Differential Regulation of Phosphoinositide 3-Kinase Adapter Subunit Variants by Insulin in Human Skeletal Muscle*

(Received for publication, March 6, 1997, and in revised form, April 24, 1997)

Peter R. Shepherd**, Barbara T. Navé†, Jorge Rincon∥, Lorraine A. Nolte‡, A. Paul Bevan*, Kenneth Siddle*, Juleen R. Zierath, and Harriet Wallberg-Henriksson

From the §Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, United Kingdom, the ¶Department of Clinical Biochemistry, University of Cambridge, Cambridge, CB2 2QR United Kingdom, and the Department of Clinical Physiology, Karolinska Hospital, Stockholm, 10401 Sweden

The role of phosphoinositide 3-kinase (PI 3-kinase) in insulin signaling was evaluated in human skeletal muscle. Insulin stimulated both antiphosphotyrosine-precipitable PI 3-kinase activity and 3-O-methylglucose transport in isolated skeletal muscle (both ~2–3-fold). Insulin stimulation of 3-O-methylglucose transport was inhibited by the PI 3-kinase inhibitor LY294002 (IC50 = 2.5 μM). The PI 3-kinase adapter subunits were purified from muscle lysates using phosphopeptide beads based on the Tyr-751 region of the platelet-derived growth factor receptor. Immunoblotting of the material adsorbed onto the phosphopeptide beads revealed the presence of p85α, p85β, p55β (p55γ/p55γi), and p50 adapter subunit isoforms. In addition, p85α-NSH2 antibodies recognized four adapter subunit variants of 54, 53, 48, and 46 kDa, the latter corresponding to the p50 splice variant. Serial immunoprecipitations demonstrated that these four proteins were associated with a large proportion of the total PI 3-kinase activity immunoprecipitated by p85α-NSH2 domain antibodies. Antibodies to p85β, p55β (p55γ/p55γi), and the p50 adapter subunit also immunoprecipitated PI 3-kinase activity from human muscle lysates. A large proportion of the total cellular pool of the 53-kDa variant, p50, and p55β was present in antiphosphotyrosine immunoprecipitates from unstimulated muscle, whereas these immunoprecipitates contained only a very small proportion of the cellular pool of p85α, p85β, and the 48-kDa variant. Insulin greatly increased the levels of the 48-kDa variant in antiphosphotyrosine immunoprecipitates and caused smaller -fold increases in the levels of p85α, p85β, and the 53-kDa variant. The levels of p50 and p55β were not significantly changed. These properties indicate mechanisms by which specificity is achieved in the PI 3-kinase signaling system.

Insulin binding to its cell-surface receptor activates a range of intracellular signaling cascades that ultimately result in the regulation of a number of important metabolic effects elicited by insulin (3–11). Specifically, several recent studies indicate that constitutive activation of PI 3-kinase is sufficient to induce stimulation of glucose uptake in adipocytes (12–14). However, many growth factors that stimulate PI 3-kinase do not stimulate glucose metabolism, suggesting that different signaling inputs have specific mechanisms for utilizing the PI 3-kinase signaling system to generate downstream responses (reviewed in Ref. 15).

Insulin and receptor tyrosine kinase-linked growth factors activate class 1 PI 3-kinases, which consist of a 110-kDa catalytic subunit linked to an adapter subunit (15). Three isoforms of the catalytic subunit have been identified: p110α, p110β, and p110γ (16–18). Of these, only p110α and p110β are present in insulin-responsive tissues. Two widely expressed ~85-kDa forms of the adapter subunit were originally characterized: p85α and p85β. These share a high degree of sequence and structural homology, with each containing two SH2 domains, two proline-rich domains (P1 and P2), an SH3 domain, and a Bcr homology domain (19). Recently, several truncated isoforms of the PI 3-kinase adapter subunit have been identified including a 55-kDa variant of p85, termed p55γ or p55γ (20, 21), which is encoded by a gene separate from p85α or p85β. Two splice variants of the p85α gene have also been reported. These are a 53–55-kDa form termed AS53 (22) or p55γ (20, 21), and a gene encoding a protein with a predicted molecular mass of 50 kDa (23, 24). PI 3-kinase purified from liver has also been reported to associate with a 46-kDa protein that cross-reacts with p85 antibodies (25). All of these truncated adapter subunit variants contain two SH2 domains and the P2 proline-rich domains, but lack the SH3 domain, the Bcr homology domain, and the P1 proline-rich domain of full-length p85.

The main mechanism by which insulin regulates PI 3-kinase activity is by tyrosine phosphorylation of specific YMXM motifs on the intracellular signaling intermediates IRS-1 and IRS-2, which enables the recruitment of PI 3-kinase via the SH2 domain on the PI 3-kinase adapter subunit (26). This increases cellular PI 3-kinase activity by a combination of increasing the catalytic activity of p110 (27, 28) and locating the p110 catalytic subunit in proximity to its substrate at the membrane.

It is not clear whether the different isoforms of the adapter subunit play independent or overlapping roles in intracellular signaling pathways. However, the possibility exists that specificity could be introduced into the PI 3-kinase system by differential recruitment of the individual adapter subunits in response to activation of different receptor tyrosine kinases. Such interactions would provide a mechanism by which multi-

---

* The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; PAGE, polyacrylamide gel electrophoresis; IRS, insulin receptor substrate.
ple signaling inputs could use PI 3-kinase in different ways to obtain specific signaling outcomes.

In this study, we have investigated the PI 3-kinase signaling system in human skeletal muscle using a unique muscle strip preparation (29). The data obtained provide strong evidence that PI 3-kinase plays a crucial role in insulin signaling to glucose transport in human skeletal muscle. Furthermore, we have focused on the role of individual variants of the PI 3-kinase adapter subunit in insulin signaling. We have identified seven variants of the PI 3-kinase adapter subunit present in human skeletal muscle, and we demonstrate that these are differentially recruited into phosphotyrosine complexes in response to insulin stimulation. This study provides evidence for mechanisms by which specificity can be obtained in the PI 3-kinase signaling system.

EXPERIMENTAL PROCEDURES

Materials—125I-Protein A, Na125I, [32P]ATP, ECL reagents, and rainbow protein molecular mass standards were from Amersham International (Buckinghamshire, United Kingdom). Phosphatidylinositol was from Lipid Products (Redhill, United Kingdom). All other chemicals were from Sigma. Monoclonal PY20 antiphosphotyrosine antibody was from Transduction Laboratories. Rabbit polyclonal antisera were raised against glutathione S-transferase fusion proteins corresponding to the SH3 domain (p85α-SH3) or the N-SH2 (p85α-NSH2) domain of human p85α as described previously (30). Monoclonal antibodies recognizing epitopes in the SH3 domain (U13) and in the N-SH2 domain (U2) of p85α and another specifically recognizing p85δ were kindly supplied by Dr. I. Gout and Prof. M. Waterfield (Ludwig Institute, London) (31). A previously described polyclonal antiserum specific to p55αTK was provided by Drs. S. Pons and M. White (Joslin Diabetes Center, Boston) (21). Antibodies to the p55δ adapter subunit and the p50 splice variant of p85α were supplied by Dr. T. Asano (24). A peptide corresponding to amino acids 735–750 of the platelet-derived growth factor receptor (GDYMDMSKDESVDYVPML) was phosphorylated at the tyrosine equivalent to residue 751 and cross-linked to Actigel (provided by Dr. B. Van Haesebroeck, Ludwig Institute, London).

Subjects—Muscle specimens were obtained from the vastus lateralis portion of the quadriceps femoris muscle from 19 healthy male subjects (age: 28.4 ± 0.7 years; weight: 76.6 ± 2.1 kg; height: 181.3 ± 1.9 cm; body mass index: 23.4 ± 0.4 kg/m²; fasting glucose: 4.9 ± 0.2 mmol/l; and serum insulin: 6.9 ± 0.4 pmol/liter). None of the subjects were taking any medication. Following an overnight fast, the subjects reported to the laboratory at 8:30 a.m. A local anesthetic (prilocain hydrochloride, 10 mg/ml) was administered subcutaneously 15 cm above the proximal border of the patella, and a 4-cm incision was made. Two muscle specimens (250 mg each) were excised for in vitro incubation, and smaller muscle strips were prepared as described (29, 32).

Stimulation of Muscle and Determination of 3-0-Methylglucose Transport—After preparation, the smaller muscle strips (~20 mg) were incubated at 35 °C for 10 min in a recovery solution containing oxygenated Krebs-Henseleit buffer supplemented with HEPES, 38 mM mannitol, 2 mM pyruvate, and 0.1% bovine serum albumin. The muscle specimens were subsequently incubated with inhibitors and insulin as indicated in the figure legends. For measurements of 3-0-methylglucose transport, the buffer contained 5 mM 3-O-[3H]methylglucose and 35 mM [14C]mannitol. For glucose transport experiments, the incubations were terminated by snap-freezing the muscle in liquid nitrogen.

Thereafter, the muscle samples were rapidly homogenized in ice-cold buffer containing 100 mM NaF and 10 mM EDTA. Cell debris was removed by centrifugation at 5000 × g for 1 min. 3-O-Methylglucose transport was calculated as described previously (33). For immunoprecipitation experiments, the muscle samples were homogenized on ice in 50 mM Tris-Cl (pH 7.4), 1% Triton X-100, 10 mM EDTA, 1 mM NaF, 1 mM NaVO4, 2 mM aprotinin, 2 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Homogenates were precleared by centrifugation at 5000 × g for 10 min prior to immunoprecipitation.

Antibody Purification and Induction—10 μl of 100 mM Tris (pH 8.0) and 25 μl of swollen protein A-agarose beads were added to 100 μl of p85α-NSH2 antiserum. The solution was mixed at 4 °C for 4 h, and the beads were collected. Beads were washed with 100 mM Tris, and the antibody was eluted with 100 mM glycine (pH 3.0) for 5 min at room temperature. The glycine buffer was collected and neutralized with 10 μl of 1 M Tris (pH 8.0). An aliquot of purified antibody (25 μl) was iodinated by sequential addition of 5 μl of Na2H18PO4/NaH2PO4 buffer (0.5 μl, pH 7.4) and 0.5 μCi of Na125I followed by 10 μl of chloramine T (0.5 mg/ml). The reaction was terminated 2 min later by the addition of 300 μl of 25 mM Tris (pH 7.4) containing 10 mM MgCl2 and 1% bovine serum albumin. Nonincorporated 125I was separated from iodinated antibody on a Bio-Rad Eoo10DG disposable desalting column, and the peak iodinated antibody fraction was used for Western blotting.

Stimulation of PI 3-Kinase Activity—PI 3-kinase activity was determined essentially as described previously (34). Incorporation of label from [γ-32P]ATP into phosphatidylinositol was determined, and the lipid product was resolved by thin-layer chromatography and quantitated on a Fuji BAS2000 phosphorimager.

Statistical Analysis—Statistical differences between treatments were analyzed using Student’s paired t test.

RESULTS

Insulin Acutely Stimulates PI 3-Kinase Activity in Human Skeletal Muscle—The ability of insulin to stimulate PI 3-kinase activity in human skeletal muscle was initially characterized. Muscle was stimulated with insulin at 1000 microunits/ml, a concentration that maximally stimulates glucose transport (data not shown). Insulin stimulation increased PI 3-kinase activity associated with phosphotyrosine-containing proteins 3.3-fold compared with basal levels, with maximal stimulation being achieved 20 min after the basal insulin (Fig. 1).

Insulin-stimulated 3-O-Methylglucose Transport Is Blocked by LY294002—Insulin increased 3-O-methylglucose transport in muscle strips 2.22-fold (basal: 0.60 ± 0.07 μmol × ml−1 × h−1; insulin: 1.33 ± 0.06 μmol × ml−1 × h−1, n = 10 strips). The insulin-induced increase in 3-O-methylglucose transport was inhibited by LY294002 in a dose-dependent manner (IC50 = 2.5 μM) (Fig. 2). The inhibition of insulin action on muscle glucose transport by LY294002 very closely matched the effect obtained with this compound on insulin-stimulated glucose transport in 3T3-L1 adipocytes (Fig. 2).

A Number of PI 3-Kinase Adapter Subunit Variants Are Expressed in Human Muscle—Immunoblotting studies of whole muscle homogenate using a polyclonal antibody to the SH3 domain of p85α consistently revealed two immunoreactive bands at 85 and 87 kDa (Fig. 3A). Subsequent immunoblotting with isofrom-specific monoclonal antibodies revealed that the faster migrating of these (lower band) represented p85α and the slower one (upper band) represented p85β (Fig. 3B). The
**Figure 2.** LY294002 blocks insulin stimulation of 3-O-methylglucose transport in human skeletal muscle. Muscle strips or 3T3-L1 adipocytes were preincubated with the indicated concentrations of LY294002 for 10 min prior to the addition of insulin at 1000 microunits/ml (muscle) or 3000 microunits/ml (adipocytes). Muscle strips were incubated a further 30 min, with 3-O-methylglucose transport being determined over the final 15 min as indicated under “Experimental Procedures.” Adipocytes were stimulated for 10 min prior to measuring 2-deoxyglucose uptake for 10 min as described previously (30). Results for adipocytes are shown as closed squares. Each point is the mean ± S.E. of three determinations. Results for muscle are shown as open circles and represent the mean ± S.E. of 10 strips (basal), two strips (3.75 μM), six strips (7.5 μM), and six strips (15 μM).

**Figure 3.** Identification of p85 isoforms present in human skeletal muscle. Muscle homogenate samples (250 μg) were separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride, incubated with the indicated antibody, and visualized by 125I-protein A for polyclonal primary antibodies or by ECL for monoclonal primary antibodies. A, immunoblotting of muscle homogenates with polyclonal antiserum specific to N-SH2 and SH3 domains of p85α; B, immunoblotting of muscle homogenates with monoclonal antibodies specific to p85α and p85β.

Polyclonal antibody raised against the N-SH2 domain revealed a prominent immunoreactive protein at 85 kDa corresponding to p85α, but did not recognize p85β. However, this antibody also recognized immunoreactive proteins at 54, 53, 48, and 46 kDa in muscle homogenates. Whereas the relative intensity of the bands varied slightly in samples from different individuals, the 48-kDa protein was consistently the most intense (Fig. 3A). Identical immunoreactive proteins were recognized by anti-serum raised against the same N-SH2 fusion protein in a separate rabbit, but these proteins were not recognized by preimmune serum from either of these rabbits (data not shown). More important, these same immunoreactive proteins were recognized by the monoclonal antibody recognizing an epitope in the N-SH2 domain (Fig. 3B), but were not recognized by monoclonal or polyclonal antibodies recognizing epitopes in the SH3 domain of p85α (Fig. 3B). The lower molecular mass bands are unlikely to represent a proteolytic product of p85α as the intensity of the bands was not dependent on the inclusion of protease inhibitors in the homogenization mixture and did not change with time of storage. Furthermore, we have previously demonstrated that the p85α-N-SH2 antisera did not recognize any comparable lower molecular mass bands in homogenates of 3T3-L1 adipocytes or 3T3-L1 fibroblasts (Ref. 30; see also Fig. 6). The fact that the lower molecular mass bands correspond closely to those of recently reported splice variants of p85α (20–25) suggests that they are in fact variants of the PI 3-kinase adapter subunit.

**Identification of PI 3-Kinase Adapter Subunit Immunoreactive Bands in Fractions Purified Using Phosphopeptide Beads**—To confirm that the lower molecular mass proteins recognized by the p85α-N-SH2 antibody were variants of p85, an affinity purification step was introduced to remove any potential nonspecific reactions. In this approach, human muscle homogenates were precipitated with phosphopeptide beads based on the amino acids surrounding Tyr-751 (p85 SH2 domain-binding region) of the platelet-derived growth factor receptor. Such phosphopeptide beads have previously been shown to have a high affinity for PI 3-kinase adapter subunits (31, 35). The specificity and selectivity of this approach are demonstrated by the fact that the p50 and p55PK antisera used in this study recognize a number of bands in human muscle homogenates, whereas in the eluate from the phosphopeptide beads, this is reduced to a single band. The protein recognized by the p55PK antisera in the phosphopeptide bead precipitates is 58 kDa, and the protein recognized by the p50 antisera is 46 kDa (Fig. 4), which correspond closely with...
components in the homogenate that were slightly immunoreactive with this antibody in muscle homogenate (Fig. 4A). Western blotting of the phosphopeptide bead eluates with the p85α-NSH2 antibody revealed protein bands at 85, 54, 53, 48, and 46 kDa, and these were of equal intensity to the corresponding bands seen with this antibody in muscle homogenate (Fig. 4A). Several other components in the homogenate that were slightly immunoreactive with the p85α-NSH2 antibody were not present in the precipitates and were thus confirmed as nonspecific reactions.

**Association of Adapter Subunit Variants with PI 3-Kinase Activity**—PI 3-kinase activity was present in PI 3-kinase adapter subunit immunoprecipitates, confirming that the antibodies were recognizing functional PI 3-kinase adapter subunits. The greatest amount of PI 3-kinase activity was found associated with antibodies specific to p50, with lesser amounts associated with p55 and still lower levels associated with p85β and p55PK (Fig. 5A).

To investigate whether the lower molecular mass protein bands recognized by the p85α-NSH2 antibody were associated with PI 3-kinase activity, serial immunoprecipitations followed by PI 3-kinase activity assays were performed. Total p85α-associated PI 3-kinase activity was immunoprecipitated from muscle homogenates using the p85α-NSH2 antibody (Fig. 5B). Immunoprecipitation of an equivalent amount of homogenate with the p85α-NSH2 antibody from an equivalent amount of homogenate (Fig. 5). This indicates that truncated but functional forms of the PI 3-kinase adapter subunit are present in human skeletal muscle.

Overall, the combination of the immunoblotting data, the phosphopeptide bead experiments, and the PI 3-kinase assays in sequential immunoprecipitations provides strong evidence that the 54-, 53-, 48-, and 46-kDa proteins recognized by the p85α-NSH2 antibody in fact represent forms of the PI 3-kinase adapter subunit containing SH2 domains, but not the SH3 domain or Bcr homology domain.

**Truncated Variants of p85 Are Differentially Recruited in Response to Insulin**—The regulation of the multiple, structurally distinct PI 3-kinase adapter subunit isoforms present in muscle had not been previously investigated. Therefore, the ability of insulin to induce the recruitment of the PI 3-kinase adapter subunit variants into antiphosphotyrosine immunoprecipitates was determined (Fig. 6). Direct blotting with iodinated p85α-NSH2 antibody was performed on antiphosphotyrosine immunoprecipitates to avoid the problems associated with secondary antibodies recognizing IgG bands. Use of the p85α-NSH2 antisera also has the advantage of allowing direct comparison of the relative effects of insulin on several forms of the PI 3-kinase adapter subunit. The p85α-NSH2 antisera revealed four immunoreactive bands in the antiphosphotyrosine immunoprecipitates at 85, 53, 48, and 46 kDa. The four proteins recognized in the antiphosphotyrosine immunoprecipitates correspond to adapter subunit-specific bands seen in p85α-NSH2 immunoblots of phosphopeptide bead eluates (Fig. 4). In these experiments, the 53-kDa protein was the most prominent immunoreactive band in antiphosphotyrosine immunoprecipitates from unstimulated muscle (Fig. 6A). The 53- and 46-kDa proteins were also readily detectable, but the 48-kDa protein was almost undetectable in antiphosphotyrosine immunoprecipitates from unstimulated muscle (Fig. 6A). The levels of the 46-kDa band, representing the p50 splice variant of p85, did not change significantly in antiphosphotyrosine immunoprecipitates following insulin stimulation (Fig. 6B). The most pronounced effect of insulin on recruitment of adapter subunit variants into antiphosphotyrosine immunoprecipitates was on levels of the 48-kDa variant (19.5-fold stimulation; p < 0.01). Insulin caused smaller -fold increases over basal levels of p85α (3.6-fold; p < 0.02) and the 53-kDa variant (1.7-fold; p < 0.02). This smaller -fold increase was
labeled with insulin. However, when viewed in terms of absolute insulin-induced increases in the levels of adapter subunit variants in antiphosphotyrosine immunoprecipitates, it is seen that the increases in the amounts of p85α and the 53- and 48-kDa forms were all very similar (Fig. 6B). In these studies, the effects of insulin on the 54-kDa protein were not established as whereas it was recognized in direct blots of muscle homogenates with iodinated p85α-NSH2 antibody, it was below the level of detection in direct blots of antiphosphotyrosine immunoprecipitates from basal and insulin-stimulated muscle (Fig. 6A). The reason for this is not clear.

As p85β and p55PIK are not recognized by the p85α-NSH2 antiserum, separate immunoblots were performed using antibodies specific to these isoforms to determine whether they associate with antiphosphotyrosine immunoprecipitates from both unstimulated and stimulated muscle. However, the concentration of wortmannin required to completely inhibit insulin-stimulated glucose transport in these cells with a similar potency. Furthermore, the potency of this compound inhibits the activity of purified PI 3-kinase (37). Wortmannin, an alternative PI 3-kinase inhibitor, has previously been used to study the role of PI 3-kinase in insulin-stimulated glucose transport in rodent muscle (11, 38–40). However, the mechanisms by which insulin stimulates glucose uptake into muscle are poorly understood. The study provides evidence that PI 3-kinase activity is essential for insulin-stimulated glucose transport in human skeletal muscle. This is based on the finding that the PI 3-kinase inhibitor LY294002 inhibits insulin-stimulated 3-O-methylglucose transport in human skeletal muscle and in 3T3-L1 adipocytes with a similar potency. Furthermore, the potency of this inhibition correlates well with the potency with which this compound inhibits the activity of purified PI 3-kinase (37).

DISCUSSION

Human skeletal muscle is a major insulin target tissue in the body as it is the major site of insulin-stimulated glucose disposal in vivo (36). However, the mechanisms by which insulin stimulates glucose uptake into muscle are poorly understood. The study provides evidence that PI 3-kinase activity is essential for insulin-stimulated glucose transport in human skeletal muscle.
Differential Regulation of PI 3-Kinase Adapter Subunits

Insulin:  
Sample P P H

**FIG. 7.** Tyrosine phosphorylation of PI 3-kinase adapter subunits cannot be detected in basal or insulin-stimulated muscle. Human muscle was incubated in the presence (+) or absence (−) of 1000 microunits/ml insulin for 20 min and then homogenized as described under "Experimental Procedures." 200 µg of total human muscle homogenate (T) and total muscle homogenate (H); 100 µg) were separated on an 8% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride, incubated with PY20 antiphosphotyrosine antibody, and visualized by 125I-protein A.

phospholipase A₃ (43). Therefore, the LY294002 data in this study strengthen the case for PI 3-kinase activity being essential for insulin stimulation of glucose transport in human skeletal muscle.

This study provides the first detailed assessment of the expression of PI 3-kinase adapter subunits and their regulation by insulin in human skeletal muscle. This is important as the effects of growth factors on PI 3-kinase are mediated through specific adapter subunits. These are p55<sub>PIK</sub> and p55<sub>b</sub>. Additionally, we demonstrate that human skeletal muscle contains five truncated forms of the PI 3-kinase adapter subunit. These are p55<sub>PIK</sub> and four variants recognized by the p58<sub>a</sub>-NSH2 antibodies. The possibility that one of the proteins recognized by the p58<sub>a</sub>-NSH2 antibodies is p55<sub>PIK</sub>p55<sub>b</sub> can be excluded as the p55<sub>PIK</sub> antiserum only recognized a single band of 58 kDa in both phosphopeptide beads (Fig. 4) and antiphosphotyrosine immunoprecipitates (Fig. 6). This also indicates that the p58<sub>a</sub>-NSH2 antiserum is highly selective for the p58<sub>a</sub> SH2 domain as it does not recognize p58<sub>b</sub> or p55<sub>PIK</sub>p55<sub>b</sub> despite the fact that the N-SH2 domains of p55<sub>PIK</sub>p55<sub>b</sub>, p58<sub>b</sub>, and p58<sub>a</sub> are >90% identical at the amino acid level. Therefore, the most likely explanation for the 54-, 53-, 48-, and 46-kDa bands is that they all contain an N-SH2 domain identical to that of p58<sub>a</sub> and are thus likely to be p58<sub>a</sub> splice variants. Both p50 (23, 24) and p55<sub>a</sub>AS53 (20, 22) are splice variants of p58<sub>a</sub> containing identical N-SH2 domains and would therefore be expected to cross-react with the p58<sub>a</sub>-NSH2 antisera with a similar affinity for p58<sub>a</sub> itself. Indeed, the 46-kDa band recognized by the p58<sub>a</sub>-NSH2 antibody in phosphopeptide bead eluates cross-reacts with an antibody specific to the p50 splice variant of p85<sub>a</sub>. The 53- and 48-kDa bands are likely to represent the two described forms of the AS53 splice variant of p85<sub>a</sub> as antibodies raised against the unique regions of AS53 recognize bands in Western blots of rat tissue (22) that have very similar characteristics to the 53- and 48-kDa bands seen in this study. The identity of the 54-kDa band cannot be explained by any known PI 3-kinase adapter subunit isoforms, although the molecular mass slightly greater than 53 kDa suggests that it could represent the variant of AS53 that has an 8-amino acid splice insert in the inter-SH2 domain (22). However, suitable reagents to assess this were not available.

Very few studies have been conducted to directly compare the function of different PI 3-kinase adapter subunits. While p85<sub>a</sub> and p85<sub>b</sub> are coded by separate genes, they are structurally very similar, containing two SH2 domains, two proline-rich domains, a single SH3 domain, and a Bcr/crasGAP homology domain (19). Despite these similarities, previous studies have suggested that differences may exist in the mechanism by which these two isoforms control the catalytic activity of the complex. For example, in some cell types, PI 3-kinase complexes containing p85<sub>b</sub> appear to be less responsive to insulin than those containing p85<sub>a</sub> (44). Furthermore, p85<sub>a</sub> is phosphorylated by p110 at serine residues (45), whereas this does not occur in p85<sub>b</sub> (31). Little was previously known about the mechanisms by which the different truncated adapter subunit isoforms are regulated. Insulin stimulation of rat muscle has been reported to cause a small increase in IRS-1 association of the AS53 splice variant of p85<sub>a</sub> (22). Furthermore, coexpression of p110, p55<sub>PIK</sub>, the insulin receptor α-subunit, and IRS-1 in S9 insect cells has been reported to cause an increase in the specific activity of PI 3-kinase associated with p55<sub>PIK</sub>, suggesting that insulin could potentially regulate this isoform (21).

This study compared the ability of insulin to induce recruitment of multiple adapter subunit isoforms into protein complexes containing tyrosine-phosphorylated proteins. The results provide evidence that there are important differences in the mechanisms by which the PI 3-kinase adapter subunit isoforms are regulated by insulin. We identified several major differences in the pattern of association of the p85 variants with tyrosine-phosphorylated protein complexes. First, there was a great variation in the proportion of the total pool of each p85 variant that associated with phosphotyrosine-containing proteins in unstimulated muscle. The levels of the 48-kDa variant associated with phosphotyrosine immunoprecipitates in unstimulated muscle were almost undetectable. Full-length p85<sub>a</sub> was readily detectable in the same immunoprecipitates, although it still represented <5% of the total pool of full-length p85<sub>a</sub>. In contrast, a much higher proportion of the total of the p55<sub>PIK</sub> isoform and the 53-kDa and p50 variants was present in antiphosphotyrosine immunoprecipitates from unstimulated muscle. The other area where major differences existed was in the ability of insulin to increase the levels of different adapter subunit variants in antiphosphotyrosine immunoprecipitates. Insulin caused no significant increase at all in the levels of the 46-kDa band or the p55<sub>PIK</sub> variant in antiphosphotyrosine immunoprecipitates. However, insulin was able to induce the recruitment of full-length p85<sub>a</sub>, p85<sub>b</sub>, and the 53- and 48-kDa variants into phosphotyrosine-containing protein complexes. This indicates that these four isoforms are the ones that contribute to PI 3-kinase-dependent insulin signaling pathways in human skeletal muscle. Insulin induced a similar increase in the absolute amount of full-length p85<sub>a</sub> and the 53- and 48-kDa forms in phosphotyrosine-containing protein complexes, indicating that these may each make similar contributions to the magnitude of the insulin stimulation of PI 3-kinase activity. However, it was striking that the ratio of the amount of adapter subunit present in these complexes in the basal and insulin-stimulated states varied greatly between the p85 variants. The biggest difference was in the case of the 48-kDa variant, where insulin increased the levels in antiphosphotyrosine immunoprecipitates >19-fold compared with basal levels, whereas the smallest fold increase was for the 53-kDa
form, where levels in antiphosphotyrosine immunoprecipitates increased only 1.7-fold. If, for example, the different adapter subunits are involved in different signaling complexes and hence different downstream responses, it is possible to see that this offers a mechanism by which insulin could use PI 3-kinase-dependent signals to modulate different PI 3-kinase-dependent downstream responses to different extents.

The mechanism by which these adapter subunit variants are differentially recruited into complexes with tyrosine-phosphorylated proteins is not clear. The fact that all the adapter subunit variants have a minimal core containing the p110-binding region and the two SH2 domains suggests that all have a similar capability to bind to tyrosine-phosphorylated proteins containing Tyr(P)-Xaa-Xaa-Met motifs. However, our results demonstrate that this is not the case in reality; thus, it appears likely that the N-terminal additions to the minimal adapter subunit core contain elements that modulate the adapter subunit’s interaction with the tyrosine-phosphorylated proteins. Consistent with this is the observation that structurally similar forms of the adapter subunit behave in a similar manner. For example, p85α and p85β both contain an SH3 domain and a Bcr domain. A small amount of the total pool of each of these isoforms is found in antiphosphotyrosine immunoprecipitates from unstimulated muscle, and insulin causes a significant increase in the levels of each of these in antiphosphotyrosine immunoprecipitates. The 53-kDa variant and p55

There are a number of potential mechanisms by which the multiple adapter subunit isoforms could introduce specificity into the PI 3-kinase signaling system. The most obvious is that this allows for a range of independently regulatable links between receptor tyrosine kinases and the PI 3-kinase system. This identifies a mechanism by which insulin could utilize specific PI 3-kinase adapter subunit isoforms to regulate particular downstream responses and thus introduce specificity into the PI 3-kinase signaling system.

Acknowledgments—We thank Prof. M. Waterfield, Dr. S. Pons, Dr. M. White, Dr. T. Asano, and Dr. Brian Holloway (Zeneca Pharmaceuticals) for providing reagents.

REFERENCES

1. Cheatham, B., and Kahn, C. R. (1995) Endocr. Rev. 16, 117–142
2. Lee, J., and Pilch, P. F. (1996) J. Physiol. 504, 419–432
3. Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M., and Holman, G. D. (1994) Biochem. J. 300, 631–633
4. Cheatham, B., Vlahos, C., Cheatham, L., Wang, L., Blegen, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
5. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3568–3572
6. Kaliman, P., Vainal, F., Testar, X., Palacin, M., and Zorzano, A. (1995) Biochem. J. 312, 471–477
7. Takiyuki, T., McDowell, H. E., Walker, T., Downes, C. P., Hundle, H. S., Vranic, M., and Klip, A. (1996) Endocrinology 136, 4315–4322
8. Kotani, K., Carrioz, A., Sakura, H., Hara, K., Robinson, L. J., Clark, S. F., Yonezawa, K., James, D. E., and Kasuga, M. (1995) Biochem. Biophys. Res. Commun. 209, 343–345
9. Shepherd, P. R., Nave, B. T., and Siddle, K. (1995) Biochem. J. 305, 25–28
10. Moua, S. K., Edgell, N. J., Welsh, G. L., Diggle, T. A., Foulston, E. J., Heesom, K. J., Proud, C. G., and Denton, R. (1995) Biochem. J. 311, 595–601
11. Nishi, L. A., Hine, J., Meagher-Klahntrum, E., Craig, B. W., Zierath, J. R., and Wallberg-Henriksson, H. (1995) Diabetes 44, 1345–1348
12. Frevert, E. U., and Kahn, B. B. (1997) Mol. Cell. Biol. 17, 190–198
13. Tanti, J. F., Greneaux, T., Grillo, S., Calleja, V., Klipp, A., Williams, L. T., VanObberghen, E., and Le Marchand-Brustel, Y. (1996) J. Biol. Chem. 271, 25227–25232
14. Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Shibasaki, Y., Kikuchi, M., Yasaki, Y., and Oka, Y. (1996) J. Biol. Chem. 271, 16987–16990
15. Shepherd, P. R., Nave, B. T., and Olfahy, S. (1996) J. Mol. Endocrinol. 17, 175–184
16. Hu, P., Mondino, A., Skolnik, E. Y., and Schlessinger, J. (1995) Mol. Cell. Biol. 15, 6777–6788
17. Hiles, I. D., Otsu, M., Volinia, S., Fruman, D. A., and Carpenter, C. L. (1996) J. Biol. Chem. 271, 395–405
18. Inukai, K., Anai, M., Vaa, S., Hosaka, T., Katagiri, H., Funaki, M., Fukushima, Y., Oghara, T., Yasaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1996) J. Biol. Chem. 271, 5317–5320
19. Pons, S., Asano, T., Glade, S., Miralle, M., Zhang, Y., Fisher, T. L., Myers, M. G., Sun, X. J., and White, M. F. (1995) Mol. Cell. Biol. 15, 4453–4465
20. Antonetti, D. A., Algenstaedt, P., and Kahn, C. R. (1996) Mol. Cell. Biol. 16, 2195–2203
21. Fruman, D. A., Cantley, L. C., and Carpenter, C. L. (1996) Genes Dev. 10, 113–121
22. Inukai, K., Funaki, M., Oghara, T., Katagiri, H., Kanda, A., Anai, M., Fukushina, Y., Hosaka, T., Suzuki, M., Shih, B., Takata, K., Yasaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1997) J. Biol. Chem. 272, 7873–7882
23. Kurosawa, T., Hazeki, O., Kikuchi, M., Honzawa, S., Shibasaki, M., Nakada, M., Ui, M., and Katada, T. (1995) Biochem. Biophys. Res. Commun. 216, 655–661
24. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
25. Latch, J. A., Myers, M. G., Shokelson, S. E., Chen, D. J., Sun, X., Miralle, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) EMBO J. 11, 3469–3471
26. Herbst, J. L., Andrews, G., Costillo, L., Lamphere, L., Gardner, J., Lienhard, G. E., and Giros, B. (1994) Biochemistry 35, 9376–9381
27. Zierath, J. R., Galuska, D., Holm, A., A., Smedgaard-Kristensen, J., and Wallberg-Henriksson, H. (1994) Diabetologia 37, 270–277
28. Nave, B. T., Haigh, R. J., Hayward, A. C., Siddle, K., and Shepherd, P. R.
Differential Regulation of PI 3-Kinase Adapter Subunits

31. Reif, K., Gout, I., Waterfield, M. D., and Cantrell, D. A. (1993) J. Biol. Chem. 268, 10780–10788
32. Zierath, J., Bang, P., Galuska, D., Hall, K., and Wallberg-Henriksson, H. (1992) FEBS Lett. 307, 379–382
33. Wallberg-Henriksson, H., Zetan, N., and Henriksson, J. (1987) J. Biol. Chem. 262, 7665–7671
34. Jackson, T. R., Stephens, L. R., and Hawkins, P. T. (1992) J. Biol. Chem. 267, 16627–16636
35. Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991) Cell 65, 91–104
36. DeFronzo, R. A. (1988) Diabetes 37, 667–687
37. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
38. LeMarchand-Brustel, Y., Gautier, N., Cormont, M., and VanObberghen, E. (1995) Endocrinology 136, 3564–3570
39. Yeh, J.-I., Gulve, E. A., Rameh, L., and Birnbaum, M. J. (1995) J. Biol. Chem. 270, 2107–2111
40. Lund, S., Holman, G. D., Schmitz, O., and Pedersen, O. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5817–5821
41. Elmendorf, J. S., Damrau-Abney, A., Smith, T. R., David, T. S., and Turinsky, J. (1995) Biochem. Biophys. Res. Commun. 206, 1147–1153
42. Nakaniishi, S., Catt, K. J., and Balla, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321
43. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995) J. Biol. Chem. 270, 25352–25355
44. Baltensperger, K., Koza, L. M., Jaspers, S. R., and Czech, M. P. (1994) J. Biol. Chem. 269, 26877–26886
45. Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Virendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S. A., and Waterfield, M. D. (1994) EMBO J. 13, 522–533
46. Bokoch, G. M., Vlahos, C. J., Wang, Y., Knaus, U. G., and Traynor-Kaplan, A. E. (1996) Biochem. J. 315, 775–779
47. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) Science 263, 1609–1612
48. Shibasaki, F., Fukami, F., Fukui, Y., and Takenawa, T. (1994) Biochem. J. 302, 551–557
49. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N., Hsuan, J., Booker, G. W., Campbell, I. D., and Waterfield, M. D. (1993) Cell 75, 25–36