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Insulin-dependent Tyrosine Phosphorylation of the *vav* Proto-oncogene Product in Cells of Hematopoietic Origin*

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Shahab Uddin‡§, Shulamit Katzav¶‖, Morris F. White, and Leonidas C. Platanias‡§ ‡‡**

*From the ‡Division of Hematology-Oncology, Loyola University of Chicago, Maywood, Illinois 60153, the §Hines Veterans Administration Medical Center, Hines, Illinois 60141, the ¶Terry Fox Molecular Oncology Group, Lady Davis Institute, Jewish General Hospital, Montreal H3T 1E2, Canada, the ‖Departments of Oncology and Medicine, McGill University, Montreal H3G 1Y6, Canada, and the **Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215*

Insulin activates the *ras* signaling pathway and promotes hematopoietic cell proliferation. One possible mediator in such signaling is the *vav* proto-oncogene product ($p95^{vav}$), which is specifically expressed in cells of hematopoietic origin and contains domains typical of guanine nucleotide exchange factors as well as Src homology 2 and Src homology 3 domains. We studied the tyrosine phosphorylation of $p95^{vav}$ in hematopoietic cells expressing insulin receptors. Immunoblotting experiments with an antiphosphotyrosine monoclonal antibody disclosed that insulin induces rapid and transient tyrosine phosphorylation of $p95^{vav}$ in the human U-266 myeloma cell line. These findings were confirmed by immunoprecipitation experiments performed with ^{32}P-labeled cells and phosphoamino acid analysis of the bands corresponding to $p95^{vav}$. Similarly, insulin-dependent tyrosine phosphorylation of $p95^{vav}$ was observed in the human IM-9 and mouse J558L hematopoietic cell lines. Furthermore, insulin treatment of cells led to the association of the Src homology 2 domain of $p95^{vav}$ with the activated β-subunit of the insulin receptor *in vitro*. Altogether, these data suggest that $p95^{vav}$ is a substrate for the insulin receptor tyrosine kinase and may be involved in an insulin signaling pathway linking receptor-generated signals to Ras or other GTP-binding proteins in cells of hematopoietic origin.

The *vav* proto-oncogene product ($p95^{vav}$) is a tyrosine kinase substrate that is specifically expressed in cells of hematopoietic origin and has regions of homology to *bcr*, *dbl*, and the yeast CDC24 guanine exchange factor as well as one SH2[1] and two SH3 domains (1–8). $p95^{vav}$ undergoes rapid tyrosine phosphorylation in response to a variety of stimuli in different cell types: in T-cells after activation of the T-cell antigen receptor or interleukin-2 stimulation (7–9); in B-cells after activation of IgM receptors (10); in activated mast cells after stimulation of the IgE receptor (8); in stem cell factor responsive cells after stimulation of the c-*kit* receptor (11); and in hematopoietic cell lines in response to interferon-α, -β, and -ω (12). Although *vav* was predicted to function as a guanine nucleotide exchange factor for Rho-like proteins, it was recently demonstrated that it exhibits guanine nucleotide exchange activity toward *ras* during T-cell activation (13), in NIH-3T3 cells transfected with the *vav* gene (14), and in antigen receptor-triggered B-cells (15).

Insulin exhibits mitogenic effects in a variety of hematopoietic cells, including myeloid (16), multiple myeloma (17), and lymphoblastoid (18) cell lines. After insulin treatment of cells, the β-subunit of the insulin receptor tyrosine kinase is activated, resulting in tyrosine phosphorylation of several cellular substrates (19). A major substrate for the insulin receptor tyrosine kinase is IRS-1 (19), which acts as a docking protein for the SH2 domains of several proteins involved in insulin signaling, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (20–23), the phosphotyrosine phosphatase Syp (24), the oncogenic protein Nck (25), and the adaptor protein Grb-2 (26–28). In this study, we examined the effect of insulin on the phosphorylation status of $p95^{vav}$. We report that $p95^{vav}$ is tyrosine-phosphorylated in response to insulin in several hematopoietic cell lines expressing the insulin receptor. We also demonstrate that $p95^{vav}$ associates via its SH2 domain with the β-subunit of the insulin receptor *in vitro*, providing evidence of direct interaction of $p95^{vav}$ with this receptor tyrosine kinase.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The human myeloma U-266 and IM-9 cell lines were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) or 10% (v/v) defined calf serum (Hyclone Laboratories, Logan, UT) and antibiotics. The mouse plasmacytoma J558L cell line (kindly provided by Dr. Hans Martin Jack, Loyola University) was grown in RPMI 1640 medium (Life Technologies, Inc.) with 10% (v/v) defined calf serum and antibiotics. The antiphosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). A rabbit polyclonal antibody against a peptide corresponding to residues 576–589 of the mouse Vav protein (identical to residues 528–541 of the human Vav protein) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An antibody against a synthetic peptide of a sequence present in the C terminus of the β-subunit of the insulin receptor was kindly provided by Dr. R. C. Kahn (Joslin Diabetes Center) and was used for immunoprecipitations. A polyclonal antibody against the β-subunit of the insulin receptor was purchased from Transduction Laboratories (Lexington, KY) and was used for immunoblotting.

Immunoprecipitations and Immunoblotting—Cells were stimulated with insulin (1 μM) for the indicated periods of time. IM-9 and J558L cells were serum-starved by incubation in serum-free RPMI 1640 medium at 37 °C for 1–2 h immediately prior to insulin stimulation. After insulin stimulation, the cells were rapidly centrifuged and lysed in phosphorylation lysis buffer (0.5–1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 200 μM sodium orthovanadate, 50 mM Hepes, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM magnesium chloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aproti-

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‡‡ Recipient of a career development award from the American Cancer Society. To whom correspondence should be addressed: Div. of Hematology-Oncology, Loyola University Chicago, Bldg. 112, 2160 South First Ave., Maywood, IL 60153. Tel.: 708-327-3304; Fax: 708-216-2319.

[1] The abbreviations used are: SH, src homology; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis.

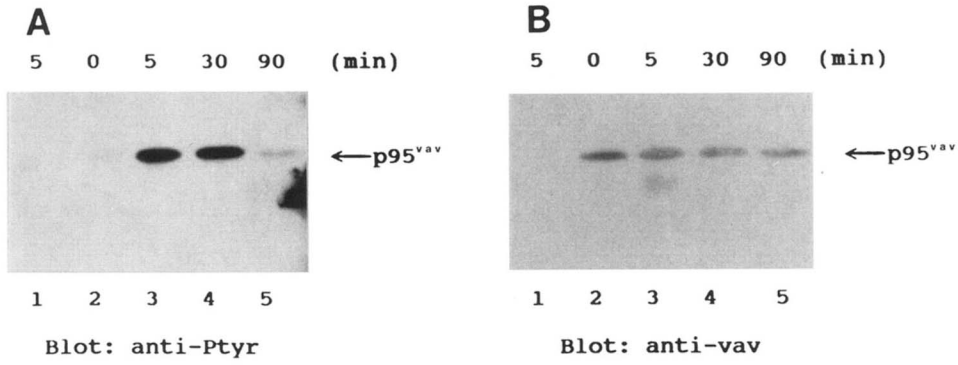

FIG. 1. **Insulin-dependent tyrosine phosphorylation of p95^{vav}.** U-266 cells were stimulated with insulin for the indicated times at 37 °C. Cell lysates were immunoprecipitated with either nonimmune rabbit immunoglobulin (*lane 1*) or an antibody against p95^{vav} as indicated (*lanes 2–5*). *A*, shown is the antiphosphotyrosine (*anti-Ptyr*) immunoblot. The tyrosine-phosphorylated form of p95^{vav} is indicated. *B*, the same blot was reprobed with an antibody against p95^{vav} to establish that equal amounts of p95^{vav} were present in all lanes.

nin). Lysates obtained from 1–5 × 10^7 cells were immunoprecipitated with a polyclonal antibody against p95^{vav}, with control purified rabbit immunoglobulin (Sigma), or with an antibody against the β-subunit of the insulin receptor. Immunoprecipitates were washed five times with phosphorylation lysis buffer containing 0.1% Triton X-100 and analyzed by SDS-PAGE. In some experiments, the cells were lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM NaF, 100 μM sodium orthovanadate, 10 μg/ml aprotinin, and 0.2–1 mM phenylmethylsulfonyl fluoride), immunoprecipitated with an antibody against the β-subunit of the insulin receptor, washed five times in RIPA buffer without sodium deoxycholate, and analyzed by SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore Corp.), and the residual binding sites on the filters were blocked by incubating with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20)/10% bovine serum albumin) for 1–3 h at room temperature or overnight at 4 °C. The filters were subsequently incubated with antiphosphotyrosine, washed with TBST, and developed using an enhanced chemiluminescence kit (ECL, Amersham Corp.) following the manufacturer's recommended procedure.

Preparation of Glutathione S-Transferase Fusion Proteins—Thr construction of the pGEX-2TK SH2 expression plasmid containing *vav*SH2 (nucleotides 2356–2673) has been previously described (29). Fusion proteins were purified from transformed *Escherichia coli* bacteria that were induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After 2 h of additional growth, bacteria were lysed by sonication in phosphate-buffered saline, pH 7.0. Lysed bacteria were spun for 25 min at 14,000 rpm at 4 °C, and the supernatant was immobilized on glutathione-Sepharose beads (Pharmacia Biotech Inc.).

Labeling of Cells with [^{32}P]Orthophosphoric Acid—U-266 cells were washed two times with phosphate-free RPMI 1640 medium and incubated for 30 min at 37 °C in phosphate-free medium. The cells were subsequently incubated for 4 h in phosphate-free medium with carrier-free [^{32}P]orthophosphoric acid (DuPont NEN) at a concentration of 0.1 mCi/ml. The labeled cells were stimulated with insulin (1 μM) for the indicated times and lysed in phosphorylation lysis buffer. Lysates obtained from 8 × 10^7 cells were immunoprecipitated either with an antibody against p95^{vav} or with control nonimmune rabbit immunoglobulin, washed five times in phosphorylation lysis buffer, and analyzed by SDS-PAGE.

In Vitro Kinase Assays—Cells were serum-starved for 2 h in serum-free RPMI 1640 medium at 37 °C. After insulin treatment, the cells were lysed either in phosphorylation lysis buffer (for immunoprecipitations with the anti-insulin receptor antibody) or in RIPA buffer (in the experiments with glutathione *S*-transferase proteins). Cell lysates were either immunoprecipitated with an antibody against the β-subunit of the insulin receptor or incubated with a GST-*vav*SH2 fusion protein or control glutathione *S*-transferase protein bound to glutathione-Sepharose beads as indicated. Immunoprecipitates of the insulin receptor on protein G-Sepharose beads or of protein complexes on glutathione-Sepharose beads were washed three times with phosphorylation lysis buffer (containing 0.1% Triton X-100) or RIPA buffer (without sodium deoxycholate), respectively, and two times with *in vitro* kinase buffer (12 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 5 mM sodium orthovanadate, and 150 mM NaCl). The immunocomplex-protein G-Sepharose beads or the protein complex-glutathione-Sepharose beads were resuspended in 30 μl of *in vitro* kinase buffer to which $MnCl_2$ at a final concentration of 2.5 mM and 10–20 μCi of [γ-^{32}P]ATP were added. The beads were incubated for 30 min at room temperature, and the reaction was terminated by adding loading buffer.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described previously (12, 30, 31).

RESULTS

Insulin Induces Tyrosine Phosphorylation of p95^{vav}—We initially performed studies to determine whether p95^{vav} is tyrosine-phosphorylated in response to insulin in the human U-266 myeloma cell line. Cells were treated with insulin, and cell lysates were immunoprecipitated with a polyclonal antibody against p95^{vav} and immunoblotted with an antiphosphotyrosine monoclonal antibody (4G10). Fig. 1*A* shows that p95^{vav} is tyrosine-phosphorylated within 5 min of insulin treatment of the cells and that the signal diminishes after 90 min of insulin treatment, demonstrating that its phosphorylation on tyrosine is rapid and transient. Reprobing of the same blot with the anti-p95^{vav} polyclonal antibody demonstrated that equal amounts of p95^{vav} were present in the immunoprecipitates of insulin-treated and -untreated cells (Fig. 1*B*). Insulin-dependent tyrosine phosphorylation of p95^{vav} was also observed when the IM-9 and J558L hematopoietic cell lines were studied (data not shown). We subsequently studied the expression and tyrosine phosphorylation of the β-subunit of the insulin receptor in these cell lines. The β-subunit of the insulin receptor was expressed and tyrosine-phosphorylated in response to insulin in U-266, J558L, and IM-9 cells (data not shown). *In vitro* kinase assays performed in IM-9 and J558L cells confirmed that the tyrosine phosphorylation of the β-subunit was due to autophosphorylation (data not shown; see also Fig. 4). As the β-subunit of the insulin receptor has a molecular mass similar to that of p95^{vav} (95 kDa), we considered the possibility that the phosphorylated band detected in anti-p95^{vav} immunoprecipitates may correspond to the β-subunit of the insulin receptor (which might have been coimmunoprecipitated by the anti-p95^{vav} antibody). This was unlikely as in antiphosphotyrosine immunoblots, the characteristics of the bands precipitated by anti-p95^{vav} or anti-insulin receptor antibodies are different (*vav* migrates as a sharp band, while the insulin receptor migrates as a diffuse band). However, as these proteins are a similar size, they could not be distinguished by antiphosphotyrosine immunoblotting of total lysates from insulin-stimulated U-266 cells.[2] To address this issue, we performed experiments in which immunoprecipitates obtained with the anti-p95^{vav} antibody were immunoblotted with an antibody against the insulin receptor. Such experiments failed to demonstrate coimmunoprecipitation of the insulin receptor by the anti-p95^{vav}

[2] S. Uddin and L. C. Platanias, unpublished observations.

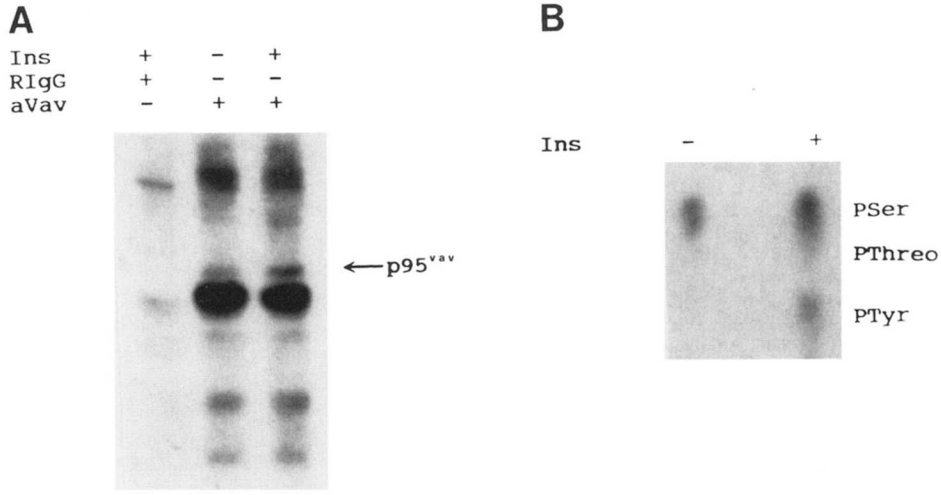

FIG. 2. **Analysis of the phosphoamino acid content of p95^{vav} before and after insulin stimulation.** *A*, ^{32}P-labeled U-266 cells (8×10^7/lane) were incubated in the presence or absence of insulin (*Ins*) for 10 min at 37 °C as indicated. Cell lysates were immunoprecipitated with an antibody against p95^{vav} (*aVav*) or control nonimmune rabbit IgG (*RIgG*) as indicated. *B*, shown are the results from phosphoamino acid analysis of p95^{vav} prior to and after insulin treatment. *PSer*, phosphoserine; *PThreo*, phosphothreonine; *PTyr*, phosphotyrosine.

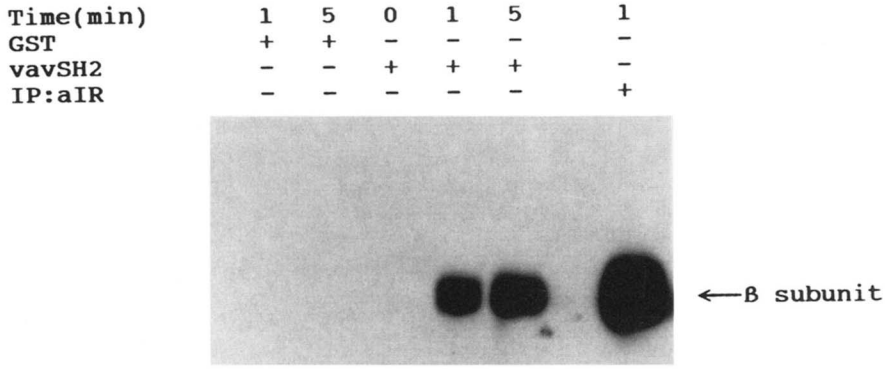

FIG. 3. **p95^{vav} associates via its SH2 domain with the β-subunit of the insulin receptor.** Serum-starved IM-9 cells (8×10^7/lane) were treated with insulin for the indicated times at 37 °C. The cells were lysed in denaturing RIPA buffer, and cell lysates were incubated with either a GST-*vav*SH2 fusion protein or glutathione *S*-transferase (*GST*) alone bound to glutathione-Sepharose beads or were immunoprecipitated (*IP*) with an antibody against a region of the C terminus of the β-subunit of the insulin receptor (*aIR*) as indicated. Proteins were separated by SDS-PAGE and immunoblotted with an antibody against the β-subunit of the insulin receptor.

antibody, excluding such a possibility (data not shown).

Analysis of the Phosphoamino Acid Content of p95^{vav} after Insulin Stimulation—To further characterize the phosphorylation of p95^{vav} in response to insulin, experiments were performed with ^{32}P-labeled U-266 cells. Cells were labeled with [^{32}P]orthophosphate, cell lysates were immunoprecipitated with the anti-p95^{vav} antibody, and the phosphoamino acid content of p95^{vav} was examined before and after insulin stimulation of the cells. Fig. 2*A* shows that p95^{vav} is phosphorylated prior to insulin treatment and that its phosphorylation increases significantly after insulin stimulation of the cells. Phosphoamino acid analysis of the bands corresponding to p95^{vav} demonstrated that significant levels of phosphorylation of p95^{vav} on serine residues were present at the base line in these cells, consistent with our previous findings (12). After insulin treatment of the cells, significant levels of phosphorylation on tyrosine were detectable, confirming the immunoblotting findings (Fig. 2*B*).

p95^{vav} Associates via Its SH2 Domain with the β-Subunit of the Insulin Receptor—As the SH2 domain of *vav* has been previously shown to associate with other tyrosine kinase receptors (7, 8), we sought to determine whether it also associates with the β-subunit of the insulin receptor tyrosine kinase. IM-9 cells were treated with insulin for the indicated times, and cell lysates were incubated with a GST-*vav*SH2 fusion protein or glutathione *S*-transferase alone bound to glutathione-Sepharose beads. The protein complexes were subsequently analyzed by SDS-PAGE and immunoblotted with an antibody against the insulin receptor (Fig. 3). Our results clearly demonstrate that the GST-*vav*SH2 protein associates with the β-subunit of the insulin receptor from cell lysates of insulin-stimulated cells (Fig. 3). To confirm that this association occurs with the activated form of the β-subunit, *in vitro* kinase assay experiments were performed. J558L cells were treated with insulin, and cell lysates were incubated with the GST-*vav*SH2 fusion protein bound to glutathione-Sepharose beads. *In vitro* kinase assays were subsequently performed in the protein complexes. The GST-*vav*SH2 fusion protein associated with a 95-kDa protein exhibiting *in vitro* kinase activity, corresponding to the autophosphorylated form of the insulin receptor β-subunit (Fig. 4*A*). Phosphoamino acid analysis of the autophosphorylated β-subunit associated with the SH2 domain of *vav* confirmed that its phosphorylation is primarily on tyrosine residues (Fig. 4*B*). Altogether, these findings suggested that *vav* associates via its SH2 domain with the activated form of the insulin receptor tyrosine kinase. They did not, however, exclude the possibility that this interaction requires adaptor proteins of the IRS signaling family. To address this issue, we performed studies to determine if *vav* is tyrosine-phosphorylated in 32D myeloid cells transfected with a full-length cDNA for the β-subunit of the insulin receptor tyrosine kinase. These cells have been previously shown to lack expression of IRS-1 and the interleukin-4-induced phosphotyrosine substrate (4PS) (34). Fig. 5*A* shows that p95^{vav} is tyrosine-phosphorylated in response to insulin stimulation of these cells. When proteins bound to the *vav*SH2 fusion protein were immunoblotted with antiphosphotyrosine, we observed that the SH2 domain of *vav* associated with a 95-kDa tyrosine-phosphorylated protein in an insulin-dependent manner (Fig. 5*B*). Reprobing of the same blot with an antibody against the β-subunit of the insulin receptor tyrosine kinase confirmed that this protein corresponds to the β-subunit (Fig. 5*C*).

DISCUSSION

The mechanisms of insulin signal transduction have been extensively studied (19). After insulin binds to its receptor, there is activation of the intrinsic tyrosine kinase activity pre-

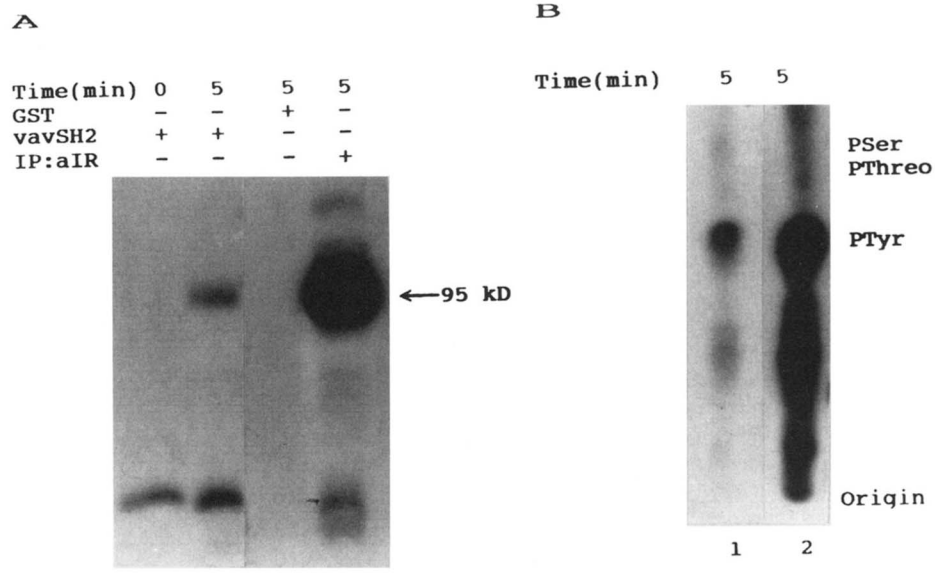

FIG. 4. **Association of the SH2 domain of p95^{vav} with the activated β-subunit of the insulin receptor detected in *in vitro* kinase assays.** *A*, cells (8×10^7/lane) were treated with insulin for the indicated time points, and cell lysates were incubated with either glutathione *S*-transferase (*GST*) alone or GST-*vav*SH2 bound to glutathione-Sepharose beads or were immunoprecipitated (*IP*) with an antibody against the β-subunit of the insulin receptor (*aIR*) as indicated and subjected to an *in vitro* kinase assay. *B*, shown are the results from phosphoamino acid analysis of the autophosphorylated insulin receptor associating with *vav*SH2 (*lane 1*) or immunoprecipitated by an anti-insulin receptor antibody (*lane 2*). *PSer*, phosphoserine; *PThreo*, phosphothreonine; *PTyr*, phosphotyrosine.

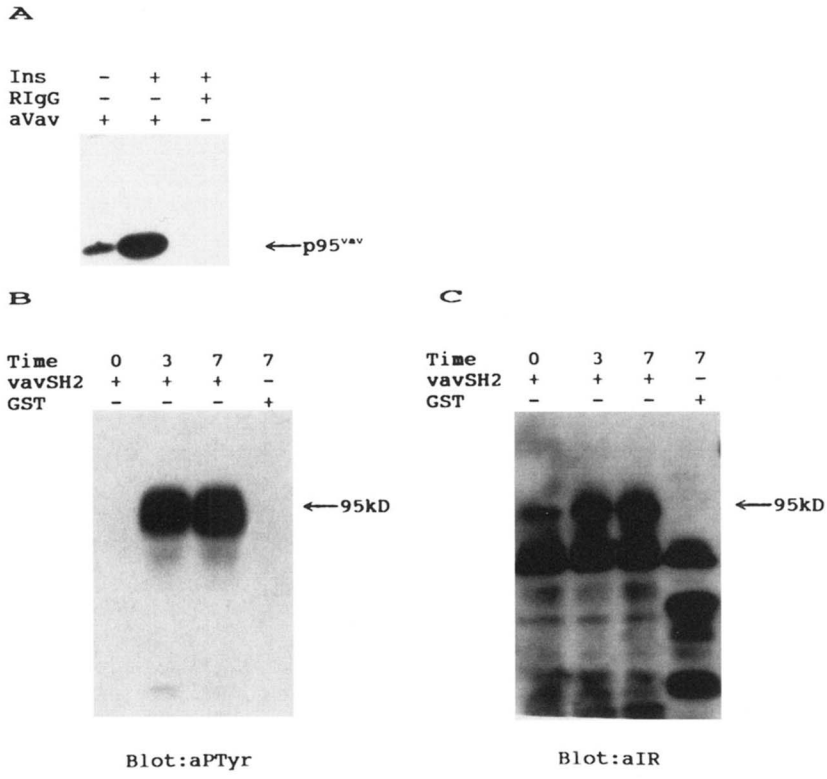

FIG. 5. **Tyrosine phosphorylation of p95^{vav} in 32D myeloid cells transfected with the β-subunit of the insulin receptor.** *A*, serum-starved cells were incubated in the presence or absence of insulin (*Ins*) for 10 min at 37 °C, and cell lysates were immunoprecipitated with an antibody against p95^{vav} (*aVav*) or control nonimmune rabbit IgG (*RIgG*) as indicated and immunoblotted with antiphosphotyrosine. *B*, serum-starved cells were treated with insulin as indicated and lysed in denaturing RIPA buffer, and cell lysates were incubated with either glutathione *S*-transferase (*GST*) alone or GST-*vav*SH2 bound to glutathione-Sepharose beads as indicated and immunoblotted with antiphosphotyrosine (*aPTyr*). *C*, the same blot shown in *B* was stripped and reblotted with an antibody against the β-subunit of the insulin receptor (*aIR*).

sent in the intracellular portion of the β-subunit (19). Activation of the insulin receptor tyrosine kinase leads to tyrosine phosphorylation of the principal substrate, IRS-1. IRS-1 acts as an SH2-docking protein for several signaling proteins, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (20–23), the tyrosine phosphatase Syp (24), the oncogenic protein Nck (25), and the adaptor protein Grb-2, which links the guanine nucleotide factor mSos to p21^{ras} (26–28). Another substrate for insulin-dependent tyrosine kinase activity is the oncogenic protein Shc, which also interacts with the adaptor protein Grb-2 (26, 32, 33). In myeloid hematopoietic cell lines, insulin induces tyrosine phosphorylation of the interleukin-4-induced phosphotyrosine substrate (4PS) (16), a functional homologue of IRS-1 that is essential for the mitogenic effects of insulin and interleukin-4 (34).

In this study, we examined the tyrosine phosphorylation status of p95^{vav} in response to insulin treatment of the hematopoietic cell lines U-266, IM-9, and J558L, which express functional insulin receptors. We found that p95^{vav} is tyrosine-phosphorylated in a rapid and transient manner in response to insulin, as determined by antiphosphotyrosine immunoblotting and ^{32}P labeling experiments. We subsequently sought to determine whether p95^{vav} associates with the β-subunit of insulin receptor. The insulin receptor could not be coimmunoprecipitated by the anti-p95^{vav} antibody, probably due to low stoichiometry of association between these two proteins. Glutathione *S*-transferase binding experiments, however, demonstrated that the SH2 domain of *vav* associates with the activated insulin receptor tyrosine kinase *in vitro* in an insulin-dependent manner. Similar results were obtained when the 32D myeloid cell line transfected with a full-length cDNA for the β-subunit of the insulin receptor was studied. These find-

ings are of considerable interest as they suggest that p95^{vav} can associate directly with the insulin receptor tyrosine kinase without the requirement of docking proteins of the IRS family. Thus, *vav* seems to be another signaling protein, in addition to Shc, that is not regulated by IRS-signaling proteins, but instead may be activated by direct interaction with the insulin receptor tyrosine kinase.

Our data suggest that p95^{vav} is involved in the signal transduction of insulin in certain cells of hematopoietic origin. The biological significance of insulin-dependent tyrosine phosphorylation of p95^{vav}, however, remains to be determined. Previous reports demonstrated that p95^{vav} acts as a guanine nucleotide exchange factor for *ras* (12–15), despite its predicted function as a guanine nucleotide exchange factor for members of the Rho family. These studies also demonstrated that tyrosine phosphorylation of *vav* is required for its guanine exchange function (12–15). In a more recent report, however, Bustelo *et al.* (35) failed to confirm that *vav* acts as a guanine exchange factor for *ras*, but instead reported that it cooperates with *ras* to induce cell transformation. Another report also demonstrated that while transformation of NIH-3T3 cells with the *ras* guanine exchange factor GRF/CDC25 induces elevated *ras*-GTP and transcriptional activation from *ras*-responsive DNA elements, transformation with *vav* does not (36). However, similarly to *ras*- and GRF-transformed cells, *vav*-transformed cells exhibit constitutive activation of mitogen-activated protein kinases (36). Based on all these reports and our data, it is tempting to hypothesize that insulin-dependent tyrosine phosphorylation of p95^{vav} is involved in a pathway regulating the activation of *ras* in hematopoietic cells or in a pathway acting synergistically with *ras* to induce mitogenesis. The relationship of such pathways with known signals leading to insulin-dependent activation of *ras* (such as the pathway involving the adaptor protein Grb-2 and the Shc protein) is unclear at this time. Interestingly, a recent report has provided evidence suggesting the existence of a complex of Shc-Grb-2-*vav* in T lymphocytes *in vivo* (37). It remains to be determined if such a complex is formed in insulin-responsive cells and, if so, how it functions to regulate *ras* activation. It is also possible that p95^{vav} has other signaling functions in addition to the regulation of GTP-binding proteins. This has been suggested by the finding that the C-terminal SH3 domain of *vav* forms a stable association with the heterogeneous ribonucleoprotein K, which is implicated in the regulation of the c-*myc* gene (38). This finding raises the possibility of direct transmission of cell-surface receptor signals through *vav* activation to the nucleus to regulate gene expression (38). Future studies should provide valuable information on the importance of such functions of *vav* in the signal transduction of insulin.

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