Insulin Substrates 1 and 2 Are Corequired for Activation of Atypical Protein Kinase C and Cbl-Dependent Phosphatidylinositol 3-Kinase during Insulin Action in Immortalized Brown Adipocytes[†]

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ABSTRACT: Phosphatidylinositol 3-kinase (PI3K)-dependent activation of atypical protein kinase C (aPKC) is required for insulin-stimulated glucose transport. Although insulin receptor substrate-1 (IRS-1) and IRS-2, among other factors, activate PI3K, there is little information on the relative roles of IRS-1and IRS-2 during aPKC activation by insulin action in specific cell types. Presently, we have used immortalized brown adipocytes in which either IRS-1 or IRS-2 has been knocked out by recombinant methods to examine IRS-1 and IRS-2 requirements for activation of aPKC. We have also used these adipocytes to see if IRS-1 and IRS-2 are required for activation of Cbl, which is required for insulin-stimulated glucose transport and has been found to function upstream of both PI3K/aPKC and Crk during thiazolidinedione action in 3T3/L1 adipocytes [Miura et al. (2003) *Biochemistry 42*, 14335]. In brown adipocytes in which either IRS-1 or IRS-2 was knocked out, insulin-induced increases in aPKC activity and glucose transport were markedly diminished. These effects of insulin on aPKC and glucose transport were fully restored by retroviral-mediated expression of IRS-1 or IRS-2 in their respective knockout cells. Knockout of IRS-1 or IRS-2 also inhibited insulin-induced increases in Cbl binding to the p85 subunit of PI3K, which, along with IRS-1/2, may be required for activation of PI3K, aPKC, and glucose transport during insulin action in 3T3/L1 adipocytes. These findings provide evidence that directly links both IRS-1 and IRS-2 to aPKC activation in immortalized brown adipocytes, and further suggest that IRS-1 and IRS-2 are required for the activation of Cbl/PI3K during insulin action in these cells.

Insulin-stimulated glucose transport is dependent upon the activation of atypical protein kinase C isoforms (aPKCs), PKC-ζ and PKC-λ, in rat adipocytes (*1*, *2*), mouse-derived 3T3/L1 adipocytes (*3*, *4*), human adipocytes (*5*), and brown adipocytes (*6*). Although it is clear that aPKC activation by insulin in these adipocytes is dependent upon the activation of phosphatidylinositol (PI) 3-kinase (3K), generation of PI-3,4,5-$(PO_4)_3$ (PIP_3) and increased action of 3-phosphoinositide-dependent protein kinase-1 (PDK1), the factors that function upstream of PI3K during insulin-stimulated increases in aPKC activity and glucose transport, are not entirely certain. In this regard, it is generally assumed that insulin receptor substrates (IRSs), in particular, IRS-1 and/or IRS-2, serve as major activators of PI3K and aPKCs during insulin-stimulated glucose transport in adipocytes. In support of this assumption, glucose transport is partially diminished in adipocytes isolated from mice in which IRS-1 has been knocked out by gene targeting methods (*7*, *8*). However, there is presently no evidence from gene knockout studies that directly links IRS-1 to aPKC activation during insulin action in adipocytes. On the other hand, defective activation of aPKCs has been observed in immortalized brown adipocytes (*6*) and hepatocytes (*9*) in which IRS-2 has been knocked out, and the loss of aPKC activation seems to be responsible for decreases in insulin-stimulated glucose transport in IRS-2-deficient brown adipocytes (*6*).

Although both IRS-1 and IRS-2 activate PI3K, they activate pools of PI3K that are at least partly different (see ref *10*). It is therefore possible that IRS-1 and IRS-2 may activate functionally separate pools of PI3K that are corequired, or perform functions other than PI3K activation during insulin-stimulated glucose transport. Also, IRS-3, IRS-4, and factors other than IRS family members may function upstream of PI3K during insulin action. In this regard, we have found that Cbl activates a relatively small pool of PI3K that potently (nearly as well as insulin) activates aPKCs and glucose transport [but not protein kinase B (PKB), which, like aPKC, functions downstream of PI3K and PDK1] during thiazolidinedione action in 3T3/L1 adipocytes (*11*, *12*). Of further note, Cbl has been found to function within a flotillin/CAP/Cbl/Crk/C3G/TC10 complex that localizes to caveolin-rich lipid raft microdomains of the plasma membrane during

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insulin-stimulated glucose transport in 3T3/L1 adipocytes (*13*−*20*). In addition, we have found that (a) insulin activates a relatively small (i.e., 15−20% relative to IRS-1-associated PI3K) pool of Cbl-associated PI3K in 3T3/L1 adipocytes, but comparable in size to that activated by thiazolidinediones (*11*) and (b) expression of Y371F or Y731F tyrosine mutants of Cbl (presumably by nullifying the function of the two canonical pYXXM motifs that are present in Cbl) inhibits the binding of Cbl, not only to Crk in the aforesaid flotillin/CAP/Cbl/Crk/C3G/TC10 complex, but also to the p85 subunit of PI3K in 3T3/L1 adipocytes during the stimulatory actions of both thiazolidinediones (*12*) and insulin (see the preceding paper in this issue) in 3T3/L1 adipocytes. Moreover, expression of these Cbl mutants inhibits the activation of Cbl-associated, but not IRS-1-associated, PI3K, aPKCs, and glucose transport during insulin action in 3T3/ L1 adipocytes (see the preceding paper in this issue). Accordingly, the possibility exists that both IRS-1/2 and Cbl may be corequired for activation of PI3K and aPKCs during insulin-stimulated glucose transport in 3T3/L1 adipocytes. The possibility also exists that IRS-1/2 may function upstream of Cbl activation, or vice versa.

To gain more insight into the roles of IRS-1 and IRS-2, vis-à-vis Cbl, in the activation of PI3K and aPKCs during insulin-stimulated glucose transport in adipocytes, we have presently used immortalized brown adipocytes in which either IRS-1 or IRS-2 has been knocked out, or knocked out and restored by retroviral gene transfer (*21*−*23*). We have found that activation of aPKC and stimulation of glucose transport by insulin are equally dependent upon both IRS-1 and IRS-2 in immortalized brown adipocytes. We have also found that insulin-induced increases in Cbl binding to the p85 subunit of PI3K and activation of Cbl-associated PI3K are markedly diminished in brown adipocytes in which either IRS-1 or IRS-2 has been knocked out. Our findings therefore suggest that both IRS-1 and IRS-2 not only function not only directly upstream of PI3K, but are also required for activation of Cbl and Cbl-associated PI3K during insulin-induced increases in aPKC activity and glucose transport in brown adipocytes.

EXPERIMENTAL PROCEDURES

Adipocyte Incubations. As described (*21*−*23*), preadipocytes of immortalized wild-type brown adipocytes and immortalized brown adipocytes in which IRS-1 or IRS-2 was knocked out, or knocked out and subsequently restored by retroviral gene transfer methods, were cultured in the presence of 20 nM insulin and 1 nM triiodothyronine in Dulbecco's minimal Eagle's medium (DMEM) containing 10% fetal bovine serum, differentiated over 48 h to mature adipocytes by addition of 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 0.125 mM indomethacin, and then incubated in the absence of hormones and differentiating agents for 48 h (*21*−*23*). In some studies, the fully differentiated adipocytes were treated during a 48 h incubation period with the indicated concentrations (given as MOI, i.e., multiplicity of infection, or viral particles per cell) of adenovirus alone or adenovirus encoding wild-type, constitutively active, or kinase-inactive PKC-λ, kinase-inactive 3-phosphoinositide-dependent protein kinase-1 (PDK1), as described (*5*, *11*). For subsequent experimental use, adipocytes were incubated for 3 h in serum-free DMEM containing 2% bovine serum albumin (BSA), then equilibrated in Krebs Ringer phosphate (KRP) medium containing 1% BSA, and ultimately treated with or without 100 nM insulin for the indicated times. After incubation, the cells were sonicated, cell lysates were prepared as described below, and the lysates were subjected to immunoprecipitation and assay for aPKCs and PKB or, as another indicator of PKB activation, used to measure immunoreactive phosphoserine-473-PKB.

Cell Lysate Preparations. As described (*5*, *11*, *12*), adipocytes were scraped, collected, and sonicated in ice-cold buffer, which, for aPKC assays, contained 0.25 M sucrose, 20 mM Tris/HCl (pH 7.5), 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL leupeptin, 10 μg/mL aprotinin, 3 mM $Na_4P_2O_7$, 3 mM Na_3VO_4, 3 mM NaF, and 1 μM LR-microcystin. For PKB assays and Cbl immunoprecipitations, the homogenizing buffer contained 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 0.1% β-mercaptoethanol, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10 mM β-glycerophosphate, 1 mM PMSF, 10 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 μM LR-microcystin. After low-speed centrifugation for 10 min at 700*g* to remove unbroken cells, debris, nuclei, and floating fat, 0.15 M NaCl, 1% Triton-X, and 0.5% Nonidet were added, and the resulting cell lysates were immunoprecipitated with antibodies that target (a) aPKCs (rabbit polyclonal antiserum from Santa Cruz Biotechnologies, Santa Cruz, CA; recognizes the C-termini of both PKC-λ and PKC-ζ), (b) PKB [mouse monoclonal antibodies from Upstate Biotechnologies, Inc. (UBI), Lake Placid, NY], or (c) Cbl (rabbit polyclonal antiserum from Santa Cruz Biotechnologies). Immunoprecipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies), and aPKCs and PKB were assayed, or Cbl immunoprecipitates were subjected to Western analyses, as described below. The lysates were also used to measure levels of immunoreactive proteins, as described below.

Assay of aPKC Activity. As described (*5*, *11*, *12*), aPKCs were immunoprecipitated from cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognizes the C-termini of both PKC-λ and PKC-ζ (unlike mouse adipocytes which contain mainly PKC-λ and little PKC-ζ, brown adipocytes contain substantial amounts of both PKC-λ and PKC-ζ), collected on Sepharose-AG beads, and incubated for 8 min at 30 °C in 100 μL of buffer containing 50 mM Tris/HCl (pH 7.5), 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM PMSF, 4 μg of phosphatidylserine (Sigma), 50 μM [γ-^{32}P]ATP (NEN Life Science Products), 5 mM $MgCl_2$, and, as substrate, 40 μM serine analogue of the PKC-ϵ pseudosubstrate (BioSource). After incubation, ^{32}P-labeled substrate was trapped on P-81 filter papers and counted.

Assay of PKB Activity/Phosphorylation. PKB enzyme activity was measured using a kit obtained from UBI, as described (*5*, *11*, *12*). In brief, PKB was immunoprecipitated with mouse monoclonal antibodies, collected on Sepharose-AG beads, and incubated as per directions in the PKB assay kit. PKB activation was also assessed by immunoblotting for phosphoserine-473 (see below).

Binding of Cbl to the p85 Subunit of PI3K and Crk. As described (*12*), Cbl was immunoprecipitated with rabbit polyclonal antiserum (Santa Cruz Biotechnologies), and the

precipitates were resolved by SDS−PAGE and blotted for the p85 subunit of PI3K, Crk, and other signaling factors (see below). Note that the recovery of immunoprecipitable Cbl was not influenced by treatments with wortmannin or insulin, or the presence or absence of IRS-1 or IRS-2.

Assay of Cbl-Dependent PI3K Activity. As described (*12*), Cbl was immunoprecipitated with rabbit polyclonal antiserum (Santa Cruz Biotechnologies), and the precipitates were examined for PI3K activity, i.e., ^{32}P incorporation into PI-3-PO_4, which was purified by thin-layer chromatography and quantified in a BioRad PhosphorImager.

Western Analyses. Western analyses were conducted as described (*5*, *11*, *12*), using the following for immunoblotting: (a) rabbit polyclonal anti-PKC-ζ/λ antiserum (Santa Cruz Biotechnologies; recognizes the C-termini of both PKC-ζ and PKC-λ); (b) mouse monoclonal anti-PKC-λ antibodies (Transduction Labs); (c) rabbit polyclonal anti-PKC-ζ antiserum (gift of Dr. Todd Sacktor); (d) rabbit polyclonal anti-PKB antiserum (UBI); (e) rabbit polyclonal anti-phosphoserine-473-PKB antiserum (New England BioLabs Inc); (f) rabbit polyclonal anti-p85/PI3K antiserum (Santa Cruz Biotechnologies); (g) rabbit polyclonal anti-Crk antiserum (Santa Cruz Biotechnologies); (h) rabbit polyclonal anti-PKB antiserum (UBI).

Statistical Methods. P values were determined by one-way ANOVA and the least significant multiple comparison method.

RESULTS

Requirements for PI3K, PDK1, and aPKCs during Insulin-Stimulated [^{3}H]-2-Deoxyglucose Uptake in Brown Adipocytes. Similar to findings in other cell types (*4*, *5*, *11*), insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes was inhibited by the PI3K inhibitor wortmannin (Figure 1). It therefore may be surmised that PI3K is required for insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes.

Operating downstream of PI3K, PDK1 has been found to be required for activation of aPKC, PKB, and glucose transport in 3T3/L1 adipocytes, L6 myotubes, and cultured human adipocytes (*5*). It was therefore of interest to find that adenoviral-mediated expression of kinase-defective forms of both PDK1 and PKC-λ inhibited insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes as effectively as wortmannin (Figure 1). Overexpression of wild-type PKC-λ, on the other hand, did not inhibit insulin-stimulated [^{3}H]-2-deoxyglucose uptake (Figures 1 and 2), and expression of constitutively active PKC-λ, if anything, enhanced basal and insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes (Figure 2). Of further note, expression of wild-type or constitutively active PKC-λ restored insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes expressing kinase-defective PDK1 (Figure 2).

From these findings, it may be surmised that PI3K, PDK1, and aPKCs are required for insulin-stimulated [^{3}H]-2-deoxyglucose uptake in wild-type brown adipocytes.

Requirements for Both IRS-1 and IRS-2 during Insulin-Stimulated [^{3}H]-2-Deoxyglucose Uptake in Immortalized Brown Adipocytes. As seen in Figure 3, relative to that in wild-type brown adipocytes, insulin-stimulated [^{3}H]-2-deoxyglucose uptake was diminished by approximately 60−70% in brown adipocytes in which either IRS-1 or IRS-2 had been knocked out. Of further note, there was full or nearly full restoration of insulin-stimulated [^{3}H]-2-deoxyglucose uptake by retroviral-mediated expression of IRS-1 or IRS-2 in their respective knockout adipocytes (Figure 3). The restorative effects of IRS-1 and IRS-2 suggested that defects in insulin-stimulated [^{3}H]-2-deoxglucose uptake observed in respective knockout adipocytes were specifically related to the absence of IRS-1 and IRS-2.

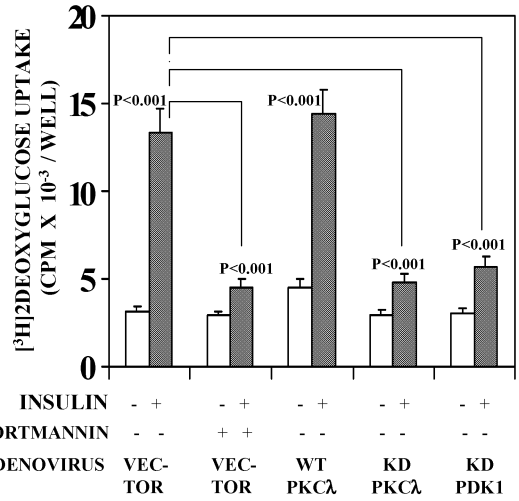

FIGURE 1: Requirements for PI3K, PDK1, and aPKC during insulin-stimulated glucose transport in immortalized brown adipocytes. Adipocytes were infected with 10 MOI of adenovirus vector alone or adenovirus encoding wild-type (WT) or kinase-defective (KD) PKC-λ or KD PDK1. After 48 h to allow time for expression (see refs *5*, *10*, *11*, and *24*), the cells were incubated in glucose-free KRP medium for 15 min with or without 100 nM wortmannin, and then for 30 min with or without 100 nM insulin, as indicated, following which uptake of [^{3}H]-2-deoxyglucose (0.05 mM) was measured over 5 min. Values are the mean ± SE of four determinations. *P* values reflect comparisons of (a) basal versus insulin-stimulated values of vector and WT PKCλ groups and (b) insulin-stimulated values of the vector-treated group versus those of groups treated with wortmannin or KD- PKCλ or KD-PDK1 groups.

Requirements for Both IRS-1 and IRS-2 during Insulin-Induced Activation of aPKCs in Immortalized Brown Adipocytes. As seen in Figure 4, insulin provoked approximately 3-fold increases in aPKC activity in brown adipocytes, and relative to that in wild-type adipocytes, the activation of aPKCs was markedly diminished in brown adipocytes in which either IRS-1 or IRS-2 had been knocked out. Of particular interest, as with [^{3}H]-2-deoxyglucose uptake, insulin effects on aPKC activation were fully restored by retroviral-mediated expression of IRS-1 or IRS-2 in their respective knockout adipocytes (Figure 4). Levels of immunoreactive PKC-ζ, PKC-λ, and total aPKCs were comparable in wild-type, IRS-1 knockout, and IRS-2 knockout adipocytes (data not shown).

Preservation of PKB Activation and Phosphorylation in Immortalized Brown Adipocytes in Which IRS-1 or IRS-2 Was Knocked Out. In contrast to aPKC activation, insulin-induced increases in both the enzyme activity of PKB and phosphorylation of serine-473 in PKB were virtually the same in wild-type brown adipocytes and brown adipocytes in which either IRS-1 or IRS-2 was knocked out (Figure 5). Levels of PKB were comparable in wild-type, IRS-1

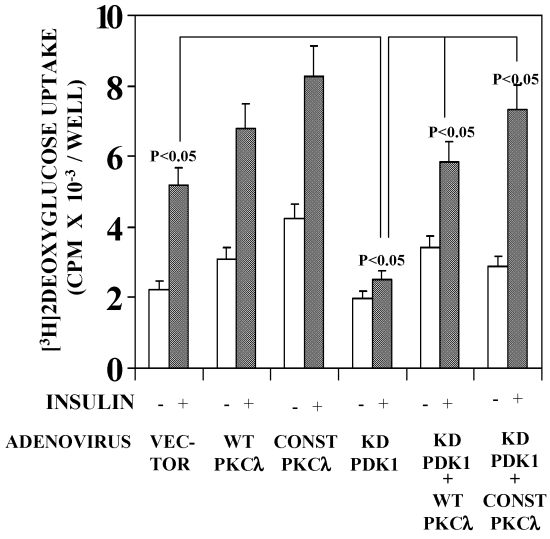

FIGURE 2: Restoration of insulin-stimulated glucose transport by expression of WT or constitutively active (CONST) PKC-λ in adipocytes inhibited by KD PDK1. As indicated in the figure, adipocytes were infected with adenovirus vector alone or adenovirus encoding KD PDK1 (10 MOI) and/or WT or CONST PKC-λ (20 MOI). After 48 h to allow time for expression (see refs *5*, *10*, and *11*), the cells were incubated in glucose-free KRP medium for 30 min with or without 100 nM insulin, as indicated, following which uptake of [^{3}H]-2-deoxyglucose (0.05 mM) was measured over 5 min. Values are the mean ± SE of four determinations. *P* values reflect comparisons of (a) basal versus insulin-stimulated values of the vector group, (b) insulin-stimulated values of the vector versus those of the KD PDK1 group, and (c) insulin-stimulated values of the KD PDK1 group versus those of the (i) KD PDK1 plus WT PKCλ and (ii) KD PDK1 plus CONST PKCλ groups.

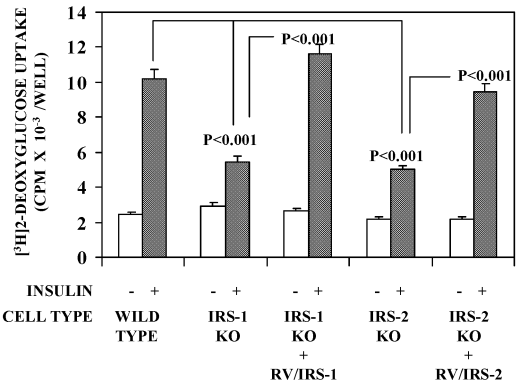

FIGURE 3: Requirements for IRS-1 and IRS-2 for insulin-stimulated glucose transport in immortalized brown adipocytes. As in Figures 1 and 2, basal and insulin-stimulated uptake of [^{3}H]-2-deoxyglucose (0.05 mM) was measured in wild-type brown adipocytes and brown adipocytes in which IRS-1 or IRS-2 was knocked out (KO), or knocked out and subsequently restored by retroviral (RV) gene transfer (see refs *21*−*23*). Values are the mean ± SE of four determinations. *P* values reflect comparisons of (a) insulin-stimulated values of the wild-type group versus those of the IRS-1 and IRS-2 KO groups and (b) insulin-stimulated values of the IRS-1 and IRS-2 KO groups versus those of their respective RV groups.

knockout, and IRS-2 knockout adipocytes (data not shown).

Activation of Cbl-Dependent PI3K and Dependency on IRS-1 and IRS-2 in Immortalized Brown Adipocytes. As in 3T3/L1 adipocytes treated with thiazolidinediones (*12*) or insulin (see the preceding paper in this issue), insulin provoked increases in Cbl-dependent PI3K activity (Figure 6A) and the binding of Cbl to the p85 subunit of PI 3-kinase

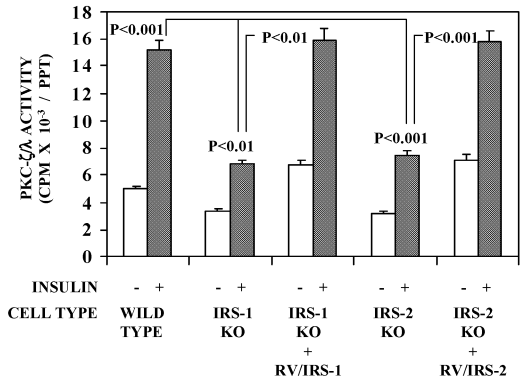

FIGURE 4: Requirements for IRS-1 and IRS-2 during activation of aPKCs by insulin in immortalized brown adipocytes. Wild-type brown adipocytes and brown adipocytes in which IRS-1 or IRS-2 was knocked out, or knocked out and subsequently restored by RV gene transfer (see refs *21*−*23*), were incubated in glucose-free KRP medium for 15 min with or without 100 nM insulin. The lysates were then examined for aPKC activity. Values are the mean ± SE of four determinations. *P* values reflect comparisons of (a) basal and insulin-stimulated values of the wild-type group, (b) insulin-stimulated values of the wild type versus those of the IRS-1 and IRS-2 KO groups, and (c) insulin-stimulated values of the IRS-1 and IRS-2 KO groups versus those of their respective RV groups.

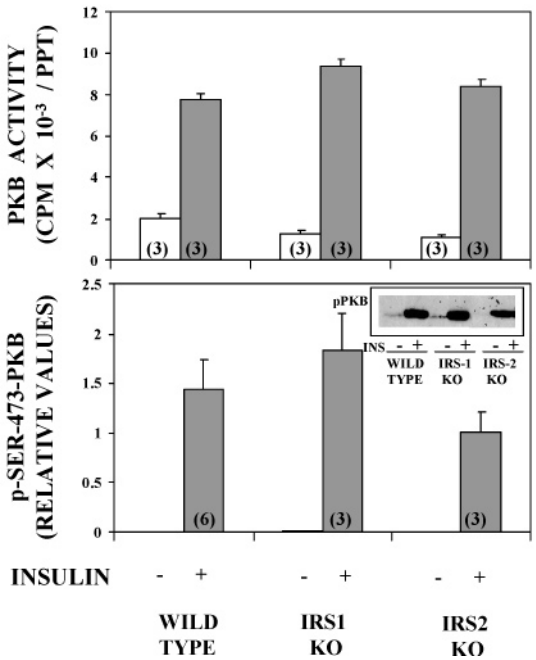

FIGURE 5: Lack of effects of knockout of IRS-1 or IRS-2 on activation (A, top) or phosphorylation (B, bottom) of PKB by insulin in immortalized brown adipocytes. Wild-type and IRS-1 and IRS-2 knockout adipocytes were incubated in glucose-free KRP medium for 15 min with or without 100 nM insulin. The lysates were then examined for PKB enzyme activity and content of phosphoserine-473-PKB. Values are the mean ± SE of *n* determinations. Representative immunoblots for phosphoserine-473-PKB are shown in the inset.

(Figure 6B) in wild-type brown adipocytes. Interestingly, these effects of insulin on Cbl binding to the p85 subunit of PI3K and Cbl-dependent PI3K activity were inhibited in adipocytes in which either IRS-1 or IRS-2 had been knocked out (Figure 6A,B).

In addition to the p85 subunit of PI3K, Cbl binding to Crk was increased by insulin in brown adipocytes (Figure 6C). However, unlike the binding to the p85 subunit of PI3K, the mean basal binding of Cbl to Crk was increased

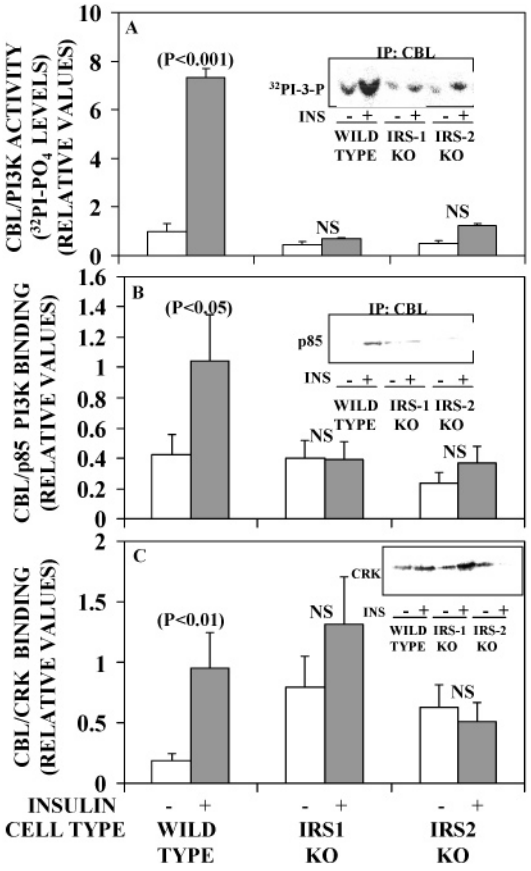

FIGURE 6: Effects of knockout of IRS-1 or IRS-2 on insulin-induced activation of Cbl-dependent PI3K activity (A) and binding of Cbl to the p85 subunit of PI3K (B) and Crk (C) in immortalized brown adipocytes. Wild-type and IRS-1 and IRS-2 KO adipocytes were incubated in glucose-free KRP medium for 15 min with or without 100 nM insulin. The lysates were then examined for Cbl-dependent PI3K activity (as thin-layer chromatography-purified $^{32}PI-PO_4$) and binding of Cbl to the p85 subunit of PI3K and Crk. Bar graph values are the mean ± SE of 4−6 determinations. Representative autoradiograms (for $^{32}PI-PO_4$) and immunoblots (for p85/PI3K and Crk) are shown in the insets. *P* values reflect comparisons between adjacent basal and insulin-stimulated values. Other differences between basal and insulin-stimulated samples were not statistically significant (NS).

(however, not significantly, due to large variations of individual samples) in adipocytes in which either IRS-1 and IRS-2 had been knocked out, and insulin did not provoke further significant increases in Cbl binding to Crk in IRS-1 or IRS-2 knockout cells (Figure 6). The complex effects of IRS-1 or IRS-2 knockout on binding of Cbl to Crk may reflect the fact that Cbl can bind to either SH2 or SH3 domains of Crk, and it is not clear how Cbl/Crk binding is regulated, and which binding mechanism is responsible for the activation of more distal factors, such as C3G and TC10.

Cbl Binding to the p85 Subunit of PI3K Is Not Dependent on PI3K Activation in Immortalized Brown Adipocytes. Since IRS-1 and IRS-2 were found to be required for insulin-induced increases in the binding of Cbl to the p85 subunit of PI3K and since IRS-1 and IRS-2 activate PI3K, we questioned if PI3K activation is necessary for increases in Cbl binding to the p85 subunit of PI3K and Crk. As seen in Figure 7, wortmannin alone was without effect, and insulin-stimulated increases in Cbl binding to both the p85 subunit

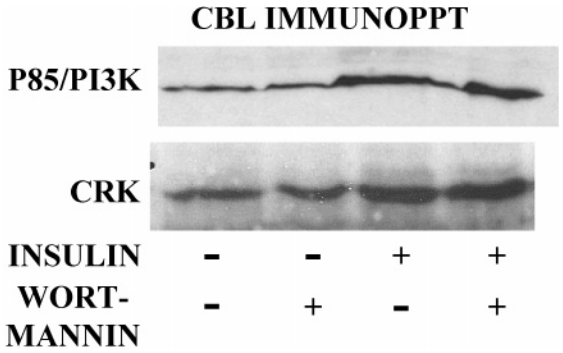

FIGURE 7: Binding of Cbl to the p85 subunit of PI3K and Crk is independent of PI3K activation in immortalized brown adipocytes. As in Figure 1, wild-type adipocytes were incubated in the presence or absence of 100 nM wortmannin (added 15 min before insulin) and 100 nM insulin, as indicated, and Cbl immunoprecipitates were examined for coimmunoprecipitation of the p85 subunit of PI3K and Crk. Shown here are immunoblots representative of 4−6 determinations.

of PI3K and Crk were not diminished by the PI3K inhibitor wortmannin.

DISCUSSION

From the present findings, it seems clear that IRS-1 and IRS-2 are corequired for activation of aPKCs and glucose transport in mouse brown adipocytes. Loss of either IRS-1 or IRS-2 led to similar marked decreases in insulin-stimulated aPKC activity and glucose transport. Whereas previous findings from gene targeting/knockout studies have shown that IRS-1 is required for insulin-stimulated glucose transport in white adipocytes (*7*, *8*) and IRS-2 is required for insulin-stimulated increases in aPKC activity and glucose transport in immortalized brown adipocytes (*6*), the present findings provide the first clear evidence, using a gene targeting/knockout approach, that both IRS-1 and IRS-2 are simultaneously corequired for activation of both aPKCs and glucose transport in brown adipocytes. Moreover, it seems likely that a similar dependency on both IRS-1 and IRS-2 exists in white adipocytes, since the inhibition of insulin-stimulated glucose transport in white adipocytes of IRS-1 knockout mice is only partial (*7*, *8*) and since we have found that aPKC, but not PKB, activation is diminished in white adipocytes of IRS-1 knockout mice (*24*).

The similar decreases in aPKC activation and glucose transport caused by knockout of either IRS-1 or IRS-2 alone were less than what might be expected if IRS-1 and IRS-2 performed entirely separate functions that were corequired for full effects of insulin on these processes. On the other hand, these decreases were considerably greater than what might be expected if IRS-1 and IRS-2 performed simply additive functions. These findings may be explained by postulating that IRS-1 and IRS-2 perform separate, as well as similar, functions that are corequired for aPKC activation and glucose transport in brown adipocytes. In keeping with this possibility, differences in the activation and function of distinct IRS-1- and IRS-2-dependent PI3K pools have been observed in other studies (*22*, *23*).

It may be noted that the present finding of full restoration of glucose transport effects of insulin in IRS-2 knockout brown adipocytes by expression of IRS-2 differs from results

of a prior study in which IRS-2-dependent restoration was only partial (*25*). The present finding of full restoration of glucose transport by IRS-2 in IRS-2 knockout brown adipocytes seems most likely to be related to the full restoration of activation of aPKCs, and perhaps the function of other related signaling factors, in the brown adipocytes presently used. On the other hand, aPKC activation was not measured in the previous study (*25*), and it is therefore uncertain if clonal differences in the ability to restore aPKC and/or other related signaling factor activation could account for differences in restoration of insulin-stimulated glucose transport.

In view of the dependence of aPKC activation on both IRS-1 and IRS-2 and in view of the fact that PKB, like aPKCs, operate downstream of PI3K and PDK1, it was surprising to find that, unlike those of aPKCs, the enzymatic activation and phosphorylation of PKB were maintained in brown adipocytes in which either IRS-1 or IRS-2 had been knocked out. In this regard, it may be noted that, whereas knockout of IRS-1 only slightly inhibits PKB phosphorylation in the immortalized brown preadipocytes (*23*), and knockout of IRS-2 has little or no effect on overall PKB activation in immortalized brown adipocytes (*6*, *25*), decreases in PKB activation have been observed in other studies of IRS-1-deficient immortalized brown adipocytes (e.g., see ref *26*). The reason for such differences in overall PKB activation in immortalized brown adipocytes in which IRS-1 has been knocked out is uncertain, but may reflect variations in different clones or culturing conditions. However, in accord with the present findings, it may be noted that a similar dissociation, i.e., a loss of aPKC, but not PKB, activation, is seen in white adipocytes of IRS-1 knockout mice (*24*).

It is important to note that, despite the fact that overall PKB activation/phosphorylation is presently not inhibited in either IRS-1 or IRS-2 knockout brown adipocytes, this does not necessarily mean that PKB activation is fully intact in these knockout cells. Indeed, Fasshauer et al. (*25*) have reported that, despite normal overall activation of PKB in IRS-2 knockout brown adipocytes, the plasma membrane localization of PKB is defective in these cells. Obviously, this localization is likely to be important for insulin-stimulated glucose transport. In this regard, it is also important to note that the plasma membrane localization of aPKC is compromised in adipocytes in which Cbl function is inhibited by expression of Y731F Cbl mutant (see the preceding paper in this issue). Analagously, as presently shown, Cbl function is compromised in IRS-1 and IRS-2 knockout brown adipocytes, and it is therefore reasonable to suggest that Cbl function is required for plasma membrane localization of PKB, as well as aPKC.

The presently observed dissociation between overall aPKC and PKB activation suggests that, unlike aPKC activation, either overall PKB activation is largely independent of IRS family members or, more likely, the requirement for overall PKB activation is largely satisfied by the remaining IRS family members, including IRS-3 or other factors that continue to activate PI3K and aPKCs in brown adipocytes in which either IRS-1 or IRS-2 has been knocked out. Implicit in the latter scenarios, IRS-2, IRS-3, and/or other factors may be sufficient for PKB activation when IRS-1 is knocked out, and vice versa, IRS-1, IRS-3, and/or other factors may be sufficient when IRS-2 is knocked out. In this respect, we have observed approximately 2-fold increases in IRS-2-dependent PI3K activation in IRS-1 knockout brown adipocytes, but no appreciable increase in IRS-1-dependent PI3K activation in IRS-2 knockout brown adipocytes (data not shown). Thus, a compensatory increase in IRS-2 activation may offset the loss of IRS-1 and contribute to overall PKB activation in IRS-1 knockout cells. On the other hand, without compensatory increases in IRS-2-dependent PI 3-kinase activity, total pY-dependent PI3K activation is diminished by approximately 30% in IRS-1 knockout brown adipocytes (*23*, *25*, *26*), and it may therefore be surmised that PKB can be fully activated at a level of total pY-dependent PI3K activation that is less than that required for aPKC activation in brown adipocytes. Needless to say, our understanding of compartmentalization of PI3K pools that are relevant to activation of PKB and aPKC is still fragmentary.

It was particularly interesting to find that insulin-induced increases in the binding of Cbl to the p85 subunit of PI3K and subsequent activation of Cbl-dependent PI3K were markedly impaired in brown adipocytes in which either IRS-1 or IRS-2 had been knocked out. These findings therefore raise the possibility that IRS-1 and IRS-2 may function upstream of, or in close association with, Cbl during insulin action. Moreover, the importance of these findings is underscored by the fact that Cbl appears to be required for aPKC activation during insulin action in 3T3/L1 adipocytes (see the preceding paper in this issue). However, we presently have little or no insight into mechanisms that IRS-1 and IRS-2 may use in functioning upstream of, or in association with, Cbl. In this regard, the recruitment of Cbl to the activated insulin receptor is thought to require the Cbl-associated protein (CAP) and APS and the subsequent formation of a flotillin/CAP/Cbl complex that occurs independently of PI3K activation (*13*−*20*). As presently reported, the binding of Cbl to both Crk and the p85 subunit of PI3K was not inhibited by wortmannin, so it is clear that activation of IRS1/2-dependent PI3K is not required for the binding of Cbl to either Crk or the p85 subunit of PI3K. On the other hand, it is possible that, independently of PI3K, IRS-1 and/or IRS-2, which are known to bind to the juxtamembranous region of the β-subunit of the insulin receptor (*27*−*29*), may be corequired along with CAP and APS for recruiting Cbl to the insulin receptor and subsequent phosphorylation of Cbl and APS (see Figure 7 in the preceding paper in this issue for a schematic). Further studies are needed to evaluate this and other possibilities.

In summary, we found that the knockout of either IRS-1 or IRS-2 in immortalized brown adipocytes markedly inhibited insulin activation of aPKCs and glucose transport, and these functions were restored by retroviral-mediated expression of IRS-1 or IRS-2 in their respective knockout cells. The absence of IRS-1 or IRS-2 in these cells also inhibited insulin-stimulated increases in the binding of Cbl to the p85 subunit of PI3K and subsequent activation of Cbl-dependent activation of PI 3-kinase. These findings suggest that both IRS-1 and IRS-2 function upstream of aPKCs and Cbl during insulin stimulation of glucose transport in brown adipocytes.

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