The type 2 vascular endothelial growth factor receptor recruits insulin receptor substrate-1 in its signalling pathway

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Vascular endothelial growth factor (VEGF) isoforms exert their biological effects through receptors that possess intrinsic tyrosine kinase activity. Whether VEGF binding to its receptors recruits insulin receptor substrate (IRS) family of docking proteins to the receptor is not known. Following incubation of mouse kidney proximal tubular epithelial cells with VEGF, we observed an increase in tyrosine phosphorylation of several proteins, including one of ≈200 kDa, suggesting possible regulation of phosphorylation of IRS proteins. VEGF augmented tyrosine phosphorylation of IRS-1 in kidney epithelial cells and rat heart endothelial cells in a time-dependent manner. In the epithelial cells, association of IRS-1 with type 2 VEGF receptor was promoted by VEGF. VEGF also increased association of IRS-1 with the p85 regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase), and PI 3-kinase activity in IRS-1 immunoprecipitates was increased in VEGF-treated cells. Incubation of epithelial cells with antisense IRS-1 oligonucleotide, but not sense oligonucleotide, reduced expression of the protein and VEGF-induced PI 3-kinase activity in IRS-1 immunoprecipitates. Additionally, VEGF-induced protein synthesis was also impaired by antisense but not sense IRS-1 oligonucleotide. These data provide the first evidence that binding of VEGF to its type 2 receptor promotes association of IRS-1 with the receptor complex. This association may account for some of the increase in VEGF-induced PI 3-kinase activity, and the increase in *de novo* protein synthesis seen in renal epithelial cells.

Key words: antisense oligonucleotide, epithelial cell, phosphoinositide 3-kinase (PI 3-kinase), protein synthesis, tyrosine phosphorylation.

INTRODUCTION

Vascular endothelial growth factors (VEGFs) are a family of polypeptides with diverse functions including control of endothelial cell differentiation, organization of blood vessels and vascular permeability [1,2]. Five different VEGFs (VEGFs A–E), and a related protein, placental growth factor, have been identified that vary in their receptor-binding properties and phenotypic effects. In addition, there are splice variants of VEGF-A with distinct properties of dimerization and binding to heparin [3].

VEGFs exert their effects by binding to cell-surface proteins, of which at least three VEGF receptors have been identified: type 1 [fms-like tyrosine kinase 1 (Flt-1)], type 2 [kinase-insert domain (KDR)/fetal liver kinase (Flk-1)], type 3 (Flt-4) and neuropilin, an accessory 'receptor' [3]. The three receptors possess intrinsic tyrosine kinase activity and share structural motifs in that they have an extracellular domain made of seven immunoglobulin-like repeats, a short hydrophobic intramembranous domain and a cytoplasmic tail consisting of a kinase domain interrupted by a kinase insert domain.

The type 2 VEGF receptor (KDR/Flk-1) contains four major autophosphorylation sites: Tyr-951 and Tyr-996 in the kinase insert domain and Tyr-1054 and Tyr-1059 in the kinase domain [4]. Several Src homology 2 (SH2) domain-containing proteins bind to the cytoplasmic domain of the receptor, and include phospholipase C-γ, Grb2, Nck, Shc and the SH2 domain-containing phosphatases SHP1 and SHP2 [5,6]. Binding of VEGF to the type 2 receptor activates a number of important signalling pathways, including phosphoinositide 3-kinase (PI 3-kinase) [7] and phospholipase C-γ, the latter, perhaps, leading to extracellular signal-regulated protein kinase 1 and 2 (ERK1/2)-type mitogen-activated protein kinase activation [8–10].

Activation of PI 3-kinase by other receptor tyrosine kinases, e.g. insulin receptor, may involve binding of an intermediary docking protein belonging to the insulin receptor substrate (IRS) family, although direct binding between the receptor and PI 3-kinase may also occur. IRS proteins are tyrosine-phosphorylated by kinase activity of the receptor, and phosphorylated tyrosines on IRS serve as docking sites for SH2 domain-containing proteins such as the p85 regulatory subunit of PI 3-kinase [11]. Alternatively, SH2 domain-containing signalling proteins may also bind directly to the receptor tyrosine kinase without intermediary proteins, e.g. platelet-derived growth factor receptor. Observations based on yeast 2-hybrid analysis have suggested that PI 3-kinase binds to the Tyr-1213 of type 1 VEGF receptor through a YVNA motif, and not the conventional YXXM sequence [12]. The YXXM sequence is not present in the cytoplasmic domain of the type 2 VEGF receptor [8,12], raising the possibility of a receptor-docking protein binding to SH2 domain-containing proteins such as the p85 subunit of PI 3-kinase [7]. It is not known whether activation of VEGF receptor signalling involves recruitment of IRS family of proteins. In this study, we examined whether IRS protein is involved in VEGF receptor signalling pathway in kidney proximal tubular epithelial cells (PTE cells) and rat heart endothelial cells. We report for the first time that binding of VEGF to the type 2 receptor promotes association of IRS-1 with the receptor complex. This association may account

Abbreviations used: VEGF, vascular endothelial growth factor; IRS, insulin receptor substrate; PI 3-kinase, phosphoinositide 3-kinase; PTE cell, proximal tubular epithelial cell; SH2, Src homology 2; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; IGF-I, insulin-like growth factor-I.

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for some of the increase in VEGF-induced PI 3-kinase activity, and the increase in *de novo* protein synthesis seen in renal epithelial cells.

EXPERIMENTAL

Cell culture

Simian virus 40-transformed murine PTE cells were kindly provided by Dr Eric Neilson (Vanderbilt University, Nashville, TN, U.S.A.). Cells were grown in Dulbecco's minimal essential medium (DMEM) containing 7% fetal bovine serum, 5 mM glucose and no insulin [13]. These cells retain characteristic properties of PTE cells *in vivo* [14]. VEGF effects were also studied in rat heart endothelial cells in culture that were also grown in DMEM containing 7% fetal bovine serum. Confluent monolayers of cells were serum-deprived in DMEM for 18 h before experiments were performed.

Tyrosine phosphorylation of proteins

In these experiments, we employed mouse VEGF-A_{165} (Sigma, St. Louis, MO, U.S.A.), a heparin-binding glycoprotein. PTE cells were incubated without serum overnight and treated with or without 20 ng/ml VEGF for 0–60 min. This concentration of VEGF was chosen based on preliminary experiments that showed in a range of concentrations (10–100 ng/ml) that 20 ng/ml VEGF maximally stimulated *de novo* protein synthesis in PTE cells. Equal amounts of proteins were separated on 7.5% SDS/PAGE and transferred to nitrocellulose membrane. After blocking in 5% non-fat dry milk, the membrane was washed three times in Tris-buffered saline containing 0.1% Tween-20. Membranes were immunoblotted with 1:2000 dilution of a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Phosphotyrosine antibody was detected by an antibody against mouse IgG linked to horseradish peroxidase. The reactive bands were detected by chemiluminescence. The membrane was stripped and probed with an anti-actin antibody to assess loading.

IRS-1 tyrosine phosphorylation

Serum-starved PTE and rat heart endothelial cells were incubated with 20 ng/ml VEGF for the indicated times. Cells were lysed in RIPA buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium ortho-vanadate, 1 mM PMSF, 0.1% aprotinin and 1% Nonidet P-40. Equal amounts of homogenates (500 μg) were immunoprecipitated with a specific antibody against IRS-1 (Upstate Biotechnology). The immunoprecipitates were separated by 7.5% SDS PAGE and transferred to nitrocellulose membrane overnight. The membrane was probed with 1:2000 dilution of a mouse monoclonal antibody against phosphotyrosine. The antibody against phosphotyrosine was detected by an antibody against horseradish peroxidase-linked mouse IgG. The reactive bands were detected by chemiluminescence. The membrane was stripped and immunoblotted with the antibody against IRS-1 to assess loading of samples and to ascertain the identity of the immunoprecipitated band.

Association between type 2 VEGF receptor complex and IRS-1

Serum-deprived quiescent PTE cells were incubated with or without 20 ng/ml VEGF for the indicated times and immunoprecipitated with antibody against IRS-1 as described above. The immune complexes were separated by 15% SDS/PAGE and transferred to nitrocellulose membrane overnight. The membrane was probed with 1:2000 dilution of an antibody against type 2 VEGF receptor (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The latter was detected by an antibody against rabbit IgG that was linked to horseradish peroxidase. The reactive bands were visualized by chemiluminescence. The membrane was stripped and reprobed with antibody against IRS-1 to assess loading of samples.

Association between IRS-1 and PI 3-kinase

Serum-deprived quiescent PTE cells were incubated with or without 20 ng/ml VEGF for the indicated times, and immunoprecipitated with antibody against IRS-1 as described above. The immune complexes were separated by 7.5% SDS/PAGE, transferred to nitrocellulose membrane and immunoblotted with 1:2000 dilution of an antibody against p85 regulatory subunit of PI 3-kinase (Santa Cruz Biotechnology). The reactive bands were visualized by chemiluminescence. The membrane was stripped and reprobed with antibody against IRS-1 to assess loading of samples.

PI 3-kinase assay

PI 3-kinase activity was assessed as described previously [13,15]. Briefly, control and VEGF-treated PTE cells were homogenized in RIPA buffer. Equal amounts of homogenates (500 μg) were immunoprecipitated using the anti-IRS-1 antibody. The immunobeads were incubated with 10 μg of PtdIns for 10 min at 25 °C in the PI 3-kinase assay buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid. Following addition of 20 mM $MgCl_2$ and 5 μCi of [γ-^{32}P]ATP to the assay mixture, the reactants were further incubated for 10 min at 25 °C. Reaction was stopped by addition of 150 μl of chloroform/methanol/11.6 M HCl mixture in a ratio of 50:100:1. The reactants were extracted with 100 μl of chloroform and the organic layer was washed with methanol/1 M HCl in a 1:1 ratio. The reaction products were dried, resuspended in chloroform, separated by silica gel 60 TLC using the solvent chloroform/methanol/28% NH_4OH/water in a ratio of 129:114:15:21. The PtdIns3*P* spots were visualized by autoradiography.

Treatment of PTE cells with IRS-1 antisense oligonucleotides

Phosphorothioate antisense and sense oligonucleotides were designed and synthesized to include the translation start site of IRS-1. The sequences chosen were 5′-TATCCGGAGGGCTC-GCCAT-3′ for the antisense and 5′-AGCAGCATGGCGAGC-CCTCCGGATA-3′ for the sense oligonucleotides [16]. PTE cells were grown in the presence of 1 μM antisense or sense oligonucleotides for 3 days prior to addition of VEGF. Changes in the amount of IRS-1 expressed by the cells were monitored by Western blotting with an antibody against IRS-1, as described above. Following 3 days of incubation with sense or antisense oligonucleotides, PTE cells were incubated with or without VEGF for 5 mins. Equal amounts of cell lysates were immunoprecipitated with anti-IRS-1 antibody, and PI 3-kinase activity was assayed as described above.

Protein synthesis assay

We have described this procedure recently [17]. PTE cells were grown in the presence of 1 μM antisense or sense oligonucleotides for 3 days prior to addition of VEGF and cells were incubated with [^{35}S]methionine at 10 μCi/ml for 6 h. Cells were then washed in PBS and lysed in RIPA buffer. Protein (20 μg) was spotted on to 3MM paper (Whatman). The filters were then washed by boiling them for 1 min in 10% trichloroacetic acid containing

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0.1 g/l methionine. This was repeated three times. The filters were then dried and immersed in scintillation fluid before determining radioactivity.

RESULTS AND DISCUSSION

VEGF increases tyrosine phosphorylation of cellular proteins

The effect of VEGF on tyrosine phosphorylation of cellular proteins was examined in PTE cells. Serum-starved PTE cells were incubated with or without VEGF. Immunoblotting of cleared cell lysates with an anti-phosphotyrosine antibody showed a significant increase in tyrosine phosphorylation of proteins within 2 min of exposure to VEGF that peaked at 30 min (Figure 1). Augmented tyrosine phosphorylation appeared to involve proteins of approx. 200, 145, 85 and 32 kDa. As the type 2 VEGF receptor (200 kDa [18]) and IRS-1 migrate in this area, the 200 kDa tyrosine-phosphorylated protein could represent the type 2 VEGF receptor and/or a member of the IRS family. Among the other proteins, tyrosine phosphorylation of the SH2 domain-containing proteins phospholipase C-γ (145 kDa) and regulatory subunit of PI 3-kinase (85 kDa) may be increased by VEGF, as has been described previously in endothelial cells [5]. Augmented tyrosine phosphorylation of proteins following VEGF is consistent with activation of tyrosine kinase activity of its receptors. Possible recruitment of IRS-1 by the VEGF receptors was further explored.

VEGF augments IRS-1 tyrosine phosphorylation

Following addition of VEGF to PTE cells, cell lysates were immunoprecipitated with an antibody against IRS-1 and immunoblotted with an anti-phosphotyrosine antibody. Tyrosine phosphorylation of IRS-1 was evident at 2 min (1.5-fold,

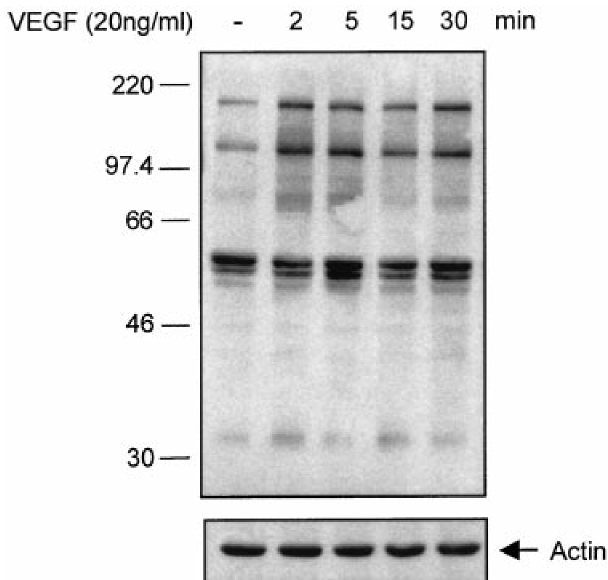

Figure 1 VEGF increases tyrosine phosphorylation of cellular proteins

Serum-starved PTE cells were treated with or without 20 ng/ml VEGF for the indicated times. Equal amounts of proteins were separated by 7.5% SDS/PAGE and transferred to nitrocellulose membrane. Membranes were immunoblotted with a 1:2000 dilution of a monoclonal anti-phosphotyrosine antibody as described in the Experimental section. Lower panel: Western blot with an anti-actin antibody that was done to assess loading. A representative blot of four individual experiments is shown. Molecular-mass markers are shown on the left.

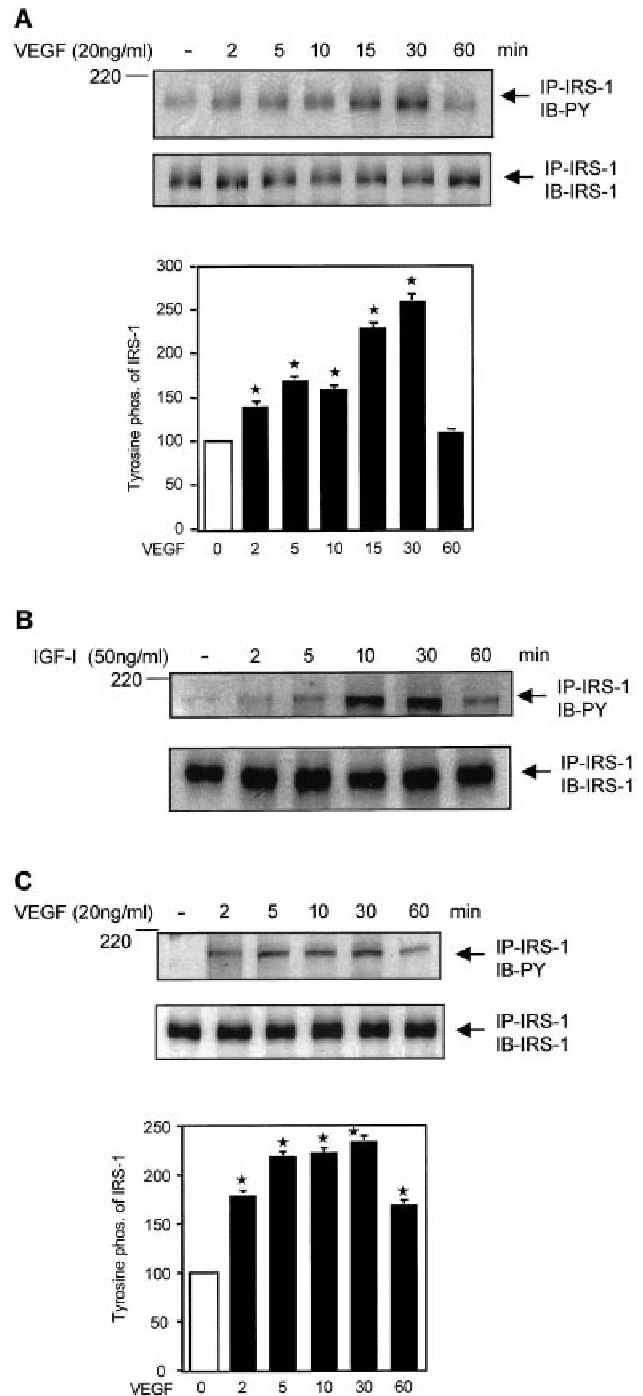

Figure 2 VEGF augments IRS-1 tyrosine phosphorylation

(**A**) Serum-starved PTE cells were incubated with or without VEGF. Equal amounts of lysates were then immunoprecipitated (IP) with IRS-1 antibody. Immunoprecipitated proteins were separated on 7.5% SDS/PAGE and transferred to nitrocellulose membrane. Membranes were immunoblotted (IB) with a 1:2000 dilution of a monoclonal anti-phosphotyrosine (PY) antibody. Lower panel: membranes were stripped and reprobed with anti-IRS-1 antibody to assess loading. Representative blots from at least three individual experiments are shown. Histogram shows composite data from three experiments expressed as a percentage of control; $^{*}P < 0.0001$. (**B**) Serum-starved PTE cells were incubated with or without IGF-I. Immunoprecipitation and immunoblotting were performed as described in (**A**). (**C**) Serum-starved rat heart endothelial cells were incubated with or without VEGF. Immunoprecipitation and immunoblotting were performed as described in (**A**). Histogram shows composite data from three experiments expressed as a percentage of control; $^{*}P < 0.0001$.

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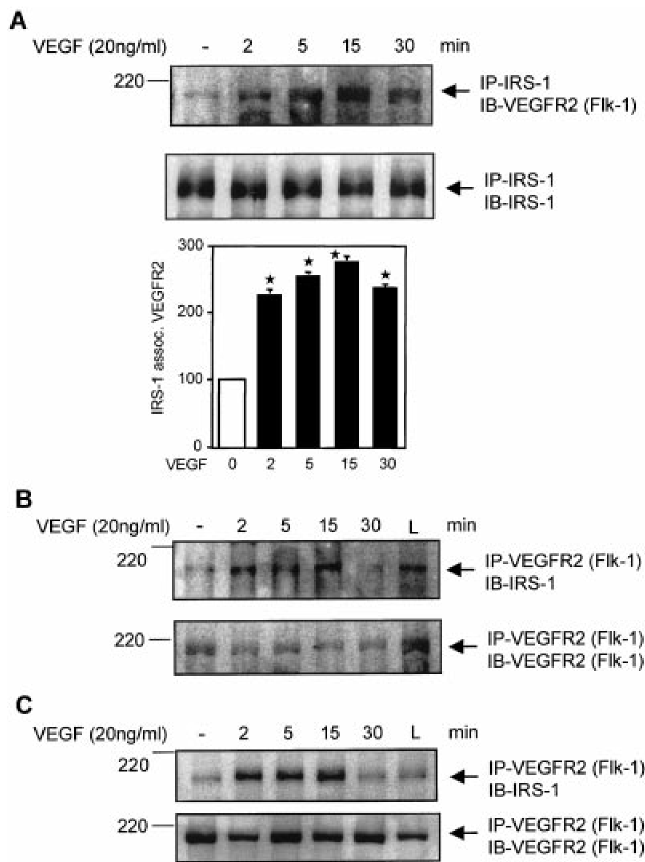

Figure 3 IRS-1 associates with type 2 VEGF receptor complex

(**A**) Equal amounts of cell lysates from serum-starved PTE cells incubated with or without VEGF were immunoprecipitated (IP) with IRS-1 antibody. Immunoprecipitated proteins were separated on 7.5% SDS/PAGE and transferred to nitrocellulose membrane. Membranes were immunoblotted (IB) with an antibody against type 2 VEGF receptor (VEGFR2). Lower panel: membranes were stripped and reprobed with anti-IRS-1 antibody to assess loading. A representative blot from three individual experiments is shown. Histogram shows composite data from three experiments expressed as a percentage of control; $^{*}P < 0.0001$. (**B**) Equal amounts of lysates from PTE cells incubated with or without VEGF were first immunoprecipitated with an antibody against type 2 VEGF receptor and immunoblotted with an antibody against IRS-1. Lysate alone was run in lane L. Lower panel: membranes were stripped and reprobed with anti-VEGF receptor antibody to assess loading. A representative blot from two individual experiments is shown. (**C**) Equal amounts of lysates from rat heart endothelial cells incubated with or without VEGF were first immunoprecipitated with an antibody against type 2 VEGF receptor and immunoblotted with an antibody against IRS-1. Lysate alone was run in lane L. Lower panel: membranes were stripped and reprobed with anti-VEGF receptor antibody to assess loading. A representative blot from two individual experiments is shown.

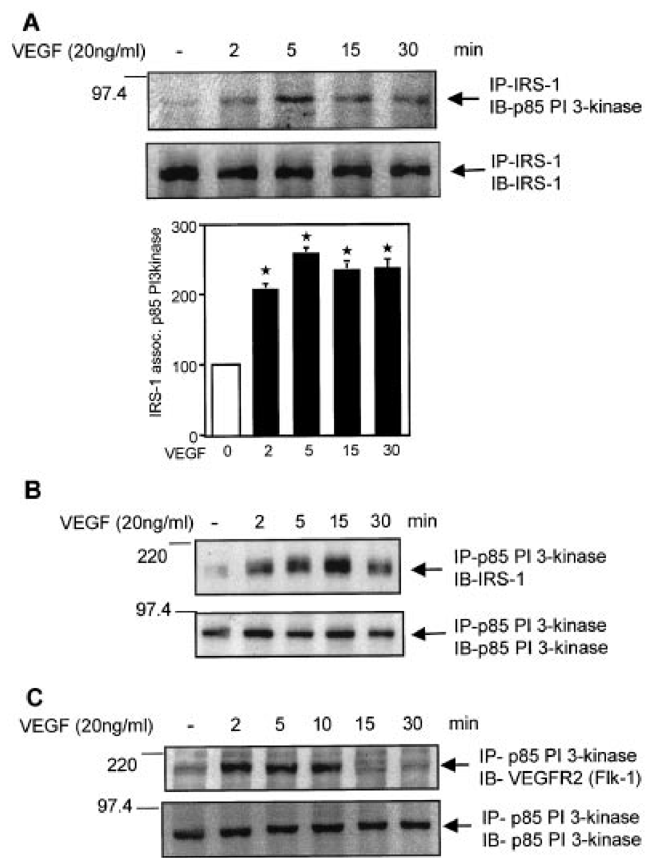

Figure 4 VEGF augments binding of PI 3-kinase to IRS-1 and type 2 VEGF receptor in PTE cells

(**A**) Equal amounts of lysates from control and VEGF-treated cells were immunoprecipitated (IP) with an IRS-1 antibody. The immune complexes were separated on 7.5% SDS/PAGE followed by immunoblotting (IB) with p85 subunit of PI 3-kinase antibody. Lower panel shows a Western blot with the anti-IRS-1 antibody that was done to assess loading. A representative blot of four individual experiments is shown. Histogram shows composite data from four experiments expressed as a percentage of control; $^{*}P < 0.0001$. (**B**) Lysates from PTE cells incubated with or without VEGF were first immunoprecipitated with an antibody against the p85 subunit of PI 3-kinase and immunoblotted with an antibody against IRS-1. Lower panel: membranes were stripped and reprobed with anti-p85 antibody to assess loading. A representative blot from two individual experiments is shown. (**C**) Lysates from PTE cells incubated with or without VEGF were first immunoprecipitated with an antibody against p85 subunit of PI 3-kinase and immunoblotted with an antibody against type 2 VEGF receptor. Lower panel: membranes were stripped and reprobed with antibody against p85 subunit antibody to assess loading. A representative blot from two individual experiments is shown.

$P < 0.0001$), peaked at 30 min (2.5-fold, $P < 0.0001$) and waned by 60 min (Figure 2A). VEGF regulation of IRS-1 phosphorylation was compared with that induced by insulin-like growth factor-I (IGF-I). Incubation with 50 ng/ml IGF-I resulted in robust induction of IRS-1 tyrosine phosphorylation that was evident at 2 min, sustained for 30 min and persistent (although at a reduced intensity) at 60 min (Figure 2B). IGF-I appeared to cause a more prolonged tyrosine phosphorylation of IRS-1 relative to VEGF. Induction of IRS-1 phosphorylation was also seen in rat heart endothelial cells within 2 min (1.75-fold, $P < 0.0001$), peaked at 30 min (2.5-fold, $P < 0.0001$) and decreased but was still evident at 60 min (Figure 2C). These data demonstrate rapid induction of tyrosine phosphorylation of IRS-1 by VEGF in both endothelial and non-endothelial cells.

Type 2 VEGF receptor complex associates with IRS-1

Stimulation of IRS-1 tyrosine phosphorylation by VEGF suggests that IRS-1, being a docking protein, may associate with one of its receptors. In preliminary experiments, type 1 VEGF receptor could not be detected by Western blotting (results not shown), although the type 2 receptor expression could be readily detected. We examined whether IRS-1 bound to the type 2 VEGF receptor. Serum-starved cells were incubated with or without VEGF and cell lysates were immunoprecipitated with an antibody against IRS-1. Immune complexes were separated on SDS/PAGE and immunoblotted with antibody against type 2 VEGF receptor. VEGF robustly induced physical association between type 2 receptor and IRS-1 that was increased by more than 2-fold at 2 min ($P < 0.0001$), and by nearly 3-fold at 15 min ($P < 0.0001$; Figure 3A). Similar results were obtained when the

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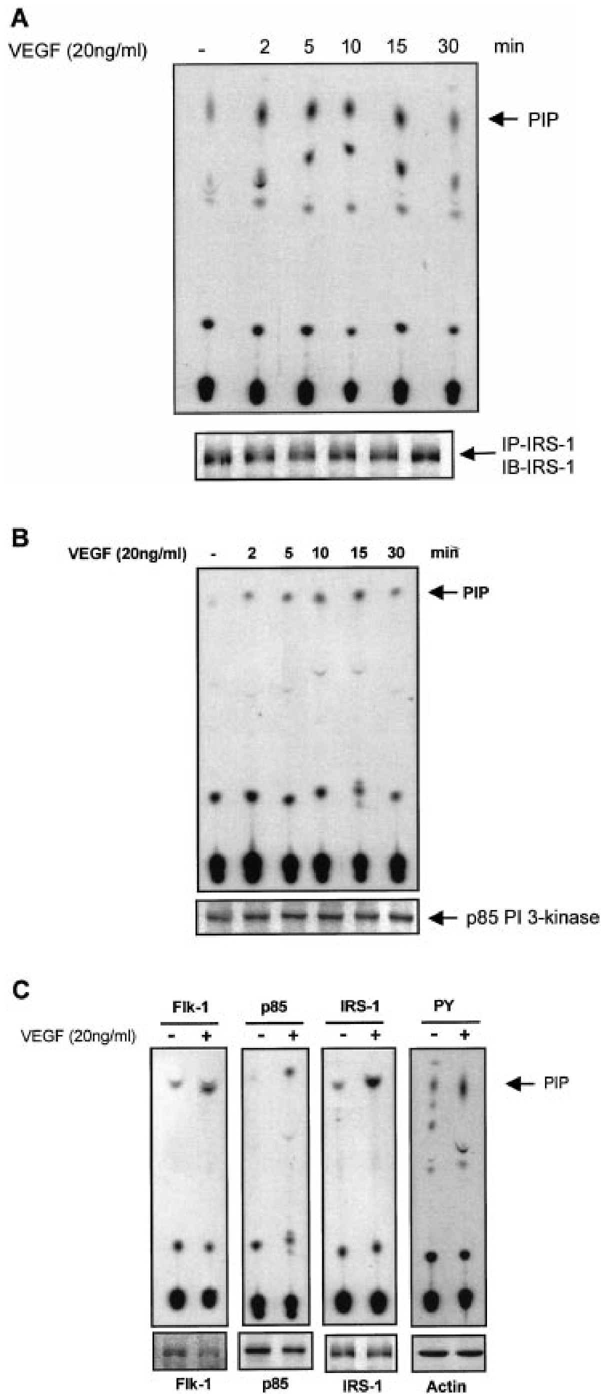

Figure 5 PI 3-kinase activity is increased in VEGF-treated cells

(**A**) IRS-1-associated PI 3-kinase activity is increased in VEGF-treated cells. PTE cells were incubated with or without VEGF and equal amount of cell lysates were immunoprecipitated (IP) with an IRS-1 antibody. PI 3-kinase activity in immune complexes was measured by using PtdIns as a substrate, as described in the Experimental section. Phospholipids were separated by TLC, and visualized by autoradiography. A representative chromatogram from four individual experiments is shown. PIP, PtdIns3*P*. Lower panel: aliquots of lysates of cells were immunoprecipitated and immunoblotted (IB) with an antibody against IRS-1 to assess relative amounts of lysates subjected to immunoprecipitation in the assay. (**B**) p85-associated PI 3-kinase activity is increased in VEGF-treated cells. The experiment was done as described in (**A**), except that cell lysates were immunoprecipitated with antibody against p85 subunit of PI 3-kinase. A representative chromatogram from two individual experiments is shown. Lower panel: aliquots of lysates of cells were immunoprecipitated and immunoblotted with an antibody against p85 to assess relative amounts of lysates subjected to immunoprecipitation in the assay. (**C**) The experiment was done as described in (**A**), except that cell lysates were immunoprecipitated with antibodies against type 2 VEGF receptor (Flk-1), p85 subunit of PI 3-kinase, IRS-1 or with anti-phosphotyrosine (PY) antibodies. A representative chromatogram from two individual experiments with each immunoprecipitating antibody is shown. Lower panels: aliquots of lysates of cells that were immunoprecipitated with an antibody against VEGFR2 (Flk-1), p85 subunit of PI 3-kinase or IRS-1 were immunoblotted with the respective antibody to assess relative amounts of lysates subjected to immunoprecipitation in the assay. Cell lysates that were immunoprecipitated with anti-phosphotyrosine antibody were immunoblotted with an antibody against actin to assess relative amounts of lysates subjected to immunoprecipitation in the assay.

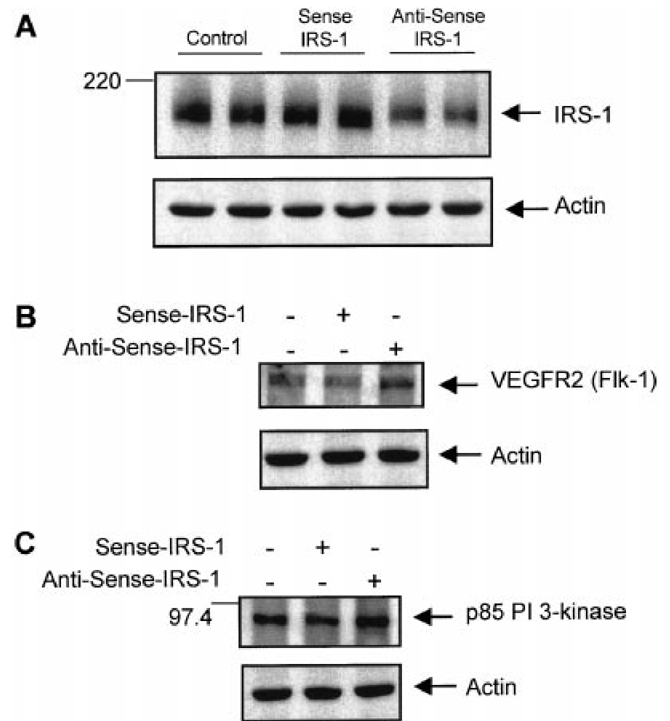

Figure 6 Effect of antisense IRS-1 oligonucleotides on the expression of IRS-1, type 2 VEGF receptor and PI 3-kinase

(**A**) Antisense IRS-1 oligonucleotide inhibits IRS-1 expression. PTE cells were grown in the presence of 1 μM phosphorothioate sense or antisense IRS-1 oligonucleotides for 3 days as described in the Experimental section. Cells were lysed in RIPA buffer, and equal amounts of homogenates (500 μg) were immunoprecipitated with antibody against IRS-1. The immunoprecipitates were separated on SDS/PAGE, and immunoblotted with IRS-1 antibody. Lower panel shows Western blotting with anti-actin as a loading control. Data from each treatment condition are shown in duplicate. A representative blot from two individual experiments is shown. (**B**, **C**) Antisense IRS-1 oligonucleotide does not inhibit expression of type 2 VEGF receptor and p85 subunit of PI 3-kinase. Lysates from cells treated with or without sense or antisense IRS-1 oligonucleotide were immunoblotted with antibody against type 2 VEGF receptor or p85 subunit of PI 3-kinase. Lower panels show Western blotting with anti-actin as a loading control. Representative blots from two individual experiments are shown.

order was reversed, i.e. immunoprecipitation with antibody against the type 2 VEGF receptor and immunoblotting with antibody against IRS-1 (Figure 3B). Similarly, in heart endothelial cells, immunoprecipitation with antibody against type 2 VEGF receptor and immunoblotting with antibody against IRS-1 also showed that VEGF promoted association between the receptor and IRS-1 (Figure 3C). Association of IRS-1 with the insulin receptor involves the phosphotyrosine binding domain of IRS-1 and an NPXY sequence in the cytoplasmic domain of the

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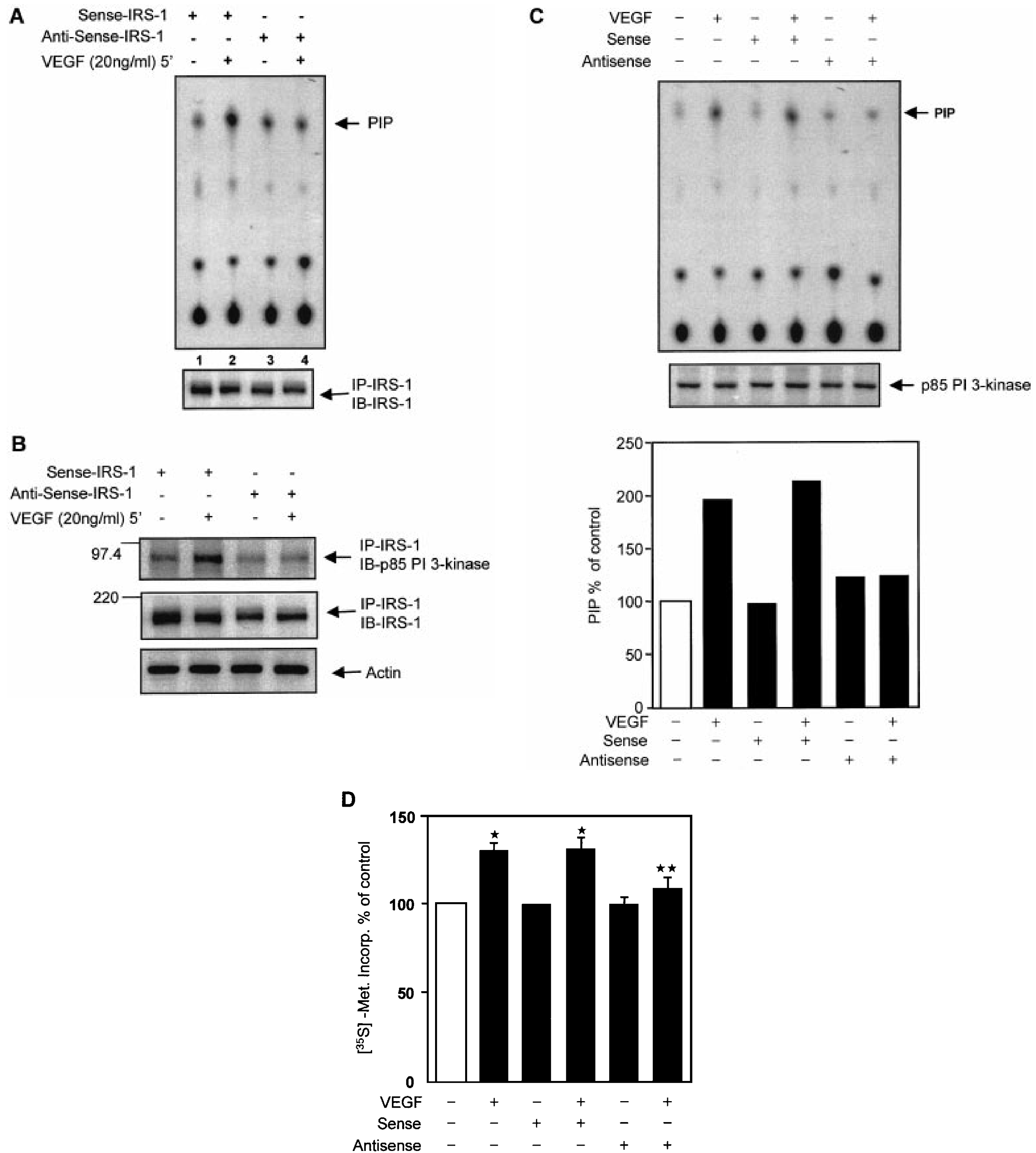

Figure 7 Effect of antisense IRS-1 oligonucleotides on VEGF-induced PI 3-kinase activity and protein synthesis in PTE cells

(**A**) Decreased expression of IRS-1 modulates VEGF-induced IRS-1 associated PI 3-kinase activity. PTE cells were grown in the presence of 1 μM phosphothioate antisense or sense IRS-1 oligonucleotides for 3 days prior to addition of VEGF. Cells were lysed in RIPA buffer and equal amounts of homogenates (500 μg) were immunoprecipitated with antibody against IRS-1. PI 3-kinase assay was performed on the immunoprecipitates as described in the Experimental section. Lower panel: aliquots of lysates of cells were immunoprecipitated (IP) and immunoblotted (IB) with an antibody against IRS-1. PIP, PtdIns3*P*. (**B**) Inhibition of IRS-1 expression affects association of p85 subunit of PI 3-kinase with IRS-1. PTE cells were grown in the presence of 1 μM antisense or sense IRS-1 oligonucleotides for 3 days prior to addition of VEGF. Cells were lysed in RIPA buffer and equal amounts of homogenates (500 μg) were immunoprecipitated with antibody against IRS-1. Immunoprecipitates were separated on a 7.5% gel and immunoblotted with an antibody against p85 subunit of PI 3-kinase. The blot was stripped and immunoblotted with an antibody against IRS-1. Lower panel: aliquots of cell lysates processed for immunoprecipitation were immunoblotted with antibody against actin to assess loading. (**C**) Involvement of IRS-1 in VEGF-induced PI 3-kinase activity. PTE cells were processed as described in (**A**), but immunoprecipitated with antibody against p-85 subunit of PI 3-kinase. PI 3-kinase assay was performed on the immunoprecipitates. Lower panel: aliquots of lysates of cells were immunoprecipitated and immunoblotted with an antibody against p85 subunit of PI 3-kinase. A representative blot from two experiments is shown. Histogram shows composite data from two experiments. (**D**) IRS-1 is involved in VEGF-induced protein synthesis in PTE cells. PTE cells were grown in the presence or absence of 1 μM antisense or sense IRS-1 oligonucleotides for 3 days prior to addition of VEGF. Cells were then labelled with [^{35}S]methionine for 6 h. Incorporation of the label into trichloroacetic acid-precipitable protein was estimated. Incorporation of [^{35}S]methionine into protein is expressed as percentage of control. Combined data from three experiments with $n = 4$ at each point are shown. Data are expressed as means $\pm$ S.E.M. $^{*}P < 0.001$ compared with sense treatment or non-oligonucleotide-treated control; $^{**}P < 0.03$ compared with sense treated with VEGF (ANOVA).

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receptor [19]. The cytoplasmic domain of type 2 VEGF receptor does not contain NPXY sequence [20], suggesting other motifs may be involved in binding to IRS-1. Previous studies have shown that some receptors that bind to IRS-1 do not express NPXY sequence. Alternative mechanisms could include IRS protein binding to the receptor via its non-phosphotyrosine-binding domains, as has been shown for IRS-2 binding to insulin receptor [21,22]. It is also possible that an as-yet unidentified protein mediates binding of IRS-1 to the VEGF receptor.

VEGF augments binding of IRS-1 to PI 3-kinase

In select receptor tyrosine kinase signalling systems, e.g. insulin receptor, downstream events of receptor activation depend in part on association of the IRS docking proteins and SH2 domain-containing proteins, e.g. p85 subunit of PI 3-kinase [11]. VEGF is known to activate PI 3-kinase, and this activation may be mediated by type 2 rather than type 1 VEGF receptor [7]. We examined whether VEGF promoted association between p85 subunit of PI 3-kinase and IRS. Serum-starved cells were incubated with or without VEGF and cell lysates were immunoprecipitated with an antibody against IRS-1. Immune complexes were separated on SDS/PAGE, transferred to nitrocellulose membrane and immunoblotted with an antibody against the p85 subunit of PI 3-kinase. Exposure to VEGF induced a rapid association between IRS-1 and p85 subunit that was evident at 2 min (2-fold increment, $P < 0.0001$), peaked at 5 min (2.5-fold increment, $P < 0.0001$) and remained elevated at 30 min (Figure 4A). Reversing the order and immunoprecipitation with anti-p85 and immunoblotting with anti-IRS-1 led to the same conclusion (Figure 4B). Similar analytic strategies showed rapid association between p85 subunit of PI 3-kinase and the type 2 VEGF receptor following exposure to VEGF (Figure 4C). Thus, our data suggest that the p85 subunit of PI 3-kinase binds to both type 2 VEGF receptor and IRS-1 in VEGF-treated PTE cells. It is unclear if mediation by IRS-1 is required for p85 subunit of PI 3-kinase to bind to type 2 VEGF receptor. Mutational studies have suggested that the p85 subunit of P1 3-kinase may bind directly to Tyr-799 and Tyr-1173 of the cytoplasmic domain of the type 2 VEGF receptor [23]. The sequence involved in this binding does not involve the conventional YXXM motif [23]. It is possible that both direct and indirect (via IRS-1) means exist for binding between PI 3-kinase and type 2 VEGF receptor.

IRS-1-associated PI 3-kinase activity is increased in VEGF-treated cells

We examined activity of the kinase in IRS-1 immunoprecipitates. PTE cells were incubated with or without VEGF and cell lysates were immunoprecipitated with an antibody against IRS-1. Immune complexes were processed for associated PI 3-kinase activity. As shown in Figure 5(A), following addition of VEGF, IRS-1-associated PI 3-kinase activity increased within 2 min and waned by 30 min. A VEGF-induced increment in PI 3-kinase activity of comparable net intensity was also seen in immunoprecipitates generated by antibodies against p85 subunit of PI 3-kinase, type 2 VEGF receptor or anti-phosphotyrosine antibodies (Figures 5B and 5C). These data demonstrate that VEGF-induced PI 3-kinase activity is at least partly accounted for by a pathway that involves IRS-1. Our data, along with previous observations [23], suggest that there may be both direct and indirect pathways of activation of PI 3-kinase by the type 2 VEGF receptor.

Antisense IRS-1 inhibits IRS-1-associated PI 3-kinase activity in PTE cells

We employed antisense strategy to more definitively test the involvement of IRS-1 in VEGF-induced PI 3-kinase activity. PTE cells were treated with 1 μM antisense or sense oligonucleotides for 3 days. Exposure of cells to antisense oligonucleotides resulted in nearly 50 % reduction of IRS-1 protein levels, whereas sense oligonucleotides had no effect on IRS-1 levels, as determined by Western blotting (Figure 6A). Incubation with either sense or antisense IRS-1 oligonucleotides did not significantly affect the expression of either type 2 VEGF receptor or p85 subunit of PI 3-kinase compared with oligonucleotide-untreated controls (Figures 6B and 6C). These data show that antisense IRS-1 oligonucleotide selectively reduced IRS-1 protein levels. IRS-1 was immunoprecipitated from equal amounts of cell lysates and PI 3-kinase activity was measured. Kinase activity in cells treated with sense oligonucleotides was increased following incubation with VEGF for 5 min (Figure 7A, compare lanes 2 and 1). Antisense IRS-1 appeared to increase basal PI 3-kinase activity, which may have been due to the non-specific presence of PI 4-kinase in the immunoprecipitates (Figure 7A, lane 3 versus lane 1). However, cells treated with VEGF for 5 min in the presence of antisense IRS-1 failed to show an increase in PI 3-kinase activity relative to control cells treated with antisense oligonucleotide alone (Figure 7A, lane 4 versus lane 3). Immunoblotting aliquots of cell lysates with antibody against IRS-1 showed reduced levels of the protein in antisense IRS-1 oligonucleotide-treated cells (Figure 7A, lower panel). These data confirm results shown in Figure 6(A), and suggest that decreased PI 3-kinase activity in antisense IRS-1 oligonucleotide-treated cells exposed to VEGF is due to reduced availability of that protein. Immunoprecipitating with antibody against IRS-1 and immunoblotting with antibody against the p85 component of PI 3-kinase showed that VEGF treatment in antisense IRS-1 oligonucleotide-treated cells was associated with reduced binding between IRS-1 and the p85 subunit (Figure 7B). Thus, reduced availability of IRS-1 to bind the p85 component of PI 3-kinase in antisense IRS-1 oligonucleotide-treated cells appears to account for the decreased association between the two proteins. PI 3-kinase activity in p85 immunoprecipitates showed that the VEGF-induced increase in the lipid kinase activity was intact in control and sense IRS-1-oligonucleotide treated cells; however, VEGF failed to stimulate kinase activity in antisense IRS-1 oligonucleotide-treated cells (Figure 7C). This was not due to a lack of availability of p85 in the lysates (Figure 7C, lower panel). Taken together, these data suggest that failure of VEGF to stimulate PI 3-kinase activity in antisense IRS-1-treated cells is due to a reduction in ambient levels of IRS-1 protein, leading to a decrease in the association between IRS-1 and the p85 component of PI 3-kinase, although the amounts of type 2 VEGF receptor and p85 proteins are unchanged.

PI 3-kinase activation by VEGF has several functional implications. PI 3-kinase activation is required for VEGF-stimulated endothelial cell proliferation [23] and angiogenesis [24]. In endothelial cells, VEGF-induced PI 3-kinase activation and an increase in downstream Akt activity are important for cell survival [7], and regulation of endothelial nitric oxide synthase activity [25,26]. Activation of PI 3-kinase is probably not needed for activation of some signalling pathways in VEGF-treated cells. For instance, VEGF stimulates the activity of ERK1/2 type mitogen-activated protein kinase in PTE cells that is independent of PI 3-kinase activation (D. Senthil and B. S. Kasinath, unpublished work), as has been shown previously in endothelial cells [9,10].

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VEGF activation of PI 3-kinase appears to be important for stimulation of *de novo* protein synthesis in PTE cells as it is abolished by LY294002, a PI 3-kinase inhibitor (D. Senthil and B. S. Kasinath, unpublished work). It is possible that IRS-1-mediated PI 3-kinase activity is involved in VEGF-induced stimulation of protein synthesis, as has been reported with insulin [27]. Accordingly, the regulatory importance of IRS-1 in VEGF stimulation of protein synthesis was examined. VEGF induced a significant increase in *de novo* protein synthesis in both control and IRS-1 sense oligonucleotide-treated cells ($P < 0.001$; Figure 7D). In antisense IRS-1 oligonucleotide-treated cells incubated with VEGF the rate of label incorporation was significantly lower than in sense IRS-1 oligonucleotide-treated cells incubated with VEGF ($P < 0.03$, see Figure 7D). These data demonstrate that recruitment of IRS-1 by the type 2 VEGF receptor facilitates an increase in protein synthesis stimulated by VEGF.

In summary, we have demonstrated for the first time that VEGF induces IRS-1 phosphorylation in endothelial and non-endothelial cells. We have further shown that following exposure of cells to VEGF, IRS-1 associates with the type 2 VEGF receptor complex and with the p85 subunit of PI 3-kinase. We have also provided evidence that VEGF stimulates association of IRS-1 with PI 3-kinase with an increase in the lipid kinase activity. Furthermore, we report that IRS-1 recruited by the type 2 receptor may participate in VEGF-stimulated protein synthesis, demonstrating a functional dimension to this association. Physical association between IRS-1 and type 2 VEGF receptor provides an efficient mechanism for amplification of VEGF effects on cell function. With IRS-1 serving as a docking protein, effects of VEGF may not be limited by endocytosis and metabolism of the type 2 VEGF receptor. Other biological events regulated by IRS-1 tyrosine phosphorylation by VEGF need further examination.

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