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THE PI3-KINASE SERINE KINASE PHOSPHORYLATES ITS p85 SUBUNIT AND IRS-1 IN PI3-KINASE/IRS-1 COMPLEXES

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SUMMARY: Insulin receptor tyrosine kinase activity accounts for tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), but the serine kinase(s) responsible for serine phosphorylation of IRS-1 is(are) unknown. *In vitro* kinase assays performed on PI3-kinase and IRS-1 immunoprecipitates demonstrated insulin-dependent serine phosphorylation of IRS-1. IRS-1 was associated with both insulin-dependent and independent serine kinases. Only the insulin-dependent serine kinase preferred Mn^{2+} over Mg^{2+} and was recovered from cell lysates containing dithiothreitol. In complexes of tyrosine phosphorylated recombinant IRS-1 and PI3-kinase, phosphorylation of IRS-1 was associated with decreased phosphorylation of the p85 subunit of PI3-kinase. These results are consistent with PI3-kinase being responsible for insulin-dependent serine phosphorylation of IRS-1 and suggest that this phosphorylation reaction may affect functions of both IRS-1 and the PI3-kinase.

Insulin and IGF-1 receptors are transmembrane tyrosine kinases which autophosphorylate and tyrosine phosphorylate one or more intracellular proteins in response to hormone (1). The best characterized substrate for insulin and IGF-1 receptors is insulin receptor substrate-1 (IRS-1) (1,2). IRS-1, which migrates between Mr 170,000 and 180,000 on SDS-PAGE, is a cytosolic protein isolated as a component of the high molecular weight receptor substrate, pp185, which has been characterized in anti-phosphotyrosine (anti-PY) immunoprecipitates by SDS-PAGE (3). This highly conserved protein contains 21 potential tyrosine phosphorylation sites and over 30 potential serine/threonine phosphorylation sites (4). Several tyrosine phosphorylated residues on IRS-1 are believed to act as docking sites for SH2 domain-containing proteins (5). Evidence indicates that at least four such proteins, including phosphatidylinositol 3-kinase (PI3-kinase) (6), Grb2/SEM5 (7), Nck (8), and SHPTP2 (9), complex to IRS-1 in a hormone-dependent manner.

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In contrast to tyrosine phosphorylation of IRS-1, insulin-dependent serine/threonine phosphorylation of this protein is poorly understood and the kinase(s) involved has not been identified. This is despite the consistent observation that insulin-dependent serine/threonine phosphorylation of IRS-1 exceeds tyrosine phosphorylation. A recent report suggests that the serine kinase activity of PI3-kinase is responsible for *in vitro* serine phosphorylation of IRS-1 (10). In this study, we further characterize the interaction of IRS-1 with the PI3-kinase serine kinase and propose that this interaction not only affects IRS-1 phosphorylation but may also affect activation of the PI3-kinase lipid kinase by altering serine phosphorylation of the p85 subunit of PI3-kinase.

MATERIALS AND METHODS : The U266 human multiple myeloma cell line was purchased from American Type Culture Collection (Rockville, MD). [γ^{32}P]-ATP and ^{32}Pi were purchased from Amersham (Arlington Heights, IL). Recombinant IRS-1, anti-phosphotyrosine antibody (4G10), anti-PI3-kinase antibody, and anti-IRS-1 antibody (polyclonal) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

In Vitro Kinase Assays: Prior to hormonal studies, U266 cells were incubated overnight in RPMI 1640 supplemented with 1% BSA. $10x10^6$ cells were incubated at 37°C for 5 min with the indicated insulin concentration in 1 ml aliquots. Cells were solubilized in ice-cold 1% Nonidet P40 (NP40), 10mM pyrophosphate, 50mM NaF, 100mM NaCl, 25mM benzamidine, 1mM PMSF, 2mM orthovanadate and 50mM Tris, pH 7.4 in the presence or absence of 1 mM dithiothreitol. PI3-kinase and IRS-1 were immunoprecipitated from cell lysates as described (11). *In vitro* kinase reactions were performed on the immunoprecipitates at room temperature by addition of 50μL of reaction buffer (0.1% Triton X-100, 50mM Tris, pH 7.4 and either 10mM $MgCl_2$ or 10mM $MnCl_2$) with 5μM [γ^{32}P]ATP (40μCi/nmol). After 10 min the reaction was stopped by addition of 1ml of ice-cold 0.1% NP-40, 20mM EDTA, 100mM NaCl and 10mM Tris, pH 7.4. Immunoprecipitates were washed extensively, proteins were separated by SDS-PAGE, and examined by autoradiography. For analysis of alkaline labile phosphoproteins, the dried gels were hydrolyzed for 1hr in 1N NaOH at 60°C, re-dried and re-analyzed by autoradiography.

Immunoprecipitation of Phosphorylated IRS-1 from *In Vitro* Kinase Assays: An *in vitro* kinase assay was performed on PI3-kinase immunoprecipitates as above. The immune complex was dissociated by boiling for 5 min in 50μL of 1% SDS, 2mM pervanadate and 50mM Tris, pH 7.4. The boiled eluates were diluted 1:100 with 0.1% Triton X-100, 10 mM pyrophosphate, 50mM NaF, 100mM NaCl, 25mM benzamidine, 2mM pervanadate and 50mM Tris, pH 7.4 and immunoprecipitated with anti-IRS-1 antibody. IRS-1 immunoprecipitates were washed twice with 500mM $LiCl_2$ buffer as above and twice with low salt buffer. Samples were separated by SDS-PAGE and analyzed as above.

Tyrosine Phosphorylation of Recombinant IRS-1: Recombinant IRS-1 was incubated with insulin receptors isolated by wheatgerm agglutinin affinity chromatography from U266 cells. The tyrosine phosphorylation reaction contained 0.1% Triton X-100, 50 mM Tris, pH 7.4, 0.3 M n-acetyl-glucosamine, 10 mM $MnCl_2$, 25 μM ATP, ± 1 μM insulin. After a 30 min incubation at 37°C, reactions were stopped with 20 mM EDTA. IRS-1 was either immunoprecipitated as described above or added to PI3-kinase immunoprecipitates for a 3 hr incubation at 4°C.

RESULTS: In an *in vitro* kinase assay performed on PI3-kinase immunoprecipitates derived from U266 cells (11,12) (figure 1A), a Mr 180,000 protein was phosphorylated only from cells pretreated with 1μM insulin. When immunoprecipitates were eluted with an SDS-containing buffer and re-immunoprecipitated with an anti-IRS-1 antibody, a Mr 180,000 phosphoprotein consistent with IRS-1 was again detected (figure 1B). Phosphoamino acid analysis of the pp185/IRS-1 band demonstrated that most ^{32}P-label was incorporated into serine residues (data not shown). Phosphoproteins specific to the PI3-kinase immunoprecipitates were observed at approximately Mr 120, 85 and 80,000, but were not hormone dependent. When the Mg^{2+} used in the *in vitro* kinase assay (figure 1A,B) was replaced with Mn^{2+}, the Mr 85,000 protein (presumeably the p85 subunit of PI3-kinase) became dominant, though the pp185/IRS-1 band remained the only insulin-dependent phosphoprotein (figure 1C). While not evident in a comparision of figures 1A and 1C, incorporation of label into pp185/IRS-1 and p85 was markedly increased with Mn^{2+}.

Immunoprecipitates of IRS-1 also contained serine kinase activity. Using the *in vitro* kinase assay, insulin-dependent phosphorylation of IRS-1 was again markedly greater with Mn^{2+} than with Mg^{2+} (figure 2). Insulin-dependent phosphorylation of the presumed p85 subunit of PI3-kinase, which was not observed in figure 1 (masked by abundant PI3-kinase not associated with IRS-1), was now clearly seen in the presence of Mn^{2+}. Insulin independent serine phosphorylation of

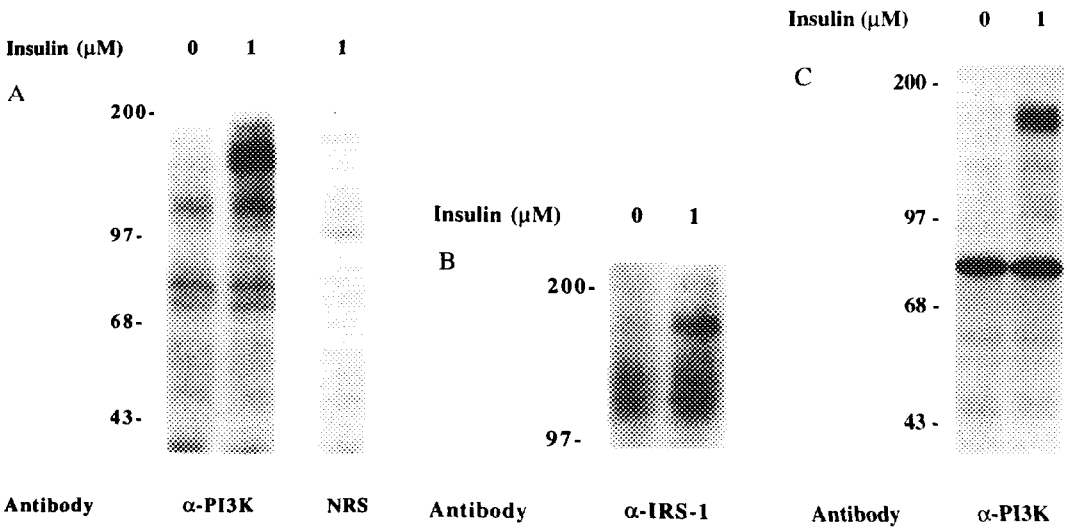

Figure 1. Insulin-dependent phosphorylation of a Mr 180,000 protein by a PI3-kinase-associated protein kinase. U266 cells were treated with or without 1μM insulin for 5 min, rapidly solubilized and immunoprecipitated with either an anti-PI3-kinase antibody (α-PI3K) or normal rabbit serum (NRS). *In vitro* kinase assays were performed on the immune complexes with (A,B) Mg^{2+} or (C) Mn^{2+} as the divalent cation as described (11). (A,C) Immune complexes were extensively washed and proteins were separated by SDS-PAGE. (B) IRS-1 was eluted from the complexes in (A) and immunoprecitated with anti-IRS-1 antibody (α-IRS-1).

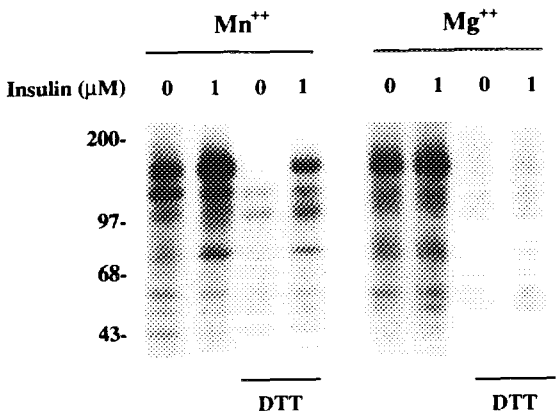

Figure 2. Effects of divalent cations and DTT on phosphorylation of IRS-1 in IRS-1 immune complexes. *In vitro* kinase assays were performed on α-IRS-1 immune complexes derived from cells treated ± insulin as described in Methods. Assays were performed in the presence of either 10mM Mn^{2+} or Mg^{2+}. Cell lysates were prepared with or without 1 mM DTT.

IRS-1 (also observed in IRS-1 immunoprecipitates by Lam *et. al.* [10]) was markedly decreased by inclusion of dithiothreitol (DTT) in the lysate buffer. Neither IRS-1 nor p85 were phosphorylated in IRS-1 immune complexes in the absence of insulin pretreatment in the presence of DTT. DTT appeared to affect the isolation of the insulin-independent kinase rather than its activity since DTT had no affect on this activity if added only to the kinase assay (data not shown).

The contribution of PI3-kinase to insulin-dependent serine phosphorylation of IRS-1 was further investigated by examining the interaction of PI3-kinase with recombinant IRS-1. Recombinant IRS-1, when tyrosine phosphorylated, displayed a marked increase in complexing to PI3-kinase from cell lysates as assessed by *in vitro* IRS-1 phosphorylation. Pre-clearing PI3-kinase from cell lysates with an anti-PI3-kinase antibody resulted in a marked decrease in phosphorylation of IRS-1 and an almost complete absence of p85 phosphorylation (figure 3).

Complexing of tyrosine phosphorylated IRS-1 to PI3-kinase appears to active the PI3-kinase lipid kinase activity (13). It is not known whether an insulin-dependent mechanism also regulates the serine kinase activity of PI3-kinase. In figure 4, increasing concentrations of recombinant IRS-1 were combined with PI3-kinase immunoprecipitated from cells which had not seen insulin. IRS-1 which had been tyrosine phosphorylated was more effectively serine phosphorylated and complexed to PI3-kinase (compare lanes 1 and 5). Importantly, phosphorylation of the p85 subunit of PI3-kinase decreased as more IRS-1 was available for association/phosphorylation. Since the *in vitro* kinase assays were performed after unbound IRS-1 was removed by washing, the association of PI3-kinase with IRS-1 appears to promote IRS-1 phosphorylation and suppress p85 phosphorylation. An additional phosphoprotein appears at Mr 120,000 in both this PI3-kinase

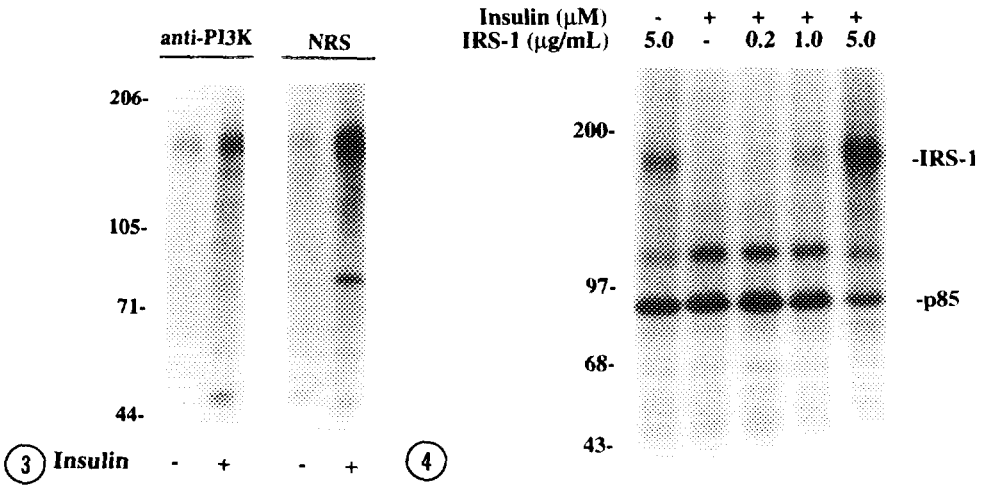

Figure 3. Phosphorylation of recombinant IRS-1 by PI3-kinase from cell lysates. Recombinant IRS-1 was tyrosine phosphorylated with insulin receptors ± 1μM insulin and immunoprecipitated. The immunoprecipitates were incubated with cell lysates (with DTT) which had been pre-cleared with either normal rabbit serum (NRS) or α-PI3-kinase. After washing the immune complexes, *in vitro* kinase assays were performed with Mn^{2+}.

Figure 4. Effect of IRS-1 on phosphorylation of the p85 subunit of PI3-kinase. Recombinant IRS-1, which was tyrosine phosphorylated as in figure 3, was incubated at the concentrations indicated with PI3-kinase immunoprecipitated from non-hormone treated cells. *In vitro* kinase assays were then performed on the immune complexes.

immunoprecipitate and in figure 1. Phosphorylation of this protein, which may be the p110 subunit of PI3-kinase is affected by IRS-1 concentrations similarly to p85.

DISCUSSION: Okadaic acid (a phosphoserine phosphatase inhibitor) reduces insulin-dependent glucose transport, PI3-kinase activation, and tyrosine phosphorylation of IRS-1 (14). These effects are paralleled by an increase in serine phosphorylation of IRS-1. IRS-1 recovered from okadaic acid-treated cells was also a poorer substrate for the insulin receptor tyrosine kinase. Casein kinase-2 phosphorylates rat IRS-1 *in vitro* at sites which correspond to *in vivo* insulin-dependent serine/threonine phosphorylation sites (15). MAP kinase also appears to phosphorylate recombinant IRS-1 (1). These observations suggest that serine phosphorylation of IRS-1 is a regulatory mechanism, but the responsible kinases remain unknown.

We have demonstrated that a serine kinase is present in PI3-kinase/IRS-1 complexes after insulin treatment, and that this kinase is capable of *in vitro* serine phosphorylation of IRS-1. All evidence is consistent with this kinase being the p110 catalytic subunit of PI3-kinase. For example, Dhand *et. al.* (16) demonstrated that the p110 subunit of PI3-kinase is a protein serine kinase which phosphorylates its

p85 subunit. Carpenter *et. al.* (17) have also reported that a protein serine kinase associates with PI3-kinase and phosphorylates the p85 and p110 subunits of PI3-kinase and casein. The recent report of Lam *et. al.* (10) used the PI3-kinase inhibitor Wortmannin to argue that the serine kinase activity of the p110 subunit of PI3-kinase is responsible for *in vitro* serine phosphorylation of IRS-1.

We have also shown that insulin-dependent phosphorylation of IRS-1 and p85 in the IRS-1/PI3-kinase complexes is preferentially a Mn^{2+}-dependent reaction. This is also consistent with the kinase being the PI3-kinase serine kinase activity. Dhand *et. al.* reported that the p110 protein serine kinase of PI3-kinase required Mn^{2+} for activity. Similarly, the PI3-kinase kinase of Carpenter *et.al.* could utilize either Mg^{2+} or Mn^{2+}, though Mn^{2+} significantly increased enzyme activity (17).

The detection of a serine kinase activity in the absence of insulin and the differential response of the insulin-dependent and independent kinase activities to divalent cations distinguish at least two serine kinases in the IRS-1/PI3-kinase immune complexes. However, only the insulin-dependent IRS-1 phosphorylation was retained when cells were lysed in the presence of DTT. Since DTT had no effect on either activity when added directly to the *in vitro* kinase assays, these results suggest that DTT decreased the association of insulin-independent kinase activity to the immunoprecipitated PI3-kinase/IRS-1 complexes.

The demonstration that PI3-kinase serine kinase has IRS-1 as a *in vitro* substrate raises the possibility that insulin-dependent PI3-kinase/IRS-1 complex formation not only activates the lipid kinase activity of PI3-kinase via SH2 domain interactions but also plays a regulatory role in insulin action by mediating serine phosphorylation of IRS-1 and PI3-kinase. It has been reported that serine phosphorylation of p85 inhibits PI3-kinase lipid kinase activity (16). Our results demonstrate that complexing of IRS-1 to PI3-kinase results in a marked decrease in phosphorylation of p85. This latter mechanism may contribute to activation of the PI3-kinase lipid kinase by decreasing the inhibitory effect of serine phosphorylation of the p85 subunit. In conclusion, our results further demonstrate the serine phosphorylation of IRS-1 by PI3-kinase and suggest a potential regulatory role for this reaction affecting both PI3-kinase and IRS-1 function.

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