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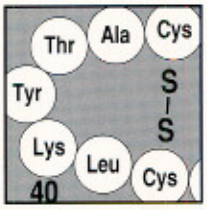

Insulin-like growth factor-1 induces rapid tyrosine phosphorylation of the *vav* proto-oncogene product

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Abstract

The *vav* proto-oncogene product ($p95^{vav}$) is specifically expressed in cells of hematopoietic origin and has one *src* homology 2 (SH2) domain, two SH3 domains, and motifs typical of guanine exchange factors. Insulin-like growth factor-1 (IGF-1) receptors are expressed on a variety of hematopoietic cells and, upon ligand binding, mediate signals regulating hematopoietic cell proliferation. We studied the phosphorylation status of $p95^{vav}$ in the U-266 human myeloma cell line, in response to IGF-1 stimulation. Immunoblotting experiments with an antiphosphotyrosine monoclonal antibody disclosed that $p95^{vav}$ is phosphorylated on tyrosine in an IGF-1-dependent manner. The tyrosine phosphorylation of $p95^{vav}$ was rapid, appearing within 5 minutes of IGF-1 treatment, and transient, diminishing by 90 minutes. Similar results were obtained when the mouse plasmacytoma J558L cell line was studied. IGF-1-dependent tyrosine phosphorylation of $p95^{vav}$ was also seen in the 32D mouse myeloid cell line that lacks expression of insulin receptor substrate (IRS) proteins, suggesting that it is not regulated by activation of the IRS-signaling system. Taken together, these data suggest that the *vav* proto-oncogene is a substrate for the IGF-1 receptor tyrosine kinase and may be involved in the signal transduction of IGF-1 in cells of hematopoietic origin.

Key words: Insulin-like growth factor-1—Tyrosine phosphorylation—*vav* proto-oncogene

Introduction

Insulin and insulin-like growth factor-1 exhibit cell growth and differentiation effects on a variety of hematopoietic cells [1–4]. For insulin and IGF-1 to exhibit their biological effects on target cells, binding to their cognate cell-surface receptors is required. The insulin and IGF-1 receptors have similar structures, consisting of homodimers of two extracellular α-subunits, which serve as the ligand binding domain(s), and homodimers of two transmembrane β-subunits, which possess intrinsic tyrosine kinase activity [5,6]. Binding of these ligands to their receptors activates the respective β-subunit tyrosine kinases, resulting in tyrosine phosphorylation of several downstream signaling elements. The major substrate for the IGF-1 and insulin receptor tyrosine kinases is the insulin receptor substrate-1 [7–9], which is tyrosine phosphorylated on multiple sites by both the insulin and IGF-1 receptors [7–11]. IRS-1 plays a critical role in the signal transduction of insulin and IGF-1 [12] by its function as an SH2-domain docking site for various downstream proteins, including the p85 regulatory subunit of the phosphatidylinositol 3′-kinase [13,14], the adaptor protein Grb-2 that provides a link to the *Ras* signaling cascade [15–17], the phosphotyrosine phosphatase SHPTP-2 [18], and the oncogenic protein *Nck* [19]. It appears, however, that these receptors also activate pathways that are IRS-1-independent. One such pathway involves the adaptor protein *Shc*, which also links the insulin receptor tyrosine kinase to *Ras* [20]. Furthermore, we have recently observed that insulin induces rapid tyrosine phosphorylation of the *vav* proto-oncogene product, a molecule that contains SH2 and SH3 domains and motifs typically present in guanine exchange factors [21]. In the current study, we sought to determine whether *vav* is a substrate for IGF-1-dependent tyrosine kinase activity. Our data establish that $p95^{vav}$ is rapidly phosphorylated on tyrosine in response to IGF-1 stimulation of hematopoietic cells, suggesting that it is a common element in the signaling pathways of insulin and IGF-1.

Materials and methods

Cells and reagents

The human myeloma U-266 cell line was grown in RPMI-1640 (Life Technologies) supplemented with 10% (vol/vol) fetal bovine serum (Life Technologies) and antibiotics. The mouse plasmacytoma J558L cell line (kindly provided by Dr. Hans Martin Jack, Loyola University) was grown in RPMI-1640 (Life Sciences) with 10% (vol/vol) defined calf serum (Hyclone) and antibiotics. The antiphosphotyrosine monoclonal antibody (4G10) was obtained from UBI (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 (Santa Cruz, CA).

Immunoprecipitations and immunoblotting

Immunoprecipitations and immunoblotting were performed as previously described [21–23]. Briefly, cells were stimulated

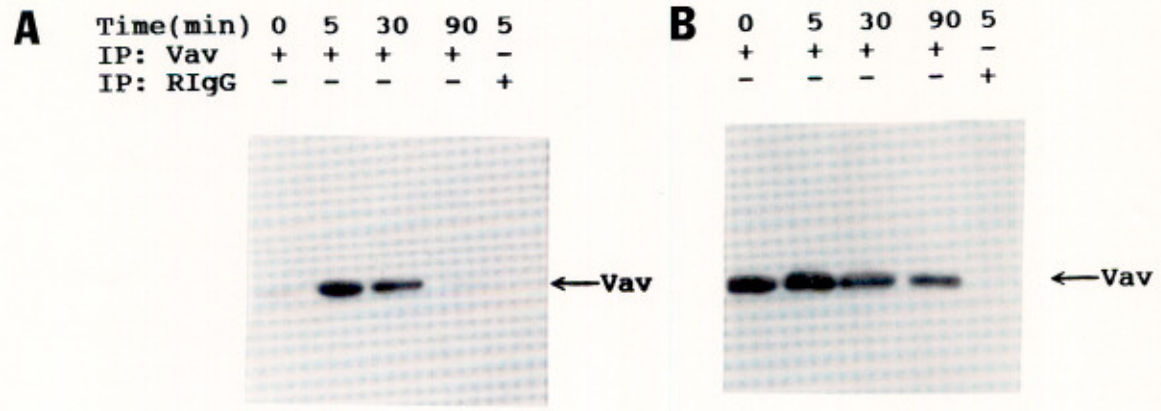


Fig. 1. IGF-1-dependent tyrosine phosphorylation of p95^{vav}. U-266 cells were stimulated with 100 nM IGF-1 for the indicated times at 37°C. Cell lysates were immunoprecipitated with either nonimmune rabbit immunoglobulin (RIgG) or an antibody against p95^{vav} as indicated. **A.** Antiphosphotyrosine immunoblot. The tyrosine phosphorylated form of p95^{vav} is indicated. **B.** The same blot was stripped and reprobed with an antibody against p95^{vav}.

with the indicated amounts of IGF-1 for the indicated periods of time. In some experiments, the cells were serum-starved for 2–3 hours immediately before IGF-1 treatment. After 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 phenylmethyl sulfonyl fluoride, and 10 µg/mL aprotinin). Cell lysates were immunoprecipitated with either a polyclonal antibody against p95^{vav} or control purified rabbit immunoglobulin (Sigma). Immunoprecipitates were washed five times with phosphorylation lysis buffer containing 0.1% Triton X-100 and analyzed by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore), 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 hours at room temperature or overnight at 4°C. The filters were subsequently incubated with antiphosphotyrosine, washed with TBST, and developed using an enhanced chemiluminescence (ECL) kit following the manufacturer's recommended procedure (Amersham, Arlington Heights, IL).

Labeling of cells with ^{32}P-orthophosphoric acid

J558L cells were washed two times with phosphate-free RPMI 1640 and incubated for 30 minutes at 37°C in phosphate-free medium. The cells were subsequently incubated for 4 hours in phosphate-free medium with carrier-free ^{32}P-orthophosphoric acid (Du Pont, New England Nuclear) at a concentration of 0.1 mCi/mL. The labeled cells were stimulated with IGF-1 for the indicated times and lysed in phosphorylation lysis buffer. Lysates cells were immunoprecipitated with either an antibody against p95^{vav} or control nonimmune rabbit immunoglobulin, washed five times in phosphorylation lysis buffer, and analyzed by SDS-PAGE.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as previously described [22,24]. Briefly, ^{32}P-labeled proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore). The membranes were rinsed three times with deionized water, dried, and subjected to autoradiography. After identification of the phosphorylated proteins, the pieces of the membrane containing the individual phosphoproteins were cut out and rewetted sequentially with methanol and deionized water. The peptides were subsequently hydrolyzed in 200 µL of 6N HCl at 110°C for 1 hour. The acid was separated from the Immobilon by centrifugation, and samples were lyophilized twice. Samples were subsequently resuspended in pH 3.5 buffer (glacial acetic acid:pyridine:water, 5:0.5:94.5, vol/vol/vol), and the individual phosphoamino acids were separated by electrophoresis in pH 3.5 buffer at 1 kV. Control phosphoserine, phosphothreonine, and phosphotyrosine were detected by reaction with ninhydrin, and radioactive phosphoaminoacids were detected by autoradiography.

Results

IGF-1 induces tyrosine phosphorylation of p95^{vav}

To determine whether IGF-1 induces tyrosine phosphorylation of p95^{vav} in the U-266 human myeloma cell line, lysates of untreated or IGF-1-treated cells were immunoprecipitated with either an anti-p95^{vav} antibody or control rabbit immunoglobulin. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with an antiphosphotyrosine monoclonal antibody (4G-10). Figure 1A shows that within 5 minutes of treatment of U-266 cells with IGF-1, p95^{vav} was strongly phosphorylated on tyrosine residues. The IGF-1-induced tyrosine phosphorylation of p95^{vav} was also clearly detectable after 30 minutes of treatment of cells and diminished to baseline levels by 90 minutes. To exclude the possibility that the intense signal detected at 5 and 30 minutes was due to variations in the amounts of immunoprecipitated protein, the same blot was stripped and reprobed with the anti-p95^{vav} polyclonal antibody. Figure 1B shows that similar amounts of vav protein were present before and after IGF-1 treatment of the cells, excluding such a possibility. Thus, *vav* is tyrosine phosphorylated during IGF-1 stimulation in a rapid and transient manner. Similarly, IGF-1-dependent tyro-

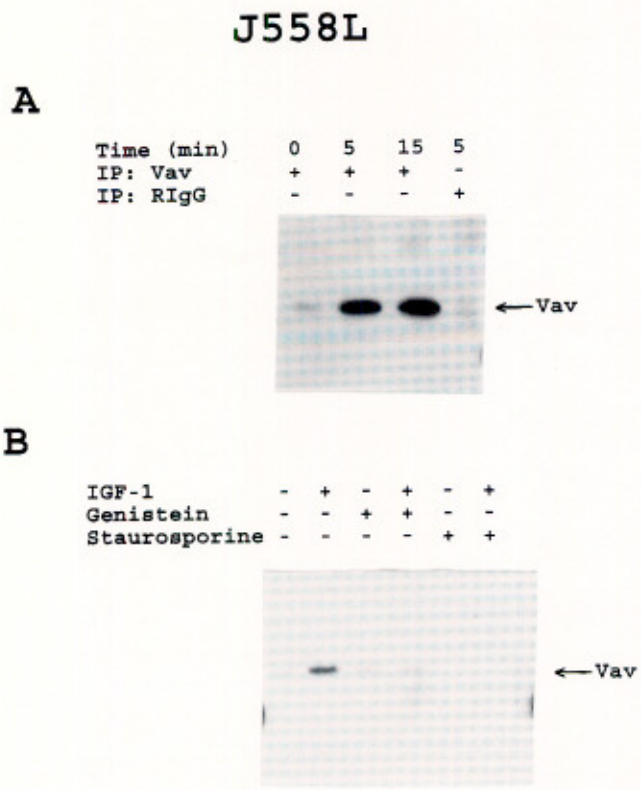


Fig. 2. IGF-1-dependent tyrosine phosphorylation of $p95^{vav}$ in J558L cells. Antiphosphotyrosine immunoblots are shown. **A.** Cells (4×10^7/lane) were stimulated with IGF-1 (200 ng/mL) for the indicated times at 37°C. Cell lysates were immunoprecipitated with either nonimmune RIgG or an antibody against $p95^{vav}$ as indicated. **B.** Cells were preincubated in serum-free medium for 2 hours alone (lanes 1–2) or in the presence of 100 µg/mL genistein (lanes 3–4) or 100 µM staurosporine (lanes 5–6) and treated with IGF-1 for 5 minutes as indicated. Cell lysates were immunoprecipitated by the anti-*vav* antibody and analyzed by SDS-PAGE.

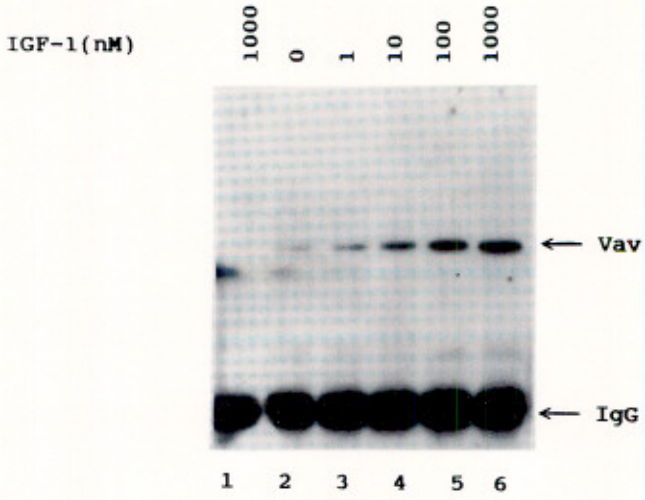


Fig. 3. Dose–response experiment of the IGF-1-induced phosphorylation of *vav*. Serum-starved cells were stimulated with indicated doses of IGF-1 for 10 minutes at 37°C. Cell lysates were immunoprecipitated with either nonimmune RIgG (lane 1) or an antibody against $p95^{vav}$ (lanes 2–6) and immunoblotted with antiphosphotyrosine.

sine phosphorylation of $p95^{vav}$ was also observed when the J558L mouse plasmacytoma cell line was studied (Fig. 2A). Furthermore, the IGF-1-induced phosphorylation of *vav* was blocked by preincubation of the cells in the presence of the tyrosine kinase inhibitors genistein or staurosporine, suggesting that it results from tyrosine kinase activation (Fig. 2B). To further establish the specificity of the process, dose–response experiments were performed. Figure 3 shows such an experiment. Tyrosine phosphorylation of *vav* was clearly inducible with as little as 10 nM IGF-1 and maximal at 1 µM. These results strongly suggest that phosphorylation of $p95^{vav}$ in these cells results from activation of the β-subunit of the IGF-1 but not the insulin receptor tyrosine kinase, since at the physiologic dose of 10 nM IGF-1, the insulin receptor is not activated by cross-reactivity.

^{32}P-labeling experiments and phosphoamino acid analysis

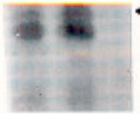
We subsequently sought to directly establish the IGF-1-dependent phosphorylation of $p95^{vav}$ on tyrosine residues by analyzing its phosphoamino acid content before and after IGF-1 treatment of cells. J558L cells were metabolically labeled with ^{32}P-orthophosphate and stimulated with IGF-1, and after cell lysis the ^{32}P-labeled proteins were immunoprecipitated by the anti-*vav* antibody. Figure 4A shows that a 95-kD band corresponding to the phosphorylated form of $p95^{vav}$ was detectable before and after stimulation of the cells. Upon IGF-1 stimulation, however, its phosphorylation increased significantly. The identities of the other phosphoproteins coimmunoprecipitated by the anti-*vav* antibody remain unknown at this time. Phosphoamino acid analysis of the bands corresponding to $p95^{vav}$ demonstrated that, prior to IGF-1 stimulation, $p95^{vav}$ is primarily phosphorylated on serine residues. After IGF-1 treatment, significant amounts of phosphorylation on tyrosine were detectable, confirming the immunoblotting findings (Fig. 4B).

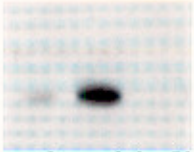
Engagement of $p95^{vav}$ in IGF-1 signaling does not require IRS proteins

We subsequently sought to determine whether tyrosine phosphorylation of *vav* during IGF-1 stimulation is dependent on the SH2-docking function of the IRS-signaling system. We determined whether *vav* is tyrosine phosphorylated in the 32D mouse myeloid cell line, which lacks expression of IRS-1, as well as the related 4PS (IRS-2) [25]. Figure 5 shows that IGF-1 induces tyrosine phosphorylation of $p95^{vav}$ in 32D cells, establishing that tyrosine phosphorylation of *vav* is not dependent on engagement of the IRS-signaling system by IGF-1.

Discussion

The *vav* proto-oncogene ($p95^{vav}$) is a 95-kD protein selectively expressed in hematopoietic cells that contains SH2 and SH3 domains and motifs typically present in guanine exchange factors [26–32]. $p95^{vav}$ is rapidly phosphorylated in response to multiple stimuli in cells of hematopoietic origin: in T cells





with type I interferons (IFNs) [24]. The tyrosine phosphorylation of *vav* after engagement of several surface receptors, and structure, suggest that it may have an important regulatory repoit, we sougiit to uetermine wnether *vav* is a substrate for IGF-1-dependent tyrosine kinase activity. Although IRS-1 is a immunoblotting experiments demonstrated that *vav* is tyrosine phosphorylated in a rapid manner in the U-266 and by ^{32}P-labeling experiments and phosphoamino acid analysis of the phosphorylated vav protein. Furthermore, our data established that engagement of *vav* in IGF-1 signaling does not require the function of IRS-signaling proteins, as demon-32D mouse myeloid cell line.

Taken together, these results clearly demonstrate that IGF- and suggest a role for *vav* in IGF-1 signaling in hematopoietic cells. However, the precise role of IGF-1-induced *vav* phospho- been made recently in our understanding of the functional role of *vav* in signal transduction pathways. Experimental evi- exchange factor for the *ras* oncogene [38–40], despite the fact a pathway involving members of the Rho family of proteins [42]. This apparent controversy may be explained by the bind to the C-terminus SH3 domain of the adaptor protein Grb-2 [43]. The vation of *ras* by IGF-1 and insulin. The C-terminus SH3 domain of *vav* may also play an important role in transducing that is part of hnRPNP particles and is involved in the transcriptional regulation of the *c-myc* gene [44]. The impor- future studies should provide valuable information about its role in IGF-1 and insulin signal transduction.

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