Role of Janus kinase-2 in insulin-mediated phosphorylation and inactivation of protein phosphatase-2A and its impact on upstream insulin signalling components

Najma BEGUM*† and Louis RAGOLIA*

*The Diabetes Research Laboratory, Winthrop University Hospital, 259 First St, Mineola, NY 11501, U.S.A., and the †School of Medicine, State University of New York at Stony Brook, NY 11794, U.S.A.

Our recent studies indicate that insulin rapidly inactivates serine/threonine protein phosphatase-2A (PP-2A) by increasing tyrosine phosphorylation on the catalytic subunit. The exact mechanism of PP-2A inactivation by insulin *in vivo* is unclear. The Janus kinase (JAK) family of non-receptor protein tyrosine kinases constitute a novel type of signal-transduction pathway which is activated in response to a wide variety of polypeptide ligands, including insulin. In this study we investigated the potential role of JAK-2 in insulin-mediated tyrosine phosphorylation and inactivation of PP-2A using the rat skeletal muscle cell line L6. Co-immunoprecipitation studies revealed that PP-2A is associated with JAK-2 in the basal state. Insulin treatment did not alter JAK-2 association with PP-2A, but did increase JAK-2-mediated tyrosine phosphorylation of the PP-2A catalytic subunit and therefore inhibited PP-2A enzymic activity. Furthermore, PP-2A is associated with phosphoinositide 3-kinase

(PI-3K) in the basal state and insulin treatment increases the catalytic activity of PI-3K bound to PP-2A. Pretreatment with AG-490, a specific JAK-2 inhibitor, and SpcAMP, a cAMP agonist, prevented the insulin-mediated increase in (i) JAK-2 kinase activity, (ii) PP-2A tyrosine phosphorylation, (iii) PP-2A inactivation and restored the enzyme activity to control levels, and (iv) PP-2A and JAK-2-associated PI-3K activity. These observations, together with the fact that insulin rapidly activates JAK-2 in L6 cells, and that this is accompanied by an increase in tyrosine phosphorylation of PP-2A in JAK-2 immunoprecipitates, suggest that insulin controls the activation status of PP-2A by tyrosine phosphorylation via JAK-2. PP-2A inactivation may result in an amplification of insulin-generated signals at the level of PI-3K.

Key words: insulin resistance, IRS-1, PI 3-kinase, TNF- α .

INTRODUCTION

Previous studies have implicated serine/threonine protein phosphatase-2A (PP-2A) in a wide variety of cellular functions, including cellular metabolism, transcription and translation, ion transport, development, cell growth and differentiation [1–4]. The activity of an enzyme participating in so many diverse cellular processes must be tightly regulated *in vivo*. Little is known however, about the mechanism of regulation of PP-2A *in vivo*. The PP-2A holoenzyme is a heterotrimer composed of catalytic (C), structural (A) and regulatory (B) subunits [1–4]. So far, over 15 regulatory subunits have been identified for PP-2A, which, in addition to regulating enzymic activity, are predicted to be involved in the subcellular targeting of the holoenzyme. In addition to regulatory subunits, PP-2A activity is also influenced by catalytic subunit phosphorylation [5–8] and carboxy-methylation [9,10].

In addition to the core phosphatase subunits, recent evidence suggests that the regulation of PP-2A activity also involves association with other proteins. For example, PP-2A has been shown to bind the β 2-adrenergic receptor [11], casein kinase 2 α [12], homeobox gene 11 [13], tau [14], translation termination eukaryotic release factor 1 [15], adenovirus e4orf4 protein [16], α 4 protein [17], neurofilament proteins [18,19] and small t and middle t antigens encoded by DNA tumour viruses [20,21]. Recent studies by Westphal et al. [22] identified a calmodulin IV (CaMIV)–PP-2A complex in which PP-2A dephosphorylates

CaMIV and functions as a negative modulator of CaMIV signalling. These studies suggest that PP-2A function is regulated by its association with other proteins in multi-protein signalling complexes.

We have previously shown that insulin rapidly inactivates PP-2A in cultured rat skeletal muscle cells by increasing tyrosine phosphorylation of the catalytic subunit. cAMP agonists counter-regulate the effect of insulin on PP-2A inhibition by blocking tyrosine phosphorylation of PP-2A. The kinase responsible for tyrosine phosphorylation of the PP-2A catalytic subunit *in vivo* and its subsequent inactivation remains unknown, however. Since the insulin receptor tyrosine kinase does not directly phosphorylate the catalytic subunit of PP-2A [5], we sought to investigate the involvement of Janus kinases (JAKs) in insulin-mediated PP-2A inactivation. The JAK family of non-receptor protein tyrosine kinases constitute a novel type of signal-transduction pathway activated in response to a wide variety of polypeptide ligands, including insulin [23]. At present, the JAK kinase family comprises four members: JAK-1, JAK-2, JAK-3 and TYK-2. JAKs are recruited to the plasma membrane upon cell activation and are required for the normal signal transduction of many important cytokines and growth factors [23]. These kinases contain a peptide sequence (LXXLL) which, based on simian virus 40 small T antigen experiments, may be part of a consensus sequence critical for PP-2A binding and complex formation with phosphoinositide 3-kinase (PI-3K) and Src-family kinases [24].

Abbreviations used: CaMIV, calmodulin IV; GK rats, Goto–Kakizaki rats; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; NIDDM, non-insulin-dependent diabetes mellitus; PI-3K, phosphoinositide 3-kinase; PP-2A, protein phosphatase-2A.

¹ To whom correspondence should be addressed (e-mail nbegum@winthrop.org).

To investigate further the exact mechanism of regulation of tyrosine phosphorylation and inactivation of PP-2A by insulin *in vivo*, we examined the potential interactions of JAK-2 with PP-2A and PI-3K after insulin and cAMP treatment of rat skeletal muscle cells and investigated the impact of PP-2A inactivation on PI-3K activity associated with PP-2A. The results of the present study suggests that PP-2A is associated with JAK-2 and PI-3K in the basal state. Insulin treatment inactivates PP-2A by increasing the tyrosine phosphorylation of the PP-2A catalytic subunit via JAK-2. Insulin stimulation also increases the catalytic activity of PI-3K bound to PP-2A. Blocking JAK-2 activity with an inhibitor as well as treatment with a cAMP agonist prevents insulin's effect on PP-2A inactivation and abolishes insulin-mediated activation of PP-2A- and JAK-2-associated PI-3K.

MATERIALS AND METHODS

Materials

Cell culture reagents, fetal bovine serum, phosphorylase kinase and phosphorylase *b* were purchased from Life Technologies (Grand Island, NY, U.S.A.). [γ - 32 P]ATP (specific activity ≥ 3000 Ci/mmol) and 125 I-Protein A were purchased from Dupont/New England Nuclear (Boston, MA, U.S.A.). SpcAMP (a cAMP agonist) and AG490 (a specific JAK-2 inhibitor) were purchased from Biomol Research (Plymouth Meeting, PA, U.S.A.). Electrophoresis and protein assay reagents were from Bio-Rad (Richmond, CA, U.S.A.). Okadaic acid was from Moana Bioproducts (Honolulu, HI, U.S.A.). Protease inhibitors, sodium orthovanadate, reagents for PI-3K assay and all other reagents were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Porcine insulin was a gift from Eli Lilly Company (Indianapolis, IN, U.S.A.). Anti-JAK-2, anti-PI-3K, anti-phosphotyrosine (clone 4G10) and antibody to the catalytic subunit of PP-2A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and UBI (Lake Placid, NY, U.S.A.).

Cell culture and treatment

The spontaneously fusing rat skeletal muscle cell line, L6, was a gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Canada). Cells were grown and maintained in α -minimal essential medium (α -MEM) containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic mixture in an atmosphere of 5% CO₂/95% air at 37 °C as previously described [25]. Completely differentiated myotubes were used for all the experiments after a 16–18 h starvation in serum-free α -MEM in the presence and the absence of a synthetic JAK-2 inhibitor, AG490 (5 μ M).

Insulin treatment and extraction of PP-2A

Serum-starved myotubes were treated in the presence and absence of insulin (10 nM) for 5–10 min. In some experiments, cells were pretreated with freshly prepared SpcAMP (100 nM) for 30 min before insulin exposure. At the end of the incubation period the medium was removed and the cells were rinsed three times with ice-cold PBS followed by the addition of PP-2A extraction buffer.

Assay of cellular PP-2A activities

Control, AG490- and insulin-treated cells were scraped off the dishes with 0.3 ml of phosphatase extraction buffer containing 20 mM imidazole/HCl (pH 7.0)/2 mM EDTA/2 mM EGTA, with 10 μ g/ml each of aprotinin, leupeptin, antipain, soy trypsin

inhibitor, 1 mM benzamidine and 1 mM PMSF. The cells were sonicated for 10 s, centrifuged at 2000 *g* for 5 min and the supernatants were assayed for phosphatase activity. The assay was performed in the presence and the absence of 2 nM okadaic acid to inhibit PP-2A activity. As detailed in our previous studies [25], the conditions of the assay allow measurement of PP-1 and PP-2A activities, and not PP-2B and PP-2C enzymes which require divalent ions. Previous studies from this laboratory have shown that okadaic acid at 2 nM inhibits only PP-2A and the remaining PP-1 activity was comparable with the activity inhibited by inhibitor-2, the heat-stable biological protein inhibitor of PP-1 [26]. PP-2A activity was calculated by subtracting the activity measured in the presence of okadaic acid from the activity measured in the absence of okadaic acid. Purified [32 P]phosphorylase 'a' was used as a substrate [27]. [32 P]-Phosphorylase 'a' was prepared by incubating [γ - 32 P]ATP with purified phosphorylase kinase and phosphorylase *b* [28].

Immunoprecipitation and Western blot analyses

Control and insulin-treated cells were rinsed with ice-cold PBS containing 2 mM sodium orthovanadate. The dishes were frozen immediately in liquid nitrogen and lysed with ice-cold buffer containing 1% Triton X-100, 100 mM NaCl, 50 mM β -glycerophosphate, 100 mM NaF, 100 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 μ M microcystin and a cocktail of protease inhibitors [25]. Cleared lysates with equal amounts of protein (0.5 mg) were immunoprecipitated overnight at 4 °C with polyclonal anti-PP-2A and anti-JAK-2 antibodies. The next day, 100 μ l of recombinant Protein A-Sepharose (50%, v/v) was added and the antibody mixture was incubated at 4 °C for 2 h with constant mixing on a rotator. The immunoprecipitates were washed extensively with lysis buffer, followed by SDS/PAGE and Western blot analyses of the membranes with (1) anti-JAK-2 antibody, (2) anti-PP-2A antibody, (3) anti-phosphotyrosine antibody, and (4) p85 PI3-kinase antibody, as detailed in the Figure legends.

Immunoprecipitation and assay of PP-2A–JAK-2-associated PI-3K activity

Serum-starved L6 cells were treated with AG490 (5 μ M) for 12 h and SpcAMP (100 nM) for 30 min and stimulated with insulin (10 nM) for 5 min or treated with insulin alone for 3 or 5 min. The cells were quickly rinsed with ice-cold PBS containing 2 mM vanadate and placed in liquid nitrogen. The frozen dishes were thawed and the cells were lysed with buffer containing 20 mM Hepes (pH 7.5), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM sodium orthovanadate, 10% glycerol, 1% Nonidet P40 and a cocktail of protease and phosphatase inhibitors described previously [25]. Insoluble material was removed by centrifugation for 15 min at 10000 *g* at 4 °C. Immunoprecipitation of cell lysates, normalized to 500 μ g of protein, was performed overnight at 4 °C with 4 μ g of anti-rabbit PP-2A antibody and 25 μ g of anti-JAK-2 antibody. As a positive control, 50–100 μ g of lysate proteins were immunoprecipitated with 1–2 μ g of anti-IRS-1 antibody. For negative control, 500 μ g of protein lysate was immunoprecipitated with 2 μ g of anti-rabbit IgG. The immunocomplexes were precipitated the next day by incubation with 50 μ l of Protein G plus/Protein-A-agarose beads (50% v/v, Calbiochem) for 2 h at 4 °C with constant shaking. The immunoprecipitates were washed exhaustively with buffers and PI-3K activity was assayed in the immunoprecipitates as described previously [29]. The reaction products were separated by TLC on oxalate-treated silica-gel 60 plates in a solvent of chloroform/methanol/water/ammonia (60:47:12.5:2). Unlabelled phos-

phatidylinositol 3-phosphate was used as a standard and visualized by iodine vapour. The [32 P]phosphatidylinositol 3-phosphate was identified by autoradiography and quantified by the cut and count technique.

Immunoprecipitation and *in vitro* kinase assay of JAK-2

Immunoprecipitated proteins bound to protein A--Sepharose were washed with kinase buffer (50 mM NaCl/5 mM MgCl₂/5 mM MnCl₂/0.1 mM sodium orthovanadate/10 mM Hepes, pH 7.4) and subsequently incubated for 30 min at 24 °C with kinase buffer containing 0.25 mCi/ml [γ - 32 P]ATP. After thorough washing with kinase buffer, the proteins were eluted by boiling with Laemmli sample buffer and separated by SDS/PAGE, transferred to PVDF and detected by autoradiography [23].

Protein assay

Proteins in the cellular extracts and lysates were quantified by the bicinchoninic acid [30] or Bradford [31] technique.

Statistics

The results are presented as means $\pm$ S.E.M. of 4–6 independent experiments each performed in triplicate at different times. Unpaired Student's *t*-test or analysis of variance was used to compare the mean values between treatments. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Association of JAK-2 with PP-2A and the effect of JAK-2 inhibitor on this association

In order to determine whether JAK-2 is involved in insulin-mediated PP-2A inactivation, we examined its association with PP-2A by immunoprecipitation and Western blot analysis. JAK-2 was chosen because of its previously reported abundant expression in skeletal muscle and its activation by insulin [23]. Immunoprecipitation of the L6 cell lysates with anti-JAK-2 antibody, followed by Western blot analysis of the immunoprecipitates, revealed a 37 kDa protein which specifically reacted with the PP-2A antibody (Figure 1). A reciprocal immunoprecipitation with anti-PP-2A antibody, followed by Western blot analysis, detected a protein which specifically reacted with JAK-2 antibody (results not shown).

In the unstimulated control cells, significant amounts of JAK-2 were associated with PP-2A (Figure 1, lane 1). Insulin treatment did not further increase JAK-2 association with PP-2A (Figure 1, compare lane 1 with lanes 2–5). Studies of the other JAK family members, JAK-1, JAK-3 and TYK-2, were negative in terms of PP-2A interaction (results not shown).

We also examined the effect of AG490, a specific JAK-2 inhibitor, and SpcAMP, a cAMP agonist, on PP-2A association with JAK-2. L6 cells were treated in the presence or absence of 5 μ M AG490 for 12 h and SpcAMP for 30 min, followed by insulin treatment for 5 min. Equal amounts of cell lysates were immunoprecipitated with JAK-2 antibody, followed by Western blot analysis of the immunoprecipitates. AG490 and SpcAMP pretreatment did not alter PP-2A association with JAK-2 (Figure 1, compare lane 6, 7 and 8 with lane 3).

JAK-2 inhibitor prevents insulin-mediated inactivation of PP-2A

To understand further the consequences of JAK-2 association with PP-2A and to examine whether insulin utilizes JAK-2 to phosphorylate and inactivate PP-2A, we measured PP-2A enzymic activity in cells pretreated with AG490. As shown in

Figure 1 JAK-2/PP-2A association and the effect of JAK-2 inhibition on PP-2A--JAK-2 complex formation

L6 cells were treated with 10 nM insulin for the indicated times. Equal amounts of cell lysate proteins (500 μ g) were immunoprecipitated at 4 °C overnight with JAK-2 antibody (25 μ g/sample). Protein A--Sepharose (100 μ l; 50%, v/v) was added and samples were incubated on a rocker for an additional 2 h. The immunoprecipitates were extensively washed and proteins were separated by SDS/PAGE. The membranes were cut across the 66 kDa marker, the top half of the membrane was probed with JAK-2 antibody and the bottom half was probed with PP-2A antibody. To evaluate the effect of the JAK-2 inhibitor and cAMP agonist, L6 cells were pretreated with AG490 (5 μ M) for 12 h and SpcAMP (1×10^{-4} M) for 30 min followed by 10 nM insulin for 5 min. In lanes 2–5, insulin treatment was for 2, 5, 10 and 30 min respectively. The effect of AG490 and SpcAMP pretreatment on JAK-2/PP-2A association is shown in lanes 7 and 8. A representative autoradiogram is shown. Similar results were obtained in three separate experiments. IP, immunoprecipitation; Ab, antibody.

Figure 2 JAK-2 inhibitor prevents insulin-mediated inactivation of PP-2A

L6 cells were serum starved in the absence or presence of AG490 (5 μ M) for 12–15 h, followed by treatment with insulin (10 nM) for 5 min. Equal amounts of proteins were assayed for PP-2A activity in the presence and absence of 1 nM okadaic acid. [32 P]Phosphorylase *a* was used as a substrate. Results are the means $\pm$ S.E.M. of four independent experiments performed in duplicate. **P* < 0.05 compared with control; ***P* < 0.05 compared with insulin.

Figure 2, treatment of L6 myotubes with 10 nM insulin for 5 min caused a 40% decrease in PP-2A activity when compared with control cells. Pretreatment with AG490 (5 μ M) for 12 h completely prevented the ability of insulin to inhibit PP-2A activity. AG490 by itself had very little effect on PP-2A enzymic activity. The reversal of insulin's inhibitory effect on PP-2A activity by AG490 was comparable with our previously reported inhibition of insulin's effect by SpcAMP [25].

Figure 3 Stimulation of JAK-2 kinase activity by insulin

Equal amounts of protein lysate prepared from L6 cells exposed to insulin with and without pretreatment with AG490 and SpcAMP were immunoprecipitated with JAK-2 antibody. The immunoprecipitates were washed with kinase buffer and assayed for kinase activity *in vitro* using [γ - 32 P]ATP. The immunoprecipitates were separated by SDS/PAGE and proteins were transferred to PVDF membrane followed by autoradiography. A representative autoradiogram is shown. Similar results were obtained in three separate experiments. Lane 1, control; lanes 2–5, insulin treated; lane 6, AG490 alone; lane 7, AG490 pretreatment followed by 5 min insulin; lane 8, SpcAMP pretreatment followed by insulin for 5 min; lane 9, insulin-treated cell lysates incubated with rat IgG instead of JAK-2 antibody to document the specificity of JAK-2 antibody. IP, immunoprecipitation; Ab, antibody

Insulin stimulates JAK-2 kinase activity

Several studies indicate that the C-terminal tyrosine kinase domain of JAK-2 contains the characteristic autophosphorylation site of tyrosine kinases [32–34]. Given that tyrosine phosphorylation of JAK-2 increases its catalytic activity [32–34], we examined whether insulin activates JAK-2 kinase directly. In this experiment, JAK-2 was immunoprecipitated and the kinase activity was measured *in vitro* using [γ - 32 P]ATP. As shown in Figure 3, insulin rapidly increases JAK-2 autophosphorylation after 2 min (Figure 3, lane 2). The phosphorylation is sustained for 30 min (Figure 3, lanes 2–5). Preincubation with AG490 for 12 h and SpcAMP for 30 min abolished insulin's effect on JAK-2 autophosphorylation (Figure 3, compare lanes 7 and 8 with lane 3).

Insulin promotes tyrosine phosphorylation of PP-2A catalytic subunit via JAK-2

It is well known that tyrosine as well as threonine phosphorylation of PP-2A catalytic subunit results in inactivation of the enzyme. However, the kinase which mediates PP-2A tyrosine phosphorylation *in vivo* and the physiological consequences of this phosphorylation remain unknown. To confirm further the hypothesis that JAK-2 inactivates PP-2A by increasing tyrosine phosphorylation of the catalytic subunit, we examined the kinetics of PP-2A tyrosine phosphorylation in cells treated with insulin in the presence and absence of AG490. As shown in Figure 4(A), insulin treatment was accompanied by a time-dependent increase in tyrosine phosphorylation of the immunoprecipitated PP-2A detected with anti-phosphotyrosine antibody. A maximal increase was observed between 2 and 10 min (Figure 4A, compare lanes 2–4 with lane 1). The effect was sustained for up to 60 min (Figure 4A, lanes 7 and 8). The kinetics of insulin-mediated tyrosine phosphorylation of PP-2A correlates with the kinetics of PP-2A inactivation by insulin reported previously [25]. Pretreatment with AG490 prevented the increased insulin-mediated tyrosine phosphorylation of PP-2A (Figure 4A, compare lane 5 with lane 3). As shown in our previous studies [25], pretreatment with SpcAMP also blocked insulin's effect on PP-2A tyrosine

Figure 4 Insulin causes tyrosine phosphorylation of PP-2A via JAK-2

(A) L6 cells were serum starved in the presence or absence of AG490 (5 μ M) followed by treatment with insulin for the indicated times. Equal amounts of cell lysates were immunoprecipitated with PP-2A antibody followed by immunoblot analyses of the immunoprecipitated proteins with anti-phosphotyrosine antibody. Lanes 2–4, insulin treatment was for 2, 5 and 10 min respectively; lanes 7–8, insulin was added for 30 and 60 min respectively; lane 5, AG490 followed by 5 min insulin treatment, and lane 6, AG490 alone. A representative autoradiogram is shown. Similar results were obtained in three independent experiments. (B) Equal amounts of cell lysate proteins were immunoprecipitated with JAK-2 antibody. The immunoprecipitates were separated by SDS/PAGE followed by Western blot analysis with anti-phosphotyrosine antibody. The blot was stripped and reprobed for PP-2A antibody. A representative autoradiogram is shown. Lane 1, control; lane 2, insulin treatment for 5 min; lane 3, AG490 alone; lane 4, AG490 followed by insulin treatment for 5 min; and lane 5, SpcAMP followed by 5 min insulin treatment. IP, immunoprecipitation; Ab, antibody.

phosphorylation and restored PP-2A enzymic activity to control levels.

We also examined PP-2A tyrosine phosphorylation in JAK-2 immunoprecipitates. As shown in Figure 4(B), treatment with insulin for 5 min increased PP-2A tyrosine phosphorylation (Figure 4B, compare lane 2 with lane 1). Pretreatment with AG490 prevented insulin's effect on PP-2A tyrosine phosphorylation (Figure 4B, compare lane 4 with lane 2). SpcAMP also inhibited insulin's effect on PP-2A tyrosine phosphorylation (Figure 4B, compare lane 5 with lane 2).

PP-2A associates with PI-3K and insulin stimulates PP-2A-associated PI-3K catalytic activity

In vitro studies indicate that purified preparations of PP-2A can inactivate PI-3K by dephosphorylation of the associated serine/threonine kinase [7]. Since insulin inhibits PP-2A activity by increasing tyrosine phosphorylation via JAK-2, we examined the impact of PP-2A inactivation on PI-3K activity associated with

Figure 5 Insulin causes the association of PP-2A with PI-3

(A) L6 cells were incubated in the presence or absence of AG490 for 12 h or SpcAMP for 30 min followed by treatment with insulin (10 nM) for 5 min. Equal amounts of cell lysate proteins (500 µg) were immunoprecipitated with PP-2A antibody. The immunoprecipitates were washed extensively and assayed for PI-3K activity using phosphatidylinositol as a substrate in the presence of [γ -³²P]ATP. The products of the PI-3K reaction were separated by TLC followed by autoradiography. A representative autoradiogram is shown. Similar results were obtained in three separate experiments. In lanes 2 and 3 insulin treatment was for 3 and 5 min respectively. As a positive control (lane 7, IRS-1), 50 µg of protein from insulin-treated cell lysates was immunoprecipitated with the insulin receptor substrate-1 (IRS-1) antibody. For the negative control (lane 8; IgG), immunoprecipitation was performed on 500 µg of lysate proteins from insulin-treated cells with 2 µg of purified anti-rabbit IgG. (B) PP-2A immunoprecipitates from (A) were separated by SDS/PAGE followed by Western blot analysis with an antibody directed against the p85 subunit of PI-3K. A representative autoradiogram is shown. Lane 1, control; lane 2, insulin; lane 3, AG490 alone; lane 4, AG490 pretreatment followed by insulin; lane 5, SpcAMP pretreatment followed by insulin; and lane 6, insulin-treated lysates immunoprecipitated with purified rat IgG as a negative control. IP, immunoprecipitation; Ab, antibody.

PP-2A (Figure 5A). In control cells, PP-2A-associated PI-3K enzymic activity was low in PP-2A immunoprecipitates (Figure 5A, lane 1). Insulin treatment of L6 cells for 3–5 min caused a rapid 40-fold increase in PI-3K activity assayed in PP-2A immunoprecipitates (Figure 5A, lanes 2 and 3). Pretreatment with AG490 completely blocked the insulin-mediated increase in PI-3K activity in PP-2A immunoprecipitates (Figure 5A, compare lane 4 with lane 3). AG490 itself did not alter basal levels of PI-3K activity associated with PP-2A (Figure 5A, compare lane 1 with lane 5). Treatment with SpcAMP also prevented the increased insulin-mediated PI-3K activity in PP-2A immunoprecipitates (Figure 5A, compare lane 6 with lane 3), presumably by causing dephosphorylation of PI-3K via an active PP-2A. As detailed in our recent studies [25], SpcAMP treatment prevents insulin-mediated PP-2A inactivation and restores PP-2A activity to control levels in L6 cells.

The effect of insulin on PP-2A-associated PI-3K activity described above was not due to differences in the amount of p85 PI-3K bound to PP-2A, but rather to an increase in PI-3K

Figure 6 Insulin increases PI-3K activity in JAK-2 immunoprecipitates

Cells exposed to AG490 and SpcAMP were treated with insulin for 5 min, as described in Figure 5(A), followed by immunoprecipitation with JAK-2 antibody and analysis of the immunoprecipitates for PI-3K activity. A representative autoradiogram is shown. Similar results were obtained in three separate experiments. IP, immunoprecipitation; Ab, antibody.

enzymic activity as evidenced by immunoblot analysis of PP-2A immunoprecipitates with p85 PI-3K antibody (Figure 5B).

Insulin increases PI-3K activity in JAK-2 immunoprecipitates

To understand further the importance of JAK-2 in modulating PP-2A interaction with PI-3K, we examined PI-3K activity in JAK-2 immunoprecipitates. As shown in Figure 6, treatment of L6 cells with insulin for 5 min increased PI-3K activity associated with JAK-2 by 20-fold (Figure 6, compare lane 1 with lane 2). Pretreatment with JAK-2 inhibitor for 12 h completely blocked the insulin-mediated increase in PI-3K activity associated with JAK-2 (Figure 6, compare lane 2 with lane 4). AG490 by itself did not alter basal PI-3K activity in JAK-2 immunoprecipitates (Figure 6, compare lane 3 with lane 1). SpcAMP, which prevents insulin-mediated PP-2A tyrosine phosphorylation and inactivation, also blocked the insulin-mediated increase in PI-3K activation in JAK-2 immunoprecipitates (Figure 6, compare lane 6 with lane 2).

JAK-2-associated PI-3K activity is decreased in adipocytes isolated from diabetic Goto-Kakizaki (GK) rats

We have recently shown that non-insulin-dependent diabetes mellitus (NIDDM) is accompanied by an increase in the basal PP-2A activity in isolated adipocytes and insulin fails to inactivate PP-2A [35]. The increase in PP-2A activity was accompanied by marked reductions in insulin-stimulated mitogen-activated protein kinase (MAPK) activation. To understand further the physiological relevance of PP-2A and JAK-2 association with PI-3K, we immunoprecipitated JAK-2 and assayed the associated PI-3K activity in adipocytes isolated from diabetic GK rats, a model for NIDDM. Insulin treatment for 5 min caused a modest 5–6-fold increase in PI-3K activity in JAK-2 immunoprecipitates in the adipocytes isolated from control rats. In contrast, adipocytes isolated from diabetic GK rats barely exhibited an increase in insulin-stimulated PI-3K activity in JAK-2 immunoprecipitates (results not shown).

DISCUSSION

The results presented in this study clearly indicate that PP-2A associates with JAK-2 in L6 cells and insulin inhibits PP-2A en-

zymic activity by increasing tyrosine phosphorylation of the PP-2A catalytic subunit via JAK-2. The time course of tyrosine phosphorylation of the PP-2A catalytic subunit by insulin demonstrated in this study correlates well with the kinetics of PP-2A inactivation reported in our previous study [25]. Blocking the kinase activity of JAK-2 with a synthetic inhibitor, AG490, as well as treatment with SpcAMP, a cAMP agonist, completely abolished insulin-mediated increase in tyrosine phosphorylation of PP-2A and restored PP-2A enzymic activity back to control levels. Thus, these results support the notion that insulin-stimulated JAK-2 phosphorylates the PP-2A catalytic subunit and causes inactivation of the phosphatase, which in turn may enhance the transmission of cellular signals through the kinase cascade. The fact that PP-2A is associated with JAK-2 under basal conditions implies that, in serum-starved cells, constitutively active PP-2A might deactivate JAK-2 or PI-3K, and/or other unknown kinases, by dephosphorylation, and thus control basal kinase activity. It is conceivable that, in a basal state, inactivation of a kinase cascade is crucial to the normal control of cell signalling. Pre-existing complexes containing active phosphatase and kinases (such as PP-2A–JAK-2, PP-2A–PI-3K shown in this study) may provide an alternative mechanism by which the appropriate basal phosphorylation state of intracellular substrates is maintained.

cAMP agonists are known to interfere with insulin receptor tyrosine phosphorylation via protein kinase A [36]. In addition, cAMP also inhibits insulin and growth-factor-stimulated MAPK signalling in most cell types [25,37–40], with the exception of Swiss 3T3 and PC12 cells (reviewed in [25]). Our previous studies have shown that SpcAMP counter-regulates insulin's effect on PP-2A inactivation by decreasing the tyrosine phosphorylation of PP-2A and restoring its catalytic activity [25]. Given that PP-2A regulates MAPK signalling, the results of this study add a new dimension to the well-known antagonism between insulin and cAMP and suggest that SpcAMP prevents PP-2A inactivation and reverses insulin's effect by inactivating JAK-2, or the kinase upstream of JAK-2, thereby blocking insulin signalling through the PI-3K cascade by dephosphorylation via an active PP-2A. Thus PP-2A inactivation/reactivation via its complexing with JAK-2 may play an important role in insulin- and cAMP-mediated signal transduction.

Three lines of compelling evidence presented in this study suggest the existence of protein kinase–PP-2A signalling complexes as a new paradigm in the control of the insulin signalling cascade. First, PP-2A associates with JAK-2 and PI-3K, as evidenced by a robust increase in PP-2A–JAK-2-associated PI-3K catalytic activity. Secondly, blocking JAK-2 activation with a synthetic inhibitor, as well as exposure of the cells to a cAMP agonist, abolishes insulin's effect on PP-2A–JAK-2-associated PI-3K activity without affecting PP-2A association with the p85 subunit of PI-3K. Thus it appears that inhibition of PP-2A catalytic activity by JAK-2-mediated tyrosine phosphorylation results in the amplification of insulin-generated signals at the level of PI-3K. Finally, JAK-2 associates with PI-3K, as evidenced by a greater than 20-fold increase in JAK-2-associated PI-3K activity. In contrast, blocking the kinase activity of JAK-2, as well as treatment with a cAMP agonist, completely abolishes the insulin-mediated increase in JAK-2-associated PI-3K activity. This presumably may be due to dephosphorylation of the PI-3K-associated serine kinase by the active PP-2A. Earlier studies by Carpenter et al. [41] have shown that PP-2A regulates PI-3K-associated serine kinase activity. Furthermore, PI-3K is seen as a complex with simian virus 40 small T antigen and PP-2A [42]. These observations, together with the fact that PP-2A association with middle T antigen inactivates PP-2A, resulting in increased

PI3-kinase activity [42], suggests that PP-2A may regulate the lipid kinase activity of PI-3K by modulating the function of the associated serine kinase. Whether or not JAK-2 associates directly with PI-3K, or associates with PI-3K via PP-2A complex formation, cannot be assessed from the results of immunoprecipitation and the assay of associated PI-3K activity. However, quantification of phosphatidylinositol 3-phosphate, the product of the PI-3K assay, revealed that PI-3K activity associated with PP-2A was 2-fold higher than the activity associated with JAK-2. Increasing the amount of JAK-2 antibody did not further increase the associated PI-3K activity. Thus it appears that PP-2A associates with PI-3K, and the activity seen in JAK-2 immunoprecipitates may be due to complex formation between PP-2A and JAK-2, which is precipitated by JAK-2 antibody. Further *in vitro* studies with recombinant proteins are warranted to clearly define these interactions.

Our observations coincide with a number of recent studies documenting PP-2A association with different kinases [12–22]. For example, studies by Heriche et al. [12] indicate that casein kinase 2 α associates with PP-2A and causes activation of PP-2A. A recent study by Westphal et al. [43] indicates that PP-2A forms complexes with p70 S6 kinase, p21-activated kinase-1 (PAK-1) and p21-activated kinase-3 (PAK-3) *in vivo*. The physiological significance of these associations is, however, unknown.

Further support for a regulatory function of PP-2A in the enhancement of insulin signalling through PI-3K comes from studies on adipocytes isolated from diabetic GK rats, a model for NIDDM. We have recently shown that these rats exhibit an elevated PP-2A basal activity in adipocytes and a marked reduction in insulin-mediated MAPK activation due to the failure of insulin to inhibit PP-2A activity [35]. Analysis of JAK-2-associated PI-3K activity in insulin-stimulated adipocytes isolated from diabetic GK rats revealed marked reductions in PI-3K activity when compared with adipocytes isolated from control rats. Thus these results suggest that PP-2A may participate in the *in vivo* regulation of PI-3K via complex formation with JAK-2. Reductions in JAK-2 kinase activity observed in aging and diabetes [23] may result in elevated PP-2A activities, which in turn interferes with insulin signalling by dephosphorylating PI-3K as well as the MAPK cascade. Our previous studies have demonstrated an inhibition of insulin-activated MAPK kinase signalling by tumour necrosis factor- α -induced insulin resistance in L6 cells [44]. The inhibition was downstream of p21^{ras} and Raf-1. This inhibitory effect was accompanied by elevations in PP-2A activity and a lack of insulin-mediated PP-2A inactivation [44]. Blocking PP-2A activity by treatment with low concentrations of okadaic acid (a PP-2A inhibitor) prevented tumour necrosis factor- α -induced inhibition of MAPK signalling and restored insulin's stimulatory effects on MAPK activation. These results clearly indicate that PP-2A may modulate insulin signalling via the kinase cascade by forming complexes with kinases.

In summary, our results suggest that PP-2A forms complexes with JAK-2 and PI-3K, and PP-2A activity in the complex is regulated by insulin, resulting in amplification of insulin signalling at the level of PI-3K.

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