Involvement of 14-3-3 Proteins in the Second Epidermal Growth Factor-induced Wave of Rac1 Activation in the Process of Cell Migration*§

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Background: The spatiotemporal regulation of Rac1 controls cell migration.

Results: EGF induced two waves of Rac1 activation in the process of cell migration.

Conclusion: 14-3-3 proteins regulate the second EGF-induced wave of Rac1 activation by interacting with RacGEF.

Significance: The second wave of Rac1 activation might be required for EGF-induced cell migration.

Immense previous efforts have elucidated the core machinery in cell migration, actin remodeling regulated by Rho family small GTPases including RhoA, Cdc42, and Rac1; however, the spatiotemporal regulation of these molecules remains largely unknown. Here, we report that EGF induces biphasic Rac1 activation in the process of cell migration, and UTKO1, a cell migration inhibitor, inhibits the second EGF-induced wave of Rac1 activation but not the first wave. To address the regulation mechanism and role of the second wave of Rac1 activation, we identified 14–3–3ζ as a target protein of UTKO1 and also showed that UTKO1 abrogated the binding of 14–3–3ζ to Tiam1 that was responsible for the second wave of Rac1 activation, suggesting that the interaction of 14–3–3ζ with Tiam1 is involved in this event. To our knowledge, this is the first report to use a chemical genetic approach to demonstrate the mechanism of temporal activation of Rac1.

The importance of cell migration is evident from the number of physiological processes that depend on the regulated movement of cells, including embryonic development, immune responses, and tissue maintenance and repair, and also from the disease states driven by aberrant cell motility, such as chronic inflammation, vascular disease, and tumor metastasis (1). Key to the capacity of the cell to migrate is dynamic reorganization of the actin cytoskeleton (2). When a cell moves, site-directed de novo nucleation and polymerization of actin drives protrusive membrane structures such as lamellipodia and filopodia, which generate the locomotive force in migrating cells (3, 4). Reorganization of the actin cytoskeleton is regulated by actin-nucleating factors, the most prominent of which is the Arp2/3 complex (5). Catalytic activation of this complex is mediated by WASP/WAVE family members, which in turn translate extracellular signals via the Rho family of small GTPases such as RhoA, Cdc42, and Rac1 (6). In particular, activation of RhoA increases cell contractility and leads to the formation of focal adhesions and stress fibers (7). Activation of Cdc42 and Rac1 propagates the formation of filopodia and lamellipodia, respectively (8, 9).

The Rho family GTPases function as binary switches that cycle between an active GTP-bound form and an inactive GDP-bound form. This cycling is regulated through three factors: guanine nucleotide exchange factor (GEF),2 GTPase-activating protein, and guanine nucleotide dissociation inhibitor (10, 11). Among them, GEF activates the Rho family GTPases by promoting the exchange of GDP with GTP, resulting in the binding of the GTPases to their effectors. A number of GEFs have been shown to transduce signals from many growth factors to the Rho family GTPases. In addition to the increasing number of GEFs, the redundant specificity of GEFs renders signaling networks controlling cell migration difficult to understand; many GEFs have been shown to take multiple Rho family GTPases as substrates, at least in vitro (11, 12). The spatiotemporal coordination of the Rho family GTPases by these molecules regulates a complicated dynamic process of cell migration.

Inhibitors of cell migration would be useful not only as tools for basic research into cell migration but also as anti-metastatic drug-leads for cancer therapy. To obtain cell migration inhibitor, UTKO1 was synthesized as a derivative of natural products moverastins, which inhibit migration of EC17 cells by inhibiting farnesylation of H-Ras (13). However, although its chemical structure is very similar to that of moverastins, its inhibitory effect on cell migration was stronger than that of the moverastins and did not involve inhibition of farnesyltransferase (14). UTKO1 also failed to inhibit MEK/ERK and the PI3K/Akt path-

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Scheme S1, and Figs. S1–S9.

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2 The abbreviations used are: GEF, guanine nucleotide exchange factor; IP, immunoprecipitation; CBB, Coomassie Brilliant Blue; EGF, epidermal growth factor.
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way generally known to regulate cell migration.\(^3\) This unique pharmacological profile of UTKO1 has drawn considerable interest, prompting us to further investigate its mechanism of action. In this report, we present evidence that EGF induces two waves of Rac1 activation in the process of cell migration and that UTKO1 inhibited only the second of these waves by targeting 14-3-3\(\zeta\). Furthermore, we showed that UTKO1 abrogated the binding of 14-3-3\(\zeta\) to Tiam1 that was responsible for the second wave of Rac1 activation, presumably resulting in the inhibition of EGF-induced cell migration.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Human cDNA for 14-3-3s (\(\alpha/\beta\), \(\epsilon\), \(\eta\), \(\gamma\), \(\nu/\theta\), \(\xi/\delta\), and \(\sigma\)) were amplified from HeLa cell cDNA and cloned into pcDNA3 (Invitrogen, San Diego, CA) with the N-terminal FLAG tag. All of the constructs were cloned into pGEX-2T (GE Healthcare, Princeton, NJ) to prepare GST fusion proteins in bacteria. Expression vectors encoding GST-fused 14-3-3\(\zeta\) mutants (\(\Delta C100, 1–145\) amino acids; \(\Delta C200, 1–45\) amino acids; and \(C50, 196–245\) amino acids) were generated by PCR using pGEX-2T/14-3-3\(\zeta\) as a template. pCS2\(^+\)/MT/Tiam1, a expression vector encoding human Tiam1 followed by 6\(\times\)Myc, was kindly provided by Dr. H. Sugimura (Hamamatsu University School of Medicine, Hamamatsu, Japan).

**Chemotaxis Chamber Assay**—Cell migration was assayed with a chemotaxis chamber (Becton Dickinson, Franklin Lakes, NJ). A431 cells suspended in DMEM supplemented with 0.2% calf serum were incubated in the upper chamber; the lower chamber contained DMEM supplemented with 0.2% calf serum in PBS. The cells attached to the lower side of the filter were counted.

**Immunoprecipitation**—A431 cells were subjected to SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining (see Fig. 3B) or immunoblotting (see Fig. 4A). Following CBB staining, the bands corresponding to the UTKO1 binding proteins were excised, and the gel pieces were destained with 50% CH\(_3\)CN in 50 mM NH\(_4\)HCO\(_3\) solution. After removal of the supernatant, cysteine residues were reduced with DTT, carbamidomethylated with iodoacetamide, and the proteins were digested with trypsin at 37 °C overnight. The tryptic peptides were recovered by sequentially adding three solvent systems containing 50% CH\(_3\)CN and 1% TFA; 20% HCOOH, 25% CH\(_3\)CN and 15% i-PrOH; and 80% CH\(_3\)CN. The supernatants were collected and pooled into one tube, reducing the volume in vacuo. The dried tryptic peptides were suspended in 2% CH\(_3\)CN and 0.1% TFA and applied to the following LC-MS/MS system. Chromatographic separation was accomplished with the MAGIC 2002 HPLC system (Michrom BioResources). Peptide samples were loaded onto a Cadenza C18 custom-packed column (0.2 × 50 mm; Michrom BioResources) and eluted using a linear gradient of 5–60% CH\(_3\)CN in 0.1% HCOOH for 30 min at a flow rate of 1 ml/min. Samples were ionized with a Nanoflow-LC ESI, and MS/MS spectrum data were obtained with an LCQ-Deca XP ion trap mass spectrometer (Thermo Electron). The Mascot data base searching software (Matrix Science) was used for the identification of B-UTKO1ox binding proteins.

**Immunoprecipitation**—A431/FLAG-14-3-3\(\zeta\) cells were transiently transfected with pCS2\(^+\)/MT/Tiam1 using Metafectene Pro (Biontex, Munich, Germany). Following 24 h of transfection, the cells were pretreated with UTKO1 for 15 min and stimulated with EGF for 12 h. The cells were collected and sonicated in IP buffer. The cell lysates were cleared by centrifugation at 15,000 \(\times\) g for 10 min at 4 °C then incubated with anti-FLAG antibody and protein A/G-agarose beads (Santa Cruz, Santa Cruz, CA) at 4 °C overnight. The immunoprecipitants were washed once with IP buffer and twice with IP buffer containing 1% Nonidet P-40. The bound proteins were eluted by boiling in SDS sample buffer for 5 min and subjected to Western blotting.

**In Vitro B-UTKO1 Pulldown Assay**—GST fusion proteins, which were expressed in the Escherichia coli DH5\(\alpha\) strain and purified using glutathione-Sepharose 4B (GE Healthcare), were incubated with B-UTKO1ox and avidin beads in 500 \(\mu\)l of IP sample buffer for 5 min. The resultant samples were subjected to Western blotting.

**Identification of B-UTKO1 Binding Proteins**—A431 cells were stimulated with EGF (30 ng/ml) for 4 h. The cells were collected and sonicated twice in immunoprecipitation (IP) buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT, and a protease inhibitor mixture) for 10 s. The cell lysates were centrifuged at 130,000 \(\times\) g for 1 h at 4 °C. The resulting supernatant was precleared twice with avidin beads (Pierce) for 1 h and incubated with biotin (50 nmol) or B-UTKO1ox (50 nmol) and avidin beads at 4 °C overnight. The beads were washed three times with IP buffer and once with PBS. The bound proteins were eluted with 2 mM biotin in PBS and concentrated by a centrifugal filter device (Ultracel (YM-10); Millipore). The resulting proteins were subjected to SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining (see Fig. 3B) or immunoblotting (see Fig. 4A). Following CBB staining, the bands corresponding to the UTKO1 binding proteins were excised, and the gel pieces were destained with 50% CH\(_3\)CN in 50 mM NH\(_4\)HCO\(_3\) solution. After removal of the supernatant, cysteine residues were reduced with DTT, carbamidomethylated with iodoacetamide, and the proteins were digested with trypsin at 37 °C overnight. The tryptic peptides were recovered by sequentially adding three solvent systems containing 50% CH\(_3\)CN and 1% TFA; 20% HCOOH, 25% CH\(_3\)CN and 15% i-PrOH; and 80% CH\(_3\)CN. The supernatants were collected and pooled into one tube, reducing the volume in vacuo. The dried tryptic peptides were suspended in 2% CH\(_3\)CN and 0.1% TFA and applied to the following LC-MS/MS system. Chromatographic separation was accomplished with the MAGIC 2002 HPLC system (Michrom BioResources). Peptide samples were loaded onto a Cadenza C18 custom-packed column (0.2 × 50 mm; Michrom BioResources) and eluted using a linear gradient of 5–60% CH\(_3\)CN in 0.1% HCOOH for 30 min at a flow rate of 1 ml/min. Samples were ionized with a Nanoflow-LC ESI, and MS/MS spectrum data were obtained with an LCQ-Deca XP ion trap mass spectrometer (Thermo Electron). The Mascot data base searching software (Matrix Science) was used for the identification of B-UTKO1ox binding proteins.

3 S. Magi and M. Imoto, unpublished observations.
buffer for 3 h. The beads were washed and eluted with 2 mM biotin in PBS. The eluted proteins were subjected to SDS-PAGE. For the competition assay, UTKO1 was added before incubating with B-UTKO1ox.

**GST Pulldown Assay**—The collected cells were sonicated twice in IP buffer for 10 s. The cell lysates were cleared by centrifugation at 15,000 × g for 15 min at 4 °C and then incubated with purified GST or GST-14-3-3ζ and glutathione-Sepharose 4B (GE Healthcare) for 2 h. The bound proteins were eluted by boiling in SDS sample buffer for 5 min and subjected to Western blotting. For Fig. 6A, purified GST-14-3-3ζ was pre-incubated with UTKO1 in a total volume of 1 ml of IP buffer for 1 h. Other experimental procedures are given in the supplemental materials.

**RESULTS**

**UTKO1 Inhibits the Second EGF-induced Wave of Lamellipodia Formation**—The formation of lamellipodia, protruding membrane structures at the leading edge of migrating cells, is key in cell migration (15). We observed transient lamellipodia formation at 5 min following EGF stimulation, as reported elsewhere (12, 16), and we also found a second wave of lamellipodia formation to be initiated at 6–9 h and reach its zenith within 12 h after EGF stimulation in human epidermoid carcinoma A431 cells (Fig. 1, A and B). Furthermore, we found that UTKO1 (Fig. 1C), a cell migration inhibitor (14), inhibited only the second wave of that as shown in Fig. 1 (D–G); we evaluated the effect of UTKO1 on the lamellipodia formation induced by treatment with EGF for 5 min and for 12 h, respectively, and found no inhibition of lamellipodia formation at 5 min; lamellipodia formation at 12 h was inhibited by UTKO1 with an IC₅₀ value of 0.78 μM. This IC₅₀ value is almost the same as that for inhibiting cell migration (0.67 μM; Fig. 1H). Similar results were obtained when TT cells, a human esophageal cancer cell line, were used in place of A431 cells (supplemental Fig. S1). Thus, although EGF induced two waves of lamellipodia formation, at 5 min and 12 h after stimulation, UTKO1 inhibited only the second wave.

**UTKO1 Inhibits the Second EGF-induced Wave of Rac1 Activation**—Lamellipodia formation has been reported to be mainly regulated by Rac1, a member of the Rho family of small GTPases (9, 17). Because lamellipodia formation was observed at 5 min and 12 h following EGF stimulation, we next examined whether EGF also induces two waves of Rac1 activation. Rac1 was rapidly and transiently activated at 2–5 min after EGF stimulation, as expected from previous published reports (12, 18), and we also found that active Rac1 began to increase from 6 h onward. This second wave of Rac1 activation was much broader than the first wave and lasted until 12 h after EGF stimulation (Fig. 2A). UTKO1 did not inhibit the first wave of Rac1 activation even when the cells were pretreated with UTKO1 for 12 h but did inhibit the second wave (Fig. 2, B and C). Similar results were obtained when TT cells were used in place of A431 cells (supplemental Fig. S2). Moreover, when UTKO1 was added at 4 h after EGF stimulation, the second EGF-induced wave of Rac1 activation, lamellipodia formation, and cell migration were all inhibited (Fig. 2, D–F). These results indicate that UTKO1 suppresses EGF-induced cell migration, possibly via inhibition of the second wave of Rac1 activation required for lamellipodia formation.

**Identification of 14-3-3ζ as a UTKO1-binding Protein**—To elucidate the mechanism for the inhibition of cell migration caused by UTKO1, we tried to identify the target protein of UTKO1 responsible for Rac1 activation at 12 h following EGF stimulation. We used biotinylated UTKO1s (B-UTKO1ox and B-UTKO1ph) (Fig. 3A and supplemental Scheme S1), which were biologically active with the same potency as UTKO1. Lysates of A431 cells stimulated with EGF for 4 h were incubated overnight with B-UTKO1ox and avidin beads. The B-UTKO1ox-bound avidin beads were precipitated and washed, and co-precipitated proteins were eluted by excess biotin. The eluted proteins were separated by SDS-PAGE and detected by CBB staining (Fig. 3B). We observed 10 major protein bands that specifically co-precipitated with B-UTKO1ox and identified these proteins by LC-MS/MS system as: 1) GRP78; 2) PDI; 3) nucleobindin-2; 4) 2-phosphohexose-hydratase α-enolase; 5) unnamed product; 6) unnamed product; 7) mutant β-actin; 8) annexin A2; 9) 14-3-3ζ; and 10) 14-3-3ζ. The peptide sequences of unnamed products 5 and 6 suggest them to be nuclear lamin proteins. Of these 10 proteins, we speculated that 14-3-3ζ might be the target of UTKO1, because it has been previously reported to relate to the formation of lamellipodia (19, 20). The binding of UTKO1 to 14-3-3ζ was confirmed by Western blotting of B-UTKO1ox- or B-UTKO1ph-bound proteins using anti-14-3-3ζ antibody, as shown in Fig. 4A. Next, to determine whether UTKO1 could bind directly to 14-3-3ζ, we performed *in vitro* B-UTKO1 pulldown experiments using purified GST-tagged 14-3-3ζ. GST-14-3-3ζ co-precipitated with B-UTKO1ox: competition was clearly observed in the presence of UTKO1 (Fig. 4B). These results suggest that UTKO1 binds directly to 14-3-3ζ. Moreover, we found that B-UTKO1ox did not bind to a C terminus deletion mutant of 14-3-3ζ, indicating that the binding of UTKO1 to 14-3-3ζ is probably via the C-terminal region (supplemental Fig. S3). 14-3-3ζ is a member of the 14-3-3 family, and at least seven different isoforms have been identified in mammalian cells (21, 22). Therefore, we prepared seven recombinant GST-14-3-3 isoforms and performed *in vitro* B-UTKO1 pulldown experiments to test each for its ability to bind to UTKO1. As a result, the ζ isoform showed the strongest binding ability to B-UTKO1ox (Fig. 4C).

Next, we performed RNAi experiments to investigate whether the loss of 14-3-3ζ could suppress EGF-induced cell migration, lamellipodia formation, and Rac1 activation in A431 cells. The successful knockdown of 14-3-3ζ by siRNA was confirmed by Western blotting (Fig. 4D). Silencing of 14-3-3ζ expression consequently suppressed EGF-induced cell migration in A431 cells (Fig. 4E). Furthermore, silencing of 14-3-3ζ expression did not inhibit EGF-induced Rac1 activation at 2 min and lamellipodia formation at 5 min but did inhibit both EGF-induced Rac1 activation and lamellipodia formation at 12 h, as shown in Fig. 4 (F–H). These results indicate that 14-3-3ζ acts upstream of the second EGF-induced wave of Rac1 activation. Additionally, EGF-induced filopodia-like structures observed in 14-3-3ζ knockdown A431 cells (Fig.
FIGURE 1. UTKO1 inhibits the second EGF-induced wave of lamellipodia formation in A431 cells. A and B, EGF-induced lamellipodia formation in A431 cells, observed under confocal microscopy (A) and counted (B). C, structure of UTKO1. D–G, effect of UTKO1 on EGF-induced lamellipodia formation. A431 cells were pretreated with the indicated concentrations of UTKO1 for 15 min and stimulated with EGF. After 5 min (D and E) or 12 h (F and G), the cells were observed under confocal microscopy (D and F) and counted (E and G). The data represent the means ± S.D. (n = 6). H, inhibitory activity of UTKO1 on EGF-induced cell migration, monitored using a chemotaxis chamber. The data represent the means ± S.D. (n = 5). Throughout, the data were representative of at least three independent studies. Arrows, lamellipodia; arrowheads, see text. Scale bar, 10 μm. For G, statistical analyses were performed with a two-tailed Student's t test. *, p = 0.00013; **, p = 1.0 × 10^{-5}. For B, E, and G, more than 300 cells were analyzed per experiment.
These filopodia-like structures might be formed as the result of a failure in the formation of a branched actin network as observed in a previously reported case (23). These observations also support the conclusion that the functional defect in 14-3-3/H9256 was induced by the treatment of cells with UTKO1. Furthermore, we showed that UTKO1 did not affect the expression levels of 14-3-3/H9256 (Fig. 4I). Taken together, these results suggest that UTKO1 binds to and inactivates 14-3-3ζ, resulting in inhibition of the second EGF-induced wave of Rac1 activation and subsequent suppression of lamellipodia formation and cell migration.

**UTKO1 Inhibits the Interaction between 14-3-3ζ and Tiam1**—Next, we examined the mechanism underlying the inhibition of Rac1 activation caused by the binding of UTKO1 to 14-3-3ζ. First, we examined the role of 14-3-3ζ in EGF-induced Rac1 activation. Because 14-3-3 proteins act as adaptor or “chaperone molecules” and interact with various cellular proteins (24), we hypothesized that a binding partner of 14-3-3ζ would regulate the second wave of Rac1 activation, and the most likely candidate binding partner is RacGEF. Thus, we investigated RacGEFs to see which would interact with 14-3-3ζ at 12 h following EGF stimulation. As shown in Fig. 5A, we verified that 14-3-3ζ interacts with both Tiam1 and βPix, as described in previous reports (19, 25, 26). We subsequently performed RNAi experiments to examine whether these GEFs are actually responsible for the second EGF-induced wave of Rac1 activation. As shown in Fig. 5B,
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FIGURE 3. Identification of UTKO1 binding proteins. A, structures and bioactivities of B-UTKO1s. Bioactivities are shown as IC50 values in chemotaxis chamber assay and in lamellipodia formation assay. B, purification of UTKO1 binding proteins. Lysates of A431 cells stimulated with EGF for 4 h were incubated with biotin (50 nmol) or B-UTKO1ox (50 nmol) and avidin beads overnight. The beads were washed, and co-precipitated proteins were eluted with 2 mM biotin. The eluted proteins were subjected to SDS-PAGE followed by CBB staining. The co-precipitated proteins for B-UTKO1ox were identified as described under *Experimental Procedures."

silencing of Tiam1 expression suppressed in part (50% suppression) the second EGF-induced wave of Rac1 activation, whereas silencing of βPix expression had almost no effect. Furthermore, knockdown of Tiam1 suppressed both the second EGF-induced wave of lamellipodia formation and cell migration (Fig. 5, C and D). We also showed that Tiam1 is not involved in the first wave of Rac1 activation (supplemental Fig. S4). Similar results were obtained when TT cells were used in place of A431 cells (supplemental Fig. S5). These results demonstrate that Tiam1 is involved in the process of EGF-induced cell migration and therefore might function as the RacGEF responsible for the second EGF-induced wave of Rac1 activation by interacting with 14-3-3ζ.

Because UTKO1 binds to 14-3-3ζ and inhibits Rac1 activation, we examined the possibility that binding of UTKO1 to 14-3-3ζ abrogates the interaction between 14-3-3ζ and Tiam1. Lysates of A431 cells stimulated with EGF for 12 h were incubated with GST-14-3-3ζ in the presence or absence of UTKO1, and Tiam1 that co-precipitated with glutathione-Sepharose 4B was detected by Western blotting using anti-Tiam1 antibody. As shown in Fig. 6A, UTKO1 reduced the amount of 14-3-3ζ-bound Tiam1, indicating that it inhibited the interaction between 14-3-3ζ and Tiam1. Inhibition of the interaction between 14-3-3ζ and its binding partner by UTKO1 is not specific, because UTKO1 also inhibited the interaction between 14-3-3ζ and βPix but did not affect the interaction between 14-3-3ζ and kinesin. Subsequently, we confirmed the inhibition of the interaction between 14-3-3ζ and Tiam1 by UTKO1 in cultured cells by immunoprecipitation assay. A431 cells stably expressing FLAG-14-3-3ζ (A431/FLAG-14-3-3ζ cells) transiently transfected with Tiam1–6×Myc were stimulated with EGF for 12 h in the presence or absence of UTKO1. The cells were lysed, and the protein complexes that were precipitated with anti-FLAG antibody were detected by Western blotting. As shown in Fig. 6B, the amount of Tiam1 bound to FLAG-14-3-3ζ was reduced in a dose-dependent manner following treatment with UTKO1, confirming that UTKO1 directly inhibits the interaction between 14-3-3ζ and Tiam1 through binding to 14-3-3ζ.

DISCUSSION

Exposure of cells to growth factors leads to a variety of cellular responses at different times after stimulation. Some signaling molecules responsible for growth factor-stimulated cellular responses are activated by exposure to growth factor not only acutely, but also at a later time. For example, platelet-derived growth factor activates PI3K and PKC within minutes of stimulation and then again after 3–7 and 5–9 h, respectively (27). In addition, it has been reported that there are two distinct times during G1 phase when Ras activity is needed for cell cycle progression (28). Moreover, in endothelial cells, biphasic activation of Rac1 has been reported, with the first peak between 1 and 2 h and the second peak at 8 h after induction by wound scratch/IL-1β (29).

We found that EGF induced distinct waves of Rac1 activation. When A431 cells are stimulated with EGF, Rac1 activation begins to wane after 5–20 min as previously reported (12, 16, 18); we also detected a second, prolonged wave of Rac1 activation 6–12 h after EGF stimulation (Fig. 2A). The regulation mechanism and role of early EGF-induced Rac1 activation have been extensively studied (12, 18, 30), but the second wave of Rac1 activation has not yet been examined.

UTKO1, an inhibitor of cell migration, was found to inhibit the second EGF-induced wave of Rac1 activation, but not the first wave (Fig. 2, B–F). Therefore, UTKO1 provides a useful chemical probe for the elucidation of the regulation mechanism and role of the second wave of Rac1 activation in cell migration. Our first step was to identify the molecular target of UTKO1 as 14-3-3ζ. This conclusion is supported by the following findings: 1) 14-3-3ζ was detected in affinity-purified proteins with biotin-conjugated UTKO1 prepared from lysates of A431 cells stimulated with EGF for 4 h (Figs. 3B and 4A); 2) biotin-conjugated UTKO1 bound directly to recombinant 14-3-3ζ (Fig. 4B); 3) knockdown of 14-3-3ζ by
siRNA inhibited the second EGF-induced wave of Rac1 activation but not the first wave (Fig. 4F); and 4) the morphology of 14-3-3/H9256-deficient A431 cells was very similar to that of UTKO1-treated A431 cells (Figs. 1F and 4G).

14-3-3ζ belongs to the 14-3-3 protein family, which is a class of highly conserved acidic proteins encoded by seven mammalian genes (α/β, ε, η, γ, π/θ, ζ/δ, and σ) (21, 22). These seven isoforms share about 50% amino acid identity; nevertheless, UTKO1 seems to bind somewhat selectively to the ζ isomer (Fig. 4C). This selectivity can be explained by our finding that UTKO1 bound to the C-terminal region of 14-3-3ζ (supplementary Fig. S3), because this segment is the most variable region of the 14-3-3 isoforms (31). Taken together, these results suggest that 14-3-3ζ is involved in the mechanism for only the second EGF-induced wave of Rac1 activation. Moreover, UTKO1 bound to and inactivated 14-3-3ζ, thereby inhibiting the second EGF-induced wave of Rac1 activation. However, we cannot exclude the possibility that other members of the 14-3-3 family are also related to the inhibition of the second EGF-induced wave of Rac1 activation caused by UTKO1.

What had remained unclear was the mechanism for the involvement of 14-3-3ζ in the second EGF-induced wave of Rac1 activation.
Rac1 activation and how UTKO1 binding to 14-3-3 related to the suppression of the second wave. 14-3-3 proteins function as molecular scaffolds by modulating the conformation of their binding partners; therefore, we speculated that the binding partner of 14-3-3 would regulate the second EGF-induced wave of Rac1 activation. Of more than 200 known binding partners of 14-3-3 proteins (25, 32, 33), Tiam1 has been reported to be a Rac-specific GEF (34). Indeed, Tiam1 in lysates from A431 cells stimulated with EGF for 12 h was confirmed to co-precipitate with GST-14-3-3 (Fig. 5A). Knockdown of Tiam1 by siRNA was found to reduce the second EGF-induced wave of Rac1 activation but not the first wave (Fig. 5B and supplemental Fig. S4A). Interestingly, the intracellular expression level of Tiam1 is quite low in unstimulated A431 cells; it gradually increased and reached its zenith within 12 h after EGF stimulation (supplemental Fig. S6). These findings might explain why Tiam1 is not involved in the first wave of Rac1 activation. The involvement of Tiam1 in the second wave was further confirmed using NSC23766, a specific inhibitor of Tiam1-mediated activation of Rac1 (35, 36): NSC23766 inhibited both EGF-induced cell migration and second wave of Rac1 activation, but not the first wave (supplemental Fig. S7). Taken together with our finding that knockdown of 14-3-3 inhibited the second EGF-induced wave of Rac1 activation but not the first wave, the second wave would require interaction between 14-3-3 and Tiam1. This interaction was disrupted by the binding of UTKO1 to 14-3-3 (Fig. 6); therefore, UTKO1 inhibited the second wave of Rac1 activation by inhibiting the interaction between 14-3-3 and Tiam1.

Recently, it has been reported that the interaction of 14-3-3 proteins with the N terminus of Tiam1 regulates protein stability (37). However, UTKO1 affected neither the stability of Tiam1 protein nor the intracellular localization of Tiam1 in our assay system (supplemental Fig. S8). Therefore, it is likely that conformational change of Tiam1 caused by interaction with

FIGURE 5. Identification of Tiam1 as a RacGEF which is responsible for the second EGF-induced wave of Rac1 activation. A, GST pulldown assay. Lysates of A431 cells stimulated with EGF for 12 h were incubated with GST or GST-14-3-3 and glutathione-Sepharose 4B. The precipitated proteins were subjected to Western blotting using the indicated antibodies. B–D, knockdown experiments. B and C, control, Tiam1, or βPix siRNA-transfected A431 cells were stimulated with EGF for 12 h. Then the cells were examined for active Rac1 by pulldown assay (B) or the cells with lamellipodia were counted (C). The data represent the means ± S.D. (n = 6). D, control, Tiam1, or βPix siRNA-transfected A431 cells were incubated in the upper chamber and stimulated with or without EGF for 24 h. Then the migrated cells were counted. The data represent the means ± S.D. (n = 5). For C and D, statistical analyses were performed with a two-tailed Student’s t test. *, p = 0.0036; **, p = 5.9 × 10⁻⁹. Throughout, the data were representative of at least three independent studies. For C, more than 300 cells were analyzed per experiment.
14-3-3ζ activates the GEF property of Tiam1 and is inhibited by UTKO1. However, Tiam1 is not the only GEF regulating the second wave of Rac1 activation, because partial activation of second wave Rac1 was still observed in Tiam1 knockdown A431 cells. Therefore, another RacGEF might be involved in second wave Rac1 activation, possibly also through an interaction with 14-3-3ζ.

βPix, another RacGEF, has also been reported to be a 14-3-3 binding partner (19, 32), and we confirmed the interaction between βPix and 14-3-3ζ. However, knockdown of βPix did not affect the second EGF-induced wave of Rac1 activation but rather enhanced it even when the cells were not stimulated with EGF (Fig. 5B and supplemental Fig. S5C). Therefore, although UTKO1 disrupted the interaction of 14-3-3ζ with βPix (Fig. 6A), this disruption is not responsible for the UTKO1-inhibited second wave of Rac1 activation.

Among the multiple GEFs, Asef and Vav2 have been shown to activate Rac1 in response to EGF (18, 38). However, in most reported experiments, Rac1 activation was detected only within minutes of EGF stimulation (12, 18, 38, 39). We did not detect any interaction between 14-3-3ζ and Asef or Vav2 at times corresponding to the first or second wave following EGF stimulation (supplemental Fig. S9). Therefore, it is likely that Asef and Vav2 function as RacGEF during the first wave of Rac1 activation but are not involved in the 14-3-3ζ-mediated EGF-induced wave of Rac1 activation. Moreover, we cannot exclude the possibility that a 14-3-3 binding partner other than RacGEF is responsible for the second wave of Rac1 activation.

The biphasic timing of Rac1 activation mirrors the biphasic formation of lamellipodia. The first wave of lamellipodia formation seems to be nonpolarized, but second wave lamellipodia are formed at the leading edge and polarized. Moreover, because inhibition of only the second wave of Rac1 activation by UTKO1, as well as NSC23766, is enough to inhibit cell migration, the second wave is required for EGF-induced cell migration. The precise role of each wave of Rac1 activation in EGF-induced cell migration is being actively investigated.

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