RESEARCH COMMUNICATION

Insulin receptor substrate 1 is phosphorylated by the serine kinase activity of phosphatidylinositol 3-kinase

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Insulin receptor substrate (IRS) 1, which is tyrosine phosphorylated in response to insulin, presents multiple serine/threonine phosphorylation sites. To search for a serine kinase activity towards IRS 1, immunoprecipitates from basal or stimulated 3T3-L1 adipocytes were used in an *in vitro* kinase assay. When IRS 1 was isolated from insulin-treated cells, serine phosphorylation of IRS 1 occurred, which we attribute to the kinase activity of the phosphatidylinositol 3-kinase (PI3-kinase). Importantly, in an *in vitro* reconstitution assay, an excess of the PI3-kinase subunit prevents this phosphorylation. Together, our results suggest that following insulin stimulation, PI3-kinase associates with IRS 1, allowing for its serine phosphorylation. This phosphorylation event could play a role in the modulation of insulin signalling.

INTRODUCTION

Cell stimulation by insulin leads to the activation of the insulin receptor tyrosine kinase and the subsequent phosphorylation of IRS 1 [1,2]. This protein contains several tyrosine phosphorylation motifs which are substrates for the insulin receptor, and bind several proteins containing SH2 (Src homology 2) domains [2,3]. In addition, IRS 1 has many potential serine/threonine phosphorylation sites and has been shown to be phosphorylated on serine and threonine residues in unstimulated cells [3,4]. Insulin induces not only the tyrosine phosphorylation of IRS 1 but also enhances its serine/threonine phosphorylation [3,4]. Although IRS 1 contains consensus sites for cyclic AMP (cAMP)-dependent protein kinase, protein kinase C, mitogen-activated protein kinase and casein kinase II [4], the kinase(s) responsible for IRS 1 phosphorylation after exposure of cells to insulin is (are) not known. One of them could be casein kinase II, which phosphorylates IRS 1 *in vitro*. Furthermore, some of the phosphopeptides phosphorylated in an insulin-sensitive manner *in vivo* correspond to peptides phosphorylated by casein kinase II [5]. The role of IRS 1 serine/threonine phosphorylation in insulin action remains ill-defined. However, a series of observations suggest that it could be a part of a feed-back loop involved in attenuation of the insulin signal. Indeed, in 3T3-L1 adipocytes, okadaic acid stimulates a serine/threonine kinase activity towards IRS 1, and the resulting hyperphosphorylation of IRS 1 has been linked to a reduction in its tyrosine phosphorylation by the insulin receptor and a blockade in insulin action [6]. It is possible that serine/threonine kinases become associated with IRS 1 after insulin stimulation and thus modulate the insulin signalling pathway. The proteins that bind to tyrosine-phosphorylated IRS 1 include only one identified kinase: phosphatidylinositol 3-kinase (PI3-kinase). This enzyme is composed of two subunits: a p85 regulatory subunit which contains SH2 domains [7–9], and a p110 subunit which is the catalytic subunit [10]. PI3-kinase is activated after binding of the p85 subunit to tyrosine-phosphorylated IRS 1 [11,12], and the p110 subunit catalyses the phosphorylation of phosphatidylinositol at the D3 position of the inositol ring. However, recent data have shown that the p110 subunit also possesses a protein kinase activity [13]. It phosphorylates the p85 PI3-kinase subunit on serine residues and this phosphorylation down-regulates the lipid kinase activity [13,14]. So far, only p85 has been recognized as a substrate for the p110 serine kinase activity [13]. In this paper, we have investigated in the insulin-responsive 3T3-L1 adipocytes whether insulin stimulation causes a serine/threonine kinase to become associated with IRS 1 and to phosphorylate IRS 1. We have examined whether PI3-kinase could have such a role.

EXPERIMENTAL PROCEDURES

Materials

Insulin was a gift from Lilly France (Paris). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) and wortmannin were from Sigma. All other biochemicals were from Sigma or Serva (Heidelberg, Germany). Antibodies to IRS 1 and to the p85 subunit of the PI3-kinase were from UBI (Lake Placid, NY, U.S.A.). PGEX 2T vector was from Pharmacia. Radiochemicals were from ICN or DuPont–New England Nuclear.

Cell culture

3T3-L1 cells were grown in 35–100 mm dishes in Dulbecco's modified Eagle's medium (DMEM) and 10% (v/v) fetal calf serum, and were induced to differentiate in adipocytes as described [6]. 3T3-L1 adipocytes were used 8–15 days after the beginning of the differentiation protocol. Four hours before each experiment the medium was changed to serum-free DMEM supplemented with 0.5% (w/v) BSA.

Abbreviations used: cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PI3-kinase, phosphatidylinositol 3-kinase; GST, glutathione S-transferase; HNTV, 30 mM Hepes, pH 7.4, 30 mM NaCl, 0.1% (v/v) Triton X-100 and 200 μM sodium orthovanadate; IRS, insulin receptor substrate; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride).

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In vitro kinase assay

3T3-L1 adipocytes (cells from one 100 mm dish/condition) were incubated without or with insulin (100 nM) for the time indicated in the Figure legends. Cells were scraped and solubilized for 40 min at 4 °C in 1 ml of stopping buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM sodium orthovanadate, 100 mM NaF, 10 mM $Na_4P_2O_7$, 10% (v/v) glycerol, 10 μg/ml aprotinin and 1 mM phenylmethanesulphonyl fluoride (PMSF)] supplemented with 1% (v/v) Nonidet-P40. Lysates were centrifuged at 12000 *g* for 10 min and the supernatants were incubated overnight at 4 °C with antibodies to IRS 1 (7.5 μg) coupled to protein A–Sepharose beads. The beads were washed twice with stopping buffer containing 0.5 M NaCl, twice with stopping buffer supplemented with 1% Nonidet-P40 and twice with 30 mM Hepes, pH 7.4, 30 mM NaCl, 0.1% (v/v) Triton X-100 and 200 μM sodium orthovanadate (HNTV). Immune pellets were resuspended in 50 μl of HNTV and the phosphorylation reaction was started with 10 μl of HNTV containing 24 mM $MnCl_2$, 48 mM $MgCl_2$ and 120 μM [γ-^{32}P]ATP (10 μCi). After 30 min at 25 °C, the reaction was stopped by addition of Laemmli buffer. Samples were boiled and proteins were separated by SDS/PAGE using 7.5% (w/v) polyacrylamide gels. These were dried and autoradiographed. When the effects of PI3-kinase inhibitors were studied, experiments were performed as described above except that wortmannin (10 or 100 nM) or DTNB (300 μM) was added to the phosphorylation mixture.

Preparation of the glutathione S-transferase (GST)–p85(263–724) bacterial fusion proteins

A bacterial fusion protein containing the 263–724 fragment of bovine p85α was obtained as follows: a *Sca*I–*Stu*I fragment (encompassing nt 785–2175, amino acids 263–724) from bovine p85α in Bluescript vector (a gift of M. D. Waterfield, Ludwig Institute, London) was subcloned into PGEX-2T vector (Pharmacia) at the *Sma*I site. Transformed bacteria (JM109) were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Bacteria were lysed by two cycles of freezing and thawing in 30 ml of 50 mM Na_2HPO_4, pH 8.0, 0.3 M NaCl, 1 mM PMSF followed by 2 min of sonication. DNAase was added for 15 min at 4 °C. Soluble fractions were applied on a Glutathione Sepharose column (Pharmacia) and the fusion protein was eluted.

In vitro phosphorylation of IRS 1 and binding of PI3-kinase

Human insulin receptors were purified using wheat germ agglutinin from NHIR cells and used to phosphorylate IRS 1 as described [6]. Briefly, IRS 1 was immunoprecipitated from 3T3-L1 adipocytes. The beads were washed twice with stopping buffer containing 1% Nonidet-P40, twice with stopping buffer containing 0.5 M NaCl and twice with HNTV. Immune pellets were incubated with insulin receptors preincubated without or with insulin (100 nM); IRS 1 was phosphorylated for 15 min at room temperature by adding 10 μl of a reaction mixture containing 40 mM Hepes, pH 7.4, 24 mM $MnCl_2$, 48 mM $MgCl_2$ and 120 μM ATP. The immune pellets were washed twice with stopping buffer containing 0.5 M NaCl and twice with stopping buffer containing 1% Nonidet-P40. Then PI3-kinase binding to IRS 1 was performed as described [6]. Briefly, lysate of unstimulated 3T3-L1 adipocytes, prepared in stopping buffer with 1% Nonidet-P40, was incubated with 40 μg of GST or GST–p85(263–724) and was incubated with the immune pellets for 10 min at room temperature. Pellets were washed and resuspended in 50 μl of HNTV and the phosphorylation reaction was started by the addition of 10 μl of 40 mM Hepes, pH 7.4, 24 mM $MnCl_2$, 48 mM $MgCl_2$ and 120 μM [γ-^{32}P]ATP (25 μCi). After 30 min at room temperature, the reaction was stopped by addition of Laemmli buffer and the proteins were analysed by SDS/PAGE and autoradiography.

Metabolic labelling of 3T3-L1 adipocytes

3T3-L1 adipocytes (cells from two 100 mm dishes/condition) were washed twice with PBS and incubated for 16 h at 37 °C in methionine- and cysteine-free DMEM/10% fetal calf serum supplemented with Tran^{35}S-label (ICN; 100 μCi/ml). Cells were washed twice with PBS and incubated for 4 h in 10 ml of methionine- and cysteine-free DMEM/0.5% BSA supplemented with Trans ^{35}S-label (100 μCi/ml). Then, cells were stimulated or not with 100 nM insulin for 7 min, washed with stopping buffer and solubilized in stopping buffer containing 1% Nonidet-P40. Proteins were immunoprecipated with antibodies to IRS 1 as described above. After washes, immune pellets were resuspended in Laemmli buffer and subjected to linear gradient (7.5–15% polyacrylamide) SDS/PAGE.

Phosphoamino acid analysis

Proteins were subjected to SDS/PAGE and transferred to poly(vinylidene difluoride) (PVDF) membranes. Sheets were autoradiographed and membrane segments containing IRS 1 or the 85 kDa subunit of the PI3-kinase were cut, hydrolysed and analysed according to Kamps and Sefton [15].

Immunoblotting experiments

Proteins were separated on SDS/PAGE, transferred to PVDF membranes, and the membranes were blocked with PBS/5% BSA for 4 h at room temperature. Membranes were incubated overnight at 4 °C with antibodies to IRS 1 (1 μg/ml saturation buffer) or to the p85 subunit of the PI3-kinase (1:1000 in saturation buffer). Sheets were then washed three times with PBS/1% Nonidet-P40 and incubated for 1 h at room temperature with ^{125}I–protein A (5×10^5 c.p.m./ml of saturation buffer). After three washes, the membranes were autoradiographed.

RESULTS AND DISCUSSION

To determine whether a kinase able to phosphorylate IRS 1 was associated with immunopurified IRS 1, 3T3-L1 adipocytes were treated for various lengths of time with insulin and proteins were immunoprecipitated with antibodies to IRS 1. The kinase assay was performed on the immune pellet as described in the Experimental procedures section. As shown in Figure 1(a), no phosphorylation was visible in immune pellets obtained from cells incubated without insulin. Following insulin treatment, two proteins, with a molecular mass of 170 and 85 kDa respectively, were phosphorylated in the anti-(IRS 1) immunoprecipitates. Two other proteins with M_rs in the range 110000–120000 were also slightly labelled. No other phosphoproteins could be detected, even after prolonged film exposure or after separation of the proteins on a gel with a higher concentration of acrylamide (results not shown). The phosphorylation of the proteins was already seen with immunoprecipitates prepared from cells incubated with insulin for 2 min and was maintained for at least 15 min (Figure 1a). The 170 kDa phosphoprotein co-migrates

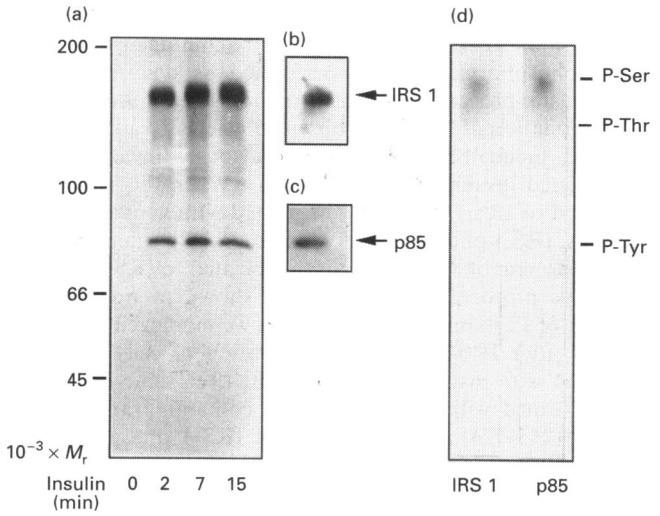

Figure 1 IRS 1 is phosphorylated by an associated serine kinase activity

(**a**) 3T3-L1 adipocytes were incubated with 100 nM insulin for 0, 2, 7 or 15 min. Proteins were solubilized and immunoprecipitated with antibodies to IRS 1. The *in vitro* kinase assay was performed on the immune pellet as described in the Experimental procedures section before performing SDS/PAGE, transfer to PVDF and autoradiography. (**b** and **c**) In parallel, aliquots of immunoprecipitates were used for immunoblotting analysis with antibodies to IRS 1 (**b**) and to the p85 subunit of the PI3-kinase (**c**). A representative autoradiogram of five independent experiments is shown. (**d**) Phosphoamino acid analysis. 3T3-L1 adipocytes were stimulated for 2 min with insulin and proteins were immunoprecipitated with antibodies to IRS 1. The *in vitro* kinase assay was performed on the immune pellet, and phosphoamino acid composition of IRS 1 and p85 was assessed by high-voltage t.l.c. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

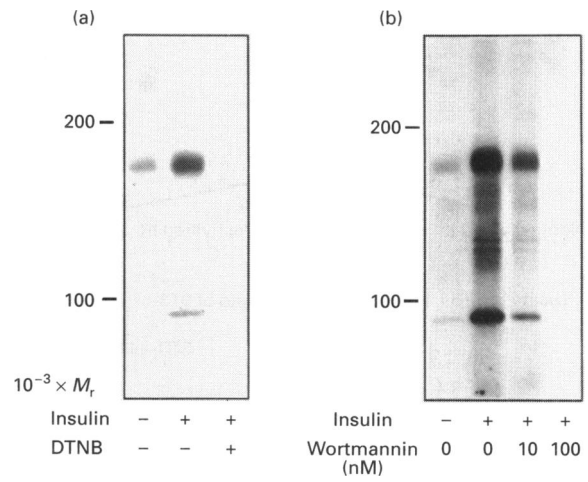

Figure 3 PI3-kinase inhibitors block the serine kinase activity towards IRS 1

3T3-L1 adipocytes were stimulated for 2 min with 100 nM insulin and proteins were immunoprecipitated with antibodies to IRS 1. The *in vitro* kinase assay was performed in the absence or presence of 300 μM DTNB (**a**) or wortmannin (10 or 100 nM) (**b**). Phosphorylated proteins were separated on SDS/PAGE and revealed by autoradiography.

with IRS 1 identified by immunoblotting with antibody to IRS 1 (Figure 1b), whereas the p85 phosphoprotein co-migrates with the regulatory subunit of the PI3-kinase detected with specific antibody in immunoblotting experiment (Figure 1c). Phosphoamino acid analysis indicated that the two proteins were phosphorylated on serine residues (Figure 1d). Because IRS 1 is devoid of kinase activity [3], this observation suggests that the increase in IRS 1 phosphorylation is attributable to a kinase activity co-immunoprecipitating with IRS 1. Although part of IRS 1 could be associated with the insulin receptor [16], it can be ruled out that IRS 1 phosphorylation resulted from the insulin-receptor tyrosine kinase activity because phosphoamino acid analysis indicated that this phosphorylation occurred only on serine residues.

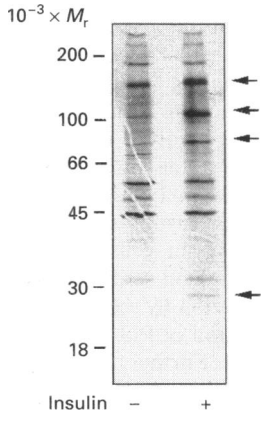

Figure 2 Association of IRS 1 with various proteins in insulin-stimulated 3T3-L1 adipocytes

3T3-L1 adipocytes were labelled for 16 h with Tran^{35}S-label, before being incubated without or with 100 nM insulin for 7 min. Solubilized proteins were immunoprecipitated with antibodies to IRS 1, separated by SDS/PAGE and visualized by autoradiography. A typical autoradiogram is shown. The arrows point to proteins associated with IRS 1 following incubation with insulin.

To identify the kinase responsible for IRS 1 phosphorylation, we have first analysed the proteins associated to IRS 1 after insulin stimulation. Therefore, 3T3-L1 adipocytes were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine, stimulated with insulin, and cell extracts were immunoprecipitated with antibodies to IRS 1. In anti-(IRS 1) immunoprecipitates obtained from cells incubated without insulin, a 170 kDa ^{35}S-labelled protein was observed, corresponding to IRS 1 (Figure 2). When cells were stimulated with insulin, the mobility of IRS 1 slightly decreased because of its tyrosine phosphorylation [12]. Further, three proteins with a molecular mass of 85, 110 and 25 kDa respectively became associated with IRS 1 (Figure 2). The 85 and 100 kDa proteins probably correspond to the two subunits of the PI3-kinase [7–10], and the 25 kDa protein to GRB2 [17]. Among the SH2-domain-containing proteins previously described to bind to tyrosine-phosphorylated IRS 1, PI3-kinase is the only kinase identified so far [1,2]. Moreover, this kinase is a dual-specificity enzyme, as its p110 catalytic subunit possesses both a lipid kinase activity and a serine protein kinase activity [13]. The following experiments were undertaken to identify PI3-kinase as the enzyme involved in IRS 1 phosphorylation in the anti-(IRS 1) immunoprecipitates. The serine protein kinase activity of p110, which has a thiol requirement for activity [13], is sensitive to the sulphydryl-modifying reagent DTNB. As shown in Figure 3, addition of 300 μM DTNB in the kinase assay inhibited in a parallel fashion the phosphorylation of p85 and IRS 1. Next, we studied the effect of wortmannin, which is considered to be a selective inhibitor of PI3-kinase, when it was used at concentrations between 10 and 100 nM [18–20]. Addition

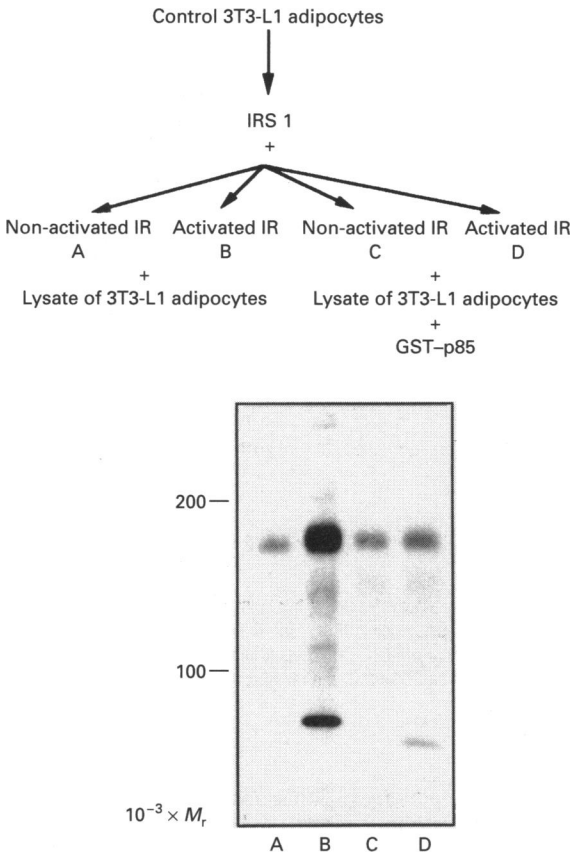

Figure 4 An excess of GST–p85 blocks the serine kinase activity towards IRS 1

IRS 1 was immunopurified from control 3T3-L1 adipocytes and phosphorylated by non-activated or activated insulin receptors (IR). Then a lysate of 3T3-L1 adipocytes, plus 40 μg of GST (lanes A and B) or GST–p85 (lanes C and D), was added and phosphorylation was performed in the presence of [γ-^{32}P]ATP, as described in the Experimental procedures section, before SDS/PAGE and autoradiography. A representative autoradiogram out of three different experiments is shown.

of wortmannin in the *in vitro* kinase assay induced a parallel reduction in the phosphorylation of both the p85 subunit of the PI3-kinase and IRS 1 (Figure 3). The dose dependency for the two processes was the same, with 60 and 95% inhibition seen at 10 and 100 nM wortmannin respectively. These inhibitory concentrations are in the range of those previously reported to inhibit lipid kinase activity of PI3-kinase, whereas inhibition of other kinases such as myosin light-chain kinase occurred only in the micromolar range [18–20]. The finding that wortmannin blocks the serine kinase activity of the PI3-kinase was in fact not unexpected, as wortmannin acts on the catalytic subunit of the enzyme [18].

To add further support for the PI3-kinase implication in IRS 1 phosphorylation, we tried to block the binding of the PI3-kinase to IRS 1 before phosphorylation of IRS 1. This can be obtained with an excess of free p85 subunit which, by competition with the p85–p110 complexes, prevents the association of this complex with tyrosine-phosphorylated IRS 1 [21] or growth-factor receptors [22]. As shown schematically in Figure 4, immunopurified IRS 1 from unstimulated cells was phosphorylated with unlabelled ATP by insulin receptors activated or not with insulin. A lysate from unstimulated 3T3-L1 adipocytes, as a source of PI3-kinase, was incubated with an excess of GST or GST–p85(263–724) and was added to the immune pellet. Then, IRS 1 phosphorylation was performed as described in the Experimental procedures section. As shown in Figure 4, a slight phosphorylation of IRS 1 was observed when a lysate of 3T3-L1 cells was incubated with IRS 1 previously incubated with unstimulated insulin receptors (lane A). When the same lysate was added to IRS 1 previously incubated with activated insulin receptors, IRS 1 phosphorylation was markedly enhanced and a phosphoprotein of 85 kDa became associated to IRS 1 (lane B). Using this protocol, which has been shown to reveal an association of PI3-kinase with IRS 1 [6], we mimicked the results obtained after IRS 1 immunoprecipitation of cells previously stimulated with insulin (Figure 1). When the same experiment was performed with a 3T3-L1 lysate containing an excess of GST–p85(263–724), the stimulation of IRS 1 phosphorylation was nearly totally abolished and p85 phosphorylation was suppressed (lane D). At the same time, we observed a slight phosphorylation of a protein with a molecular mass corresponding to GST–p85(263–724). Thus, an excess of GST–p85(263–724) is able to prevent the association of a functional p85–p110 PI3-kinase complex to IRS 1, and concomitantly the phosphorylation of IRS 1 is markedly reduced. The basal IRS 1 phosphorylation was not prevented after addition of GST–p85(263–724) (lane C), suggesting that it was not attributable to PI3-kinase.

Taken together, our results strongly suggest that the serine phosphorylation of IRS 1 is mediated by PI3-kinase. Although, so far, p85 has been considered as the only substrate for the p110 serine kinase [13], our results indicate that p110 is able to catalyse phosphorylation reactions towards another protein. The p110 serine kinase activity did not phosphorylate proteins such as myelin basic protein (results not shown), enolase or histone [13]. Further, GRB2, which is also associated to IRS 1 ([17] and Figure 2), was not phosphorylated in our *in vitro* kinase assay (results not shown). The binding of p85 to p110 appears to be required for the serine phosphorylation of p85 [13], suggesting that the substrate must be brought into close proximity to the active site of the enzyme. Thus, insulin-induced tyrosine phosphorylation of IRS 1, which allows for the binding of PI3-kinase to IRS 1, might also favour the subsequent serine phosphorylation of IRS 1 by the p110 catalytic subunit of PI3-kinase.

The data presented in this paper were obtained *in vitro*, and thus a key question is whether such a process is also occurring *in vivo*. However, it is tempting to suggest that it might play a role in regulation of insulin action. Serine/threonine phosphorylation events are known to be involved in down-regulation of insulin signalling. For instance, serine/threonine phosphorylation of the insulin receptor by protein kinase C or by cAMP-dependent protein kinase inhibits insulin-stimulated receptor tyrosine phosphorylation [23–28]. In cells treated with okadaic acid, phosphorylation of IRS 1 on serine/threonine residues reduces its tyrosine phosphorylation by the insulin receptor, leading to a reduction in the activation of PI3-kinase and in the stimulation of glucose transport [6]. Further, in adipocytes and fibroblasts, an increase in intracellular cAMP level attenuates the insulin-induced activation of mitogen-activated protein kinase [29,30]. Finally, in 3T3-L1 adipocytes which are desensitized to insulin action because of long-term treatment with the hormone, the amount of IRS 1 is reduced and its degree of serine phosphorylation is increased [31,32]. Interestingly, PI3-kinase activation seems to be required for this process, as a PI3-kinase inhibitor blocks the insulin-induced degradation of IRS 1 [33]. Serine/threonine phosphorylation of IRS 1 by PI3-kinase might

render IRS 1 more sensitive to degradation by proteases and thus might be involved in the down-regulation of insulin action.

Note added in proof (received 20 September 1994)

During the submission of this report, another study appeared on the phosphorylation of IRS 1 by the PI3-kinase serine kinase [34].

This work was supported in part by the Institut National de la Santé et de la Recherche Médicale (France), the University of Nice, and grant no. 2111 from the Association pour la Recherche contre le Cancer (ARC). We thank Dr. M. Cormont for critical reading of the manuscript. We thank M. D. Waterfield (Ludwig Institute, London) for the gift of the cDNA coding for the bovine p85α subunit of the PI3-kinase. The illustration work of A. Grima and G. Visciano is acknowledged.

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Received 7 July 1994/30 August 1994; accepted 8 September 1994