

## **Insulin-like Growth Factor Induces Phosphorylation of Immunoreactive Insulin Receptor Substrate and Its Association with Phosphatidylinositol-3 Kinase in Human Thymocytes**

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### **Summary**

Insulin receptor substrate 1 (IRS-1) is the principle cellular substrate for insulin and insulin-like growth factor I (IGF-I) receptor signaling. After phosphorylation of tyrosine residues within the YMXM or YXXM motifs, IRS-1 associates with phosphatidylinositol-3 kinase (PI3K). This signaling pathway and the presence of an IRS-1-like molecule have been demonstrated in IRS-1-transfected and in nontransfected hematopoietic cell lines, respectively. IGF-I has been implicated in lymphocyte development and function, and recently, we showed that functional type-I IGF receptors are present on human thymocytes and peripheral T cells. In this study, we addressed IGF-I signal transduction in nontransformed, freshly isolated, human thymocytes, as well as in blood T cells. Using Western blot analysis, we found that IGF-I induced phosphorylation of a 160–180-kD protein (pp170) in human thymocytes and that phosphorylated pp170 becomes associated with PI3K and is recognized by anti-IRS-1. In blood T cells, this immunoreactive IRS-1 (irIRS-1) is less abundantly expressed than in thymocytes, as assessed with immunoblotting. As a consequence, phosphorylated pp170 was not or hardly detectable after stimulation with IGF-I, and irIRS-1 was not detected in PI3K immunoprecipitates from lysates of IGF-I-stimulated T cells. However, IGF-I induced the tyrosine phosphorylation of other cellular proteins, indicating that differential expression of irIRS-1 contributes to a distinct signaling pathway in T cells.

**I**nsulin receptor substrate 1 (IRS-1) is a common element in insulin and type I insulin-like growth factor (IGF) receptor signaling. This cytosolic protein contains multiple potential serine, threonine, and tyrosine phosphorylation sites (1, 2) and undergoes tyrosine phosphorylation immediately after stimulation with insulin or IGF-I (1, 3–5). Several tyrosine-phosphorylated sites are included in the consensus sequences YMXM and YXXM, which represent recognition motifs for SH2 domain-containing target proteins. IRS-1 associates with phosphatidylinositol-3 kinase (PI3K) via the SH2 domains of the 85-kD regulatory subunit (1), resulting in the activation of the enzyme (6). In addition, IRS-1 is a docking protein for other regulatory enzymes such as growth factor receptor-bound protein 2 and SH2 containing tyrosine phosphatase 2 (7).

Recently, IRS-1 has been implicated in IL-4 and insulin signaling in hematopoietic cells that were transfected with IRS-1 (8, 9). Furthermore, IL-4, insulin, and IGF-I induced phosphorylation of an IRS-1-like molecule, 4PS, in IL-3-dependent murine hematopoietic cell lines and its associa-

tion with PI3K (8). In addition, IL-7 activates PI3K in human thymocytes (10). The use of IRS-1-like molecules and PI3K as common elements in IGF-I and cytokine signaling might explain the cytokine-like activity of IGF-I on lymphocyte development and function (11–14). The signal transduction pathways for IGF-I have been studied mainly in cell lines or transfected cell lines expressing high levels of receptors or IRS-1. In this study, we addressed the role of IRS-1 in IGF-I signaling in freshly isolated human thymocytes and mature peripheral T cells.

We have demonstrated previously that functional type-I IGF receptors are expressed on all developmental stages of human thymocytes and on the majority of peripheral T cells (14, 15). The *in vitro* proliferation of both thymocytes (15a) and peripheral T cells (16–19) is stimulated by IGF-I. Others showed that the presence of type I IGF receptors in T cells is essential for progression to the S phase of the cell cycle (20). In this study, we demonstrate that immunoreactive IRS-1 (irIRS-1) is the main tyrosine kinase substrate in IGF-I signaling in thymocytes, which, after stimulation, associates with

PI3K. However, in peripheral T cells, *irIRS-1* is less abundantly expressed and, in contrast to several other cellular proteins, not or hardly phosphorylated after IGF-I stimulation.

## Materials and Methods

**Reagents.** Recombinant human IGF-I was a gift from Dr. Jeantran (Lilly Research Laboratories, Indianapolis, IN). A polyclonal Ab to IRS-1 was kindly provided to us by Dr. J. A. Maassen (Department of Medical Biochemistry, University of Leiden, The Netherlands). This Ab is directed against a peptide corresponding to the amino acid sequence 581–860 of rat IRS-1. Rabbit anti-PI3K, directed against the p85 subunit, was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and antiphosphotyrosine (anti-PY, clone PY20) was from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated donkey anti-rabbit IgG, peroxidase-conjugated sheep anti-mouse IgG, and nitrocellulose membranes (Hybond C, 0.45  $\mu$ m) were obtained from Amersham International (Amersham, UK). Protein A was purchased from Pharmacia (Uppsala, Sweden).

**Cells.** Thymic tissue was obtained from patients undergoing cardiac surgery. The patients ranged in age from 5 d to 5.5 yr. The tissue was cut into small pieces in MEM, and single cell suspensions were obtained by pressing these pieces against a stainless steel mesh. Dead cells and erythrocytes were removed by centrifugation on Ficoll-Paque (Pharmacia) density gradients (1.077 g/ml) at 1,000 *g* for 20 min at room temperature. PBMCs were purified from heparinized blood from healthy adult donors by centrifugation on Ficoll-Paque, as described for thymocytes. T cells were isolated from PBMC by rosetting with sheep erythrocytes that were pretreated with 2-aminoethyl-isothiuronium bromide (Sigma Chemical Co., St. Louis, MO) (21).

**Cell Stimulation.** Purified thymocytes and T cells ( $10^8$  cells/ml) were taken into MEM containing 0.2% BSA and preincubated at 37°C for 1 h. After incubation with  $10^{-8}$  M IGF-I, 1 ml ice-cold PBS was added to 0.4 ml cell suspension, and the cells were centrifuged for 30 s at 10,000 *g* at 5°C.

**Immunoprecipitation.**  $40 \times 10^6$  cells were lysed in 100  $\mu$ l ice-cold lysis buffer (1% digitonin, 10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 10 mM NaF, 0.4 mM EDTA, 0.4 mM  $\text{Na}_3\text{VO}_2$ , 0.1 mM phenyl-methylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptide, and 1  $\mu$ g/ml  $\alpha$ 1-antitrypsin) at 5°C for 15 min. Insoluble material was pelleted at 10,000 *g* for 15 min at 5°C, and supernatants were incubated with anti-PI3K (1:20) for 2 h at 5°C. Immunocomplexes were precipitated with protein A beads (1% vol/vol). Immunoprecipitates were subsequently washed six times with buffer (containing 0.2% digitonin and otherwise of the same composition as lysis buffer) and denatured in Laemmli sample buffer containing 2-ME (22).

**Immunoblotting.** For detection of tyrosine phosphorylated proteins, IRS-1 and PI3K in thymocytes and T cells, whole cells were solubilized in Laemmli sample buffer containing 2-ME. Proteins were resolved by SDS-PAGE using a 4% stacking gel and a 7% or 10% separating gel (100  $\times$  70  $\times$  1 mm) at 100 V, and transferred to nitrocellulose membranes in Towbin buffer containing 20% methanol (23) using a semi-dry transfer cell (Bio-Rad Laboratories, Richmond, CA) at 0.8 mA/cm<sup>2</sup> for 2 h at room temperature. The membranes were blocked overnight at 5°C in TBS (25 mM Tris, pH 7.5, 125 mM NaCl) containing 3% BSA (Promega Corp., Madison, WI). Blots were incubated with anti-PY (0.4  $\mu$ g/ml) in TBS containing 0.02% Tween 20 and 1% BSA for 2 h at 5°C. Subsequently, the blots were washed twice for 10 min in

TBS containing 0.02% Tween 20 and incubated with peroxidase-conjugated anti-mouse IgG (1:20,000). After two washing steps, the blots were exposed to detection solution for enhanced chemical luminescence and autoradiographed according to the manufacturers procedure (Amersham International). Subsequently, antibodies were removed by stripping in 62 mM Tris, pH 6.7, containing 2% SDS and 100 mM  $\beta$ -mercaptoethanol for 30 min at 50°C. After re-blocking in TBS/3% BSA (Sigma Chemical Co.), blots were incubated with anti-IRS-1 (1:1000) in TBS/1% BSA and stained with peroxidase-conjugated anti-rabbit IgG (1:20,000). After detection, blots were stripped, reblocked, and immunoblotted with anti-PI3K (1:2,000) according to the same procedure. The specificity of anti-PY was confirmed by inhibiting binding with phenylphosphate.

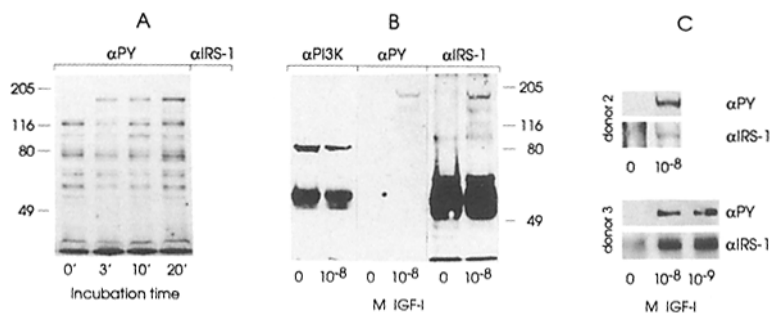
## Results and Discussion

**Tyrosine Phosphorylation of *irIRS-1* and Association with PI3K in Thymocytes.** We first investigated the effect of IGF-I on the tyrosine phosphorylation state of cellular proteins in thymocytes. It appeared that a high molecular weight protein, which was not detectable with anti-PY before incubation with IGF-I, was markedly phosphorylated within 3 min of incubation. The degree of phosphorylation of this protein was further increased after 10 and 20 min (Fig. 1 A). This result was confirmed with cells from three donors. In thymocytes obtained from two other donors, the phosphorylation at 10 min was decreased compared with that after 3 min of incubation (data not shown).

A high molecular weight band, designated pp170, which was detected by anti-IRS-1, comigrated with a phosphorylated band at 160–180 kD (Fig. 1 A). This is in accordance with other observations showing that IRS-1 migrates as a 165–185-kD protein on a PAGE gel (3, 4, 24). To test whether pp170 associated with PI3K after stimulation with IGF-I, cell lysates were immunoprecipitated with anti-PI3K antibodies and immunoblotted with anti-PY, anti-IRS-1, and anti-PI3K. Fig. 1 B shows that approximately equal amounts of the p85 subunit of PI3K were precipitated. Furthermore, pp170 was detected with both anti-PY and anti-IRS-1 after a 5-min stimulation with IGF-I. In contrast, pp170 was not detected in anti-PI3K immunoprecipitates with anti-PY or anti-IRS-1 before stimulation with IGF-I (Fig. 1 B). As depicted in Fig. 1 C, coprecipitation of pp170 with PI3K was also markedly increased after stimulation with IGF-I in thymocytes from two other donors.

These results indicate that IGF-I induces the association of PI3K with *irIRS-1* in human thymocytes. Furthermore, *irIRS-1* is the major tyrosine-phosphorylated substrate in response to IGF-I. The presence of an IRS-1-like molecule, 4PS, has been demonstrated in the IL-3-dependent murine hematopoietic cell lines FDC-P1 and FDC-P2. 4PS was phosphorylated after stimulation with IGF-I, insulin, or IL-4, and it appeared to be similar but not identical to IRS-1 as assessed by V8 protease digestion (8). Further investigations are necessary to identify *irIRS-1* in human thymocytes and to address the role of this molecule in cytokine signaling.

**Tyrosine Phosphorylation in Peripheral T Cells.** Because several functions of mature T cells can be influenced by IGF-I (16,



**Figure 1.** IGF-I-induced tyrosine phosphorylation and association of irIRS-1 with PI3K in thymocytes. (A) Tyrosine phosphorylation.  $40 \times 10^6$  cells were incubated in the absence of IGF-I or in the presence of  $10^{-8}$  M IGF-I. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-PY ( $\alpha$ PY) or anti-IRS-1 ( $\alpha$ IRS-1). (B) Association of irIRS-1 with PI3K in thymocytes from donor 1. Cells were incubated for 10 min with or without  $10^{-8}$  M IGF-I. Cell lysates were immunoprecipitated with antibodies to PI3K ( $\alpha$ PI3K). Precipitated proteins were revealed by Western blotting and antibodies to PY, IRS-1, and PI3K. (C) Association of irIRS-1 with PI3K in thymocytes from donors 2 and 3. Thymo-

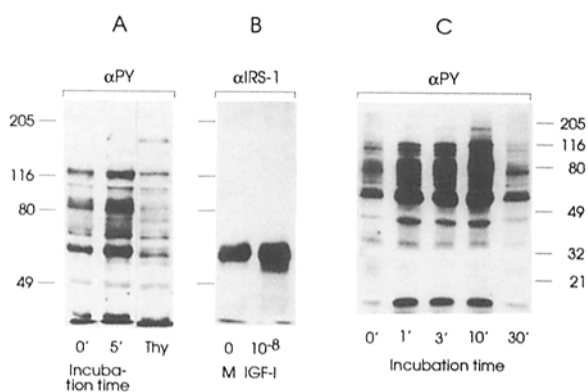
cytes were incubated with different concentrations of IGF-I, and cell lysates were immunoprecipitated with  $\alpha$ PI3K as described for Fig. 1 B. The irIRS-1 in anti-PI3K immunoprecipitates was stained with  $\alpha$ PY or  $\alpha$ IRS-1. Molecular masses of prestained marker proteins (Bio Rad Laboratories) are indicated in kilodaltons.

17), we also addressed the role of IRS-1 in purified peripheral blood T cells. We demonstrated that the majority of blood T cells possess approximately the same number of type I IGF receptors with the same  $K_d$  as human thymocytes (14; Kooijman, R., et al., manuscript submitted for publication). As depicted in Fig. 2 A, IGF-I increased the phosphorylation of several proteins. However, tyrosine phosphorylation of pp170 could not be detected in peripheral T cells after stimulation with IGF-I (Fig. 2 A). Even when higher concentrations of IGF-I ( $10^{-7}$  M) and longer incubation times (up to 30 min) were used, phosphorylated pp170 was not detected (data not shown). Furthermore, irIRS-1 was not found in immunoprecipitates with anti-PI3K after stimulation with IGF-I (Fig. 2 B). To address the ability of IGF-I to induce tyrosine phosphorylation of pp170 in blood T cells, immunoblots were also incubated with an alternative anti-PY Ab, AB-2. Using this Ab, we detected a relatively weak phosphorylation of pp170 in IGF-I-stimulated T cells from two out of four donors (Fig. 2 C). It should be noted that IGF-I

incubation of peripheral T cells does increase the tyrosine phosphorylation state of a number of cellular proteins. This effect is already evident after 1 min of incubation and includes phosphoproteins of 55, 85, and 130 kD (Fig. 2, A and C).

Overall, these data indicate that in peripheral blood T cells, IRS-1 is not a principle substrate in IGF-I signaling but that other as yet not defined signaling routes mediate the effects of IGF-I in T cell function. The identity of the tyrosine kinase substrates in T cells remains to be established. Potential candidate molecules are the substrates that are tyrosine phosphorylated by the insulin receptor such as the p85 subunit of PI3K (25). However, the phosphorylated 85-kD protein in peripheral T cells is not the p85 subunit of PI3K because it was not detected in anti-PI3K immunoprecipitates with anti-PY antibodies (data not shown). The 55-kD phosphoprotein in T cells might be one of the 52–62 kD proteins that are tyrosine phosphorylated in insulin-treated cells. In Chinese hamster ovary cells, the 52-kD SH<sub>2</sub>-containing protein (SHC) was phosphorylated in response to insulin (26). Furthermore, insulin-induced phosphorylation of 60–62-kD proteins was found in Chinese hamster ovary cells and in rat adipocytes. These proteins were associated either with GTPase-activating protein (26–28) or with PI3K (27, 29). Notably, the 55-kD protein that was phosphorylated in response to IGF-I in T cells was not detected in PI3K immunoprecipitates (data not shown). Finally, the 130-kD substrate may be an ecto-ATPase (pp120/HA4) that can be phosphorylated by the insulin receptor in hepatoma cells (30, 31). Interestingly, extracellular ATPase activity has been found on human peripheral T cells (32). Furthermore, tyrosine phosphorylation of substrates with a comparable molecular weight occurred after stimulation of T cells via the CD2 or CD3 pathway (33, 34).

*Expression of irIRS-1 and PI3K in Thymocytes and T Cells.* The low or absent induction of tyrosine-phosphorylated pp170 in peripheral T cells could either be the result of a low cellular expression of IRS-1 or, alternatively, of the inability of IGF-I to induce IRS-1 phosphorylation. We therefore compared the expression of this molecule in T cells with its expression in thymocytes. It appeared that there was much less irIRS-1 in T cells compared with thymocytes. Whereas irIRS-1 was always detected in whole cell lysates of thymocytes, in T cells, irIRS-1 was hardly or not detectable (Fig.

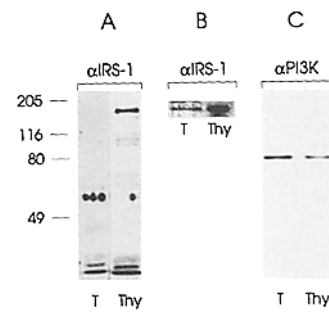


**Figure 2.** IGF-I-induced tyrosine phosphorylation in human peripheral blood T cells and precipitation of proteins with anti-PI3K. (A) Phosphorylation status of cellular proteins before and after stimulation of T cells with IGF-I. Phosphorylated cellular proteins from stimulated thymocytes were run on the same gel (Thy). (B) Precipitation with anti-PI3K. Experiments were performed as described in Fig. 1. The data in Fig. 2, A and B, are representative of four independent experiments. (C) Phosphorylation status of cellular proteins before and after stimulation with IGF-I as measured with the AB-2 anti-PY Ab (0.4  $\mu$ g/ml; Oncogene Science, Manhasset, NY). With this Ab, phosphorylation of pp170 was detected in two out of four independent experiments.

3). The weak band that was detected in T cells from two out of four donors suggests that the phosphorylated pp170 in Fig. 2 C is IRS-1 or an IRS-1-like tyrosine kinase substrate. In contrast to the expression of IRS-1, the level of PI3K in T cells was higher than in thymocytes (Fig. 3 C).

Taken together, our data demonstrate that IGF-I signaling in thymocytes differs from the signaling pathway in peripheral T cells. In thymocytes, pp170 is the principle tyrosine kinase substrate, whereas in T cells, pp170 is either not phosphorylated or only a minor substrate. This difference appears to be a consequence of differential expression of irIRS-1 in thymocytes and T cells. Although we did not find any differences in irIRS-1 expression or in IGF-I signaling between thymocytes from 5-d-old and 5-yr-old children, we cannot formally exclude the possibility that the differences in signaling between thymocytes and T cells are age related rather than dependent on the developmental T cell stage.

The differential effects of IGF-I on tyrosine phosphorylation in thymocytes and T cells are remarkable because IGF-I can stimulate the proliferation of both thymocytes and T cells (15a, 19). Possibly, a low level of irIRS-1 phosphorylation is sufficient for the effects in T cells. Alternatively, the stimulating effect of IGF-I might be effected at different control points of the cell cycle. For instance, we found that IGF-I potentiates the transition of T cells from the G<sub>0</sub> to the G<sub>1</sub> phase of the cell cycle, and not the proliferation of IL-2-de-



**Figure 3.** Expression of irIRS-1 and PI3K in thymocytes and peripheral blood T cells. 10<sup>6</sup> cells were resolved in sample buffer and subjected to SDS-PAGE. IRS-1 and PI3K were detected by immunoblotting and incubation with antibodies to IRS-1 and PI3K. The expression of immunoreactive IRS-1 was variable and detected in T cells from two of five donors. (A) Donor without detectable IRS-1. (B) Donor with low levels of IRS-1. In Fig. 3 B, the contrast

was enhanced using a computer program (Photopaint; Corel Corp., Ottawa, Canada). (C) PI3K was always present in peripheral T cells from all five donors tested.

pendently growing T cells (Kooijman, R., et al., manuscript in preparation for publication). On the other hand, in thymocytes, IGF-I does stimulate the proliferation of cycling cells (15a).

Type-I IGF receptors are differentially expressed on different activation stages of peripheral T cells (15), indicating that responsiveness for IGF-I in different maturation and activation stages can be regulated by differential receptor expression. The data presented here are suggestive of differential postreceptor signaling pathways in different T cell stages.

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