Reconstitution of Phosphoinositide 3-Kinase-dependent Insulin Signaling in a Cell-free System*

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Early insulin signaling events were examined in a novel cell-free assay utilizing subcellular fractions derived from 3T3-L1 adipocytes. The following cellular processes were observed in vitro in a manner dependent on insulin, time of incubation, and exogenous ATP: 1) autophosphorylation and activation of the insulin receptor; 2) tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1); 3) association of tyrosine-phosphorylated IRS-1 with phosphoinositide 3-kinase 3); 4) activation of the kinase Akt via its phosphorylation on Thr-308 and Ser-473; and 5) phosphorylation of glycogen synthase kinase-3 by activated Akt. The activation of Akt in vitro was abolished in the presence of the phosphoinositide 3-kinase inhibitor, wortmannin, thus recapitulating the most notable regulatory feature of Akt observed in vivo. Evidence is presented indicating that the critical spatial compartmentalization of signaling molecules necessary for efficient signal transduction is likely to be preserved in the cell-free system. Additionally, data are provided demonstrating that full Akt activation in this system is dependent on plasma membrane-associated IRS-1, cannot be mediated by robust cytosol-specific tyrosine phosphorylation of IRS-1, and occurs in the complete absence of detectable IRS-2 phosphorylation in the cytosol and plasma membrane.

Insulin initiates multiple signaling pathways leading to numerous responses that regulate carbohydrate, fat, and protein metabolism (1). Hormone binding induces a conformational change in the insulin receptor that activates its intrinsic tyrosine kinase through an autophosphorylation mechanism. The activated receptor can then phosphorylate several intracellular protein substrates, most notably the insulin receptor substrate (IRS)1 proteins (2, 3). Tyrosine-phosphorylated IRS proteins can recruit and activate the downstream effector, phosphoinositide 3-kinase (PI 3-kinase), which generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) using inositol-containing phospholipids resident in the plasma membrane as substrates (4). Many of the metabolic effects of insulin are absolutely dependent on PI 3-kinase activation. For example, insulin stimulation of glucose transport via translocation of the glucose transporter isofrom Glut4 is completely blocked by the PI 3-kinase inhibitor wortmannin (5).

In vitro assays have proven to be enormously useful for many areas of cell biology. The earliest studies, to our knowledge, in which this experimental approach was applied to the investigation of insulin action occurred during the late 1970s when L. Jarett and co-workers noted that the direct addition of insulin to a purified adipocyte plasma membrane fraction resulted in numerous effects, including alterations in the phosphorylation of several proteins (6) and increased calcium binding by the plasma membrane (7). These investigators had very few guideposts available at the time for interpreting their observations in a molecular context. Indeed, their work predated the cloning of the cDNA encoding the insulin receptor, which occurred in 1985 (8, 9). More recently, the laboratory of C. R. Kahn employed subcellular fractions from 3T3-L1 adipocytes to reconstitute 1) the dynamic association of IRS-1/2 and PI 3-kinase with various cellular compartments (10) and 2) the binding of Glut4 vesicles to the plasma membrane (11). These investigators employed components derived from cells that were treated in vivo with or without insulin (100 nm for 10 min at 37 °C). The extent of manipulations that can be performed in their assay may, thus, be potentially limited due to the likelihood of the insulin-dependent process under investigation already having occurred in vivo before the time the components of their assay are recombined in vitro. This limitation may explain why the insulin-stimulated association of Glut4 vesicles with the plasma membrane that they observed in vitro was wortmannin-insensitive and did not require ATP or cytosol (11).

During the current era in which complete genomic sequence data can be used in conjunction with sensitive proteomic techniques, it may be possible to comprehensively catalog all of the factors that are involved in bringing about the cellular responses elicited by insulin. As such information accrues, the challenge will increasingly shift toward untangling the web of functional interrelationships that exist among the identified factors. Efforts to devise in vitro assays that reconstitute cellular processes should be maintained to keep pace with the rate of discovery in insulin action. In this report we describe a novel in vitro assay reconstituting key aspects of PI 3-kinase-dependent insulin signaling using subcellular components isolated from 3T3-L1 adipocytes. Our data derived from this assay reinforce the functional significance of spatial compartmentalization exhibited by signaling components in the PI 3-kinase pathway.

EXPERIMENTAL PROCEDURES

Cell Culture of 3T3-L1 Adipocytes—3T3-L1 preadipocytes obtained from the American Type Culture Collection were grown to confluence and...
In Vitro Reconstitution of Insulin Signaling

RESULTS AND DISCUSSION

Characterization of the Cell-free System—We have attempted to reconstitute key aspects of the insulin-signaling pathway using subcellular fractions derived from 3T3-L1 adipocytes. We chose to use fully differentiated 3T3-L1 adipocytes as the source material because they are highly responsive to insulin. These cells typically exhibit a

48 h later subjected to differentiation as described previously (12). 3T3-L1 adipocytes were used 10–14 days after initiating differentiation.

Isolation of Subcellular Components—Mature 3T3-L1 adipocytes grown on 10-cm dishes were serum-starved overnight. The cells were then rapidly washed 3 times with ice-cold serum-free Dulbecco's modified Eagle's medium and maintained further for 15 min at 4 °C in serum-free Dulbecco's modified Eagle's medium in the absence or presence of 1 μM insulin. Cells were then washed 3 times with ice-cold phosphate-buffered saline, scraped in 2 ml of ice-cold HES buffer (50 mM Hepes, pH 7.4, 255 mM sucrose, and 1 mM EDTA) dialyzed containing protease inhibitors (0.082 trypsin inhibitory units/ml aprotinin (Sigma), 1 μg/ml leupeptin, 1 μg/ml benzamidine, 1 μg/ml antipain, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 μM phenylmethylsulfonyl fluoride), and then homogenized at 4 °C by passing the cells 10 times through a Yamato SC homogenizer at a speed of 1200 rpm. The plasma membrane (PM) fraction was obtained by differential centrifugation and sucrose cushion flotation as described previously (13) and designated as either PM (−ins) or PM (+ins) according to whether the starting cell source was exposed to insulin. The low density microsomes (LDM) fraction was obtained from basal cells as described previously (13). PM and LDM subsequent to their isolation were resuspended in IC buffer (20 mM Hepes, pH 7.4, 140 mM potassium glutamate, 5 mM NaCl, 1 mM EGTA, and protease inhibitors). A highly concentrated cytosol (CTY) fraction was prepared by washing the 3T3-L1 adipocytes 3 times with ice-cold IC buffer then removing the buffer as much as possible by aspiration followed by cell scraping and homogenizing with a ball-bearing homogenizer. The supernatant was recovered after an ultracentrifuge spin for 1 h at 200,000 × g. For the preparation of immunodepleted CTY, pre-cleared CTY was incubated for 1.5 h at 4 °C with protein A-Sepharose bound with the appropriate antibodies. A polyclonal rabbit antibody raised against the RNA-binding protein syncrip served as the control.

In Vitro Assay—Samples were prepared on ice by mixing in various combinations LDM (~2.5 mg/ml final concentration), CYT (~3 mg/ml final concentration), and PM (+ or − ins) (~0.5 mg/ml final concentration). Reaction volumes ranging from 100 to 200 μl were adjusted as necessary with IC buffer. Reactions were initiated with the addition of either ATP regenerating system (final reaction concentrations: 2.5 mM ATP, 8 mM creatine phosphate, 30 units/ml creatine phosphokinase, and 5 mM MgCl2) or an ATP-depleting system (final reaction concentrations, 25 units/ml hexokinase and 5 mM glucose). Samples were rotated for 0–15 min in a 37 °C incubator. The reactions were quenched by the addition of an equal volume of buffer A (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 100 mM NaF, and 10 mM sodium pyrophosphate) either containing 2% SDS and 1 mM EDTA (for samples to be run directly on SDS-PAGE) or 2% Triton X-100 and 40 mM EDTA (for samples to be immunoprecipitated). For certain in vitro reactions, as indicated, some of the following were also added (final concentrations): 1 mM DTT, 150 μM sodium vanadate, 1 μM microcystine-LR (Calbiochem), 100 μM wortmannin (Calbiochem), or 1 μg/100 μl reaction volume of recombinant human insulin receptor cytoplasmic β subunit-GST fusion protein (Calbiochem; 407697).

Immunoblot Analysis and Immunoprecipitation—Protein samples from the in vitro assay were subjected to SDS-PAGE and transferred to nitrocellulose. Phospho-specific antibodies recognizing the phosphorylated forms of Akt or glycogen synthase kinase-3 (GSK-3) were obtained from Cell Signaling Technology. The monoclonal anti-phosphotyrosine antibody PY20 was purchased from BD Biosciences. The monoclonal anti-phospho-Akt antibody (Cell Signaling Technology) was purchased from Upstate Biotechnology. The Akt3 antibody used for immunoblot analyses was from Santa Cruz Biotechnology.

FIG. 1. Reconstitution of early insulin signaling events in a cell-free system. A, scheme of the in vitro assay. See “Experimental Procedures” for details. B, subcellular distribution of insulin signaling molecules. Fully differentiated 3T3-L1 adipocytes were fractionated by differential centrifugation as described under “Experimental Procedures.” PM, LDM, and CYT fractions were separated by SDS-PAGE (50 μg of protein) and analyzed by immunoblot analysis using each of the designated primary antibodies. PM (+ ins) and PM (−ins) denote plasma membrane fractions that were derived from cells pre-exposed or not to insulin on ice before the fractionation. IR, insulin receptor; PDK, phosphoinositide-dependent kinase.

receptor and IRS-1) (15–17) and target molecules (such as the insulin-responsive glucose transporter Glut4) (12, 18) are dramatically increased. Subcellular fractionation protocols exist for adipocytes that allow the reproducible recovery of distinct subcellular components with relative ease (13, 19, 20). An outline of our basic in vitro assay is provided in Fig. 1A. Fully differentiated 3T3-L1 adipocytes in the basal state were first cooled rapidly by washing with ice-cold media and then maintained at 4 °C in the presence or absence of 1 μM insulin. The cold temperature incubation allowed insulin to bind its cell surface receptor but prohibited subsequent intracellular signaling events. After the cold temperature incubation, purified PM fractions were obtained by differential centrifugation and sucrose cushion flotation. The PM fractions are referred to as PM (−ins) or PM (+ins) according to whether or not the cell source was exposed to insulin. Basal cells were also used to obtain the LDM and CYT. The LDM is enriched in endosomes, the Golgi apparatus, and insulin-responsive Glut4-containing vesicles as well as certain insulin-signaling molecules such as IRS-1 and PI 3-kinase (21). In vitro reactions (~100–200 μl) were prepared by mixing various combinations of the 3T3-L1 subcellular fractions (PM (−ins) or PM (+ins), LDM, or CYT) on ice. The reactions were initiated by the addition of an ATP regenerating system and then rotated for up to 15 min in a 37 °C incubator, thereby allowing insulin, carried through to this point in reactions containing PM (+ins) via high affinity interaction with its receptor, to exert its effects. The concentrations of PM, LDM, and CYT protein in a typical reaction were 0.5, 2.5, and 3 μg/ml, respectively. The delay in kinetics from what is observed in vivo is likely due in part to the time required for the temperature of the samples to rise from 4 to 37 °C and to the dilution of components in the cell-free system.

The starting subcellular fractions were examined for the presence of insulin signaling molecules by immunoblot analysis (Fig. 1B). The insulin receptor was highly enriched in the PM fraction, and the amount did not vary with exposure to insulin.
IRS-1 and IRS-2 were found mainly in the LDM and, to a lesser extent, the cytosol fraction, as previously reported (10). IRS-3, which is present in primary adipocytes, is not expressed in 3T3-L1 adipocytes (22). In contrast to the IRS proteins, Gab1 (another insulin receptor substrate) (23) was found exclusively in the cytosol. The p85 subunit of PI 3-kinase was present to a significant degree in all three subcellular fractions. Phosphatidylinositol-dependent kinase-1 was mainly found in the cytosol. Akt1–3 isoforms were present almost exclusively in the cytosolic fraction.

We were particularly interested in reconstituting insulin-dependent processes downstream of PI 3-kinase. The generation of PIP3 by PI 3-kinase leads to the activation of Akt by phosphorylation of two of its residues, Thr-308 and Ser-473 (24) (numbers corresponding to the Akt1 isoform). Akt in turn can phosphorylate GSK-3 on Ser-21 (for the α isoform) or Ser-9 (for the β isoform) (25). The phosphorylation status of Akt and GSK-3 in our in vitro system was examined by immunoblot analysis using appropriate phospho-specific antibodies. The phospho-specific Akt antibodies used in this study (Cell Signaling Technology) are capable of detecting both Akt1 and Akt2 phosphorylation (26), although Akt2 has been reported to be the major isoform in 3T3-L1 adipocytes (26, 27). As shown in Fig. 2, Akt was properly phosphorylated in vitro on both Thr-308 and Ser-473 in response to insulin in reactions containing PM (+ins), LDM, and CYT in the presence of 150 μM sodium vanadate and 1 μM microcystin-LR (lane 3). The occurrence of insulin-dependent signaling suggested that the cytoplasmic domain of the insulin receptor, which contains the intrinsic tyrosine kinase, was properly oriented with respect to the membrane to access its substrates in the cell-free system. This conforms to our estimate using alkaline phosphatase activity as an ecto-domain marker (28, 29), that 70% of the PM vesicles exhibited an inside-out orientation (data not shown). Moreover, the PM vesicles were apparently sealed, because Akt was not significantly activated by the direct addition of insulin to in vitro reactions containing PM(−ins) (lane 2). The addition of 100 nM wortmannin (lanes 4–6) or the absence of ATP (lanes 7–9) completely suppressed the insulin response. Reactions containing PM alone (lanes 10–12), CYT alone (lanes 13 and 14), and LDM alone (lanes 15 and 16) failed to result in insulin-stimulated phosphorylation of Akt. The absence of insulin-stimulated Akt phosphorylation in reactions containing PM, LDM, or CYT alone as well as the absolute requirement for exogenous ATP indicates that the system was not significantly contaminated by unbroken cells. Inhibition by wortmannin indicates that the insulin-stimulated Akt phosphorylation observed in vitro was PI 3-kinase-dependent.

We attempted to optimize the conditions that elicited the maximal insulin response with regard to Akt and GSK-3 phosphorylation by performing the in vitro reactions in the absence or presence of phosphatase inhibitors (Fig. 3). Despite being enriched in signaling molecules such as PI 3-kinase and IRS-1/2 (21), the LDM appeared to be dispensable for the phosphorylation of Akt. In fact, a stronger insulin-stimulated signal was consistently observed for both Akt phosphorylation sites in reactions excluding the LDM in the absence of the broad specificity Ser/Thr phosphatase inhibitor, microcystin-LR. This suggested that LDM might contain a phosphatase activity capable of acting on Akt. Generally, the tyrosine phosphatase inhibitor vanadate appeared to elevate the signal for both Akt phosphorylation sites in reactions containing PM(−ins). However, at the same time, the corresponding control (basal) signal in reactions containing PM(−ins) was also elevated, thus blunting the discernable insulin response. Microcystin also increased the insulin-stimulated signal for both Akt phosphorylation sites. However, this phosphatase inhibitor elevated the basal signal only for the Ser-473 site. Some of the in vitro reactions were also performed in the presence of 1 mM DTT, which was included to mimic the reducing environment found...
inside cells (Fig. 3; lower two rows). DTT appeared to inhibit the phospho-Akt signal in most cases. There are at least two reasons to explain why DTT failed to facilitate signaling. First, a reducing environment such as that provided by DTT is required for optimal activity of certain tyrosine phosphatases, which can be expected to down-regulate insulin signaling (30, 31). Second, DTT is known to inhibit the phosphatase-countering activity of vanadate (32). The insulin-stimulated phosphorylation of GSK-3(α/β) on Ser-(21/9) mirrored that of Akt (Fig. 3). The empirical comparison of various conditions demonstrated that the optimal reaction for observing insulin-responsive phosphorylation of Akt and GSK-3 (i.e. the most dramatic fold difference between basal and insulin-stimulated signals) contained PM and CYT but excluded DTT, vanadate, and microcystin. Under these conditions both phosphorylation and dephosphorylation reactions could occur because phosphatase inhibitors were not necessary for detecting insulin-stimulated Akt phosphorylation.

The time course for insulin receptor-mediated tyrosine phosphorylation under the optimal conditions described above was ascertained by immunoblot analysis using an anti-phosphotyrosine antibody. Two bands at ~160 and ~95 KDa appeared in response to insulin, corresponding to the molecular mass of IRS-1/2 and the β subunit of the insulin receptor, respectively (Fig. 4A). Phosphotyrosine signals were completely absent when ATP was depleted from the reaction (Fig. 4A, last four lanes), thus ruling out the possibility of significant reaction contamination by intact cells. The identities of the two insulin-dependent phosphotyrosine bands were confirmed by solubilizing the reaction with 1% Triton X-100 and then immunoprecipitating with an antibody recognizing either the β subunit of the insulin receptor or IRS-1 (Fig. 4B). For both of these proteins, the phosphotyrosine signal peaked at 2.5 min from the start of the reaction and somewhat decreased thereafter, presumably due to dephosphorylation. We also examined the in vitro recruitment of PI 3-kinase to tyrosine-phosphorylated adaptor proteins. After solubilizing the reaction with 1% Triton X-100, tyrosine-phosphorylated proteins capable of co-immunoprecipitating with the p85 subunit of PI 3-kinase were detected by immunoblot analysis (Fig. 4B, bottom panel). Insulin stimulated the association of PI 3-kinase with a tyrosine-phosphorylated protein doublet corresponding to the molecular mass of IRS-1 and IRS-2, mimicking what occurs in vivo (10, 33). A minor population of PI 3-kinase was found to be complexed with a protein of ~95 KDa, which may be the autophosphorylated β subunit of the insulin receptor. It is important to note that others have observed an in vivo association between PI 3-kinase and the activated insulin receptor (34, 35).

The time course for the phosphorylation of Akt was assessed by immunoblot analysis using phospho-Akt-specific antibodies (Fig. 5A). Akt phosphorylation exhibited kinetics delayed relative to that of IRS-1 tyrosine phosphorylation, with the insulin-stimulated signal peaking at ~10–15 min from the start of the reaction. The time course for GSK-3 phosphorylation was similar to that of Akt (Fig. 5A). As was observed for tyrosine phosphorylation, the phosphorylation of Akt and GSK-3 in vitro was completely dependent on exogenous ATP (Fig. 5A, last four lanes). Also, the addition of 100 nM wortmannin to the reaction completely abrogated insulin-stimulated Akt phosphorylation on Thr-308 and Ser-473 and GSK-3(α/β) phosphorylation (Fig. 5B). This indicates that the in vitro kinase activities targeting both Akt sites and the subsequent GSK phosphorylation were PI 3-kinase-dependent, recapitulating in vivo characteristics (24).

Akt has been shown to translocate from the cytosol to the PM in response to insulin in intact cells (26). In the basal state, Akt1–3 isoforms were found almost exclusively in the cytosolic...
Akt3 antibodies. Immunoblot analysis of CYT and CYT(-Akt) using Akt1, Akt2, and Akt3 antibodies collectively immunodepleted. INSULIN + or − refers to whether the PM used in the reactions was derived from cells pretreated or not on ice with insulin as described under "Experimental Procedures." Samples were subjected to immunoblot analysis using phospho-Akt and phospho-GSK-3 antibodies. B, immunoblot analysis of CYT and CYT(-Akt) using Akt1, Akt2, and Akt3 antibodies.

Fig. 6. Depletion of cytosolic Akt1–3 completely inhibits insulin-stimulated phosphorylation of Akt in reactions containing PM and CYT. A, in vitro reactions containing different combinations of PM and CYT were incubated at 37 °C for 15 min. CYT(-Akt) refers to cytosol from which Akt1, Akt2, and Akt3 were collectively immunodepleted. INSULIN + or − refers to whether the PM used in the reactions was derived from cells pretreated or not on ice with insulin as described under "Experimental Procedures." Samples were subjected to immunoblot analysis using phospho-Akt and phospho-GSK-3 antibodies. B, immunoblot analysis of CYT and CYT(-Akt) using Akt1, Akt2, and Akt3 antibodies.

fraction in our system (Fig. 1B). It was possible, however, that by incubating cells on ice with insulin before isolating the subcellular fractions, the system was "primed," causing a residual amount of Akt1–3 to translocate to the PM and be phosphorylated after the addition of ATP. By immunodepleting Akt from the cytosol, it could be determined which form of Akt, cytosolic or PM-associated, was phosphorylated in the cell-free assay. As shown in Fig. 6A, each of the Akt isoforms could be collectively immunodepleted from the cytosol. In vitro reactions containing various combinations of PM(+/−ins), CYT, and CYT(-Akt) revealed that insulin-dependent Akt and GSK-3 phosphorylation required the presence of both PM and CYT (Fig. 6B). No detectable Akt or GSK-3 phosphorylation was observed with PM alone. Immunodepleting Akt1–3 from the cytosol almost completely blocked insulin-dependent Akt and GSK-3 phosphorylation. These results indicate that Akt phosphorylated during the cell-free incubation originated from the cytosolic fraction, thus recapitulating what appears to occur in vivo.

Insulin-stimulated Akt Activation Does Not Require Cytosolic IRS Proteins in the Cell-free Assay—The preceding data demonstrate that early insulin signaling events dependent on PI 3-kinase up to and including Akt and GSK-3 phosphorylation appear to be faithfully reconstituted in the in vitro system. A cell-free system offers several advantages that would be extremely difficult or impossible to address in a satisfactory manner using an intact cell system. In particular, facile experimental access to all components of our system allows manipulations such as the introduction of membrane-impermeant reagents or the depletion of cellular factors, as was illustrated in Fig. 6 with Akt. As a further demonstration of this principle, we added a soluble recombinant insulin receptor kinase domain fusion protein (derived from the catalytic β subunit) to an in vitro reaction containing PM(+/−ins) and CYT. The fusion protein was robustly tyrosine-phosphorylated in the absence of insulin, reflective of its constitutive activity (Fig. 7A). The signal derived from the insulin receptor fusion protein (72 kDa) was in vast excess relative to that derived from the native insulin receptor β subunit (95 kDa) in a parallel reaction containing PM(+ins) and CYT. The insulin receptor fusion protein was capable of phosphorylating IRS-1 (Fig. 7B). The level of tyrosine-phosphorylated IRS-1 was considerably greater in the reaction containing the insulin receptor fusion protein as compared with the reaction in which in vitro signaling was initiated by insulin according to our basic protocol. IRS-1/2 phosphorylated by the insulin receptor fusion protein was found in a complex with the p85 regulatory subunit of PI 3-kinase as demonstrated by co-immunoprecipitation (Fig. 7C), thereby establishing that the insulin receptor fusion protein was indeed acting on physiologically relevant sites of substrate molecules. The tyrosine-phosphorylated insulin receptor fusion protein was also associated with a large amount of p85 (Fig. 7C). Despite the successful propagation of these early steps of insulin signaling, the addition of the insulin receptor fusion protein
failed to activate downstream Akt, i.e. neither Thr-308 nor Ser-473 was phosphorylated (Fig. 7D). In a parallel reaction containing PM(+ins) and CYT, Akt was efficiently phosphorylated at both regulatory sites.

The preceding data suggest that the signal from the insulin receptor must originate at the plasma membrane in order for Akt to be activated efficiently. The activated insulin receptor soluble form can phosphorylate its physiological substrates, but the resulting signaling complexes, despite being present at abundant levels, are likely to be aberrantly localized and incapable of stimulating further downstream signaling. The most probable impediment to signaling initiated by the insulin receptor fusion protein is at the level of PI3 generation. Under these circumstances, the activated PI 3-kinase in complex with IRS proteins may have limited access to its phosphoinositide substrate present in the inner leaflet of the plasma membrane. Apparently, random intermolecular encounters lead to inefficient signal transduction. In vivo, the signaling components are likely to be spatially segregated in such a way as to be poised for rapid action upon insulin stimulus. Diffusion constraints are likely to be greatly exacerbated in an in vitro system in which the cellular components are diluted by several orders of magnitude relative to the native intracellular milieu. Thus, the PI 3-kinase-dependent Akt activation in the in vitro system is likely to reflect the preservation of signaling compartmentalization that takes place in vivo at the interface between the cytosolic face of the plasma membrane and the cytoplasm.

Soluble adaptor proteins could be uncoupled from downstream signaling events using another approach. As shown in Fig. 8A, CYT can be successfully immuno-depleted of Gab1, IRS-1, IRS-2, or both IRS-1 and IRS-2 in combination. In vitro reactions were performed using PM(+ins) mixed with the various immuno-depleted CYT. In the control reaction containing PM(+ins) and CYT mock-immuno-depleted with an irrelevant antibody, the normal pattern of phosphotyrosine bands was observed by immunoblot analysis (Fig. 8B). Close inspection of the broad signal centered at ~160 kDa revealed two bands in close apposition. Removal of IRS-1 from CYT resulted in the absence of the lower phosphotyrosine band, whereas removal of IRS-2 resulted in the absence of the upper phosphotyrosine band (Fig. 8B). This result is consistent with the slower reported electrophoretic mobility of IRS-2 relative to IRS-1. As expected, removal of both IRS-1 and IRS-2 from CYT (CYT-IRS1/2) resulted in the absence of the broad ~160-kDa insulin-stimulated phosphotyrosine band (Fig. 8B). Removal of Gab1 from CYT did not noticeably alter the pattern of insulin-stimulated phosphotyrosine bands (Fig. 8B). Downstream signaling to Akt was then assessed by immunoblot analysis using phospho-Akt antibodies. The removal of soluble adaptor proteins had no effect on insulin-stimulated Akt phosphorylation. Both Thr-308 and Ser-473 were phosphorylated normally in all of the immuno-depleted reactions (Fig. 8C). This experiment provides results complementary to that depicted in Fig. 7 and further reinforces the notion that IRS proteins in the cytosolic fraction are not conduits for productive signaling to PI 3-kinase in the cell-free system.

There are several possible explanations for these findings. IRS proteins may be entirely dispensable for signaling to Akt. Other adaptor proteins may be responsible for recruiting the PI 3-kinase activity necessary for Akt signaling. Alternatively, Akt signaling may be stimulated by a subpopulation of PI 3-kinase directly recruited to the activated insulin receptor in a manner similar to that for other growth factor receptors. Another possibility is that insulin activation of Akt may involve IRS proteins, but only those constitutively associated with the PM. In this regard, it is notable that readily detectable amounts of IRS-1, IRS-2, and p85 are reproducibly present in the PM derived from our fractionation protocol as demonstrated in Fig. 1B and Fig. 8A.

Correlation between the Insulin-stimulated Tyrosine Phosphorylation of PM-associated IRS-1 and the Activation of Akt.—To address the possibility that IRS proteins constitutively associated with the PM are responsible for propagating the insulin-derived signal that results in Akt phosphorylation, we performed a time course experiment for insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation, specifically examining the subpopulation of IRS proteins associated with the PM. In vitro reactions containing PM(+ins) but without cytosol were initiated with the addition of ATP, incubated at 37 °C, and then quenched after varying periods of time (Fig. 9). Immunoblot analysis using an anti-phosphotyrosine antibody revealed a band of ~95 kDa, corresponding to the β subunit of the insulin receptor, that appeared in response to insulin and subsequently peaked 2.5 min after the start of the reaction (Fig. 9A). Reactions quenched before the incubation at 37 °C (0 min) or incubated for 2.5 min at 37 °C without the addition of ATP (−ATP) exhibited no significant tyrosine phosphorylation. Quenched samples were also heated (100 °C) in 1% SDS, diluted with Triton X-100, and then immunoprecipitated with antibodies directed against IRS-1 or IRS-2. Immunoprecipitated IRS-1 and IRS-2 were then analyzed for phosphotyrosine content (Fig. 9B). Both IRS-1 and IRS-2 were transiently tyrosine-phosphorylated in an insulin-dependent manner that peaked at 2.5 min and decreased thereafter. Little or no significant tyrosine phosphorylation was observed with either isoform at 0 min or at 2.5 min at 37 °C in the absence of exogenous ATP.
The above results indicate that activated endogenous insulin receptor can tyrosine phosphorylate the subpopulation of IRS-1/2 molecules constitutively associated with the PM in our in vitro reaction. Next, we tested whether the exogenous soluble insulin receptor fusion protein could phosphorylate PM-associ-ated IRS-1/2. In vitro reactions containing PM in the absence or presence of CYT were incubated at 37 °C for 3.5 min. Samples were quenched, immunoprecipitated with antibodies directed against IRS-1 or IRS-2, and then analyzed for phosphotyrosine content by immunoblot analysis (Fig. 10A). In the absence of CYT, the endogenous insulin receptor (PM +ins) but not the exogenous soluble insulin receptor (IR, PM(-ins)) could tyrosine phosphorylate PM-associated IRS-1 (Fig. 10A, upper left panel). With the addition of CYT to the reactions (Fig. 10A, upper right panel), IRS-1 was tyrosine-phosphorylated by both the activated endogenous receptor (PM(+ins)) and the exogenous soluble insulin receptor (IR, PM(-ins)). In contrast, IRS-2 was tyrosine-phosphorylated by both the endogenous and soluble insulin receptors in both the absence and presence of CYT (Fig. 10A, lower panels). In the absence of CYT (Fig. 10A, bottom left panel), a diffuse tyrosine phosphorylation pattern was observed for IRS-2 in reactions containing the soluble insulin receptor (IR, PM(-ins)) compared with reactions containing the activated endogenous receptor (PM(+ins)). It is not known whether the diffuse phosphorylation pattern is due to aberrantly phosphorylated IRS-2 or represents other tyrosine-phosphorylated proteins that co-immunoprecipitate with IRS-2. Heating the samples in 1% SDS did not prevent the co-immunoprecipitation of the soluble exogenous insulin receptor with IRS-2 (Fig. 10A, lower panels).

Collectively, these results suggest the following mechanism that allows insulin to stimulate Akt in the cell-free system. The signal from the insulin receptor must originate at the plasma membrane in order for Akt to be activated efficiently. Insulin-stimulated tyrosine phosphorylation of PM-associated IRS-1, but not IRS-2, appears to be the critical conduit in the signaling pathway leading to the generation of PIP3 in the inner leaflet of the plasma membrane. Tyrosine phosphorylation of cytosolic IRS-1/2 and their subsequent activation of cytosolic PI 3-kinase fail to efficiently generate PIP3 necessary for the stimulation of Akt phosphorylation. The acute increase in PIP3 at the PM leads to the dual phosphorylation of cytosolic Akt on Thr-308 and Ser-473 by the phosphoinositide-dependent kinase(s) 1/2 (36–38). Key aspects of our cell-free signaling model are supported by several in vivo observations. Expression of a membrane-anchored constitutively active human insulin receptor kinase (39, 40) but not a cytoplasmic form resulted in enhanced 2-deoxyglucose uptake in transfected cells despite the fact that the cytoplasmic form was 20 times more active in vitro (40). The importance of IRS-1 as opposed to IRS-2 in the activation of Akt is in agreement with a study that examined insulin signaling in brown adipose cell lines derived from wild type and IRS-2 knockout mice (41). Insulin-stimulated glucose uptake was reduced by 50% in the IRS-2 knockout cells compared with the wild type controls. IRS-2-associated PI 3-kinase activity was completely suppressed, and the phosphotyrosine-associated PI 3-kinase activity was reduced 30% in the IRS-2 knockout adipocytes. In contrast, insulin activation of Akt as well as the downstream targets of GSK-3 and p70S6 kinase were unaffected by the absence of IRS-2. Other lines of investigation support the concept of IRS compartmentalization in insulin.
signaling. For example, the expression of a membrane-targeted IRS-1 construct appears to inhibit cell proliferation but enhances signaling through Akt despite less extensive insulin-stimulated tyrosine phosphorylation as well as dramatically decreased PI 3-kinase binding relative to wild type IRS-1 (41). The exact site of action for the IRS proteins is uncertain. They appear to partition in a regulated manner between the cytosol and intracellular membranes (10, 42). Further characterization of the intracellular membranes in 3T3-L1 adipocytes indicates that IRS proteins associate with a detergent-insoluble multi-protein complex, possibly the cytoskeleton (21). It has been proposed that anchoring IRS-1/2 to the cytoskeleton may be of considerable importance to insulin signal transmission by allowing IRS proteins to be localized in close proximity to the insulin receptor at the PM (21, 43). Regulated release of IRS proteins from this subcellular location to the cytosol results in a large reduction of insulin-stimulated tyrosine phosphorylation of IRS-1/2 in 3T3-L1 adipocytes (43). These observations completely support the contention that PM-associated, but not cytosolic IRS proteins, are important in insulin signal transduction.

We should note that, although all of the observed phosphorylation events occurred entirely in vitro, including the auto-phosphorylation of the insulin receptor, we cannot rule out the possibility that some priming event, such as the recruitment of a small amount of IRS proteins to the PM, took place when intact cells were incubated on ice with insulin before the isolation of the subcellular fractions. It has been shown that treatment of 3T3-L1 adipocytes with insulin at 4°C results in the autophosphorylation of the insulin receptor and the subsequent tyrosine phosphorylation of IRS-1, although the absolute amount of IRS-1 at the PM was not insulin-dependent (42). In our hands, measurable differences between basal and insulin-treated cells in the amount of IRS proteins in the PM fraction were not reproducibly observed. Nevertheless, this caveat has no impact on any of the conclusions presented herein regarding the mechanism of insulin signaling, nor does it impact significantly on the utility of the cell-free assay to investigate signaling events.

In summary, we have developed a novel cell-free assay reconstituting a PI 3-kinase-dependent signaling pathway stimulated by insulin. The generation of PIP3 in vitro requires PI 3-kinase to be activated by the receptor and recruited to the proper intracellular location, i.e. the cytoplasmic face of the plasma membrane (4). The complexity of this dynamic process is difficult to contend with from a technical standpoint. To our knowledge, this assay represents the first successful in vitro reconstitution of the salient features of PI 3-kinase-dependent insulin signaling, starting from the beginning of the pathway, i.e. insulin receptor activation. The experimental flexibility provided by this cell-free assay can be exploited to further examine the insulin-signaling pathway in mechanistic detail.

Note Added in Proof—An interesting study by Roth and co-workers (Vainshtein, I., Kovacina, K. S., and Roth, R. A. (2001) J. Biol. Chem. 276, 8073–8078) focusing on the role of the pleckstrin homology domain of IRS-1 in localizing PI 3-kinase came to our attention after the submission of our paper. Their data, consistent with our own, also highlight the importance of PM-localized IRS-1/PI 3-kinase in the downstream activation of Akt.

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