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EGF Increases TRPM6 Activity and Surface Expression

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ABSTRACT

Recent identification of a mutation in the *EGF* gene that causes isolated recessive hypomagnesemia led to the finding that EGF increases the activity of the epithelial magnesium (Mg^{2+}) channel transient receptor potential M6 (TRPM6). To investigate the molecular mechanism mediating this effect, we performed whole-cell patch-clamp recordings of TRPM6 expressed in human embryonic kidney 293 (HEK293) cells. Stimulation of the EGF receptor increased current through TRPM6 but not TRPM7. The carboxy-terminal α-kinase domain of TRPM6 did not participate in the EGF receptor–mediated increase in channel activity. This activation relied on both the Src family of tyrosine kinases and the downstream effector Rac1. Activation of Rac1 increased the mobility of TRPM6, assessed by fluorescence recovery after photobleaching, and a constitutively active mutant of Rac1 mimicked the stimulatory effect of EGF on TRPM6 mobility and activity. Ultimately, TRPM6 activation resulted from increased cell surface abundance. In contrast, dominant negative Rac1 decreased TRPM6 mobility, abrogated current development, and prevented the EGF-mediated increase in channel activity. In summary, EGF-mediated stimulation of TRPM6 occurs *via* signaling through Src kinases and Rac1, thereby redistributing endomembrane TRPM6 to the plasma membrane. These results describe a regulatory mechanism for transepithelial Mg^{2+} transport and consequently whole-body Mg^{2+} homeostasis.

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Recently we described a mutation in the EGF gene, encoding pro-EGF, which is responsible for a rare form of renal magnesium (Mg^{2+}) wasting, isolated recessive hypomagnesemia.[1] A link between a defect in EGF and renal Mg^{2+} wasting was made by demonstrating that pro-EGF is expressed in the distal convoluted tubule (DCT), where regulated transcellular Mg^{2+} reabsorption occurs *via* transient receptor potential M6 (TRPM6). Furthermore, supernatant from the basolateral compartment of polarized epithelial cells expressing pro-EGF was able to activate TRPM6, whereas supernatant from cells expressing the mutant pro-EGF was unable to. The EGF receptor (EGFR/ErbB1) is expressed in the basolateral membrane of DCT, suggesting an autocrine/paracrine activation of TRPM6 by EGF through its receptor. This has not been confirmed, however; neither have the molecular details of this activation been elucidated.

EGFR activation mediates its downstream effects *via* numerous signaling cascades, including the extracellular signal–regulated kinase (ERK) limb of the mitogen-activated protein kinase (MAPK) superfamily, protein kinase A (PKA), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and the phospholipases C and D pathways.[2] Activation of the EGFR in DCT could therefore result in phosphorylation and consequently activation of the previously described target proteins. That EGFR signaling is implicit to Mg^{2+} homeostasis is further supported by the observation that patients treated with cetuximab, a mAb

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directed against the EGFR, develop hypomagnesemia[1,3,4]; however, the signaling pathway(s) downstream of EGFR activation, which stimulates TRPM6-mediated Mg^{2+} influx, remains to be determined.

The aim of this study was to ascertain the downstream signaling events, after EGFR engagement, and ultimately the mechanism responsible for increased TRPM6 activity. Elucidation of the molecular details responsible for EGFR-mediated stimulation of TRPM6 will facilitate the development of treatments for hypomagnesemia in general and during cetuximab treatment. Using electrophysiologic measurements in combination with biochemical and live cell imaging techniques, we provide evidence that EGF stimulates TRPM6 through the specific activation of the EGFR. This activates the Src family of tyrosine kinases and the small Rho-GTPase, Rac1, which results in a redistribution of vesicular TRPM6 to the plasma membrane.

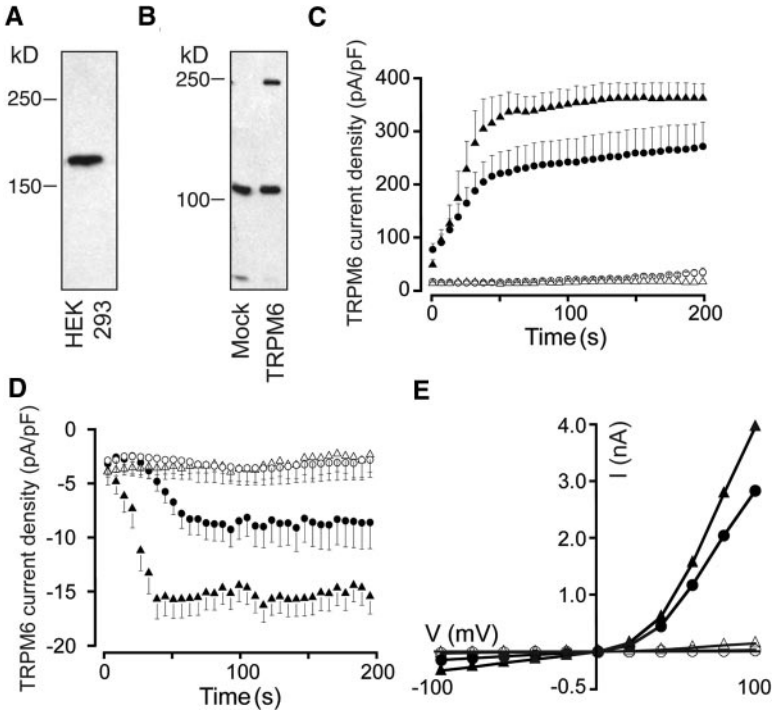

Figure 1. EGF treatment of HEK293 cells stimulates TRPM6 current. (A) EGFR protein expression in HEK293 cells by immunoblot analysis. (B) Immunoblot of TRPM6, using the HA tag, in transiently transfected HEK293 cells. (C and D) Time course of the current development (pA/pF) at +80 mV (C) and −80 mV (D) of TRPM6 transfected HEK293 cells with (▲) or without (●) EGF pretreatment (10 nM, 30 min, 37°C) and empty vector–transfected HEK293 cells with (△) or without (○) EGF pretreatment (n = 11 to 15 cells). (E) Representative current–voltage relations of TRPM6-transfected HEK293 cells with (▲) or without (●) EGF pretreatment and mock-transfected HEK293 cells with (△) or without (○) EGF pretreatment after 200 s.

RESULTS

EGF Activates TRPM6 Current in HEK293

The presence of the EGFR was first confirmed in HEK293 cells by immunoblotting, where it was detected as a single immunopositive band with the expected molecular size of 170 kD (Figure 1A). Thus, HEK293 cells provide an excellent model to study the effect of EGF on TRPM6 activation. TRPM6 has been expressed and characterized in this model system, where it is detectable at the appropriate molecular weight of 230 kD (Figure 1B).[5] We then assessed the effect of EGF on TRPM6 current activity using whole-cell recordings. We analyzed all time courses at +80 and −80 mV for a 200-s period. EGF preincubation stimulated TRPM6 current by 34 ± 6 and 65 ± 8% at +80 and −80 mV, respectively, compared with TRPM6 current in the absence of EGF (Figure 1, C and D). TRPM6 displayed an outwardly rectifying current–voltage relation with a reversal potential close to 0 mV. This was unaltered by EGF pretreatment (Figure 1E).

EGF-Stimulated TRPM6 Current Is Specifically Mediated by the EGFR

To address whether the stimulatory action of EGF was specific to activation of the EGFR, we added the receptor antagonist AG1478 (also named tyrphostin) before treatment with EGF. Incubation of TRPM6-expressing HEK293 cells with AG1478 prevented the EGF stimulatory effect (Figure 2A). To confirm that EGFR stimulation mediated the observed increase in TRPM6 current, we used a specific tyrosine kinase receptor inhibitor, RG13022, to block EGFR activation.[6] After a 10-min exposure, EGF failed to elicit an increase in TRPM6 activity (Figure 2B). In fact, incubation with either of these compounds reduced baseline TRPM6 activity (Figure 2, A and B). We also examined TRPM7, the closest homologue of TRPM6, for an EGF-mediated increase in activity. Consistent with the notion that EGFR engagement by EGF is specific for TRPM6 activation, treatment with EGF did not affect TRPM7 activity (Figure 2C).

Stimulation of TRPM6 by EGF Does not Require the α-Kinase Domain

TRPM6 uniquely encompasses an α-kinase domain with a channel. To determine whether the α-kinase domain is necessary for EGFR-mediated stimulation of TRPM6 activity, we treated HEK293 cells transiently expressing an α-kinase–truncated TRPM6 mutant (TRPM6 Δ-kinase)[7] with EGF and subjected them to whole-cell recordings. EGFR stimulation by EGF was able to increase TRPM6 channel activity, even in the absence of the α-kinase domain. EGF increased the current in TRPM6 Δ-kinase transfected cells by 75 ± 11 and 73 ± 8% at +80 and −80 mV, respectively (Figure 3A).

Stimulation of TRPM6 by EGF Involves Both Src-Family Kinases and MAPK

Src family tyrosine kinases, including Fyn and Lyn, are important downstream signaling intermediates of the EGFR. They

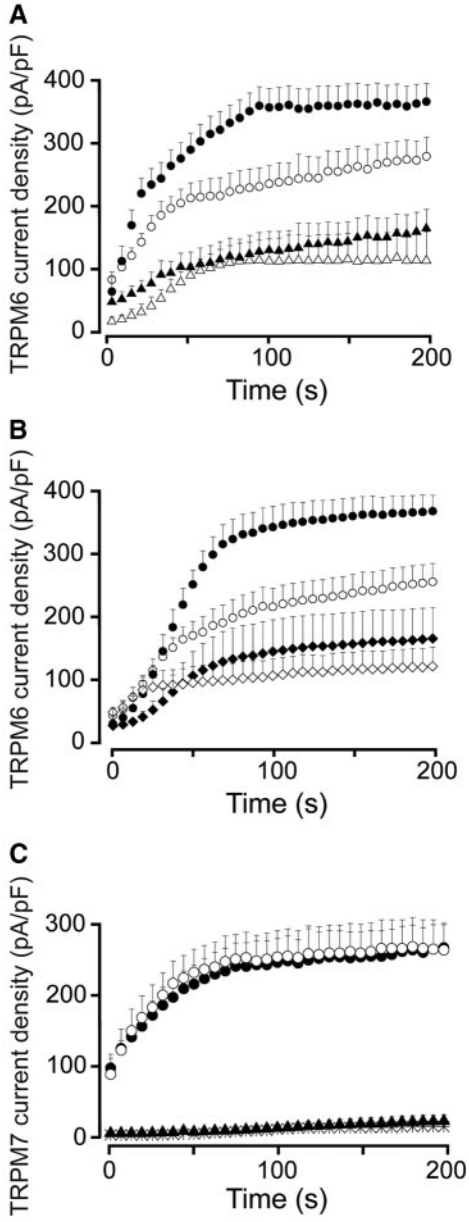

Figure 2. EGFR stimulation by EGF mediates TRPM6 current increase. (A) Time course of the TRPM6 current development (pA/pF) at +80 mV (in transiently transfected HEK293 cells) pretreated with AG1478 (tyrphostin, 1 μM, 1 h) alone (△) or before EGF pretreatment (▲) in comparison with nontreated (○) or EGF-treated (●) cells (n = 9 to 13 cells). (B) Time course of TRPM6 current development (pA/pF) at +80 mV in transiently transfected HEK293 cells pretreated with RG13022 (tyrosine kinase inhibitor, 50 μM, 10 min) alone (◇) or before EGF pretreatment (◆) relative to nontreated (○) or EGF-treated (●) cells (n = 11 to 14 cells). (C) Time course of the TRPM7 current development (pA/pF) at +80 mV after transient transfection of HEK293 cells with (●; n = 21) or without (○; n = 16) EGF pretreatment and empty vector–transfected HEK293 cells with (▲) or without (☆) EGF pretreatment (n = 9 to 14 cells).

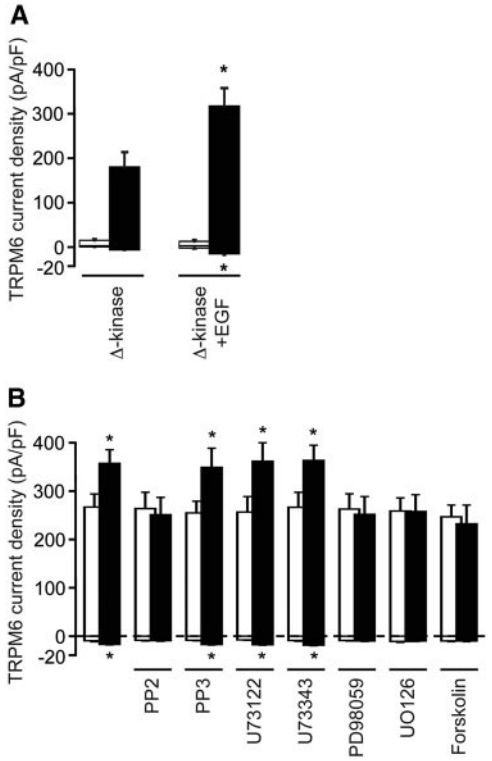

Figure 3. EGF mediates an Src-family kinase and MAPK-dependent activation of TRPM6 independent of the α-kinase domain. (A) Histogram summarizing the current density (pA/pF) at +80 mV and −80 mV after 200 s in mock-transfected (□) or TRPM6 Δ-kinase–transfected (■) HEK293 cells in the presence or absence of EGF as indicated. *$P < 0.05$ *versus* cells transfected with Δ-kinase in the absence of EGF treatment (n = 13 to 19 cells). (B) Histogram summarizing the averaged current density (pA/pF) at +80 mV and −80 mV after 200 s, of TRPM6-transfected HEK293 cells, pretreated with PP2 (5 μM, 10 min), PP3 (5 μM, 10 min), U73122 (10 μM, 15 min), U17343 (10 μM, 15 min), PD98059 (15 μM, 25 min), UO126 (50 μM, 25 min), or forskolin (10 μM, 15 min) without (□) or with (■) EGF pretreatment (10 nM, 30 min). *$P < 0.05$ *versus* TRPM6 current in the absence of EGF treatment (n = 9 to 25 cells).

are also known to regulate ion channel activity.[8] We therefore examined whether Src-family tyrosine kinases were necessary for TRPM6 activation by EGF. Preincubation with PP2, a compound that selectively inhibits Src kinases,[9] prevented the stimulatory effect of EGF, whereas its inactive analog, PP3, failed to prevent EGF from stimulating TRPM6 activity (Figure 3B).

Next, we conducted a series of experiments to identify which signaling pathway(s), downstream of the Src family kinases, is necessary for TRPM6 activation. The involvement of phospholipase Cγ (PLCγ) was excluded using the PLC inhibitor U73122 and its inactive analog, U73343 (Figure 3B). We then evaluated the putative role of the MEK1/2 and ERK1/2 limb of the MAPK superfamily. To this end, we preincubated HEK293 cells with selective inhibitors of MEK1/2 and ERK1/2 activation, UO126 and PD98059, respectively. Treatment with either UO126 or PD98059 prevented EGF from increasing

TRPM6 activity, implicating the ERK1/2 pathway in this signaling cascade (Figure 3B). None of these agents had an effect on TRPM6 in the absence of EGF. Stimulation of ERK1/2 by the EGFR is preceded by Raf-1 activation, an event that can be blocked by PKA.[10] To confirm that ERK1/2 activation was necessary for the EGF-mediated increase in TRPM6 activity, we pretreated TRPM6-transfected HEK293 cells with forskolin, thereby activating PKA. Application of forskolin had no effect on TRPM6 current; it did, however, prevent EGF-mediated activation (Figure 3B). These findings support the conclusion that TRPM6 activation after EGFR engagement is mediated *via* activation of the MAPK pathway.

Stimulation of TRPM6 by EGF Depends on PI3K and Rac1

A downstream target of Src-family kinases is the PI3K/Akt pathway. To address whether PI3K is involved in the EGF-mediated stimulation of TRPM6, we preincubated cells with the PI3K inhibitor LY294005 or wortmannin. Preincubation with either compound prevented the EGF-induced activation of TRPM6 (Figure 4A). Neither compound altered the TRPM6 current when used in isolation (data not shown). A common PI3K effector, implicated in cytoskeletal remodeling and membrane traffic, is Rac1.[11–13] We therefore sought to ascertain whether modulation of Rac1 activity would alter baseline or the EGF-mediated increase in TRPM6 current. HEK293 cells were transiently co-transfected with either a dominant negative mutant of Rac1 (Rac1-T17N) and TRPM6 or a constitutively active mutant of Rac1 (Rac1-G12V) and TRPM6 and then subjected to patch-clamp analysis. Control cells were transiently co-transfected with empty vector and TRPM6. Cells coexpressing Rac1-T17N and TRPM6 displayed significantly decreased TRPM6 currents compared with TRPM6-transfected cells (Figure 4, B and C). In these cells, EGF pretreatment did not restore TRPM6 current. In contrast, cells co-transfected with Rac1-G12V and TRPM6 exerted a significant increase in TRPM6 current (Figure 4, B and C). The addition of EGF to HEK293 cells coexpressing Rac1-G12V and TRPM6 did not further increase the TRPM6 current.

Activation of Rac1 can induce actin cytoskeletal rearrangements. To assess whether this downstream effect of Rac1 was responsible for the EGF-mediated activation of TRPM6, we incubated the cells with cytochalasin D, alone or in combination with EGF, before obtaining whole-cell recordings. Cytochalasin D pretreatment alone inhibited TRPM6 activity; however, it failed to prevent the stimulation of TRPM6 activity by EGF (Figure 4D).

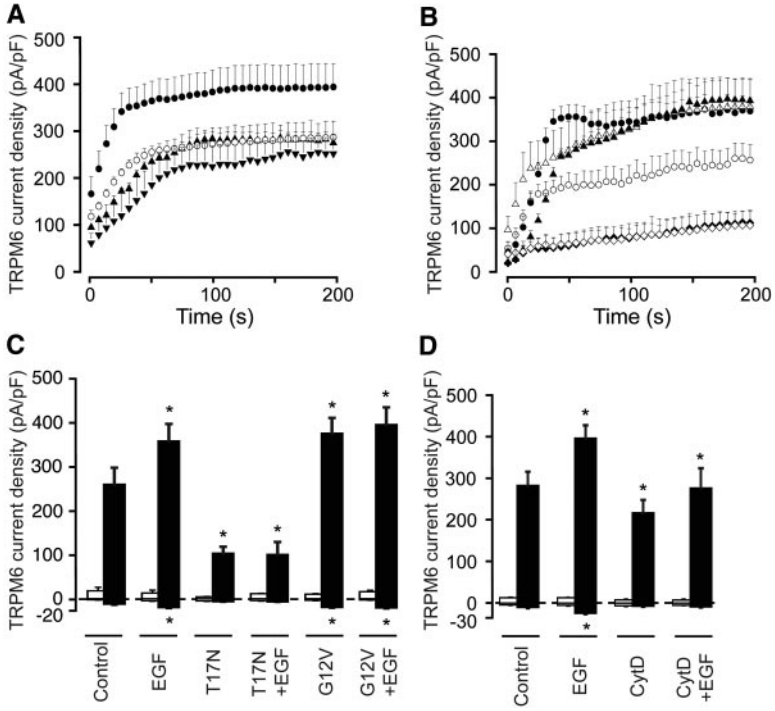

Figure 4. Stimulation of TRPM6 by EGF depends on PI3K, Rac1, and the actin cytoskeleton. (A) Time course of the current development (pA/pF) at +80 mV of TRPM6-transfected HEK293 cells pretreated with wortmannin (10 nM, 20 min; ▲) or LY492005 (50 μM, 20 min; ▼) before treatment with EGF. For comparison, cells that were not treated at all (○) or treated just with EGF (●) are included (n = 9 to 13 cells). (B) Time course of the current development (pA/pF) at +80 mV of TRPM6 and empty vector (circles), TRPM6 and Rac1-G12V (triangles), and TRPM6 and Rac1-T17N (diamonds) transfected HEK293 cells, pretreated (filled symbols) or not (open symbols) with EGF (n = 13 to 19 cells). (C) Histogram summarizing the current density (pA/pF) at +80 and −80 mV of TRPM6 and empty vector (Control), TRPM6 and Rac1-T17N (T17N), and TRPM6 and Rac1-G12V (G12V) transfected HEK293 cells pretreated or not with EGF as indicated. □, Experimental conditions in which HEK293 cells were transfected with the empty vector; ■, HEK293 cells transfected with TRPM6 in the absence or presence of Rac1 mutants. *$P <$ 0.05 *versus* TRPM6 current in empty vector–transfected, nontreated conditions (n = 13 to 19 cells). (D) Histogram summarizing the current density (pA/pF) at +80 and −80 mV of TRPM6-transfected HEK293 cells pretreated or not with EGF or cytochalasin D (CytD; 10 μM, 40 min) as indicated. □, Experimental conditions in which HEK293 cells were transfected with empty vector; ■ HEK293 cells transfected with TRPM6. *$P <$ 0.05 *versus* TRPM6 current under nontreated conditions (n = 14 to 16 cells).

EGF Increases Cell Surface Expression of TRPM6

Implicit to the observed alteration in TRPM6 activity by Rac1 is the notion that EGFR signaling promotes the mobility of TRPM6, presumably by redistributing endomembrane channels into the plasma membrane. To this end, we measured the mobility of TRPM6 in HEK293 cells. This was accomplished with a GFP-fused TRPM6 construct and the imaging technique fluorescence recovery after photobleaching (FRAP). GFP-TRPM6 can be expressed and detected in HEK293 cells by immunoblotting and fluorescence microscopy (Figure 5, A and B). We evaluated the electro-

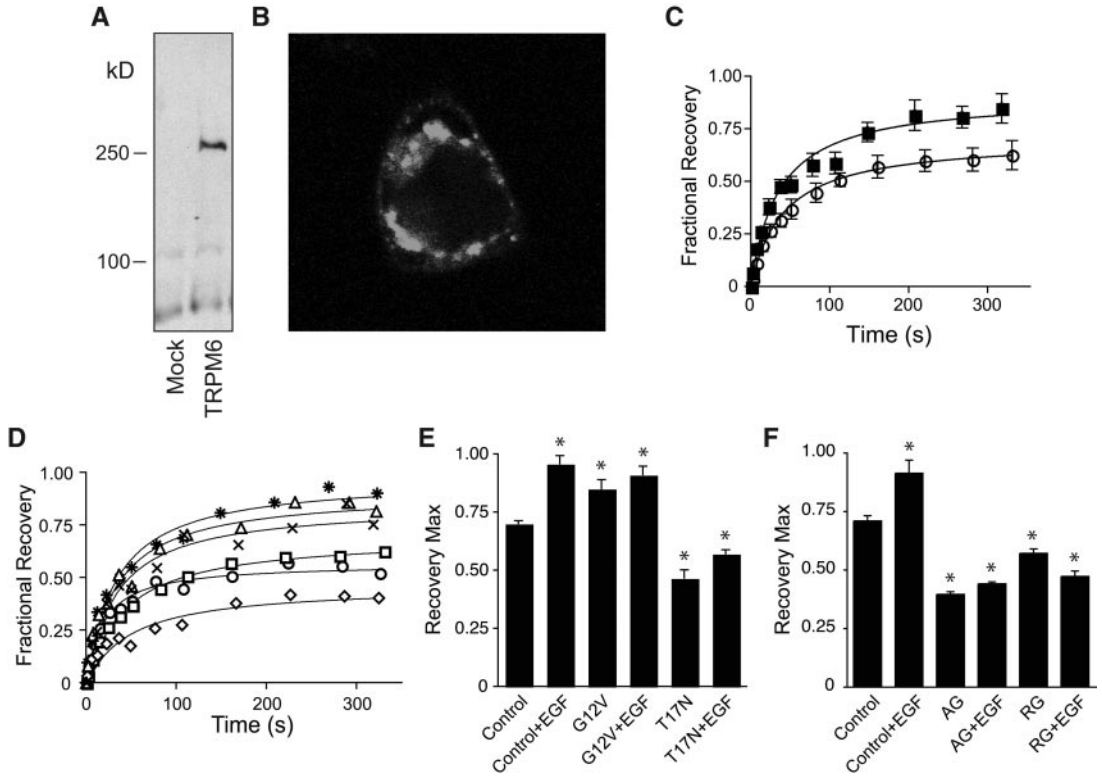

Figure 5. EGF increases the mobility of TRPM6. (A) Immunoblot of GFP-TRPM6 transiently transfected in HEK293 cells. (B) A confocal slice through a representative cell. (C) A plot of fluorescence recovery over time in the presence (■) or absence (○) of EGF. (D) Similar curves of cells co-transfected with GFP-TRPM6 and Rac1-G12V, Rac1-T17N, or mock in the presence or absence of EGF (□, mock; △, mock + EGF; ×, Rac1-G12V; *, Rac1-G12V + EGF; ◇, Rac1-T17N; ○, Rac1-T17N + EGF). (E) A histogram displaying the final recovery of TRPM6 in the presence or absence of EGF when co-transfected with mock (Control), Rac1-G12V, or Rac1-T17N as indicated. (F) A histogram displaying the final recovery of TRPM6 in the presence or absence of EGF after treatment with AG1478/tyrphostin (AG) or RG13022 (RG) as indicated. *$P < 0.05$ *versus* TRPM6 final recovery without EGF treatment in the presence of mock co-transfection ($n = 14$ to 21 cells).

physiologic characteristics of GFP-TRPM6 by patch-clamp analysis, and they displayed a similar baseline and EGF-stimulated current (data not shown). As is evident from Figure 5, C and E, EGF treatment increased both the rate and the fraction of endomembrane TRPM6 that is mobile. That Rac1 alters the mobility of TRPM6 was further supported by repeating these measurements after co-transfection with either Rac1-G12V, the constitutively active mutant, or Rac1-T17N, the dominant negative mutant (Figure 5, D and E). Consistent with the whole-cell patch-clamp analysis (Figure 4C), Rac1-G12V increased both the rate of recovery and the mobile fraction of TRPM6 (Figure 5, D and E). Moreover, EGF treatment failed to increase further the mobility or the mobile fraction. Conversely, a decreased mobile fraction is observed in cells co-transfected with Rac1-T17N. EGF treatment in the presence of Rac1-T17N failed even to return the mobile fraction of TRPM6 to that of the control (Figure 5, D and E). These observations correspond to decreased channel activity irrespective of EGF treatment (Figure 4C). Furthermore, pretreatment with either the EGFR antagonist AG1478 or the tyrosine kinase receptor inhibitor RG13022 not only prevented the EGF-mediated increase in mobile fraction of TRPM6 but also decreased the baseline mobile fraction of the channel (Figure 5F). Taken together, these findings support the concept that EGFR activation stimulates Rac1, alters cytoskeletal dynamics, and consequently increases the mobility of endomembrane TRPM6.

Given that TRPM6 current is enhanced by EGF treatment together with the FRAP results, this suggests that endomembrane TRPM6 is moving into the plasma membrane, thereby increasing the number of active channels there. To confirm that this was occurring, we performed cell surface biotinylation experiments using the fused GFP for immunodetection. We used a TRPM6 stably expressing the renal epithelial cell line MDCK to perform these studies (referred to hereafter as MDCK-GFP-TRPM6). Quantification of cell surface expressed TRPM6 by this method revealed a significant increase in the cell surface expression of TRPM6 after incubation with EGF (Figure 6).

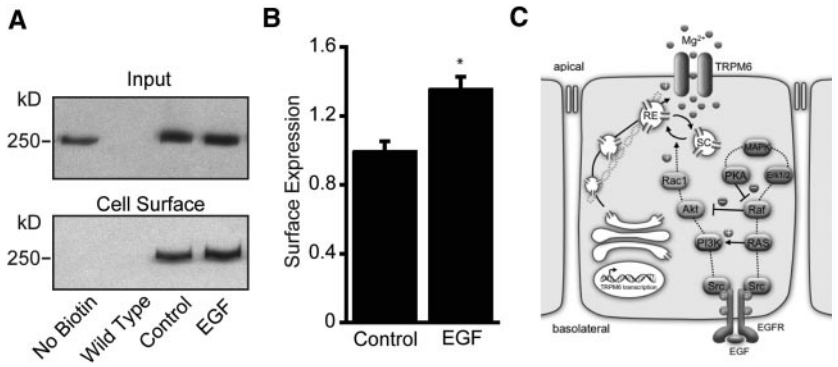

Figure 6. EGF increases the cell surface fraction of TRPM6. (A) Representative immunoblots of total cellular TRPM6, the input (top), and the biotinylated, cell surface fraction (bottom) after treatment of GFP-TRPM6-MDCK cells with EGF (30 min, 10 nM) or vehicle (Control). Nontransfected MDCK cells (Wild Type) and cells that were not treated with biotin (No Biotin) are included for comparison. (B) Quantification of experiment in A. $*P < 0.05$ *versus* vehicle treated condition ($n = 11$ per condition). (C) EGFR stimulation by EGF leads to tyrosine kinase, Src, PI3K, and Rac1 activation. The last effector alters the mobility of endomembrane TRPM6, potentially redistributing the channel from within a storage compartment (SC) to a recycling endomembrane compartment (RE). Ultimately, an increase in the plasma membrane insertion of TRPM6 is achieved and Mg^{2+} influx through the channel is thereby increased.

DISCUSSION

This study delineates the molecular details of TRPM6 stimulation by EGF, from receptor to channel. First, we demonstrated that EGF specifically activates TRPM6 *via* engagement of its receptor, the EGFR. We used a combination of live cell imaging and biochemical and electrophysiologic approaches to elucidate the downstream signaling events. This revealed TRPM6 stimulation to be dependent on the activation of the Src family of tyrosine kinases, the ERK/MEK pathway, and PI3K. Alterations in endomembrane traffic were implicated as the responsible mechanism because constitutively active Rac1 increased TRPM6 current independent of EGF, whereas dominant negative Rac1 abrogated this effect. We further used FRAP to confirm that EGF increases the mobility of TRPM6, an effect that is also Rac1 dependent. Ultimately, we showed that EGF activates TRPM6 by increasing the number of channels at the plasma membrane. Together, these findings clearly outline the activation of TRPM6 by EGF (Figure 6C), a process fundamental to Mg^{2+} homeostasis.

EGFR Signaling Pathway and TRP Channel Regulation

Our study is the first to demonstrate that EGF regulates the melastatin subfamily of TRP channels. Furthermore, the effect is specific to member TRPM6, because no increase in TRPM7 current, the closest homologue, was observed upon EGF application. To confirm that EGF specifically activates the EGFR, we demonstrated the presence of this receptor in our model system and then showed an inhibition of the observed effect when the receptor was blocked with selective antagonists. Further evidence that EGFR engagement is required for TRPM6 activation was provided by the demonstration that inhibition of the receptor's tyrosine kinase activity prevented both EGF-induced increase in TRPM6 mobility and activity. Thus, EGF-mediated activation of TRPM6 is novel within this subfamily of channels and requires the specific engagement of the EGFR.

Among EGFR effectors, PLCγ is a known regulator of TRPC channel activity.[11,14] Furthermore, TRPM7 has been shown to interact with PLCγ and β isoforms[15] and to be inactivated by PIP_2 hydrolysis[16]; however, inhibition of PLCγ failed to prevent EGF-mediated TRPM6 regulation, suggesting that PLCγ is not necessary for the activation of TRPM6 by EGF. Thus, PLCγ is a potential discriminator between EGF regulation of TRPM6 and other TRP channels.[14,15] Furthermore, because TRPM7 is activated by PLCγ and not EGF, this finding highlights the specificity of the EGF signaling cascade for TRPM6. Of note, at -80 mV, the absolute current of TRPM6 is small and therefore likely represents a significant, albeit small, leak current. Because of this, we used only the much larger current at $+80$ mV as a readout of channel activity.[17]

Inhibition of PI3K activity prevented EGF from activating TRPM6. We therefore investigated the role of the small Rho GTPase Rac1, a common effector of PI3K.[18] Dominant negative Rac1 (T17N) abrogated TRPM6 current development and prevented the EGF-mediated increase in TRPM6 activity. In contrast, a constitutively active mutant of Rac1 (G12V) mimicked the EGF stimulation of TRPM6 activity. Both PI3K and Rac1 components have been implicated in TRPC5 and TRPV1 stimulation upon EGF and nerve growth factor application, respectively[11,19]; therefore, in addition to the regulation of brain function such as neurite extension and pain perception through TRP channels, our findings implicate Rac1 in renal Mg^{2+} reabsorption *via* the regulation of TRPM6 channel activation, independent of the constitutively expressed TRPM7.

Regulation of TRPM6 at the Plasma Membrane

The requirement of PI3K and Rac1 for EGF-mediated activation of TRPM6 suggests that altered endomembrane traffic and potentially redistribution to the plasma membrane are responsible for the increased current observed.[20,21] To test this hypothesis, we measured the mobility of TRPM6 before and after treatment with EGF. This increased both the rate that endomembrane TRPM6 was moving and the fraction that was mobile. Indeed, the application of EGF seemed to mobilize an immobile subset of TRPM6 (Figure 5, C, E, and F). The coexpression of Rac1 mutants altered the mobile fraction of TRPM6, in parallel to the observed changes in activity. Moreover, coexpression of these constructs prevented the EGF-mediated alteration in TRPM6 mobility. Furthermore, applica-

tion of the tyrosine kinase inhibitors produced changes in TRPM6 mobility that were parallel to the alteration in activity observed. That EGF activation ultimately leads to increased expression at the plasma membrane was substantiated by cell surface biotinylation analysis. Together, these observations provide strong evidence that EGF increases the abundance of TRPM6 at the plasma membrane.

What, then, explains the increased abundance of TRPM6 at the plasma membrane? We propose that TRPM6 activation subsequent to EGFR stimulation regulates the amount of recycling TRPM6 and/or the balance between constitutive exocytosis and endocytosis in favor of TRPM6 accumulation at the cell surface. This hypothesis is supported by our FRAP analysis, which demonstrates that EGFR activation increases the mobility of endomembrane TRPM6 and releases a pool of immobile or poorly mobile endomembrane channels. The total number of channels within the plasma membrane is determined by the combined effects of membrane insertion, retrieval, and number of recycling channels. EGFR activation likely increases the number of recycling channels and the balance between endocytosis and exocytosis to favor increased plasma membrane expression. Consistent with an effect of EGF on the recycling kinetics of the TRP family of channels, EGF prevents the internalization of plasma membrane TRPC3.[22]

Potential Role(s) of EGF-Mediated Regulation of TRPM6

Our findings implicate both Raf-1 and the ERK1/2 limb of the MAPK superfamily in EGF-mediated activation of TRPM6. EGF is known to alter ion homeostasis through inhibition of the amiloride-sensitive Na^+ reabsorption from collecting duct. This also occurs downstream of ERK1/2 activation.[23] In kidney, TRPM6 and EGF are predominantly expressed in DCT, the nephron segment responsible for regulated, transepithelial Ca^{2+} and Mg^{2+} reabsorption.[24] TRPM6 mediates and regulates this latter process, a conclusion supported by its unique permeation characteristics, carrying almost exclusively divalent cations with a higher affinity for Mg^{2+} than Ca^{2+}[5,25] and its specific localization to Mg^{2+}-transporting epithelia. That this process is regulated by the MAPK is consistent with previous findings; however, in DCT, EGF seems to activate TRPM6 uniquely, to the exclusion of TRPM7, TRPV5, and TRPV6 (Figure 2C; data not shown). TRPV5 mediates active transcellular Ca^{2+} reabsorption from the distal part of the nephron; hence, EGFR signaling confers a precision to the control of Mg^{2+} homeostasis and can therefore be defined as a magnesiotropic hormone.

The specificity of this pathway is highlighted by clinical examples. The patient we originally described[1] displayed clinical symptoms secondary to hypomagnesemia and lacked evidence of altered Ca^{2+} homeostasis. Furthermore, treatment with the anti-EGFR antibody cetuximab specifically alters plasma Mg^{2+} levels.[1,3] The work described herein delineates the pathway between EGFR engagement and apical Mg^{2+} influx through TRPM6, a process whose specificity is mediated by the restricted location of TRPM6 to the EGF-expressing portion of the nephron and unique signaling within this location. This, in turn, provides specificity to the maintenance of Mg^{2+} homeostasis. Both the Src family of tyrosine kinases and the small Rho GTPase Rac1 are central to this. The increased activity of TRPM6 is ultimately the result of increased cell surface expression. These findings provide molecular insight into a novel mechanism specifically regulating transepithelial Mg^{2+} transport and consequently whole-body Mg^{2+} homeostasis.

CONCISE METHODS

Cell Culture and Transfection

HEK293 cells were cultured in DMEM with 10% FCS and transfected as described previously.[25] Electrophysiologic recordings, live cell imaging, and biochemical assays were performed 48 h after transfection. MDCK cells were maintained in DMEM with 10% FCS. MDCK cells were stably transfected with GFP-TRPM6, generated by subcloning TRPM6 into the GFP vector as detailed online. To select a stable line (MDCK-GFP-TRPM6), we cloned the cells by limiting dilution in the presence of 500 μg/ml G418 and screened them by immunofluorescence and immunoblotting for expression of GFP-tagged TRPM6. The experiments were performed at least 72 h after the monolayers had reached confluence.

Electrophysiology

We made electrophysiologic recordings 48 h after transfection of HEK293 cells. We measured whole-cell currents with an EPC-10 amplifier (HEKA, Lambrecht, Germany); electrode resistances were 3 to 5 MΩ, and we monitored capacitance and access resistances continuously. Please refer to additional on-line information about patch-clamp experiments.

Fluorescence Recovery after Photobleaching

The experiments used a similar approach to that described previously.[26] A detailed description of the method is available on-line.

Cell Surface Biotinylation

We incubated MDCK-GFP-TRPM6 cells at 37°C in the presence or absence of EGF (10 nM, 30 min) before placing them on ice to inhibit endo- and exocytosis. We performed biotinylation on ice and lysed cells as described previously.[7] Subsequently, cell surface proteins were precipitated with neutravidin-agarose beads. We probed the purified biotinylated surface proteins for the presence of TRPM6 by immunoblotting with an anti-GFP antibody (Sigma-Aldrich, St. Louis, MO).

Statistical Analysis

Values are expressed as means ± SEM. Statistical significance between groups was determined by ANOVA followed by pair-wise comparison using the method of Scheffé. Differences in means with $P < 0.05$ were considered statistically significant. The statistical analysis was performed using the SPSS software (SPSS, Chicago, IL). Detailed descriptions of Concise Methods are available on-line.

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DISCLOSURES

None.

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