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Activation of G proteins by GIV-GEF is a pivot point for insulin resistance and sensitivity

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ABSTRACT Insulin resistance (IR) is a metabolic disorder characterized by impaired insulin signaling and cellular glucose uptake. The current paradigm for insulin signaling centers upon the insulin receptor (InsR) and its substrate IRS1; the latter is believed to be the sole conduit for postreceptor signaling. Here we challenge that paradigm and show that GIV/Girdin, a guanine exchange factor (GEF) for the trimeric G protein G\textsubscript{i}, is another major hierarchical conduit for the metabolic insulin response. By virtue of its ability to directly bind InsR, IRS1, and phosphoinositide 3-kinase, GIV serves as a key hub in the immediate postreceptor level, which coordinately enhances the metabolic insulin response and glucose uptake in myotubes via its GEF function. Site-directed mutagenesis or phosphoinhibition of GIV-GEF by the fatty acid/protein kinase C theta pathway triggers IR. Insulin sensitizers reverse phosphoinhibition of GIV and reinstate insulin sensitivity. We also provide evidence for such reversible regulation of GIV-GEF in skeletal muscles from patients with IR. Thus GIV is an essential upstream component that couples InsR to G-protein signaling to enhance the metabolic insulin response, and impairment of such coupling triggers IR. We also provide evidence that GIV-GEF serves as a therapeutic target for exogenous manipulation of physiological insulin response and reversal of IR in skeletal muscles.

INTRODUCTION Insulin resistance (IR) is a metabolic disorder in which adipocytes and muscle cells fail to take up and metabolize glucose in response to the hormone insulin. Although IR is a hallmark of type 2 diabetes mellitus (T2DM), IR alone in the absence of T2DM significantly increases the risk for stroke, heart failure, and atherosclerosis (Carter, 2005; Rundek \textit{et al.}, 2010).

Although multiple etiologic factors contribute to the pathogenesis of IR (Saltiel and Kahn, 2001), they all ultimately converge to suppress critical components of metabolic insulin signaling. Insulin binds its receptors (InsR, IGF1R), triggering receptor autophosphorylation and subsequent tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), among others. This leads to the recruitment and activation of G proteins by GIV-GEF.
activation of Src homology 2 (SH2) proteins such as p85α (PI3-kinase) and downstream activation of Akt (Taniyagiuchi et al., 2006). Akt triggers the translocation of the 12-transmembrane glucose transporter 4 (GLUT4) to the plasma membrane (PM) by phosphorylating the Rab GTase-activating protein (GAP) AS160 (Minea et al., 2005). Among the many adaptors that relay signals within the insulin cascade, IRS1 is widely believed to serve as the major node for orchestrating metabolic insulin signaling (Taniyagiuchi et al., 2006).

Besides IRS1, metabolic insulin signaling also relies on the activation of heterotrimeric G proteins, another major hub in eukaryotic signal transduction. InsRs are functionally coupled to the pertussis toxin–sensitive Gα/o proteins, for example, insulin can trigger their activation (Rothenberg and Kahn, 1988; Ciaraldi and Maisel, 1989), localization (Gohla et al., 2007), and phosphorylation (O’Brien et al., 1987; Krupinski et al., 1988). Activation of Gi augments insulin sensitivity (Chen et al., 1997; Song et al., 2001), enhances tyrosine phosphorylation of both InsR and IRS1 (Moxham and Malbon, 1996), and triggers efficient translocation of GLUT4 storage vesicles (GSVs) to the PM (Ciaraldi and Maisel, 1989; Kanoh et al., 2000; Song et al., 2001). Although multiple clues consistently point to a critical role of Gi activation in the insulin response, what couples and activates Gi downstream of InsR and how such activation may cross-talk with IRS1-dependent insulin signaling and trigger downstream metabolic events remain unknown. Additionally, little is known about how G-protein pathways are altered in IR.

With regard to the pathogenesis of IR, suppression of metabolic insulin signaling via the IRS1/Pi3K pathway is an invariable hallmark (Kahn and Flier, 2000; Pessin and Saltiel, 2000; Le Roith and Zick, 2001). Such suppression occurs via common mechanisms that involve cellular accumulation of lipid metabolites (acyl-CoAs, ceramides, diacylglycerol, etc.), which activate, among many other kinases, the critical protein kinase C-theta (PKCθ; Griffin et al., 1999; Yu et al., 2002). PKCθ-dependent phosphorynbihobination of IRS1 at Ser1-101 (Li et al., 2004) is considered an important event that triggers lipid-induced IR. PKCθ expression levels are increased in the skeletal muscles of obese diabetics and hold an inverse relationship to insulin sensitivity (Schmitz-Peiffer et al., 1997; Yu et al., 2002), and PKCθ−/− null mice demonstrate a protective effect against IR despite a high-fat diet (Kim et al., 2004). These studies and many others have shaped the paradigm that IR is triggered when IRS1 is phosphorybhibited by kinases like PKCθ. However, some recent studies have revealed inconsistencies in this pathway, summarized in Hoehn et al., 2008). Emerging evidence indicates that IRS1 is insufficient for orchestrating the insulin response (Krook et al., 1996) and that multiple receptor tyrosine kinases (RTKs) can trigger IR independent of IRS1 (Hoehn et al., 2008). These studies raise the possibility that major unidentified signaling nodes exist within the insulin signaling cascade, whose inhibition via the fatty acid/PKCθ pathway triggers IR.

In this study, we define a single, multimodular signal transducer, GIV, as a critical node in metabolic insulin signaling. GIV is a guanine nucleotide exchange factor (GEF) that activates Gαi2/3 (Garci-Marcos et al., 2009); contains a SH2-like domain that directly binds InsR (Lin et al., 2014); is a direct substrate of InsR, which phosphorylates GIV at Y1764 (Lin et al., 2011); is a bona fide enhancer of the Pi3K-Akt pathway downstream of InsR and other RTKs (Lin et al., 2011); and is a substrate for PKCθ. The latter phosphorylates and inhibits signaling via the GIV-Gαi axis (Lopez-Sanchez et al., 2013). Furthermore, a recent study has indicated that GIV may serve as a major regulator of the metabolic insulin response in skeletal muscle (Hartung et al., 2013); overexpression of GIV in myoblasts leads to hyperphosphorylation of IRS1 and enhanced glucose uptake, whereas depletion of GIV suppresses both. Despite these insights, the molecular mechanisms that enable GIV to enhance the metabolic insulin-IRS1 response in physiology or mechanisms that derail this pathway in the setting of IR remained unknown. On the basis of its ability to cross-talk with all these key mediators of metabolic insulin signaling, we asked whether GIV is a key determinant of insulin sensitivity in physiology and whether its phosphoryregulation by PKCθ triggers IR.

RESULTS

Activation of Gαi by GIV-GEF is required for glucose uptake in skeletal muscles

To determine the role of GIV-GEF in IR, we used differentiated L6 rat skeletal myotubes. Our rationale for this choice was guided by two facts: 1) although both adipocytes and skeletal muscles are sites for IR, full-length GIV is expressed more abundantly in skeletal muscles than in mature adipocytes (Uhlen et al., 2010); and 2) a recent study showed that levels of expression of GIV mRNA in skeletal muscle biopsies from normal subjects tracks with insulin sensitivity, as measured by a hyperinsulinemic–euglycemic clamp (Hartung et al., 2013). We found that depletion of GIV in L6 myotubes (by ~80–85%; Figure 1A) reduced the efficiency of glucose uptake by ~50% (Figure 1B, as determined by a well-established fluorometric assay (Yamamoto et al., 2006). This defect was rescued by stably expressing small interfering RNA (siRNA)-resistant wild-type GIV (GIV-WT) but not the GEF-deficient F1685A mutant of GIV (GIV-FA), which can neither bind nor activate Gαi (Garci-Marcos et al., 2009; Figure 1, C and D). It is noteworthy that the levels of stable expression of GIV-WT or mutants in GIV-depleted L6 myotubes were similar to the levels of endogenous GIV in these cells (Figure 1C), indicating that the effects observed are not merely due to overexpression of GIV at nonphysiological levels. These findings indicate that GIV is required for glucose uptake in skeletal muscles and that its GEF domain is essential.

Next we asked whether phosphorynbihobination of GIV’s GEF at Ser-1689 by PKCθ (Lopez-Sanchez et al., 2013) also inhibits glucose uptake. We found that glucose uptake in cells expressing the constitutively phosphorybhibited S1689D mutant of GIV (GIV-SD) was half as efficient compared with those expressing GIV-WT (Figure 1, C and D), indicating that phosphorynbihobination of GIV’s GEF function by PKCθ impairs glucose uptake in response to insulin. These findings were reproduced in HeLa cells (Supplemental Figure S1), indicating that the effect of GIV-GEF we observe on glucose uptake may not be a restricted only to L6 myotubes but may represent a fundamental effect on IR.

To further pinpoint the impairment of Gi activation by GIV-GEF as the cause, we monitored glucose uptake in L6 myotubes stably expressing either wild-type (Gαi3-WT) or a dominant-negative W258F mutant of Gαi3, henceforth referred to as Gαi3-WF (Figure 1E), which cannot bind or be activated by GIV but localizes and interacts with Gβγ, G protein–coupled receptors (GPCRs), and Gαi regulators similar to Gαi3-WT (Garci-Marcos et al., 2010). We analyzed Gαi3 (and not Gαi1,2), because it is the most abundant Gαi subunit expressed in skeletal muscles, as confirmed by proteomics (Hwang et al., 2010). Glucose uptake was reduced in cells expressing Gαi3-WF compared with those expressing Gαi3-WT (Figure 1F), confirming that GIV drives efficient glucose transport after insulin stimulation via its ability to activate Gαi proteins.

GIV binds ligand-activated InsRβ and modulates multiple tiers of metabolic insulin signaling via its GEF function

To determine how GIV’s GEF function affects the insulin signaling cascade, we analyzed key components of metabolic insulin signaling
A similar analysis comparing L6 myotubes expressing GIV-WT or GIV-SD revealed that phosphoinhibition of GIV’s GEF activity by PKCθ affects several of these key upstream events. Compared with L6 myotubes responding to insulin. Immunoblotting (IB) for phospho-proteins revealed that insulin triggers activation of PKCθ at 5 min, coincides with peak autophosphorylation of InsRβ, and is followed by sustained phosphoactivation of IRS1 and Akt and phosphoinhibition of the GSV-associated Rab-GAP AS160 (Miinea et al., 2005). Activation of PKCθ was initiated by 5 min and sustained up to 30 min, and inhibitory phosphorylation of GIV at S1689 by PKCθ peaked at 30 min. The time line of these events is consistent with the previously described role of this phospho event in the termination of GIV’s GEF activity and disengaging GIV from Gαi (Lopez-Sanchez et al., 2013). A similar analysis comparing L6 myotubes expressing GIV-WT or GIV-SD revealed that phosphoinhibition of GIV’s GEF activity by PKCθ affects several of these key upstream events. Compared with
FIGURE 2: GIV-GEF binds and enhances autophosphorylation of InsRβ and downstream metabolic insulin response. (A) Lysates of serum-starved L6 myotubes stimulated with insulin were analyzed for various components of metabolic insulin signaling by IB. (B) Lysates of serum-starved or insulin-stimulated L6 myotubes stably expressing GIV-WT or GIV-SD were analyzed for activation of GIV, IRS1, InsRβ, and Akt by IB. (C) Immunoprecipitation was carried out on lysates (right) of insulin-treated L6 myotubes with anti-pInsRβ or control immunoglobulin G (IgG). Bound immune complexes (left) were analyzed for activated GIV, IRS1, and InsRβ by IB. (D and E) Serum-starved Cos7 cells were stimulated with insulin, fixed, and subsequently stained for active GIV (pY1764-GIV; red, D), active IRS1 (pY632-IRS1; red, E), active InsRβ (pY1150/51-InsRβ; green), and 4′,6-diamidino-2-phenylindole (DAPI)/DNA (blue). Scale bar: 10 μm. (F) Serum-starved control (Scr shRNA) or GIV-depleted (GIV shRNA) Cos7 cells were stimulated with insulin, fixed, and stained for active InsRβ (pY1150/51-InsRβ; green) and Gsi3 (red) and analyzed by dSTORM microscopy. A high degree
L6-GIV-WT cell lines, global suppression of the insulin response was encountered in L6-GIV-SD cells, starting with the most upstream event, that is, suppressed autophosphorylation of InsRβ at Y1150 and Y1151, which are essential for maximal phosphorylation of substrate proteins (Flores-Riveros et al., 1989; Figure 2B). At the immediate postreceptor level, phosphoactivation of GIV and IRS1 was suppressed, and Akt phosphorylation downstream was impaired (Figure 2B). Similar findings were noted also in paired HeLa-GIV-WT versus HeLa-GIV-SD cells (Supplemental Figure S2A).

Previous work showed that GIV's C-terminal SH2-like domain directly binds autophosphorylated cytoplasmic tails of multiple RTKs, including InsRβ (Lin et al., 2014; Middle et al., 2015). In L6 myotubes, active GIV[pY1764] coimmunoprecipitated with ligand-activated InsRβ-IRS1 complexes (Figure 2C). Active GIV[pY1764] also colocalized with the autophosphorylated InsRβ at PM microdomains (Figure 2D), where activated IRS1 adaptors coexist (Figure 2E). These findings suggest that the InsRβ-GIV-IRS1 complexes we observe in Figure 2C are likely assembled at the PM. To determine whether GIV links Gαi proteins to ligand-activated InsRβ at these PM microdomains, we used direct stochastic optical reconstruction microscopy (dSTORM). This mode of imaging achieves a spatial resolution of ~25 nm, and a high degree of colocalization between endogenous proteins indicates they are likely to interact (Huang et al., 2010). In control cells, but not in GIV-depleted cells, Gαi3 and ligand-activated InsRβ showed a high degree of colocalization along the PM (Figure 2F, yellow pixels), indicating that active InsRβ and Gαi3 come within proximity to each other exclusively in the presence of GIV-GEF. As expected, the impairment of autophosphorylation of InsRβ in GEF-deficient L6-GIV-SD mutant cells was also accompanied by defects in downstream activation of Akt and phosphoactivation of its target Rab-GAP, AS160 (Matsubara et al., 2015; Figure 2G and Supplemental Figure S2B). Taken together, these results demonstrate that GIV binds to ligand-activated InsRβ-IRS1 complexes on microdomains at the PM and links Gαi to such complexes. The presence or absence of a functional GIV-GEF, via which GIV links and activates Gαi in the vicinity of RTKs, appears to be a key determinant of whether multiple tiers within the metabolic insulin signaling cascade are activated maximally, beginning with the autophosphorylation and activation of InsRβ (Figure 2H).

GIV directly binds and modulates the localization and functional phosphorylation of IRS1

Next we investigated how GIV may affect the phosphorylation/activation of IRS1, which is a major adaptor for the metabolic insulin responses. Because the hypophosphorylation of IRS1 observed by immunoblotting lysates of L6 myotubes and HeLa cells expressing the GEF-deficient SD phosphomimetic mutant does not provide enough information about the spatial and temporal dynamics of IRS1 phospho-dephosphorylation in cells, we used a genetically encoded fluorescent biosensor, phocus-2nes (Sato et al., 2002) in fluorescence resonance energy transfer (FRET) studies. This biosensor shows energy transfer only when Y941 on IRS1 is phosphorylated and presents a docking site for the N-SH2 domain of p85α (PI3K), thereby providing a readout of the function of such phosphorylation (Figure 3A). In cells expressing GIV-WT, we observed a significant increase in FRET efficiency at/near the PM (F.E. 0.34 ± 0.08) within 5 min after insulin stimulation; however, in cells expressing GIV-SD, that response was blunted (F.E. 0.06 ± 0.03; Figure 3, B and C, and Supplemental Figure S3A), confirming that phosphoinhibition of GIV-GEF impairs functional phosphorylation of IRS1 at the PM. Because functional phosphorylation of IRS1 involves several steps, we asked whether GIV is required for the two earliest ones, that is, translocation of IRS1 from cytosol to the PM and its subsequent phosphorylation at that location in response to insulin. Compared with control cells, both steps were impaired in GIV-depleted cells (Figure 3, D and E, and Supplemental Figure S3, B and C). Coimmunoprecipitation studies on control or GIV-depleted cells further confirmed that recruitment of IRS1 to the activated InsRβ was impaired in the absence of GIV (Figure 3F). To further pinpoint whether GIV's GEF function is essential for the recruitment of IRS1 to ligand-activated InsRβ, we first looked for insulin-triggered translocation of IRS1 from the cytosol to the PM in Cos7 cells expressing WT or SD GIV (Supplemental Figure S3D). We found that IRS1 localized to the PM in cells expressing GIV-WT exclusively after ligand stimulation, whereas localization at the PM was suppressed in cells expressing GIV-SD. Next we analyzed receptor-bound complexes by immunoprecipitation assays in GIV-depleted HeLa cells stably expressing WT or SD GIV mutant (Supplemental Figure S3D). In HeLa-GIV-WT cells, ligand stimulation triggered robust autophosphorylation of InsR (pY1150, 1151), which coincided with the recruitment of pY1IRS1, GIV, and Gαi3 (Figure 3G). Consistent with our prior observations in L6-GIV-SD cells (Figure 2B), autophosphorylation of InsR was suppressed also in HeLa-GIV-SD cells, and receptor-bound complexes (InsR-GIV-G protein or InsR-IRS1) were decreased. These results not only confirm the role of GIV's GEF function in enhancing the recruitment of IRS1 to ligand-activated InsR but also demonstrate the inhibitory effect of pS1689 GIV on both Insr-IRS1 and Insr-Gαi3 complexes.

Because of the global effect of GIV depletion we observed on IRS1 localization, recruitment, and phosphoactivation, we next asked whether GIV binds IRS1. GIV coimmunoprecipitated with IRS1 before and after insulin stimulation (Figure 4A), indicating that the GIV-IRS1 interaction is constitutive. Pull-down assays with recombinant proteins showed that His-GIV-CT specifically bound Gαi from the cytosol to the PM in Cos7 cells expressing WT or SD GIV, and Gαi3 (Figure 3G). Consistent with our prior observations in L6-GIV-SD cells (Figure 2B), autophosphorylation of InsR was suppressed also in HeLa-GIV-SD cells, and receptor-bound complexes (InsR-GIV-G protein or InsR-IRS1) were decreased. These results not only confirm the role of GIV's GEF function in enhancing the recruitment of IRS1 to ligand-activated InsR but also demonstrate the inhibitory effect of pS1689 GIV on both Insr-IRS1 and Insr-Gαi3 complexes.

of colocalization was observed, as determined by the presence of yellow pixels in the merged images. (G) Lysates of starved and insulin-stimulated L6 myotubes stably expressing GIV-WT or GIV-SD were analyzed for activation of IRS1, AS160, Akt, and tubulin by IB. (H) Schematic illustrating how the presence or absence of a functional GIV-GEF, via which GIV links and activates Gi in the vicinity of InsRβ, dictates the intensity of metabolic insulin signaling, beginning with the activation and autophosphorylation of InsRβ.
FIGURE 3: GIV-GEF directly binds and regulates the localization and activation of IRS1. (A) Schematic for the biosensor phocus-2nes is shown. Energy transfer from CFP to YFP occurs only when Y941 is phosphorylated and the N-SH2 domain of p85α binds the phosphotyrosine ligand. (B and C) Serum-starved Cos7 cells coexpressing phocus-2nes with either GIV-WT-FLAG or GIV-SD-FLAG were stimulated with insulin, fixed, stained for FLAG (far red), and analyzed for FRET using confocal microscopy. Images panels display (from left to right, B) CFP, YFP, FLAG (GIV-WT), and intensities of acceptor emission due to FRET in each pixel 5 min after insulin stimulation. Image panels of serum-starved (0 min) cells are shown in Supplemental Figure S3A. Bar graph (C) displays the FRET efficiency observed in GIV-WT versus GIV-SD cells at 0 and 5 min. The analysis represents five regions of interest from 4 to 6 cells/experiment (three independent experiments). Error bars = mean ± SD. (D) Serum-starved control (sh Control) or GIV-depleted (sh GIV) Cos7 cells expressing IRS1-HA were stimulated with insulin, fixed, stained for HA (green) and DAPI/DNA (blue), and
GIV-GEF is a target for the antagonist actions of fatty acids and insulin sensitizers

Because PKCθ is the kinase that orchestrates lipid-induced IR (Haasch et al., 2006), we next asked whether fatty acids induce IR in part by phosphoinhibition of GIV-GEF at S1689 by PKCθ. When we induced IR in L6 cells using albumin-conjugated sodium palmitate (PA), which is known to activate PKCθ (Griffin et al., 1999), we found that phosphorylation of GIV at S1689 was enhanced, GIV’s ability to bind Gi was reduced, and phosphorylation of GIV at Y1764 was suppressed (Figure 5A and Supplemental Figure S5A), indicating that PA induces phosphoinhibition of GIV-GEF and concomitantly suppresses tyrosine-based signaling via GIV. PA requires PKCθ to exert such phosphoinhibition, because inhibition of PKCθ abolished phosphoinhibition of GIV-GEF (Figure 5B). When PA-treated, insulin-resistant L6 cells were incubated with pioglitazone (Pio), an insulin sensitizer in the thiazolidinedione (TZD) class of drugs, phosphorylation of GIV at S1689 was reduced, and tyrosine phosphorylation of GIV was enhanced, indicating that Pio antagonized both the effects of PA and effectively reversed the phosphoinhibition of GIV-GEF by PKCθ (Figure 5A). Consistent with its role as a true insulin sensitizer, GIV-GEF is a target for the antagonist actions of fatty acids and insulin sensitizers.
FIGURE 5: Phosphoinhibition of GIV-GEF by PKCθ is required for PA-induced IR and dephosphorylation of GIV-GEF is essential for the action of Pio. (A) Lysates of L6 myotubes treated (+) or not (−) with PA alone or a combination of PA and Pio were analyzed for phosphorylation of GIV at S1689 and Y1764 and total (t)GIV by IB. (B) Lysates of L6 myotubes treated (+) or not (−) with PA alone or a combination of PA and a pseudosubstrate PKCθ inhibitor were analyzed for phosphorylation of GIV at S1689 (pS1689 GIV), total (t)GIV, and tubulin by IB. (C) L6 myotubes stably expressing siRNA-resistant GIV-WT or GIV-SA were depleted of endogenous GIV by siRNA, treated with PA (+) or vehicle control (−), and subsequently analyzed for insulin-stimulated glucose uptake by fluorometric assay. Bar graph displays fold change in glucose uptake compared with starved controls (y-axis). Error bars represent mean ± SD; n = 3. (D) L6 myotubes stably expressing siRNA-resistant GIV-WT or GIV-SD were depleted of endogenous GIV by siRNA, treated (+) or not (−) with Pio, and subsequently stimulated with insulin before lysis. Lysates were analyzed for activation of GIV (pY1764 GIV) and Akt (pS473Akt) by IB. (E) L6 myotubes stably expressing siRNA-resistant GIV-WT or GIV-SD were depleted of endogenous GIV by siRNA, treated (+) or not (−) with Pio, and subsequently analyzed for insulin-stimulated glucose uptake by fluorometric assay. Bar graph displays fold change compared with starved controls (y-axis). Error bars represent mean ± SD; n = 3. (F) Equal aliquots of lysates of vastus lateralis muscle biopsies from obese T2DM subjects,
sensitizer that improves insulin action in peripheral tissues, Pio also enhanced tyrosine phosphorylation of GIV and Akt signaling triggered by insulin in insulin-sensitive L6 cells never exposed to PA (Supplemental Figure S5, B and C).

We then investigated whether phosphoinhibition of GIV-GEF by PKCθ in L6 myotubes plays a role in mediating the antagonistic effects of PA and Pio in the induction and reversal of IR, respectively. PA induced IR in L6-GIV-WT cells, as determined by a blunted glucose-uptake response to insulin (Figure 5C). However, L6 cells expressing a nonphosphorylatable GIV S1689A mutant (L6-GIV-SA) were resistant to PA, that is, these cells remained sensitive to insulin regardless of PA treatment and demonstrated higher glucose uptake compared with L6-GIV-WT cells (Figure 5C). These results demonstrate that the selective inhibition of GIV-GEF by PKCθ via phosphorylation of a single Ser-1689 is an essential mechanism by which PA triggers IR in L6 myotubes. As for Pio, we found that it reinstated insulin signaling in PA-treated, insulin resistant L6-GIV-WT cells, as determined by restored tyrosine phosphorylation of GIV and Akt signaling (Figure 5D and Supplemental Figure SSD). However, L6 cells expressing a constitutively phosphoinhibited GIV SD mutant (L6-GIV-SD) were resistant to Pio, that is, these cells showed no discernible enhancement of signaling compared with L6-GIV-WT cells (Figure 5D and Supplemental Figure SSD). Furthermore, Pio reversed the PA-induced IR state in L6-GIV-WT cells, but not in L6-GIV-SD cells, as determined by glucose uptake after insulin stimulation (Figure 5E). Because Pio is known to improve insulin sensitivity in muscle tissue in part by antagonizing the activity of protein kinases such as PKCθ (Markova et al., 2010), our results demonstrate that reversal of phosphoinhibition of GIV-GEF by PKCθ on Ser-1689 is an essential mechanism via which Pio reverses IR and sensitizes L6 myotubes to the action of insulin. The inability to reverse such phosphoinhibition (as in the case of the GIV-SD mutant, which mimics a constitutive phosphoinhibited state) makes cells nonresponsive to the insulin-sensitizing actions of Pio.

The physiological significance of these observations in cultured L6 myotubes was confirmed by findings in patients with IR, in whom chronic treatment with Pio reduced the phosphoinhibition of GIV-GEF and enhanced phosphorylation of Akt in skeletal muscles (vastus lateralis) of obese T2DM patients (Figure 5F). Moreover, patients with polycystic ovarian syndrome (PCOS) in whom IR was clinically reversed by Pio therapy, that is, responders (as determined by 24-h hyperinsulinemic–euglycemic clamp) showed a significant reduction in phosphoinhibition of GIV at S1689 in their muscles. By contrast, PCOS patients who failed the Pio treatment trial (i.e., nonresponders) had high pretreatment and/or posttreatment levels of phosphoinhibition of GIV-GEF (Figure 5, G and H). Taken together, our results demonstrate that a single phospho event (PKCθ-dependent phosphorylation of GIV at S1689), which selectively inhibits GIV-GEF and therefore abolishes activation of Gi by GIV, is a common pivot point for both PA and Pio to exert their antagonistic actions in IR. Phosphorylation at S1689 is essential for PA to induce IR, whereas dephosphorylation is required for Pio to enhance tyrosine phosphorylation of IRS1 and GIV, restore Akt signaling, and reinstate insulin sensitivity (Figure 5I).

Cell-permeant GIV-derived peptides can effectively reverse IR in skeletal muscle

We next asked whether GIV-GEF can serve as a therapeutic target for exogenous modulation of IR. To investigate this, we used recently validated recombinant, cell-permeable TAT-tagged GIV-CT peptides (WT and GEF-deficient FA mutant peptides spanning GIV’s GEF and SH2-like domains; Figure 6, A and B). These peptides offer a nongenetic approach for exogenous manipulation of GIV-GEF–dependent signaling programs and cellular phenotypes in diverse cells and a variety of pathophysiological processes (Ma et al., 2015). L6 myotubes homogeneously took up TAT peptides (~90% efficiency of uptake; Figure 6C). Uptake of GIV-CT-WT peptides was associated with enhancement of stress-fiber formation and phosphorylation of IRS1 and Akt proteins in response to insulin (Figure 6, C and D). However, uptake of GIV-CT-FA peptides disrupted the actin stress fibers, as shown previously in other cell lines, and suppressed IRS1 and Akt phosphorylation in response to insulin. Consistent with these signaling programs, insulin-stimulated glucose uptake in the basal state was unaffected by GIV-CT-WT peptides but was significantly inhibited by GIV-CT-FA peptides (Figure 6E). GIV-CT-WT, but not the FA mutant peptides, effectively reversed PA-induced IR (Figure 6F), and ~800 nM of WT peptide was as effective as 50 μM Pio in reversing such IR (Figure 6G). These studies demonstrate that cell-permeant GIV-CT peptides can enhance metabolic insulin signaling and reverse IR effectively in a GEF-dependent way.

DISCUSSION

Phosphoinhibition of GIV-GEF by PKCθ triggers lipid-induced IR; dephosphorylation of GIV-GEF reinstates insulin sensitivity

The fundamental discovery in this work is that GIV-GEF plays a major role as a dominant conduit for insulin response in the skeletal muscle, and identification of key phospho events that allow this GEF to serve as a decisive pivot/node for cellular insulin response in physiology and disease (see Figure 7). In lean individuals, insulin triggers activation of GIV by tyrosine phosphorylation (pY), GIV’s GEF function is turned “on,” and Gai is activated, metabolic insulin signaling is initiated through the InsR/IRS1/Pi3K/Akt signaling axis, culminating in efficient exocytosis of GSVs and subsequent uptake of glucose. In the obese, circulating free fatty acids trigger the accumulation of diacyl glycerol (DAG) and activation of PKCθ, which in turn phosphorylates GIV’s GEF motif at Ser-1689, which in turn phosphorylates IRS1 and GIV, restore Akt signaling, and reinstate insulin sensitivity.
FIGURE 6: Cell-permeant TAT-GIV-CT-WT peptides, but not FA mutant peptides, effectively reverse lipid-induced IR in skeletal muscles. (A) Design of the cell-permeant TAT-GIV-CT peptides is shown. TAT-PTD was fused to His and HA tags and coupled via a linker (GGSGHSG) to the C-terminus of GIV (aa 1660–1870). (B) Purified recombinant TAT-GIV-CT peptides were analyzed by Coomassie blue staining and by IB with anti GIV-CT and anti-His antibodies. (C) L6 myotubes were treated with TAT-GIV-CT-WT or FA peptides and cultured overnight in low serum conditions (0.2% fetal bovine serum) before fixation. Fixed cells were stained for His (green), phalloidin (F-actin, red), and DAPI/DNA (blue) and analyzed by confocal microscopy. (D) L6 myotubes were treated with TAT-GIV-CT-WT or FA peptides, starved, and stimulated with insulin before lysis. Equal aliquots of lysates were analyzed for transduction of TAT-peptides with anti-His antibody, activation of IRS1 (pY632-IRS1) and Akt (pS473) by IB. (E) L6 myotubes were treated with

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TAT-GIV-CT-WT or FA peptides, starved, and subsequently analyzed for insulin-stimulated glucose uptake by fluorometric assay. Bar graph displays fold change in uptake compared with starved controls (y-axis). Error bars represent mean ± SD. (F) L6 myotubes were treated (+) or not (−) with PA to induce IR, then transduced with TAT-GIV-CT-WT or FA peptides, and subsequently analyzed for insulin-stimulated glucose uptake by fluorometric assay. Bar graph displays fold change in uptake compared with starved controls (y-axis). Error bars represent mean ± SD. (G) L6 myotubes were treated (+) or not (−) with PA to induce IR, then either treated with Pio or transduced with TAT-GIV-CT-WT peptides (as indicated), and subsequently analyzed for insulin-stimulated glucose uptake by fluorometric assay. Bar graph displays fold change in uptake compared with starved controls (y-axis). Error bars represent mean ± SD.

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and selectively turns “off” the GEF function. Consequently, Gxi remains inactive, and a majority of the key elements of metabolic insulin signaling are suppressed, thereby triggering IR.

We also provide evidence that reversible phosphorylation of GIV at S1689 by PKCθ and the ability of this single phospho event to modulate the InsR-GIV-Gxi signaling axis are critical determinants of cellular insulin responses. We demonstrated that this phospho event alone is sufficient to mimic fatty acid–induced IR and that fatty acids require such phosphorylation to induce IR. Although this study dissected the interplay between GIV and PKCθ in lipid-induced IR, because GIV can intercept signaling downstream of multiple classes of receptors (GPCRs and RTKs) and non-RTKs alike (reviewed in Garcia-Marcos et al., 2015) and is an enhancer of STAT3 as well as its transcriptional target (Dunkel et al., 2012), it is possible that GIV is a central node for other major triggers of IR, that is, inflammation, suppression of adiponectin, leptin resistance, and so on, which also require activation of PKCθ (Itani et al., 2000; Lin et al., 2000; Shulman, 2000; Anderson et al., 2006) and/or the AKT-STAT3 pathway (Mashili et al., 2013; Wunderlich et al., 2013).

We also show that TZDs like Pio release the phosphoinhibition on GIV-GEF and restore its function. Such restoration was essential for TZD action, because TZDs failed to reverse IR and reestablish insulin sensitivity in cells expressing the constitutively phosphomimic GIV-S1689D mutant. Because chronic TZD therapy does not suppress PKCθ (Markova et al., 2010), the reduction in levels of GIV phosphorylation at S1689 we observed after TZD therapy is likely to be a consequence of dephosphorylation by one of the many S/T phosphatases that are activated by TZDs in a PPARγ-dependent manner (Altiok et al., 1997; Pugazhenthi and Khandelwal, 1998; Sharma et al., 2004; Cho et al., 2006). Regardless of the mechanism(s) involved, our results indicate that GIV is a major target of TZDs that can account, in part, for TZD action on skeletal muscle. We conclude that reversible phosphorylation at S1689 and inhibition of the GEF function, via which GIV activates Gxi, serves as a molecular switch for flipping skeletal muscles between insulin-sensitive and insulin-resistant states. Because GIV specifically binds Gxi and not Gαq/11 (Le-Niculescu et al., 2005), these findings do not account for the previously described role of yet another G protein, Gαq/11, in insulin resistance (Imamura et al., 1999).

GIV’s GEF function modulates several tiers within the metabolic insulin signaling cascade

We demonstrated that activation of Gxi by GIV impacts many tiers within the insulin signaling cascade and that phosphoinhibition of GIV’s GEF function antagonizes them all. We previously showed that GIV directly binds autophosphorylated cytoplasmic tails of ligand-activated InsR via its C-terminal SH2-like module (Lin et al., 2014). Both the SH2-like module and GIV’s GEF functions are critical for coupling of G proteins to ligand-activated InsR (Garci-Marcos et al., 2011; Lin et al., 2014; Middel et al., 2015). We show here that, at the level of the receptor, activation of Gxi via GIV’s GEF motif is required for maximal autophosphorylation and activation of InsRβ and recruitment and phosphoactivation of its major substrate, IRS1. The sites of autophosphorylation on InsRβ that GIV enhanced, Y1150 and Y1151, are required for maximal activation of the InsRβ kinase (White et al., 1988), and a failure to activate InsRβ kinase in skeletal muscles has been implicated in IR (Maegawa et al., 1991; Nolan et al., 1994; Goodyear et al., 1995). Although it is unclear how activation of Gxi by GIV may enhance receptor autophosphorylation, it is not entirely surprising, because activation of Gxi has previously been implicated in the enhancement of InsR autophosphorylation (Kreuzer et al., 2004) and because we previously showed that activation of Gxi by GIV’s GEF function can similarly enhance autophosphorylation of yet another RTK, EGFR (Ghosh et al., 2010). In both instances, suppression of protein tyrosine phosphatases (PTPs) has been implicated as the mechanism for enhanced receptor autophosphorylation (Moxham and Malbon, 1996; Lin et al., 2014). Because GIV directly binds ligand-activated InsRβ (Lin et al., 2014) and triggers the formation of InsRβ-Gxi complexes at the PM, it is possible that the formation of such InsRβ-GIV-Gxi complexes suppresses the recruitment and/or activation of key PTPases. We conclude that the GIV-Gxi axis enhances cellular insulin response by increasing InsRβ kinase activity and autophosphorylation, two upstream events in insulin signaling.

At the immediate postreceptor level, we demonstrate that GIV binds and modulates the functions of IRS1. Activation of Gxi by GIV enhanced the recruitment of IRS1 to the ligand-activated receptors at the PM, triggered robust tyrosine phosphorylation at Y632 and Y941 on IRS1, and enhanced the formation of IRS1-p85α/P13K complexes. Unlike the ligand-dependent nature of InsRβ-GIV or InsRβ-IRS1 (Sun et al., 1991) interactions, the GIV-IRS1 interaction was constitutive. We also provide evidence that this binding was direct and that it involved the C-terminal region of GIV and the N-terminal region of IRS1. The latter contains a phosphotyrosine-binding domain (PTB) that is responsible for the direct interaction of IRS1 with InsRβ (Eck et al., 1996). Our findings of InsRβ-GIV-IRS1 complexes upon insulin stimulation suggest that GIV may bind IRS1 at a distinct site where the autophosphorylated cytoplasmic tail of InsRβ docks within the PTB. The enhanced recruitment of IRS1 to InsRβ in the presence of GIV suggests that GIV may serve as a signal amplifier at the immediate postreceptor level by facilitating the recruitment of more IRS1 adaptors per activated InsR. Based on recent experimental evidence that questions the exclusivity of IRS1 for InsRβ (Knowlden et al., 2008) and that shows GIV is capable of binding multiple RTKs (e.g., EGFR, PDGFR, VEGFR; Lin et al., 2014; Lopez-Sanchez et al., 2014), it is possible that GIV provides the necessary molecular basis for IRS1 to serve as a common conduit for metabolic response observed downstream of receptors other than InsRβ.

Although the precise mechanism of GIV-IRS1 interaction remains uncertain, this interaction adds GIV to the lengthy list of proteins that IRS1 scaffolds within the insulin signaling cascade (White, 2006). The finding that GIV enhanced tyrosine phosphorylation of IRS1 is consistent with the concomitant increase in the kinase activity of InsRβ and enhanced recruitment of IRS1 to the PM, the latter is a prerequisite for maximal tyrosine phosphorylation of IRS1 (Myers et al., 1995; Volovitch et al., 1995). We conclude that GIV is required for maximal PM recruitment and tyrosine phosphorylation of IRS1, both key events implicated in metabolic insulin signaling via IRS1. In doing so, and by virtue of its ability to directly bind and bring together several other components of the metabolic insulin response (InsR, IRS1, G proteins, actin, P13K, Akt), GIV serves as an integral hub at the immediate postreceptor that fine-tunes IRS1-dependent metabolic insulin signaling.

Further downstream, the P13K-Akt signaling pathway was maximally enhanced in the presence of an intact GIV-GEF, and a major pathway downstream of Akt was triggered, that is, phosphoinhibition of the Rab-GAP AS160. Prior studies have demonstrated that docking of GSVs at the PM requires activation of Rab proteins (Miinea et al., 2005; Sun et al., 2010; Lansley et al., 2012) in response to insulin (Bai et al., 2007). By triggering the phosphoinhibition of Rab-GAP AS160, GIV’s GEF function is likely to affect the exocytosis of GSVs via potentiation of Rab GTPases. We conclude that GIV functionally interacts with and enhances key signaling pathways...
events that can also coordinate membrane trafficking within the insulin response cascade, and in doing so, it delineates a molecular basis for the observed engagement between these events during insulin-triggered glucose uptake into cells (Leto and Saltiel, 2012). Although this study has specifically dissected the role of GIV's GEF function in coordinating key signaling events that comprise the metabolic insulin response, it is notable that the GEF motif merely represents a stretch of ~30–35 aa within a 1871-aa-long, multimodu-
lar protein made up of several other key functional modules that may take part in other key aspects of glucose uptake. One such well-defined module is GIV's SH2-like domain, which is necessary and sufficient for GIV to directly bind the autophosphorylated cyto-
plasmic tail of InsR; without a functional SH2-like domain, GIV can neither bind InsR nor facilitate the formation of InsR–G protein com-
plexes (Lin et al., 2014). Another such module, whose boundaries remain to be defined, but which appears to be functionally distinct from the GEF module, is a region within GIV's C-terminus that di-
rectly binds IRS1 (Figure 4B); it is possible that selective inhibition of GIV-IRS1 interaction may also impair the metabolic insulin response and glucose uptake. Our own recent findings that GIV regulates cargo trafficking from the ER–Golgi intermediate compartment (ERGIC) to the Golgi (Lo et al., 2015) raises the possibility that GIV may also play a role in regulating GLUT4 trafficking from the Golgi to GSVs. Additionally, GIV is also known to regulate clathrin-medi-
ad endocytosis and endocytic trafficking (Beas et al., 2012; Weng et al., 2014), two processes closely intertwined with and key deter-
ninants of the kinetics of GLUT4 trafficking, glucose uptake, and
down-regulation of insulin receptor signaling. Because the actin cy-
toskeleton has also been described as a tether for GSVs (Stokli et al., 2011), it is possible that another previously characterized module that enables GIV to remodel the cortical actin cytoskeleton further aids in GSV exocytosis and glucose uptake (Enomoto et al., 2005; Ghosh et al., 2010). Thus, it is likely that many of GIV's mod-
ules, not just its C-terminal GEF motif, may play a role in integrating signaling events with vesicular trafficking and cytoskeletal changes to orchestrate glucose uptake after insulin stimulation.

Selective modulation of GIV-GEF emerges as a therapeutic strategy for reversal of IR

We found that cell-penetrable GIV peptides were as effective as TZDs in their ability to reverse fatty acid–induced IR in a GEF-depen-
dent way, and activation of Gxα via GIV's GEF thus mimics the action and matches the potency of TZDs. Because postprandial lipotoxicity can also suppress GIV expression in skeletal muscles (Supplemental Figure S6), our results using cell-permeant peptides suggest that replenishing GIV-CT (with active GEF) by gene therapy may be a viable strategy for the treatment of IR. Other strategies include ago-
nists of GIV's GEF function, antagonists of the inhibitory phospho-
etion event on GIV triggered by PKCθ, or activation of phosphatases that dephosphorylate GIV—all approaches that may serve as more re-
efined, effective, and precise therapeutic strategies to reverse IR in skeletal muscle. GIV expressed in adipocytes is also likely to en-
hance the metabolic insulin response in adipose tissue, the second major site of IR. However, phosphoinhibition of GIV-GEF by PKCθ is unlikely to be a trigger for IR, because this kinase is undetectable in adipocytes (Fleming et al., 1998). Instead, mechanisms such as tran-
scriptional repression, single nucleotide polymorphisms (SNPs), or posttranslational modifications (splice variants) that reduce the lev-
els of full-length GIV may play a role. Further studies are required to determine how IR is triggered in adipocytes and whether the GIV-
targeted approaches we show here can reverse IR also in the adi-
pose tissue.

In conclusion, we have defined activation of Gxα by GIV's GEF function as a central node that coordinately enhances the physiological insulin response and how its deregulation heralds IR. Because this node also serves as the point of convergence for the antagonistic ac-
tions of fatty acids and insulin sensitizers, selective modulation of this node emerges as a promising and precise strategy to treat T2DM and other conditions in which IR plays a central pathophysiological role.

MATERIALS AND METHODS

Detailed methods are presented in the Supplemental Materials.

Cell culture, transfection, IB, immunofluorescence, and protein–protein interaction assays

These assays were carried out exactly as described before (Ghosh et al., 2008, 2010). All Odyssey images were processed using Im-
ageJ software (National Institutes of Health [NIH], Bethesda, MD) and were assembled for presentation using Photoshop and Illustra-
tor software (Adobe Systems, San Jose, CA).

dSTORM and FRET imaging

Direct Stochastic Optical Reconstruction Microscopy imaging was performed to reveal the interaction endogenous Gxα3 and active
InsRβ at molecular level (Huang et al., 2010). Control short hairpin
RNA (shRNA) and GIV shRNA Cos7 stable cells were starved and
stained with 100 nM insulin and stained with anti-Gxα3 (1:30; Cal-
biochem, San Diego, CA) and phosho-InRβ antibodies (1:100; Santa
Cruz Biotechnologies, Dallas, TX).

FRET assays were performed using the intracellular phosphoryla-
tion biosensors custom (phoclus-2nes) designed by Yoshio Umeza-
wa’s group at the University of Tokyo (Sato et al., 2002). GIV shRNA
Cos7 stable cells were transfected with phocus-2nes and GIV-WT-
FLAG or GIV-S1689D-FLAG. Cells were starved and stimulated with
insulin and were stained with anti-FLAG antibody following the stan-
dard immunofluorescence protocol.

Patient samples

Biopsies of vastus lateralis muscle used for GIV phosphorylation
analysis were collected in the Special Diagnostic and Treatment Unit
of the Veterans Affairs Medical Center (San Diego, CA) and the Gen-
eral Clinical Research Center, University of California, San Diego
(UCSD). Muscle samples were collected from healthy, normal, cy-
cling women or women with PCOS before and after a course of
therapy with Pio (45 mg/d, for 6 mo; Aroda et al., 2009). Collec-
tion and storage of muscle samples and measurement of eGDR
were performed as described previously (Thorburn et al., 1990). The
experimental protocol was approved by the Human Research Pro-
tection Program of the UCSD.

Data analysis and statistics

All experiments were repeated at least three times, and results
were presented either as one representative experiment or as
mean ± SD or SEM. Statistical significance was assessed with the
two-tailed Student’s t test.

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