Akt2 regulates Rac1 activity in the insulin-dependent signaling pathway leading to GLUT4 translocation to the plasma membrane in skeletal muscle cells

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Highlights:

· The role of Akt in the activation of Rac1 in insulin signaling is examined in myocytes.

· Insulin-induced Rac1 activation was sensitive to the effect of an Akt inhibitor.

· Activated PI3K-induced GLUT4 translocation was suppressed by Rac1 knockdown.

· Activated Rac1-induced GLUT4 translocation was not suppressed by Akt2 knockdown.

·Insulin-induced Rac1 activation was suppressed by Akt2 knockdown.

Keywords: Akt2; glucose uptake; GTPase; insulin; Rac1; skeletal muscle

Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; Myr-p110, myristoylated p110; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA.

Abstract

The small GTPase Rac1 plays a pivotal role in insulin-stimulated glucose uptake in skeletal muscle, which is mediated by GLUT4 translocation to the plasma membrane. However, regulatory mechanisms for Rac1 and its role in the signaling pathway composed of phosphoinositide 3-kinase and the serine/threonine kinase Akt remain obscure. Here, we investigate the role of Akt in the regulation of Rac1 in myocytes. Insulin-induced, but not constitutively activated Rac1-induced, GLUT4 translocation was suppressed by Akt inhibitor IV. Insulin-induced Rac1 activation, on the other hand, was completely inhibited by this inhibitor. Constitutively activated phosphoinositide 3-kinase induced Rac1 activation and GLUT4 translocation. This GLUT4 translocation was almost completely suppressed by Rac1 knockdown. Furthermore, constitutively activated phosphoinositide 3-kinase-induced, but not constitutively activated Rac1-induced, GLUT4 translocation was suppressed by Akt2 knockdown. Finally, insulin-induced Rac1 activation was indeed inhibited by Akt2 knockdown. Together, these results reveal a novel regulatory mechanism involving Akt2 for insulin-dependent Rac1 activation.

1. Introduction

Insulin-dependent glucose uptake is critical in the maintenance of glucose homeostasis, defects in which may result in type 2 diabetes. The glucose transporter GLUT4 is expressed in muscle and adipose tissues, playing a key role in facilitative glucose uptake. Under basal conditions, GLUT4 is retained in intracellular storage compartments termed GLUT4 storage vesicles. Following insulin stimulation, exocytosis of GLUT4 from these compartments to the plasma membrane is accelerated, leading to a net accumulation of GLUT4 on the cell surface [1-3].

While our understanding of the precise mechanisms for the regulation of insulin-induced GLUT4 redistribution remains incomplete, many protein molecules have been implicated in this signaling. In particular, a phosphoinositide 3-kinase (PI3K)-dependent protein kinase cascade consisting of PI3K, PDK1, and Akt2 has a pivotal role in regulating GLUT4 translocation. In response to insulin stimulation, PI3K is recruited to the insulin receptor signaling complex through the direct interaction with the phosphorylated insulin receptor substrate 1 protein. PI3K subsequently yields 3-phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate, which in turn induces plasma membrane translocation and activation of PDK1. PDK1 and the mammalian target of rapamycin complex 2 phosphorylate and activate Akt2. Akt2 is involved in several GLUT4 exocytic processes conceivably by phosphorylating specific targets.

The Akt substrate of 160 kDa (AS160, also termed TCB1D4) [4] has been characterized by its GTPase-activating protein (GAP) activity toward Rab GTPases, which play a crucial role in membrane trafficking [5]. Phosphorylation-dependent interaction with the 14-3-3 protein and consequent decline of the GAP activity of AS160 are likely to be required for GLUT4 translocation [6,7]. In addition to AS160, Akt substrates including the C2 domain-containing phosphoprotein CDP138 and the

ARF6 guanine nucleotide exchange factor (GEF) Grp1, have recently been identified as key molecules that link the Akt pathway to the regulation of GLUT4 translocation [8,9]. However, the precise role for Akt2 in insulin-dependent glucose uptake remains incompletely understood.

Rho family small GTPases regulate a wide variety of cellular processes, including proliferation, survival, differentiation, movement, division, polarization, and adhesion [10,11]. In adipocytes, the Rho family member TC10 has been implicated in insulin-stimulated glucose uptake [12]. However, in muscle cells, the expression level of TC10 is low, thereby suggesting that TC10 may not play a pivotal role [13]. Instead, we and others demonstrated that another Rho family member Rac1 is critically involved in insulin signaling that leads to glucose uptake in skeletal muscle [14-16]. Furthermore, the Dbl family GEF FLJ00068 has been identified as a GEF responsible for the regulation of Rac1 specifically in insulin signaling [15]. Recently, it has been shown that the Ras family GTPase RalA acts as a critical component of the signaling pathway downstream of Rac1, although the precise mechanisms underlying the regulation of RalA remain obscure [17].

In this study, we show that the activation of Rac1 is a critical event downstream of the PI3K-Akt kinase cascade in insulin regulation of glucose transport in muscle cells. Of particular note is that insulin-induced Rac1 activation was totally sensitive to the inhibition of Akt. The present results highlight a pivotal role of Rac1 in Akt-mediated insulin signaling and propose a novel mechanism for insulin-dependent regulation of Rac1.

2. Materials and methods

2.1 Materials

Antibodies against Myc (mouse monoclonal (sc-40)) and hemagglutinin (HA) (rat monoclonal (11 867 423 001)) epitope tags were purchased from Santa Cruz Biotechnology and Roche Applied Science, respectively. Antibodies against the V5 epitope tag (rabbit polyclonal (V8137)) and tubulin (mouse monoclonal (T4026)) were purchased from Sigma-Aldrich. An anti-Rac1 (mouse monoclonal (610650)) antibody was purchased from BD Biosciences. Anti-phospho-(Ser/Thr) Akt substrate (rabbit polyclonal (9611)), anti-phospho-(Thr308) Akt (rabbit polyclonal (9275)), anti-phospho-(Ser473) Akt (mouse IgG2b monoclonal (4051)), anti-Akt (rabbit polyclonal (9272)) antibodies were purchased from Cell Signaling Technology. Anti-mouse IgG, anti-rabbit IgG, and anti-rat IgG antibodies conjugated with Alexa Fluor® 488/546/647 were purchased from Life Technologies. Anti-mouse IgG (NA9310) and anti-rabbit IgG (NA9340) antibodies conjugated with horseradish peroxidase were purchased from GE Healthcare. Akt inhibitor IV (124011) and wortmannin (681675) were purchased from Calbiochem. Insulin (I5500) was purchased from Sigma-Aldrich.

2.2 Cell culture and RNA interference

L6-GLUT4 cells (see below) were cultivated in minimum essential medium Eagle (M4655; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Cell Culture Bioscience), 1 mM sodium pyruvate, 100 IU /ml penicillin, and 100 μg/ml streptomycin. Prior to measurement of GLUT4 translocation, cells were starved in serum-free medium for 2 h. A mixture of three small interfering RNA (siRNA) duplexes against rat Rac1 (duplex-1, 5’-AGACGGAGCCGUUGGUAAATT-3’; duplex-2, 5’-CCUGUUAAGAAGAGGAAGATT-3’; duplex-3,

5’-CCAAUGAACCAGUCAGUAATT-3’) was purchased from Cosmo Bio (Japan). siRNAs against rat Akt1 (5’-CCUCAAGAAUGAUGGCACCUUUAUU-3’) and rat Akt2 (5’-GGAGUGUGUGGACAGUGAATT-3’) were purchased from Life Technologies and Qiagen, respectively. The control siRNA (1022076) was purchased from Qiagen. Cells were transfected with siRNA (200 nM) using the Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer’s instructions.

2.3 Adenovirus infection

Adenoviruses for the expression of 3×HA-tagged Rac1(WT) and 3×HA-tagged Rac1(G12V) were generated and used to infect L6-GLUT4 cells by using the Adenovirus Cre/loxP kit (Takara Bio, Japan) according to the manufacturer’s instructions. A recombinant adenovirus for the N-terminally myristoylated catalytic subunit of bovine PI3Kα (Myr-p110) was kindly provided by Dr. Wataru Ogawa (Kobe University Graduate School of Medicine) [18].

2.4 Immunofluorescent microscopy and GLUT4 reporter assay

The exofacial GLUT4 reporter GLUT4*myc*7-green fluorescent protein (GFP) was previously described [19]. The L6-GLUT4 cell line (derived from L6 rat myoblasts) stably expresses this reporter [15,17]. For the detection of the cell surface-exposed GLUT4 reporter, formaldehyde-fixed cells were treated with an anti-Myc antibody before permeabilization. After washing three times with phosphate-buffered saline (-), cells were permeabilized with 0.1% (v/v) Triton X-100 and then counterstained with another primary antibody for the identification of the cells that express a specific ectopically expressed or endogenous protein. Images were obtained using a 40×oil objective on confocal laser-scanning microscope (LSM 700; Carl Zeiss) and processed by the Zeiss LSM Image Browser, version 3.5. The relative

amount of GLUT4*myc*7-GFP exposed at the plasma membrane was estimated from the ratio of Myc and GFP fluorescence signal intensities. Intensities of Myc and GFP fluorescence signals in regions of interest were quantified using ImageJ software, and ratios of Myc and GFP signals (Myc/GFP) were calculated [15,16]. The fluorescence intensity per unit area was determined in more than 5 cells from one image as shown in the figure. Values of at least 20 cells in total obtained from 4 to 6 different images for each condition were subjected to statistical analysis (Student's t test).

2.5 Immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred on to a 0.45 μm pore size polyvinylidene difluoride membrane (GE Healthcare). The membrane was stained with respective primary antibodies and an horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody, followed by visualization by enhanced chemiluminescence detection reagents (GE Healthcare).

2.6 Pull down assay for activated Rac1

Serum-starved L6-GLUT4 cells were stimulated with insulin (100 nM) for 5 min and harvested with pull down assay buffer [50 mM Tris-HCl (pH 7.4), 0.5% (v/v) Nonidet P-40, 0.15 M NaCl, 20 mM $MgCl_2$, and protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan)]. Lysates were centrifuged at 18,000×*g* for 1 min, and supernatants were added to glutathione *S*-transferase (GST)-Myc-PAK1(67-150) (purified from *Escherichia coli* transformants as described in Ref. 15) immobilized on glutathione-Sepharose beads and incubated at 4°C for 30 min. Glutathione-Sepharose beads were washed twice with pull down assay buffer, and activated Rac1 bound to the beads was detected by immunoblotting using an anti-Rac1 antibody.

2.7 Overlay assay (in situ detection) for activated Rac1

Triple HA-tagged Rac1(WT) was expressed in L6 cells through adenoviral infection. Cells were fixed in overlay assay buffer [50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 20 mM $MgCl_2$, and 0.05% (v/v) Tween 20] supplemented with 2% (w/v) paraformaldehyde on ice for 1 min and washed three times with overlay assay buffer. Cells were then incubated with GST-PAK1(67-150)-3×V5 (10 μg/ml; purified from *Escherichia coli* transformants as described in Ref. 15), dissolved in overlay assay buffer supplemented with 0.1% (v/v) Triton X-100 and 50 μg/ml bovine serum albumin) on ice for 10 min. After washing three times with wash overlay assay buffer, cells were fixed again in overlay assay buffer supplemented with 2% (w/v) paraformaldehyde on ice for 5 min. Subsequently, cells were washed three times with phosphate-buffered saline (-) supplemented with 0.05% (v/v) Tween20. GST-PAK1(67-150)-3×V5 was detected with an anti-V5 antibody and a secondary antibody conjugated with Alexa Fluor® 647 and then visualized by confocal laser-scanning microscopy as described above. The activity of Rac1 was determined as the ratio of V5 and HA fluorescence signal intensities. Intensities of V5 and HA fluorescence signals in regions of interest were quantified using ImageJ software, and ratios of V5 and HA signals (V5/HA) were calculated. The fluorescence intensity per unit area was determined in more than 5 cells from one image as shown in the figure. Values of at least 20 cells in total obtained from 4 to 6 different images for each condition were subjected to statistical analysis (Student's t test)

2.8 Reverse transcription-polymerase chain reaction

The total cellular RNA was isolated from L6-GLUT4 cells using the Sepasol-RNA I super reagent (Nacalai Tesque, Japan) according to the manufacturer's instructions. cDNAs were synthesized using the SuperScript III first-strand synthesis system for reverse transcription-polymerase chain reaction (Life Technologies) and then

amplified using the Expand High Fidelity polymerase chain reaction System (Roche Applied Science) and specific primers (Operon) [5’-CCATGAACGACGTAGCCATT-3’ and 5’-GTCCATCAGCCACAGTCTGA-3’ for Akt1, 5’-CAGCGTGTTAATGCTGCCAC-3’ and 5’-GATAGCCCGTATCCACTCTT-3’ for Akt2, and 5’-ATGGATGACGATATCGCTGCGC-3’ and 5’-CAGGTCCAGACGCAGGATGGC-3’ for β-actin] according to the manufacturer’s instructions.

3. Results

L6-GLUT4 cells harbor the GLUT4 reporter GLUT4*myc*7-GFP, which is detected by an anti-Myc antibody only when translocated and exposed onto the cell surface. In this study, this cell line was employed to clarify the role of Akt in Rac1-mediated plasma membrane translocation of GLUT4. The Akt-specific inhibitor Akt inhibitor IV [20] potently inhibited insulin-induced phosphorylation of Thr-308 and Ser-473 of Akt, which is known to be critical for Akt activation [21,22], in L6-GLUT4 cells (Fig. 1A). Under these conditions, insulin-dependent GLUT4 translocation to the plasma membrane was inhibited almost completely, confirming the important role of Akt (Figs. 1B and C). A constitutively activated mutant of Rac1, Rac1(G12V), also caused the translocation of GLUT4 to the plasma membrane as previously described (Ueda et al., 2008) (Figs. 1D and E). In marked contrast to insulin stimulation, Rac1(G12V)-dependent GLUT4 translocation was totally insensitive to the prior treatment with Akt inhibitor IV (Figs. 1D and E). Therefore, Akt may act upstream of Rac1 in this signaling.

Next, we examined the effect of Akt inhibitor IV on insulin-induced Rac1 activation (Fig. 2). Insulin rapidly caused the activation of Rac1 as determined by the pull down assay (Fig. 2A). The overlay assay (*in situ* detection) for activated Rac1 using GST-PAK1(67-150)-3×V5 as an activation-specific probe showed that Rac1 in the membrane ruffling area is particularly activated in response to insulin stimulation (Fig. 2B). Treatment of cells with Akt inhibitor IV totally abrogated this Rac1 activation, supporting the notion that Akt is an upstream regulator for Rac1 in the insulin-dependent signal transduction pathway (Figs. 2A, B, and C).

PI3K is activated downstream of the insulin receptor, yielding 3-phosphoinositides which in turn cause the recruitment of Akt to the plasma membrane and its activation [21,22]. Given that insulin-induced Rac1 activation is sensitive to Akt

inhibitor IV (Fig. 2), PI3K may also be implicated in the activation of Rac1. Insulin-induced activation of Rac1 as evaluated by the pull down assay was diminished when cells were treated with the PI3K inhibitor wortmannin prior to insulin stimulation (Fig. 3A). N-terminal myristoylation is known to render the catalytic subunit (p110) of PI3Kα constitutively active [17,18]. Myristoylated p110 (Myr-p110), when ectopically expressed in L6-GLUT4 cells, indeed activated Rac1 similarly to insulin stimulation (Fig. 3B). This result, together with the effect of wortmannin on Rac1 activation (Fig. 3A, also see Ref. 15), suggests a regulatory role for PI3K upstream of Rac1. Furthermore, ectopically expressed Myr-p110 stimulates GLUT4 translocation to the plasma membrane (Figs. 4B and C, also see Ref. 17). This GLUT4 translocation by Myr-p110 was suppressed almost completely following knockdown of endogenous Rac1 by the specific siRNA, suggesting again that Rac1 serves as a key molecule downstream of PI3K in insulin signaling (Figs. 4A, B and C).

Among Akt family members, Akt1 and Akt2 are expressed at a high level in skeletal muscle [21], and thus we next examined the effect of specific knockdown of these Akt subtypes (Fig. 5A). Myr-p110-induced GLUT4 translocation to the plasma membrane was suppressed when the expression of Akt2 was specifically downregulated (Figs. 5B and C), consistent with previous reports that Akt2 is primarily responsible for insulin-dependent glucose uptake [21-24]. On the other hand, specific knockdown of Akt1 had virtually no effect on GLUT4 translocation by Myr-p110 (data not shown). In contrast to Myr-p110, Rac1(G12V) stimulated GLUT4 translocation in a manner totally independent of Akt2 expression, suggesting that Rac1 acts downstream of Akt2 (Figs. 5D and E). To further confirm this notion, the effect of Akt2 knockdown on insulin-dependent activation of Rac1 was then examined. As expected, insulin-dependent Rac1 activation as determined by the overlay assay was abrogated when the expression of Akt2 was downregulated (Fig. 6). Taken together, it is likely that Rac1 is regulated downstream of Akt2 in insulin-induced signaling that leads to

GLUT4 translocation to the plasma membrane.

4. Discussion

We and others have identified Rac1 as a skeletal muscle-specific regulator of insulin signaling that leads to glucose uptake [14-16]. However, the mechanism underlying Rac1 activation in insulin signaling remains largely unknown. Particularly, it is unclear whether the activation of Rac1 depends on the well-documented key enzyme Akt2 in insulin signaling. Our previous observation that ectopic expression of constitutively activated Rac1 in L6 myoblasts did not affect the phosphorylation level of Akt suggests that Akt does not act downstream of Rac1 [15]. Moreover, phosphorylation of Akt in mouse gastrocnemius muscle in response to intravenously injected insulin was not reduced by skeletal muscle-specific *rac1* knockout, which further supports the above notion [16].

In this study, we demonstrate that Rac1 is indeed activated downstream of Akt2 by the use of a specific inhibitor and the siRNA-based knockdown approach. This is a remarkable finding because critical signaling elements downstream of Akt2 that are responsible for trafficking, docking, and fusion of GLUT4 vesicles are still unknown, although an important role of Akt2 is well established. One of the best-characterized Akt substrates in this signaling is AS160, phosphorylation in which is believed to cause the accumulation of GTP-bound forms of a subset of Rab proteins, enhancing the GLUT4 vesicle trafficking [4,6,7]. However, it is reasonable to think that phosphorylation of AS160 and inactivation of its GAP activity toward Rab proteins do not fully explain the functional role of Akt2 because the accumulation of the GTP-bound form of Rab subtypes by itself is presumably insufficient for the induction of the whole process of GLUT4 translocation. Rather, phosphorylation of other substrates and their functional modification by Akt2 may also be important. Actually, a newly identified Akt substrate CDP138 has been reported to have a critical role in insulin-stimulated GLUT4 insertion into the plasma membrane [8]. Another Akt

substrate Grp1 was found to regulate GLUT4 vesicle formation, a previously unrecognized target of insulin actin, as a GEF for Arf6 [9]. Our current finding that Rac1 also acts under the control of Akt2 in insulin signaling, although Rac1 may not be phosphorylated and regulated by Akt2 directly, further suggests a complexity of signal transduction pathways for the induction of GLUT4 redistribution.

Thus, it is important to clarify the precise mechanisms whereby Akt2 regulates Rac1 activity. FLJ00068 has been identified as a GEF specifically involved in insulin-dependent Rac1 activation [15], and therefore it is quite likely that Akt2 exerts its regulatory function through FLJ00068. FLJ00068, however, may not be phosphorylated by Akt2 because no consensus motif for Akt phosphorylation was found in the FLJ00068 sequence. Alternatively, a still unknown mechanism may account for Akt2-dependent regulation of FLJ00068. It is also possible that as yet unidentified proteins are involved in the regulation of FLJ00068 downstream of Akt2. Another GEF for Rac1 termed P-Rex1 has been identified as a PI3K-dependent regulator of actin cytoskeletal rearrangements in response to insulin in adipocytes [25]. Although the role of Rac1 in insulin-induced GLUT4 translocation in adipocytes remains incompletely understood, P-Rex1 was reported to facilitate not only rearrangements of the actin network, but also GLUT4 trafficking, in a Rac1-dependent manner [25]. Thus, it is possible that Rac1 exerts universally conserved function, including the regulation of actin remodeling, in insulin-responsive cells, although the mechanisms by which upstream signals control Rac1 activity may differ between cell types. Another possible link between Akt2 and Rac1 in muscle cells is RhoGAP22, which acts as a GAP for Rac1, and is regulated through phosphorylation by Akt in insulin signaling [26].

The precise role of Rac1 in the induction of glucose uptake by insulin remains incompletely understood. In cultured muscle cells, insulin-stimulated actin remodeling is evident, and is required for GLUT4 translocation [14,27]. The important role for cortical actin filaments in insulin-stimulated GLUT4 translocation was also

demonstrated in isolated rat skeletal muscle, although insulin-dependent actin remodeling was hardly detectable by immunofluorescent microscopy [23]. Considering that Rac1 regulates dynamic reorganization of cortical actin filaments in various types of cells [10,11], it is reasonable to think that an important role of Rac1 in insulin signaling is the regulation of actin cytoskeleton. In fact, Rac1 was shown to be involved in the regulation of insulin-induced actin cytoskeletal remodeling, which is required specifically for the retention of GLUT4 vesicles near the plasma membrane [14,28].

However, Rac1 may also exert actin-independent functions because ectopic expression of a constitutively activated form of Rac1 or its GEF FLJ00068 sufficiently induce GLUT4 translocation [15,16]. Recently, we have shown that the activation of the small GTPase RalA is a critical event for GLUT4 translocation that occurs downstream of Rac1 in muscle cells [17]. In adipocytes, a RalA-dependent mechanism for GLUT4 vesicle trafficking has been clarified [29], which may also operate in muscle cells. The involvement of the serine/threonine kinase PAK1 in insulin-stimulated GLUT4 translocation in skeletal muscle has recently been revealed by the analysis of PAK1 knockout mice [30]. Given that PAK1 is a downstream effector of Rac1, the regulation of PAK1 activity may be another role of Rac1 in insulin signaling.

Collectively, the present study showing that insulin-dependent Rac1 activation in muscle cells is mediated by Akt2 highlights Rac1 as a previously unidentified major downstream element that exerts diverse functions of Akt2. Further investigation will be needed to fully elucidate the role and regulatory mechanisms for Rac1 in insulin signaling.

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Figure legends

Fig. 1. Inhibition of insulin-induced, but not constitutively activated Rac1-induced, cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP by Akt inhibitor IV. (A) L6-GLUT4 cells were treated with or without Akt inhibitor IV (10 μM) for 2 h, and then stimulated with or without insulin (100 nM) for 5 min. Phosphorylation of Akt at Thr-308 and Ser-473 was assessed by immunoblotting using their respective phospho-specific antibodies. (B) L6-GLUT4 cells were treated with or without Akt inhibitor IV (10 μM) for 2 h, and then stimulated with or without insulin (100 nM) for 20 min. Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP was assessed by immunostaining of the exofacial Myc epitope tag. Images were acquired from the focal plane as depicted in the schematic diagram. Scale bar, 50 μm. (C) Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP shown in (B) was quantified. Data are shown as means±S.E. (n=4). *P<0.001. (D) Rac1(G12V) was ectopically expressed in L6-GLUT4 cells, which were then treated with or without Akt inhibitor IV (10 μM) for 2 h. Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP was assessed by immunostaining of the exofacial Myc epitope tag. The expression of Rac1(G12V) was detected by immunostaining of the HA epitope tag. Images were acquired from the focal plane as depicted in the schematic diagram in Fig. 1B. Scale bar, 50 μm. (E) Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP in L6-GLUT4 cells in which Rac1(G12V) was not expressed (“-”) and L6-GLUT4 cells in which Rac1(G12V) was expressed (“Rac1(G12V)”) was quantified. Data are shown as means±S.E. (n=6). *P<0.001.

Fig. 2. Inhibition of insulin-induced Rac1 activation by Akt inhibitor IV. (A) L6 cells were treated with or without Akt inhibitor IV (10 μM) for 2 h, and then stimulated with or without insulin (100 nM) for 5 min. The activation of Rac1 was assessed by the

pull-down assay. (B) L6 cells were treated with or without Akt inhibitor IV (5 μM) for 2 h, and then stimulated with or without insulin (100 nM) for 20 min. The activation of Rac1 was assessed by the overlay assay. The expression of Rac1(WT) was detected by immunostaining of the HA epitope tag. Activated Rac1 was detected by immunostaining of the V5 epitope tag of the activation-specific probe (GST-PAK1(67-150)-3×V5). The nuclear signal in immunostaining with the anti-V5 antibody is not specific to Rac1. Images were acquired from the focal plane as depicted in the schematic diagram. Scale bar, 50 μm. (C) The activation of Rac1 shown in (B) was quantified. Data are shown as means±S.E. (n=4～6). *P<0.001.

Fig. 3. Role of PI3K in insulin-induced Rac1 activation. (A) L6 cells were treated with or without wortmannin (100 nM) for 2 h, and then stimulated with or without insulin (100 nM) for 5 min. Phosphorylation of Akt at Ser-473 was assessed by immunoblotting using a phospho-specific antibody. The activation of Rac1 was assessed by the pull-down assay. (B) The activation of Rac1 induced by constitutively activated PI3K. The activation of Rac1 following stimulation with or without insulin (100 nM) for 5 min and ectopic expression of constitutively activated PI3K ("Myr-p110") was assessed by the pull-down assay. In both upper ("Rac1·GTP") and lower ("Rac1 input") pictures, respectively, three lanes were derived from the same blot with the same exposure, but were nonadjacent in original images.

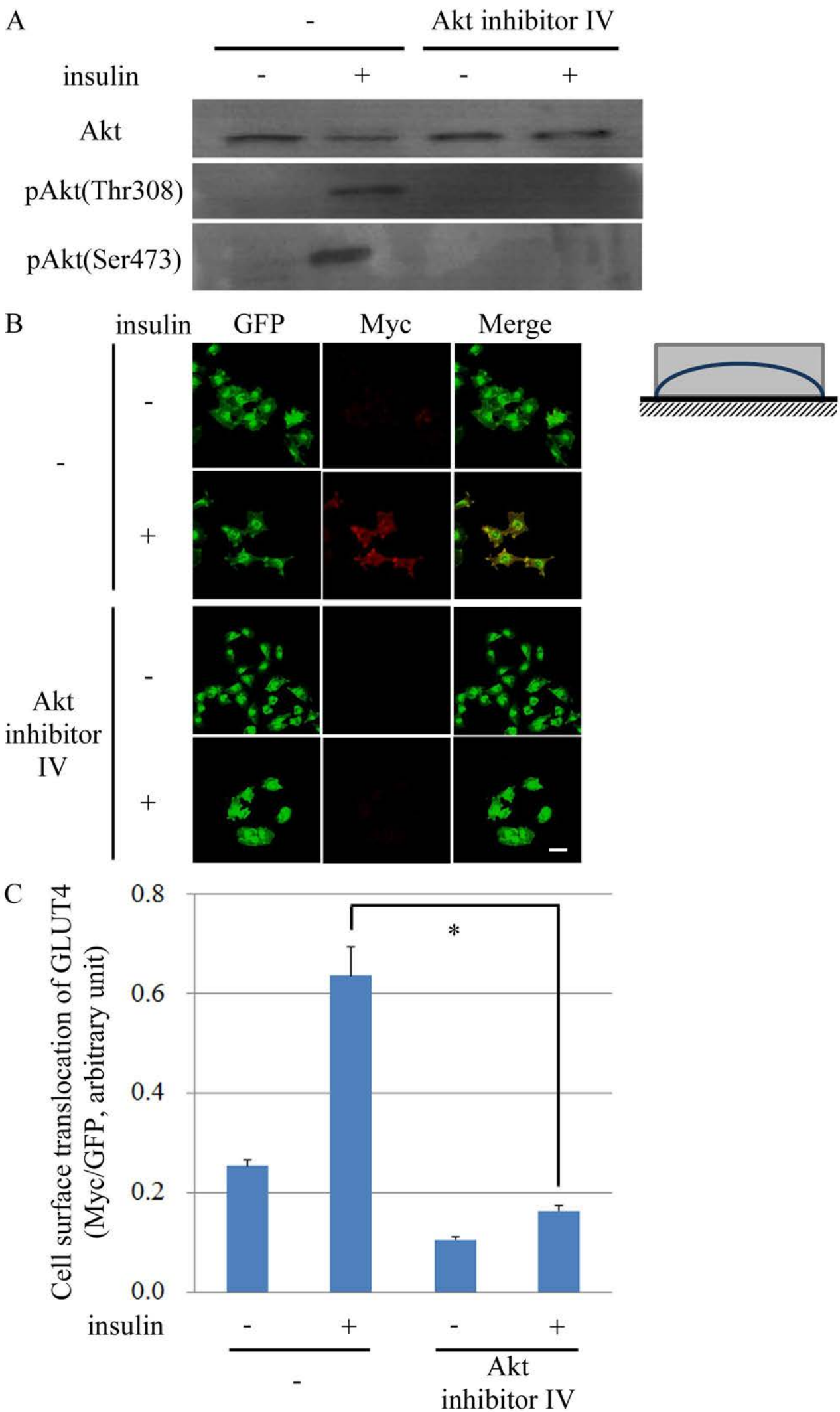
Fig. 4. Inhibition of constitutively activated PI3K-induced cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP by Rac1 knockdown. (A) L6-GLUT4 cells were treated with the control or Rac1-specific siRNA and infected with the mock ("-") or Myr-p110-expressing ("Myr-p110") recombinant adenovirus. The expression of endogenous Rac1 and tubulin (as a loading control) was assessed by immunoblotting using anti-Rac1 and anti-tubulin antibodies, respectively. (B) L6-GLUT4 cells were

treated with the control or Rac1-specific siRNA and infected with the mock ("-") or Myr-p110-expressing ("Myr-p110") recombinant adenovirus. Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP was assessed by immunostaining of the exofacial Myc epitope tag. The expression of Myr-p110 was detected by immunostaining of phosphorylated Akt substrates. Images were acquired from the focal plane as depicted in the schematic diagram in Fig. 1B. Scale bar, 50 μm. (C) Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP shown in (B) was quantified. Data are shown as means±S.E. (n=6). *P<0.001.

Fig. 5. Inhibition of constitutively activated PI3K-induced, but not constitutively activated Rac1-induced, cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP by Akt2 knockdown. (A) L6-GLUT4 cells were treated with the control, Akt1-specific, or Akt2-specific siRNA. The expression of endogenous Akt1, Akt2, and β-actin (as a loading control) was assessed by reverse transcription-polymerase chain reaction (33 cycles). (B) L6-GLUT4 cells were treated with the control or Akt2-specific siRNA and infected with the mock ("-") or Myr-p110-expressing ("Myr-p110") recombinant adenovirus. Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP was assessed by immunostaining of the exofacial Myc epitope tag. The expression of Myr-p110 was detected by immunostaining of phosphorylated Akt substrates. Images were acquired from the focal plane as depicted in the schematic diagram in Fig. 1B. Scale bar, 50 μm. (C) Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP shown in (B) was quantified. Data are shown as means±S.E. (n=4). *P<0.001. (D) L6-GLUT4 cells were treated with the control or Akt2-specific siRNA and infected with the mock ("-") or Rac1(G12V)-expressing ("Rac1(G12V)") recombinant adenovirus. Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP was assessed by immunostaining of the exofacial Myc epitope tag. The expression of Rac1(G12V) was

detected by immunostaining of the HA epitope tag. Images were acquired from the focal plane as depicted in the schematic diagram in Fig. 1B. Scale bar, 50 μm. (E) Cell surface translocation of the GLUT4 reporter GLUT4*myc*7-GFP shown in (D) was quantified. Data are shown as means±S.E. (n=6). *P<0.001.

Fig. 6. Inhibition of insulin-induced Rac1 activation by Akt2 knockdown. (A) L6 cells were treated with the control or Akt2-specific siRNA, and then stimulated with or without insulin (100 nM) for 20 min. The activation of Rac1 was assessed by the overlay assay. The expression of Rac1(WT) was detected by immunostaining of the HA epitope tag. Activated Rac1 was detected by immunostaining of the V5 epitope tag of the activation-specific probe (GST-PAK1(67-150)-3×V5). The nuclear signal in immunostaining with the anti-V5 antibody is not specific to Rac1. Images were acquired from the focal plane as depicted in the schematic diagram in Fig. 2B. Scale bar, 50 μm. (B) The activation of Rac1 shown in (A) was quantified. Data are shown as means±S.E. (n=4). *P<0.001.



Nozaki *et al.* Fig. 1

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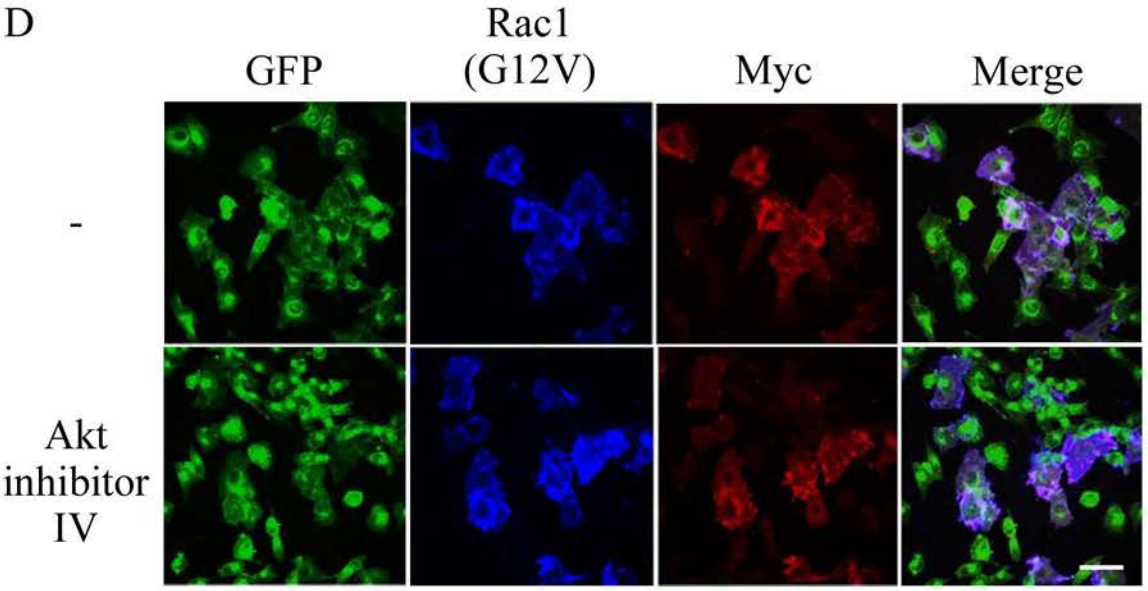

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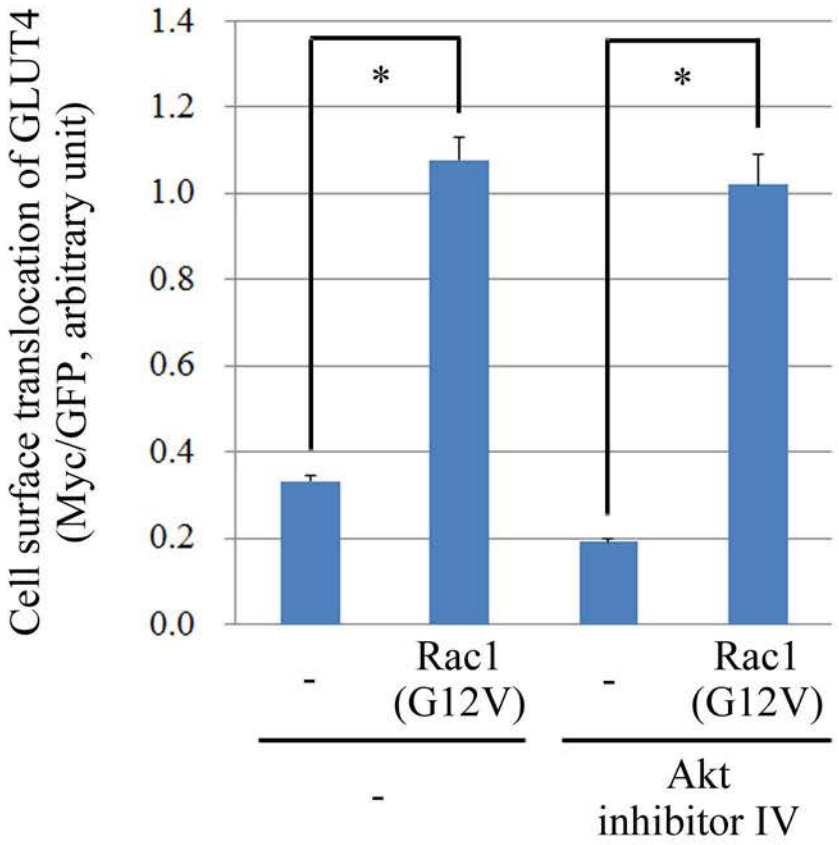
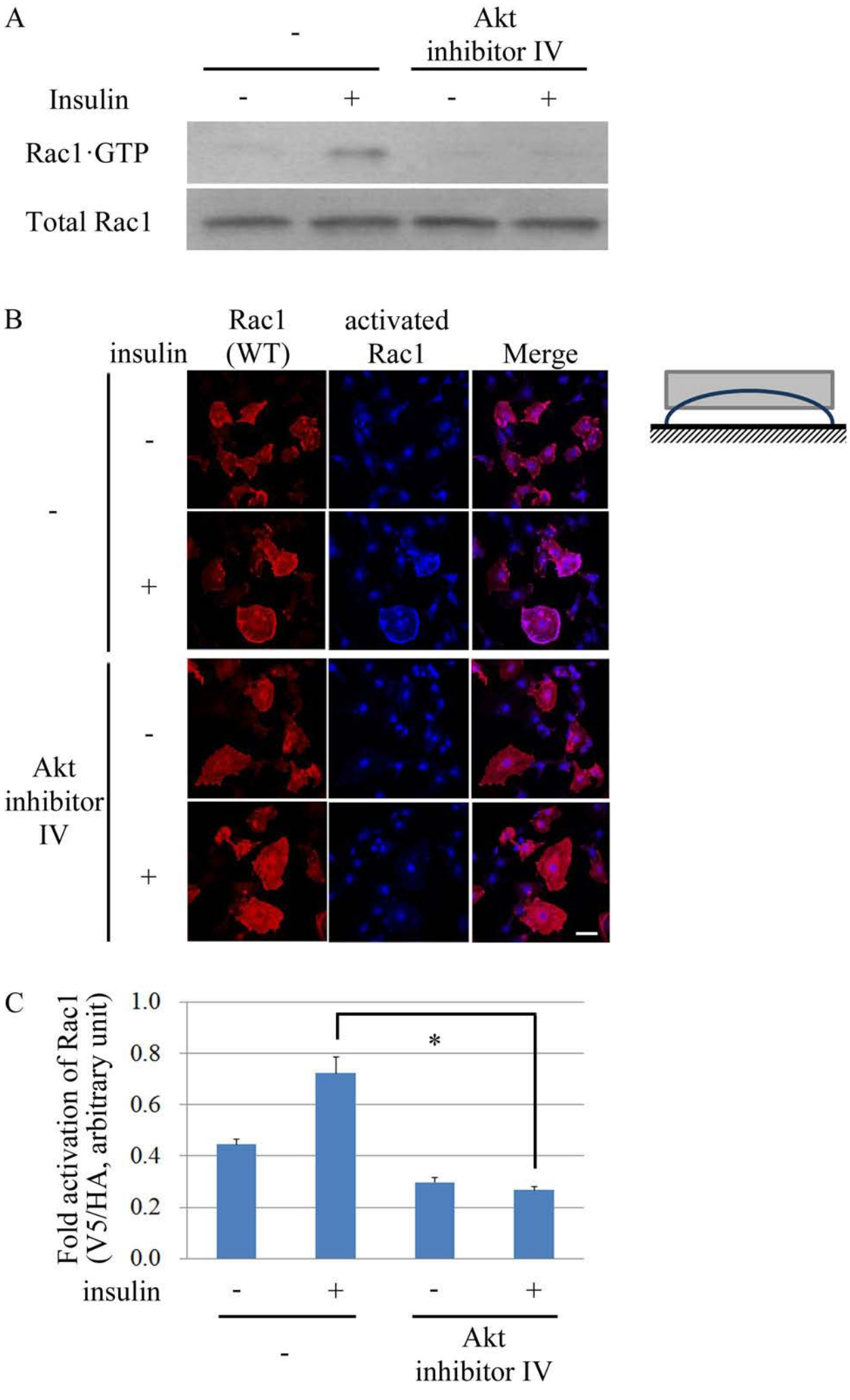
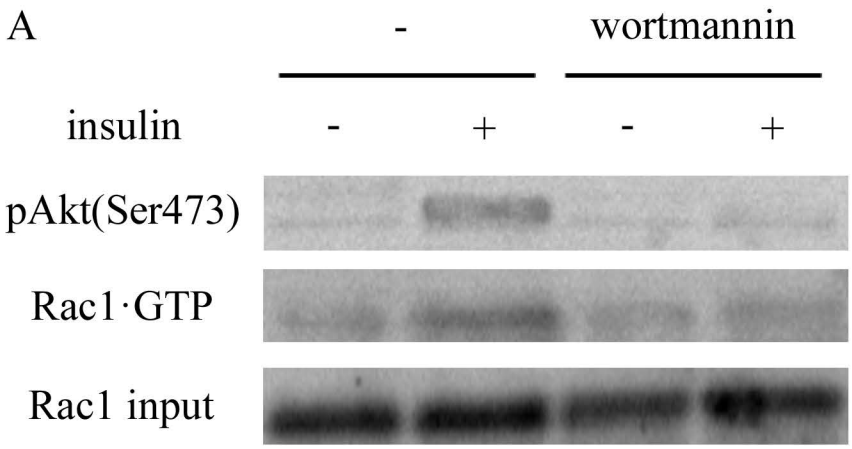
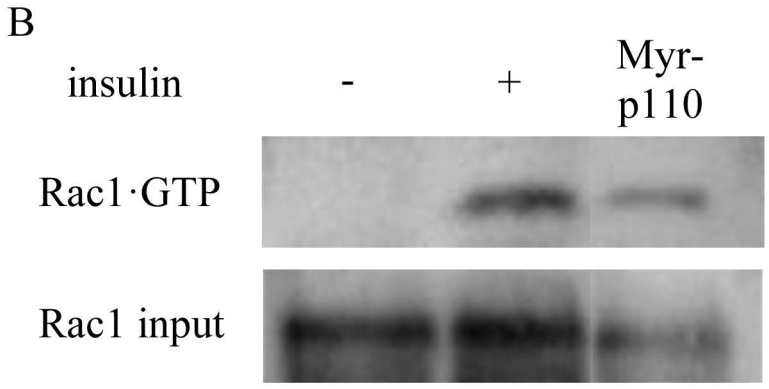


Nozaki *et al.* Fig. 1

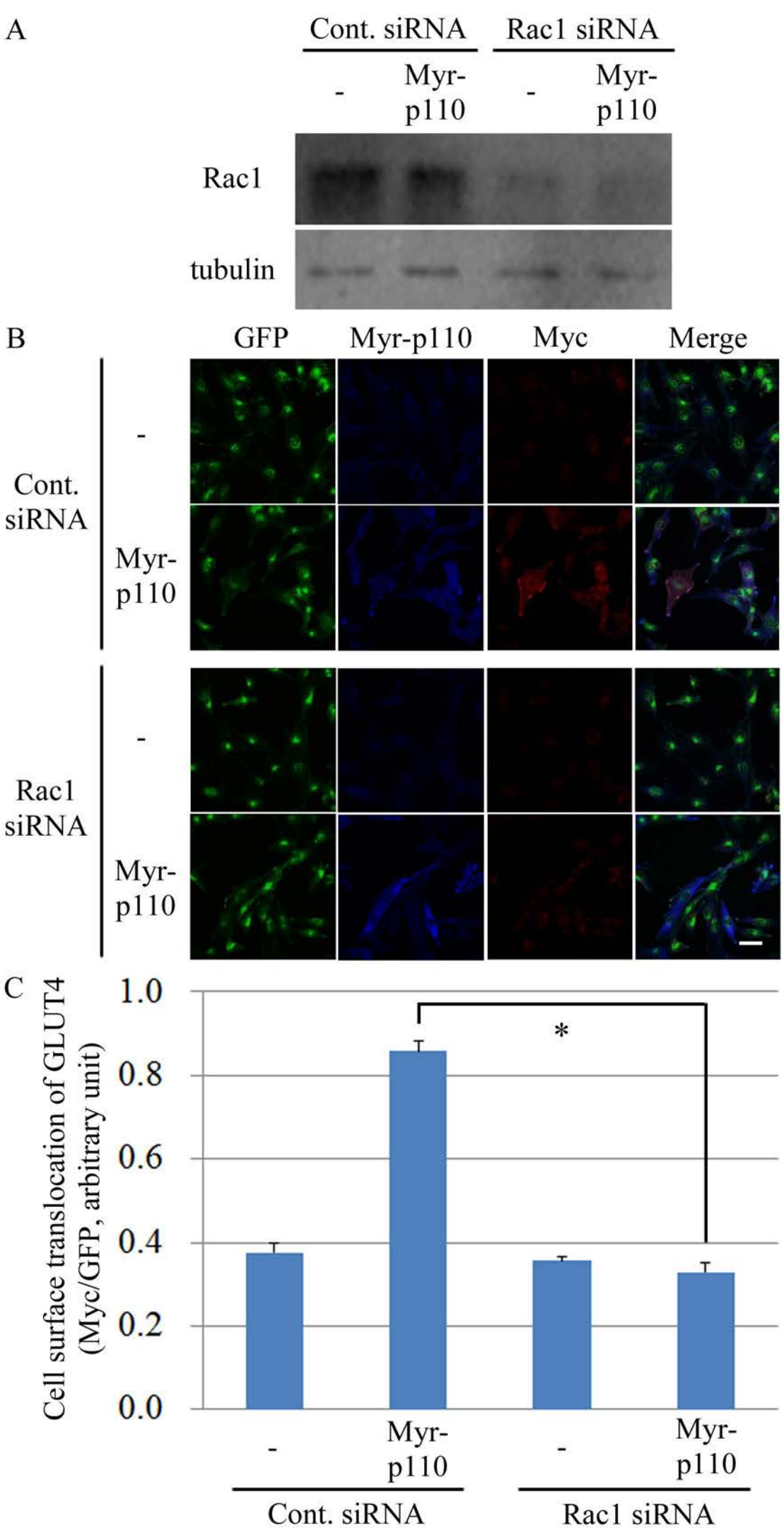


Nozaki *et al.* Fig. 2

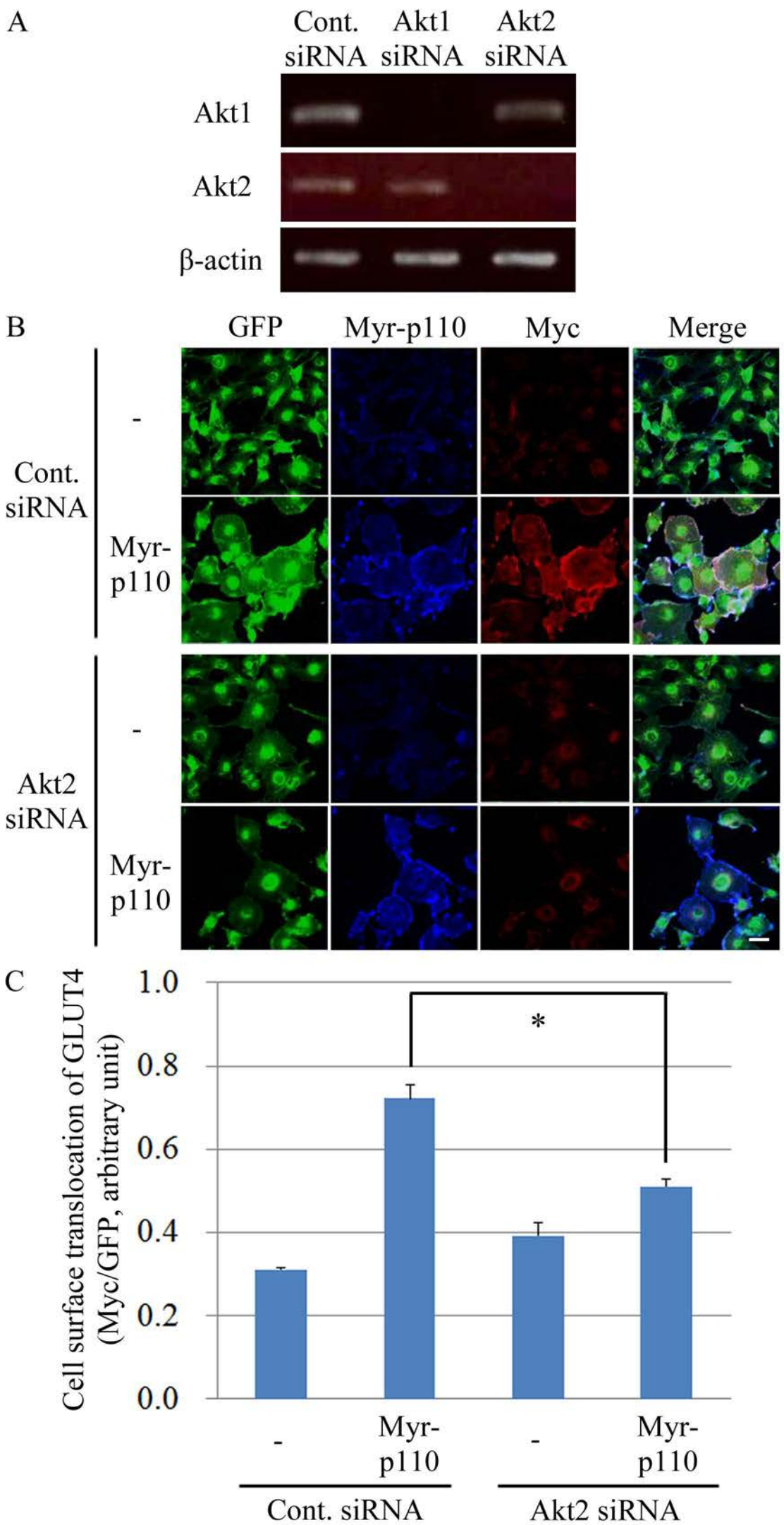




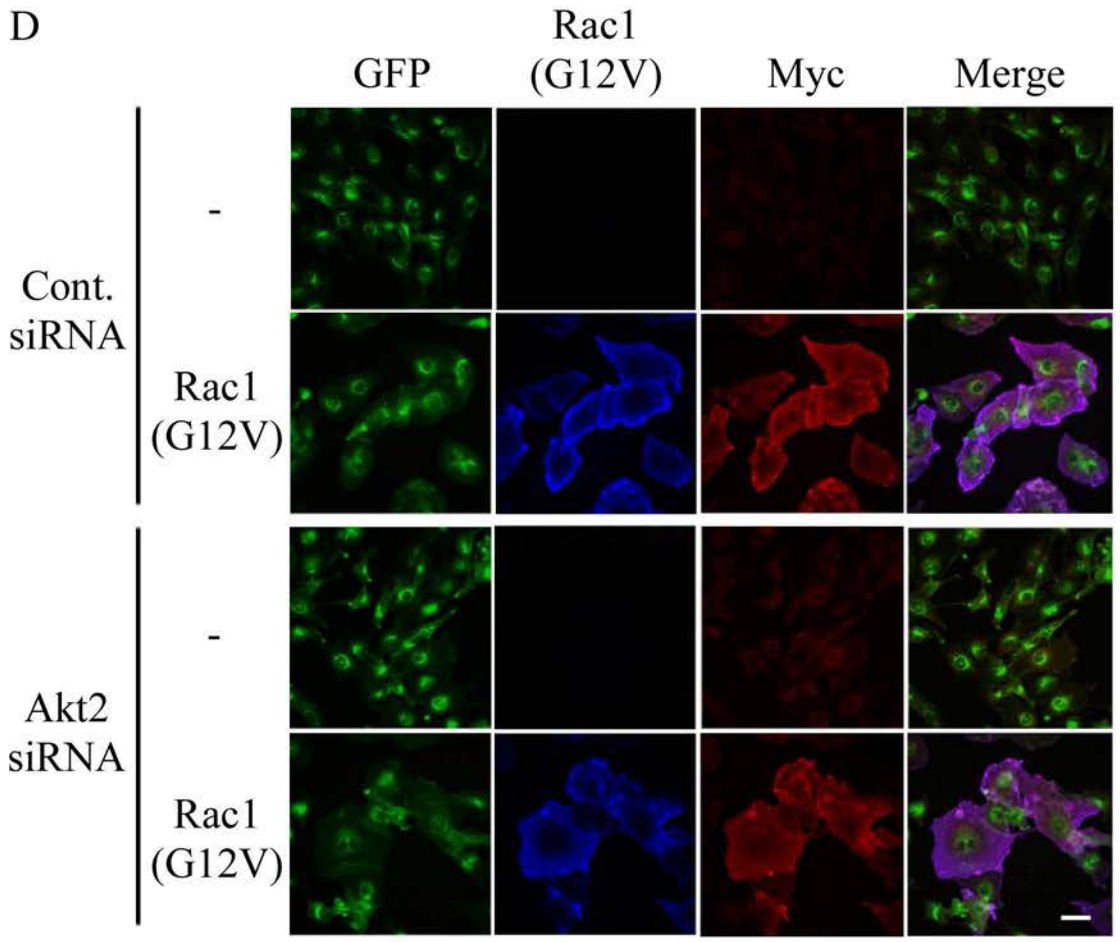
Nozaki *et al.* Fig. 3

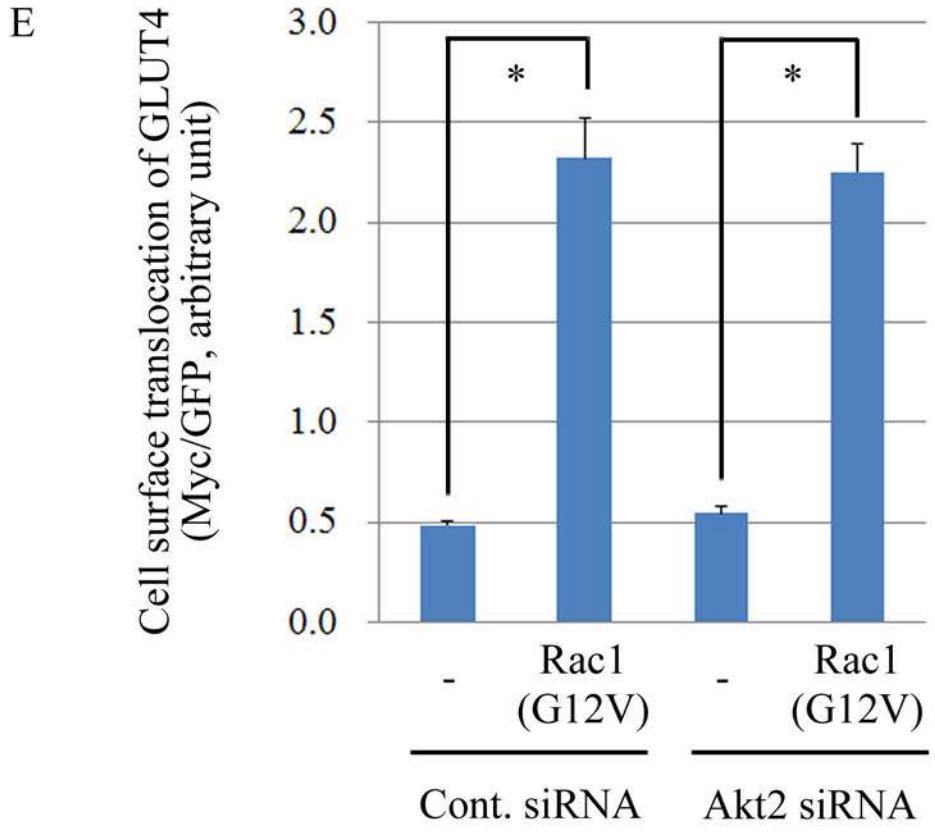


Nozaki *et al.* Fig. 4

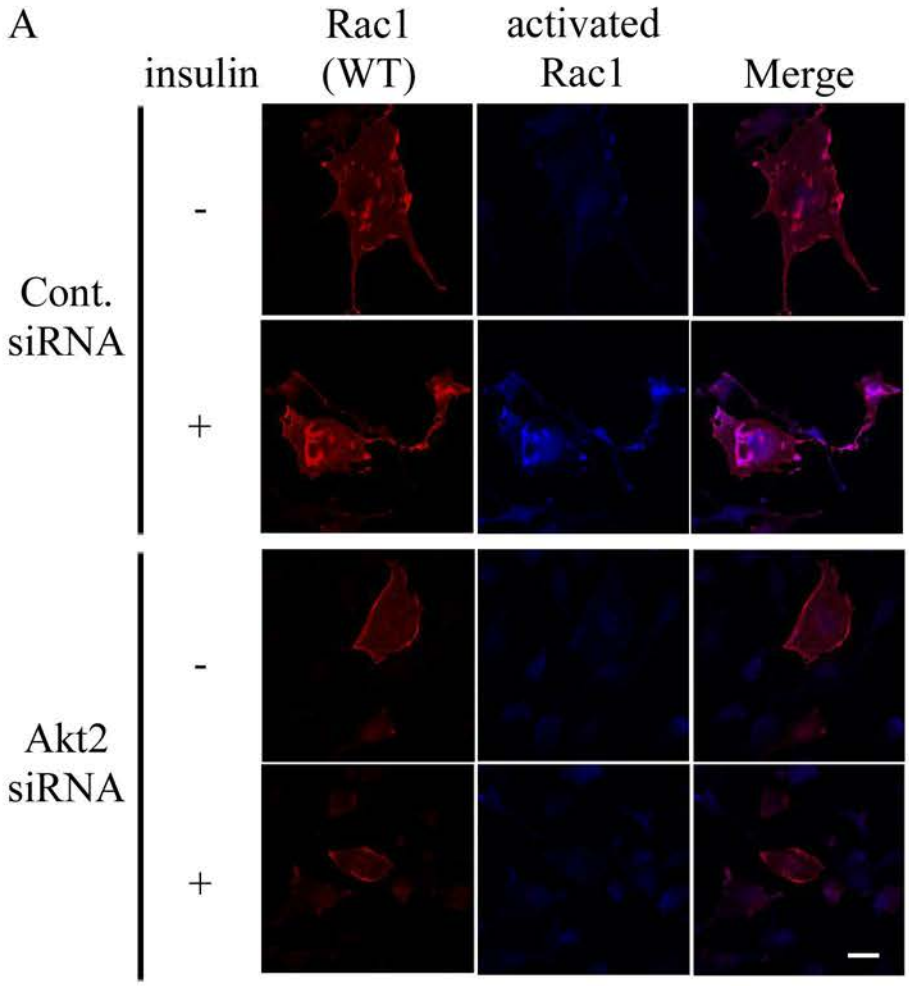


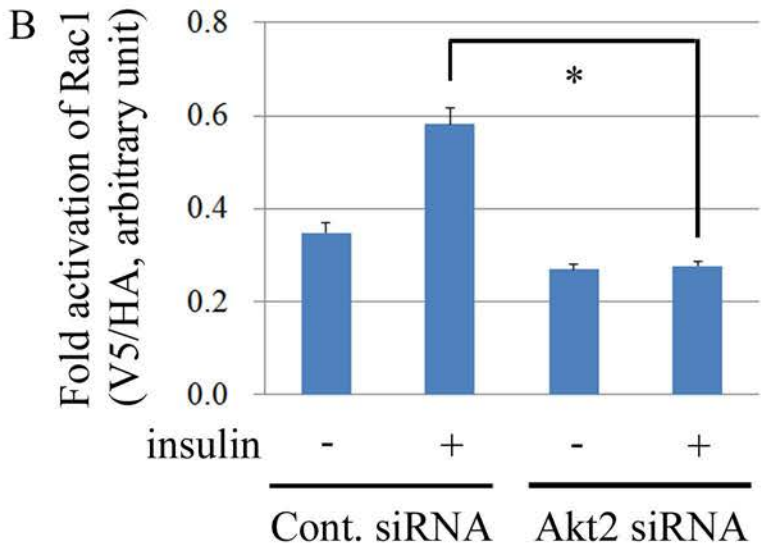
Nozaki *et al.* Fig. 5





Nozaki *et al.* Fig. 5





Nozaki *et al.* Fig. 6