Total RNA isolated from the kidneys of P2 wild-type, XLKO, rptXL<sub>a</sub>, and XLKO:rptXL<sub>a</sub> mice was prepared with the RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized with the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs) as previously described (54). qRT-PCR analysis was performed with specific primers (table S1) and FastStart Universal SYBR Green Master (Roche) with Actb (b-actin) as a reference gene.

**Immunofluorescence microscopy**

Kidneys were isolated from P2 XLKO and wild-type littersmates, fixed, and serially sectioned at 5 μm. Sections were incubated with polyclonal antiserum against mouse XL<sub>a</sub><sub>s</sub> at 1:500 dilution, antibody specific for the N-terminal region of G<sub>a</sub><sub>s</sub>, encoded by exon 1 (1:500) (a gift of S. Mollner, Germany) (4), or antibody against calbindin D-28K (Sigma) at 4°C overnight. Sections were then incubated with Alexa Fluor 568–conjugated donkey anti-rabbit antibody and Alexa Fluor 488–conjugated phalloidin or with Alexa Fluor 488–conjugated goat anti-mouse antibody (Life Technologies) at room temperature for 1 hour (24). Immunoreactivity was visualized and analyzed with a Zeiss LSM 510 confocal microscope and Zeiss Zen software.

**Measurement of serum biochemistries**

Blood samples for the analysis of serum concentrations of phosphate, Ca<sup>2+</sup>, PTH, and 1,25(OH)<sub>2</sub>D were obtained from the carotid artery and were processed as described previously (25). Serum FGF23 concentrations were measured with the Mouse/Rat FGF-23 (C-Term) ELISA Kit (Immutopics). To determine the effect of PTH on cAMP generation in vivo, urine was collected 15 min after mice were injected subcutaneously with vehicle or PTH(1–34) (50 nmol/kg), and the concentration of cAMP was quantified as described previously (14). The PTH-induced reduction in the concentration of serum phosphate was measured 2 hours after mice were injected with PTH(1–34) or vehicle as previously described (25).

**Isolation and Western blotting analysis of renal brush border membranes**

Brush border membrane portions were isolated from the kidneys of P2 mice as described previously (55) with minor modifications. Isolated brush border membrane proteins were lysed in a tris-buffered solution containing 150 mM NaCl and 1% Triton and a protease inhibitor cocktail (Roche). Measurement of protein concentrations in the samples and Western blotting analysis with antibodies specific for Npt2a, Npt2c (56), and G<sub>a</sub><sub>s</sub> (Millipore) were performed as described previously (25).

**Isolation of proximal tubule–enriched cortices and IP<sub>1</sub> assays**

Proximal tubule–enriched renal cortices were prepared by collagenase digestion of P2 mouse kidneys and isolation on a Percoll gradient as described previously (37). Proximal tubules from P2 wild-type, XLKO, or rptXL<sub>a</sub> mice were treated with different concentrations of PTH(1–34) for 30 min in the presence of LiCl<sub>2</sub> and then were analyzed to determine the amount of IP<sub>1</sub>, a downstream metabolite of IP<sub>3</sub>, with the IP-One HTRF assay kit (Cis-bio). HEK 293 cells stably expressing the PTHR [a gift of T. Gardella, Massachusetts General Hospital (MGH)] were transfected with plasmids encoding XL<sub>a</sub><sub>s</sub> or XL<sub>a</sub><sub>s</sub>-R543H and incubated for 30 min in the presence or absence of PTH(1–34) before IP<sub>1</sub> concentrations were measured. Parental wild-type and G<sub>a</sub><sub>q/11</sub>−/− HEK 293 cells (a gift of A. Inoue, Tohoku University) were transfected with pcDNA or plasmids encoding XL<sub>a</sub><sub>s</sub>, G<sub>a</sub><sub>q</sub>, or G<sub>a</sub><sub>11</sub> and then incubated for 30 min in the presence or absence of thrombin before IP<sub>1</sub> concentrations were measured.

**cAMP signaling assays**

Signaling through the cAMP and PKA pathway was assessed in experiments with HEK 293 cells stably transfected with the GloSensor cAMP reporter plasmid and with plasmid encoding PTHR and then transiently transfected with plasmids encoding XL<sub>a</sub><sub>s</sub> or XL<sub>a</sub><sub>s</sub>-R543H. For assays, confluent cells in 96-well plates were treated with luciferin and 2 mM 3-isobutyl-1-methylxanthine for 30 min before luminescence was measured as previously described (58).

**Cell fractionation and analysis of PKC isoforms**

Proximal tubule–enriched renal cortices were subjected to cell fractionation by ultracentrifuge to separate cytosolic and membrane fractions (59). Protein lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Western blots were then incubated with antibodies directed against specific PKC isoforms, phosphorylated substrates of PKA, and phosphorylated ERK1/2 (Cell Signaling). Antibodies against actin (Santa Cruz Biotechnology) and the Na<sub>K</sub>-ATPase (Cell Signaling) were used to identify cytosolic and membrane fractions. Densitometric analysis of Western blots was performed with ImageJ software.

**Statistical analysis**

The means ± SEM of multiple independent measurements were calculated. To determine statistical significance, Leven’s F test was first performed to assess equality of variances. If variances were equal, the Student’s t test (two-tailed) was used. If variances were unequal, the equal variance ANOVA test was used. Welch’s t test (two-tailed) was used. Statistical significance is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. XL<sub>a</sub><sub>s</sub> is located in the kidney at early postnatal stages, and XLKO mice do not have defects in renal structure.
Fig. S2. The abundances of Npt2a protein and Slc20a2 mRNA are similar between P2 wild-type and XLKO mice.  
Fig. S3. Increased renal Gru abundance in P2 XLKO kidneys.  
Fig. S4. The abundances of PKC protein expressed G proteins.

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Data and materials availability: The use of XLKO mice requires a materials transfer agreement (MTA) from the Babraham Institute, UK. The use of the G\textsubscript{\alpha}s\textsubscript{11}\textsubscript{q/11} HEK 293 cells requires a MTA from Tohoku University, Japan.

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The G protein $\alpha$ subunit variant XL$\alpha_s$ promotes inositol 1,4,5-trisphosphate signaling and mediates the renal actions of parathyroid hormone in vivo
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An extra-large signaling role
G protein–coupled receptors (GPCRs) stimulate heterotrimeric G proteins. G proteins of the $G_\alpha$ family stimulate generation of the second messenger cAMP, whereas $G_{q/11}$ proteins stimulate generation of the second messenger IP$_3$. The gene encoding the $G_\alpha_s$ subunit ($G\alpha_s$) also encodes a structurally similar, extra-large variant, XL$\alpha_s$, which also stimulates cAMP generation. He et al. found that the effects of parathyroid hormone (PTH), which stimulates both cAMP and IP$_3$ production by activating a GPCR that couples to both $G_\beta\gamma$ and $G_{q/11}$, on the kidney were impaired in XL$\alpha_s$-deficient mice. However, rather than producing substantially less cAMP, kidney cells from the XL$\alpha_s$-deficient mice had a defect in generating IP$_3$, which was restored when the mice were crossed with transgenic mice overexpressing XL$\alpha_s$ in renal proximal tubules. Together, these data suggest that XL $\alpha_s$ plays a physiological role in stimulating IP$_3$ signaling.

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