Tankyrase Is a Golgi-associated Mitogen-activated Protein Kinase Substrate That Interacts with IRAP in GLUT4 Vesicles*

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The poly(ADP-ribose) polymerase tankyrase was originally described as a telomeric protein whose catalytic activity was proposed to regulate telomere function. Subsequent studies revealed that most tankyrase is actually extranuclear, but a discordant pattern of cytoplasmic targeting was reported. Here we used fractionation and immunofluorescence to show in 3T3-L1 fibroblasts that tankyrase is a peripheral membrane protein associated with the Golgi. We further colocalized tankyrase with GLUT4 storage vesicles in the juxtanuclear region of adipocytes. Consistent with this colocalization, we found that tankyrase binds specifically to a resident protein of GLUT4 vesicles, IRAP (insulin-responsive amino peptidase). The binding of tankyrase to IRAP involves the ankyrin repeats of tankyrase and a defined sequence (RQSPDG101) in the IRAP cytosolic domain (IRAP1–109). Tankyrase is a novel signaling target of mitogen-activated protein kinase (MAPK); it is stoichiometrically phosphorylated upon insulin stimulation. Phosphorylation enhances the poly(ADP-ribose) polymerase activity of tankyrase but apparently does not mediate the acute effect of insulin on GLUT4 targeting. Taken together, tankyrase is a novel target of MAPK signaling in the Golgi, where it is tethered to GLUT4 vesicles by binding to IRAP. We speculate that tankyrase may be involved in the long term effect of the MAPK cascade on the metabolism of GLUT4 vesicles.

Tankyrase is an ADP-ribose transferase with certain features of both signaling and cytoskeletal proteins (1). Besides the PARP3 domain, which catalyzes poly(ADP-ribose)ylation of substrate proteins (1), the sterile a module (SAM) in tankyrase is shared by signaling molecules such as the MAPK kinase kinase Byr2 (2), whereas its ANK domain containing 24 ankyrin repeats is homologous to the cytoskeletal protein ankyrin (3). Tankyrase was identified in a yeast two-hybrid screen where its ANK domain interacts with a telomeric protein, TRF-1 (telomere repeat binding factor-1) (1). Tankyrase was initially described as a telomeric protein, and its PARP activity was proposed to regulate telomere function (1). Subsequent data revealed that tankyrase targeting is cell cycle-dependent and, surprisingly, that only a minute fraction is found at the telomeres (4). In mitotic HeLa cells tankyrase is highly concentrated around the centrosomes. During interphase, the reported pattern varies dramatically with fixation methods (4). With formaldehyde fixation, tankyrase appears to decorate the cytoplasmic side of the nuclear envelope. With methanol fixation, however, a punctate pattern was observed in a cluster near the nucleus (4). Of note, the identity of this juxtanuclear localization was not addressed. Given the complex targeting pattern of tankyrase, we hypothesized that it might interact with proteins besides TRF-1 in extranuclear compartments.

A compartment of interest is defined by “GLUT4 vesicles”, i.e. endocytic vesicles in myocytes and adipocytes that contain the glucose transporter GLUT4, and IRAP (the insulin-responsive amino peptidase) (5, 6). In insulin-deprived adipocytes, most GLUT4 vesicles are sequestered intracellularly in the trans-Golgi reticulum and in scattered cytosolic sites. Upon insulin stimulation, a major fraction of GLUT4 vesicles in the trans-Golgi reticulum, and to a smaller extent in cytosolic sites, translocate toward the cell surface (7, 8). The translocation inserts GLUT4 and IRAP into plasma membrane, where GLUT4 can facilitate glucose uptake and IRAP can hydrolyze its extracellular substrates (9, 10). Following insulin withdrawal, both GLUT4 and IRAP are internalized by endocytosis and resequestered in GLUT4 vesicles. This reversible translocation of GLUT4 allows insulin to regulate glucose utilization, but the physiological function of IRAP remains unclear (10). Because IRAP can hydrolyze vasopressin and other vasoactive peptides (9), impaired translocation of IRAP has been implicated in vascular complications caused by insulin resistance (11).

The targeting machinery for GLUT4 vesicles has not been completely identified, but it presumably interacts with domains in GLUT4 and IRAP that specify their insulin-responsive targeting. The relevant domain(s) within GLUT4 has not been mapped conclusively, presumably because of the multi-spanning membrane topology of GLUT4 (12–14). By contrast, IRAP is a type II transmembrane protein with a single cytosolic domain (aa 1–109) that clearly confers insulin responsiveness to its targeting (15). Curiously, overexpression of this IRAP domain causes GLUT4 translocation in adipocytes, suggesting that it can saturate a targeting machinery that also regulates GLUT4 exocytosis (16). The IRAP cytosolic domain is therefore an ideal handle for identifying components of the machinery that targets GLUT4 vesicles.

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This study shows that the IRAP cytosolic domain binds to tankyrase in vitro and in vivo. Consistent with the binding, we also show that tankyrase colocalizes with a significant pool of GLUT4 vesicles in 3T3-L1 adipocytes. In contrast to previous studies, we consistently localize tankyrase to the Golgi in 3T3-L1 cells. Tankyrase is likely an important signaling molecule, because it is stoichiometrically phosphorylated by MAPK upon insulin stimulation. The phosphorylation of tankyrase by MAPK increases its PARP activity in vitro. However, the phosphorylation is apparently not involved mechanistically in the acute effect of insulin on GLUT4 translocation. Instead, we propose that tankyrase may mediate the long term regulation of GLUT4 vesicles by the MAPK cascade.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Human tankyrase cDNA in pTT20 (1) was FLAG-tagged N-terminally using standard techniques to yield pFLAG-tankyrase. pFLAG-tankyrase-2 encoding full-length human tankyrase-2 with an N-terminal FLAG tag will be described elsewhere. pAnkG-AR encoding all 24 ANK repeats of ankyrin G (aa 1–850) was constructed by recombining pA3–29 and pA3–19 (3). For pIRAP-myc, cDNA encoding human IRAP was amplified by polymerase chain reaction (PCR) with a skeletal muscle cDNA library and inserted into pcDNA3 MT (17) between BamHI and ClaI sites to tag it C-terminally with six myc epitopes. Bacterial vectors expressing GST fused C-terminally to human TRF-1 (aa 1–68), human IRAP (aa 2–109, 78–109, or 96–101), or rat GLUT4 C-terminal tail (aa 813–831) showing no homology to tankyrase. Antibody concentrations were determined using an albumin standard (Pierce). For immunoblot analyses, day 8–16 adipocytes were serum-starved in DMEM containing 10% fetal bovine serum for 2 h and pretreated with U0126 (30 μM, 30 min; Promega), Wortmannin (1 μM, 1 h, Sigma), or LY294002 (100 μM, 30 min; Calbiochem) when indicated before a 10-min stimulation with insulin (1 μg/ml), PDGF-BB (50 ng/ml, Sigma), or EGF (100 ng/ml; Upstate Biotechnology) at 37 °C. At 4 °C thereafter, cells were washed with PBS and lysed in buffer A (0.5 μl/15 ml plate). After preincubating with 40 μg of GST in buffer A at 4 °C for 2 h, tankyrase was affinity purified overnight using 40 μg of wild-type GST-IRAP-aa78–109 or GST-IRAP-aa2–17 containing a G101A substitution. The precipitant was washed in buffer N (150 mM NaCl, 50 mM Tris, pH 8, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, and 20 mM β-glycerophosphate). The precipitants were washed with buffer A (1 ml) four times and separated in 6.5% SDS gels for autoradiography. For antibody characterization, BOSC cells were similarly transfected with pFLAG-tankyrase, pIRAP-myc, or both vectors using a calcium phosphate transient protocol.

For coimmunoprecipitation, BOSC cells (19) were transfected with pFLAG-tankyrase, pIRAP-myc, or both vectors using a calcium phosphate kit as recommended by the manufacturer (Invitrogen). Cells were lysed at 48 h in buffer A and clarified at 13,000 × g for 4 °C at 10 min. The supernatant was incubated with resins (6 μl/10 cm plate) containing anti-FLAG M2 antibody (Sigma) or anti-myc 9E10 antibody (Jackson) and incubated with antibodies against tankyrase (T1S, 1:150, BioRad), phospho-T2S (1:1500, 9E10, Covance), IRAP (1:2500), FTCD (1:400), and against tankyrase. The supernatant was centrifuged at 50,000 × g for 20 min to recover high density microsomes. The supernatant was again centrifuged at 160,000 × g for 70 min to separate low density microsomes from soluble proteins. For solubility studies, fibroblasts grown in DMEM containing 10% fetal bovine serum were Dounce-homogenized in 100 mM Na₂CO₃ (pH 11.5) or in 1 × NaCl, 1 mM EDTA, and 50 mM Tris, pH 8 (750 μM/15 cm plate) at 4 °C, and soluble proteins were separated from the insoluble by centrifugation at 300,000 × g for 30 min. For PARP assays, serum-starved and insulin-stimulated fibroblasts were washed in PBS and lysed in buffer A (0.5 μl/15 ml plate). After preincubating with 40 μg of GST in buffer A at 4 °C for 2 h, tankyrase was affinity precipitated overnight using 40 μg of wild-type GST-IRAP-aa2–17 containing a G101A substitution. The precipitant was washed in buffer N (150 mM NaCl, 50 mM Tris, pH 8, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, and the same protease inhibitors as in buffer A but without benzamide) and incubated with 1 μM [adenosine-32P]NAD (4 Ci/mmol) in 100 μl of buffer N at 37 °C for 90 min. The products were resolved in 6.5%/10% discontinuous gradient gels and detected by autoradiography and phosphorimaging (Fuji). The products were also immunoblotted with antibodies against polymers of ADP-ribose (SA216, 1:1500, BioRad) and against tankyrase (T1S).

**Adipocyte Lysates**—3T3-L1 adipocytes were differentiated from fibroblasts as described (16). Except for specified modifications, day 8–16 adipocytes were serum-starved in DMEM for 6–15 h and pretreated with U0126 (30 μM, 30 min; Promega), Wortmannin (1 μM, 1 h, Sigma), or LY294002 (100 μM, 30 min; Calbiochem) when indicated before a 10-min stimulation with insulin (1 μg/ml), PDGF-BB (50 ng/ml, Sigma), or EGF (100 ng/ml; Upstate Biotechnology) at 37 °C. At 4 °C thereafter, cells were washed with PBS and lysed in buffer A (1 ml/10 cm plate). Lysates were clarified at 14,000 × g for 10 min and incubated with Sepharose beads containing GST-IRAP-aa78–109 (20 μg/particle lysates) for 8–28 h. The precipitants were washed in buffer A (1 ml three times) and immunoblotted for tankyrase (~1 plate equivalent/lane). For phosphatase treatment, affinity purified tankyrase was incubated at 30 °C with protein phosphatase-1 (3 units; New England Biolabs) in 60 μl of manufacturer’s buffer. For in vitro phosphorylation, affinity purified tankyrase was incubated at 30 °C with activated p42 MAPK (New England Biolabs) and [γ-32P]ATP (100 μM at 0.3 Ci/mmol) in 55 μl of 10 mM MgCl₂, 0.5 mM dithiothreitol, 1.25 mM EGTA, 25 mM β-glycerophosphate, and 50 μM sodium orthovanadate. For in vivo 32P-labeling, cells were maintained in phosphate/pyruvate-free DMEM for 2 h and then labeled with [32P]PiP3 (300 Ci/mmol) in the same medium for 2 h. The lysates were preincubated by incubating with Sepharose beads containing GST (50 μg/particle lysates) for 30 min prior to affinity purification using GST-IRAP-aa78–109.

**RESULTS**

**Tankyrase Binds to the Cytosolic Domain of IRAP**—To identify novel components of the targeting machinery for GLUT4 vesicles, we screened a skeletal muscle cDNA library for proteins that interact with the IRAP cytosolic domain (aa 1–109)
in a yeast two-hybrid system. This revealed a specific interaction of IRAP with the ANK repeat domain (aa 1–810) of tankyrase-2, a novel tankyrase homologue to be described elsewhere. The ANK domain of tankyrase-2 shows 95% similarity to tankyrase, suggesting that tankyrase may also interact with the IRAP cytosolic domain.

The interaction was verified in vitro, where residues containing GST fused to the entire IRAP cytosolic domain (IRAP2–109) efficiently precipitated [35S]labeled tankyrase (Fig. 1A, lane 5). Fig. 1A also shows that GST fused to the juxtamembranous hexapeptide RQSPDG (IRAP96–101) bound tankyrase as efficiently as GST fused to the entire IRAP cytosolic domain (lane 4), whereas the binding was abolished when this hexapeptide was shortened from either end (data not shown). We also examined the binding of tankyrase to the telomere repeat binding factor, TRF-1, because TRF-11–68 has been shown to interact with tankyrase, as detected by indirect immunofluorescence. In formaldehyde-fixed 3T3-L1 fibroblasts, wide field microscopy showed that most tankyrase was excluded from the nucleus and formed a Golgi-like pattern near the nucleus (Fig. 4A). This juxtanuclear pattern was tankyrase-specific, because it was not observed with control immunoglobulin (Fig. 4B). To show that tankyrase is indeed localized to the Golgi regardless of fixation methods, cells were also methanol-fixed and co-stained for tankyrase and the Golgi marker FTCD (21). Therefore, our data consistently localized tankyrase to the Golgi.

FIG. 1. Tankyrase binds to TRF-1,68 and IRAP2–109 in vitro. A, tankyrase labeled in vitro with [35S]methionine was incubated with resin containing GST (lane 1), GST-IRAP2–52 (lane 2), GST-IRAP55–82 (lane 3), GST-IRAP96–101 (lane 4), GST-IRAP2–109 (lane 5), or GST-TRF-1,58 (lane 6) as described under “Experimental Procedures.” [35S]Tankyrase bound to resin was resolved by SDS-PAGE and autoradiographed. For comparison, lane 7 contained 40% of the input [35S]tankyrase. B, similar to A except that the GST fusion proteins were incubated with [35S]labeled ANK domain of ankyrin G (aa 1–850). C, GST fusion proteins were resolved in a 12% SDS gel and stained with Coomassie Blue (10 μg/lane).

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FIG. 2. Tankyrase co-immunoprecipitates with IRAP in transfected cells. BOSC cells were transfected with pFLAG-tankyrase, pIRAP-myc, or both vectors (lanes 1–3, respectively) as described under “Experimental Procedures.” Lysates were immunoprecipitated with an anti-FLAG affinity resin and immunoblotted with anti-FLAG (A) or anti-myc antibody (B). Lanes 1–3 also show that methanol preserved the juxtanuclear pattern of tankyrase (Fig. 4C). The tankyrase antibody used in previous immunofluorescence studies revealed a discordant pattern of tankyrase targeting that varied drastically with fixatives (4). Moreover, the antibody was raised against a region in tankyrase (aa 973–1149) (1) that shows 68% identity to tankyrase-2 (aa 822–996) and thus can potentially cross-react with tankyrase-2. We therefore used the tankyrase-specific antisera, T1S, to re-examine tankyrase localization by indirect immunofluorescence.

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**Tankyrase Is Dispersed in Vivo by Golgi-disrupting Agents—**The tankyrase antibody used in previous immunofluorescence studies revealed a discordant pattern of tankyrase targeting that varied drastically with fixatives (4). Moreover, the antibody was raised against a region in tankyrase (aa 973–1149) (1) that shows 68% identity to tankyrase-2 (aa 822–996) and thus can potentially cross-react with tankyrase-2. We therefore used the tankyrase-specific antisera, T1S, to re-examine tankyrase localization by indirect immunofluorescence.

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**Tankyrase Is Dispersed in Vivo by Golgi-disrupting Agents—**The Golgi apparatus is disintegrated in cells treated with brefeldin A or nocodazole (22). These agents were therefore used to confirm the Golgi localization of tankyrase. In brefelin-treated 3T3-L1 fibroblasts, tankyrase was dispersed into a fine, punctate pattern throughout the cytosol (Fig. 4D). By comparison, tankyrase was dispersed into a coarser granular pattern with nocodazole treatment (Fig. 4E), presumably because nocodazole disrupted the Golgi using a different mecha-
nism (22). Of note, even with the disruption of the Golgi, tankyrase remained virtually excluded from the nucleus (Fig. 4, \(D\) and \(E\)).

**Tankyrase Cofractionates with a Golgi Marker—**

Golgi membranes cofractionate with the low density microsomes when cellular components are resolved by differential centrifugation (23). To confirm the Golgi localization of tankyrase, we fractionated 3T3-L1 fibroblasts and recovered the majority of tankyrase in the low density microsome fraction (Fig. 5A, lane 3, upper panel). The soluble fraction also contained a significant amount of tankyrase, suggesting that tankyrase was not completely membrane-associated (Fig. 5A, lane 4, upper panel). Like tankyrase, the Golgi marker FTCD was recovered in both the low density microsome and soluble fractions, consistent with FTCD being a peripheral membrane protein in the Golgi (Fig. 5A, lanes 3 and 4, lower panel, and Ref. 21).

We also examined the solubility of tankyrase to see whether it associated with membranes as a peripheral or an integral membrane protein. Peripheral, but not integral, membrane proteins can be extracted by alkaline solutions of Na2CO3 (24). Furthermore, only peripheral membrane proteins are soluble in the absence of detergents (24). Fig. 5B shows that tankyrase was mostly soluble in a high salt buffer that contained no detergent (lanes 1 and 2), and it was quantitatively extracted by Na2CO3 (lanes 3 and 4) as described under “Experimental Procedures,” and soluble proteins (S, lanes 1 and 3) were separated from pellets (P, lanes 2 and 4). Equivalent amounts of each fraction were immunoblotted for tankyrase with T1S (upper panel) and for IRAP (lower panel).

**Tankyrase Colocalizes with Juxtanuclear GLUT4 in Adipocytes—**

Previous studies have shown that a significant pool of GLUT4 vesicles are closely associated with the trans-Golgi network (7, 8). Because tankyrase is a Golgi-associated protein that binds to IRAP (a resident of GLUT4 vesicles), we expected it to colocalize with GLUT4 near the Golgi. 3T3-L1 adipocytes were therefore co-stained for tankyrase and GLUT4, and 0.8-μm optical sections were obtained using confocal micros-
copy. Fig. 6A shows that in serum-starved adipocytes, tankyrase colocalized with the juxtanuclear pool of GLUT4 but not with the GLUT4 scattered in peripheral regions of the cytoplasm. Upon insulin stimulation, some GLUT4 was recruited from intracellular sites toward the cell surface (arrows in Fig. 6B). However, insulin did not affect the Golgi localization of tankyrase or its colocalization with the GLUT4 that remained near the nucleus (Fig. 6B). The observed colocalization was GLUT4-specific, because GLUT1 did not colocalize with tankyrase as shown in a wide field micrograph of adipocytes (Fig. 6C). We could not address the colocalization of tankyrase with IRAP by indirect immunofluorescence, because available antibodies for both proteins were all raised in rabbits.

Tankyrase Is a MAPK Substrate—Given that tankyrase binds to IRAP and colocalizes with a pool of GLUT4 vesicles, we hypothesized that tankyrase might constitute the insulin-regulated targeting machinery for GLUT4. We therefore examined the effect of insulin on endogenous tankyrase in 3T3-L1 cells. Fig. 7A shows that tankyrase obtained from serum-starved fibroblasts migrated as a 165-kDa protein (lanes 1). Interestingly, the apparent \( M_r \) shifted to 175 kDa within 10 min of insulin stimulation (lanes 2). Similar experiments in adipocytes showed that insulin, PDGF, and EGF elicited an indistinguishable \( M_r \) shift in tankyrase (Fig. 7B, lanes 1, 2, 6, and 7). The completeness of the shift indicated a stoichiometric modification on tankyrase upon growth factor stimulation. These factors (insulin, PDGF, and EGF) activate two major kinase pathways: the phosphatidylinositol 3-kinase pathway, which can be blocked by LY294002 or wortmannin, and the MAPK cascade, where certain MAPK kinases (e.g. MAPK kinases 1 and 2) can be inhibited by U0126 (25, 26). Among these inhibitors, only U0126 blocked the insulin-induced \( M_r \) shift of tankyrase (Fig. 7B, lanes 3–5), suggesting that tankyrase is a target of the MAPK cascade.

The \( M_r \) of tankyrase can be increased by auto-poly(ADP-ribosylation) (1). However, the insulin-induced 165 kDa to 175 kDa shift was entirely due to phosphorylation by MAPK for several reasons. First, the 175-kDa form of tankyrase affinity purified from insulin-stimulated cells was quantitatively converted to 165 kDa by \( \text{in vitro} \) phosphatase treatment (Fig. 7C). Conversely, the 165-kDa form of tankyrase affinity purified from serum-starved cells was shifted to 175 kDa \( \text{in vivo} \) in the presence of activated MAPK and \( \text{[32P]} \text{ATP} \). In this kinase reaction, tankyrase became \( \text{32P} \)-labeled as expected (data not shown). Interestingly, both the \( \text{in vitro} \) phosphatase and kinase reactions involved a transitional species of tankyrase with a distinct \( M_r \) (arrowheads in Fig. 7, C and D). This suggests that MAPK phosphorylated the same residues of tankyrase \( \text{in vitro} \) as were phosphorylated \( \text{in vivo} \) upon insulin stimulation. Of note, equivalent amounts of tankyrase were affinity precipitated from serum-starved versus insulin-stimulated cells by GST-IRAP \( \text{78–109} \) (Fig. 7B, lanes 1 and 2, and data not shown), suggesting that tankyrase phosphorylation did not affect its binding to GST-IRAP \( \text{78–109} \). Whether binding to endogenous IRAP is affected by tankyrase phosphorylation remains to be addressed, because we have been unable to consistently co-immunoprecipitate endogenous IRAP with tankyrase.

Tankyrase phosphorylation was further characterized in adipocytes labeled with \( \text{[32P]} \text{in vitro} \), again using GST-IRAP \( \text{78–109} \) to affinity purify tankyrase. Fig. 8A shows that insulin in-
tankyrase and GST-IRAP (lanes 3 and 4), and the increase was abolished by the MAPK kinase inhibitor U0126 (lane 5), consistent with tankyrase being a MAPK sub- 
strate. In keeping with the serine/threonine specificity of MAPKs (27), phosphoamino acid analysis of tankyrase revealed only phosphoserine residues (Fig. 8B). Taken together, our data show that tankyrase is quantitatively phosphorylated on certain serine residues by MAPK upon stimulation with insulin, PDGF, and EGF.

The PARP Activity of Tankyrase Is Enhanced by Phosphorylation—Having identified tankyrase as a novel MAPK sub- 
strate, we wondered whether tankyrase phosphorylation might serve to regulate its PARP activity. This would allow tankyrase to transduce MAPK signaling into poly(ADP-ribosyl)ation of effector proteins. The physiological substrates of tankyrase remain unidentified. However, tankyrase can use NAD as a cofactor in vitro to poly(ADP-ribosyl)ate itself (i.e. autoami- 
ification) and its interacting protein, TRF-1 (1). Another candidate substrate besides TRF-1 is IRAP, because IRAP also in- 
teracts with tankyrase (Figs. 1 and 2). To determine whether phosphorylation affects the PARP activity of tankyrase, endog- enous tankyrase was affinity purified from 3T3-L1 fibroblasts (lanes 3 and 4), and the labeling resulted from poly(ADP-ribose)ylation. To show that the PARP reaction is tankyrase-dependent, we introduced a Gly101-to-Ala mutation into the tankyrase-binding site (96RQSPDG101) of GST-IRAP2–109. This mutant GST-IRAP2–109 failed to precipitate any tankyrase protein or PARP activity (Fig. 9, lanes 5 and 6, and data not shown). Our data therefore indicate that the PARP activity of tankyrase toward itself (automodification) and GST-IRAP was enhanced by MAPK phosphorylation. Similar results were obtained when tankyrase was affinity purified using GST-TRF-11–68 instead of GST-IRAP (data not shown).

DISCUSSION

In an effort to identify proteins that interact with GLUT4 vesicles, we discovered that tankyrase binds to the cytosolic domain of IRAP, a resident protein of GLUT4 vesicles. Our data suggest that tankyrase is a peripheral membrane protein in the Golgi, and it colocalizes with a subpopulation of GLUT4 vesicles. We also show that tankyrase is a novel target of MAPK cascade, and its PARP activity is enhanced by MAPK phosphorylation following insulin stimulation.

Previous studies revealed that tankyrase is overwhelmingly extranuclear but did not provide a congruent pattern of target- 
ing (4). Using an antibody that does not cross-react with tankyrase-2, we consistently localized tankyrase to the Golgi region in 3T3-L1 fibroblasts and adipocytes. The Golgi localization as seen by immunofluorescence is also collaborated by subcellular fractionation. Consistent with the Golgi targeting, tankyrase colocalizes with the juxtanuclear pool of GLUT4 vesicles, which are known to closely associate with the trans-Golgi network (7). We suspect that the colocalization of tankyrase with GLUT4 reflects tankyrase binding to IRAP that resides in GLUT4 vesicles.

Tankyrase interacts with a membrane-proximal hexapeptide (aa 96–101) in the IRAP cytosolic domain (IRAP1–109). This
GLUT4 Vesicles Associate with a MAPK Substrate in the Golgi

A membrane-proximal heptapeptide in α-Na,K-ATPase and also binds to other unrelated proteins (3, 29, 30). Ankyrin is a peripheral membrane protein that tethers membrane channels and receptors to the submembranous cytoskeleton (31), whereas its Golgi isoforms are implicated in vesicular trafficking by tethering Golgi membranes to motor proteins (32). By analogy, IRAP binding may enable tankyrase to tether GLUT4 vesicles to cytoskeletal or motor proteins. Given that ANK domains can bind to diverse proteins (29), tankyrase may provide a tethered scaffold where GLUT4 vesicles interact with signaling molecules or targeting machinery.

To explain how insulin induces GLUT4 translocation, an intracellular retention system has been postulated that sequesters GLUT4 vesicles in the basal state and releases them upon insulin stimulation (5). The retention can apparently be saturated by both the IRAP cytosolic domain and the GLUT4 C-terminal cytosolic tail, because either domain when overexpressed can cause GLUT4 translocation (16, 33). Our results do not implicate tankyrase as a component of this retention machinery, because tankyrase binds to the IRAP cytosolic domain in a region (aa 96–101) distinct from those that cause GLUT4 translocation when overexpressed (aa 52–55–82) (16). Secondly, tankyrase does not bind to the GLUT4 C-terminal cytosolic tail in vitro. Lastly, despite causing tankyrase phosphorylation, insulin does not regulate binding of tankyrase to GST-IRAP in vitro. It remains possible that endogenous IRAP binding to tankyrase is regulated by insulin, perhaps through reversible modifications on IRAP. However, this seems unlikely given that insulin does not abolish the colocalization of tankyrase with GLUT4 in vivo (Fig. 6B).

The phosphorylation of tankyrase upon insulin stimulation is stoichiometric, suggesting that tankyrase is an important insulin signaling target. However, despite the proximity of tankyrase to GLUT4, tankyrase phosphorylation and GLUT4 translocation are apparently regulated by divergent signaling cascades: tankyrase phosphorylation depends on MAPK, whereas GLUT4 translocation depends on phosphatidylinositol 3-kinase (10, 34). Moreover, PDGF and EGF stimulate translocation are apparently regulated by divergent signaling pathways: tankyrase phosphorylation depends on MAPK, whereas GLUT4 translocation depends on phosphatidylinositol 3-kinase (10, 34). Moreover, PDGF and EGF stimulate translocation when overexpressed (aa 96–101) distinct from those that cause GLUT4 translocation when overexpressed (aa 52–55–82) (16). Secondly, tankyrase does not bind to the GLUT4 C-terminal cytosolic tail in vitro. Lastly, despite causing tankyrase phosphorylation, insulin does not regulate binding of tankyrase to GST-IRAP in vitro. It remains possible that endogenous IRAP binding to tankyrase is regulated by insulin, perhaps through reversible modifications on IRAP. However, this seems unlikely given that insulin does not abolish the colocalization of tankyrase with GLUT4 in vivo (Fig. 6B).

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Although the ras-MAPK cascade does not mediate the acute effect of insulin on GLUT4 targeting, it apparently affects long term regulation of GLUT4 metabolism. When the cascade is constitutively activated by mutant alleles of ras or MAPK kinase, adipocytes recruit more GLUT4 to their cell surface despite a lowered cellular GLUT4 content (Refs. 35 and 36, but also see Refs. 37 and 38). No molecular mechanism has been proposed to link the MAPK cascade directly with GLUT4 vesicles. Given that tankyrase is a MAPK target in close proximity to GLUT4, its PARP activity may conceivably couple the MAPK cascade to GLUT4 vesicles. Our data show that phosphorylation by MAPK alone does not explain how insulin stimulates GLUT4 translocation.

A precedent for ADP-ribosylation to regulate Golgi dynamics involves the G protein BARS (brefeldin ΔADP-ribosylation substrate). BARS is a multifunctional protein that can acylate lysophosphatidic acid in the Golgi membranes to promote...
membrane fission in vitro. Interestingly, this activity is inhibited when BARS is ADP-ribosylated in vivo by an unknown ADP-ribose transferase (39, 40). By analogy, the PARP activity of tankyrase seems poised to regulate Golgi dynamics and in particular the targeting of GLUT4 vesicles.

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