Communication

Interferon α Induces Rapid Tyrosine Phosphorylation of the vav Proto-oncogene Product in Hematopoietic Cells*

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The vav proto-oncogene product (p95\textsuperscript{vav}) is specifically expressed in cells of the hematopoietic system, contains one Src homology 2 and two Src homology 3 domains, and is a substrate for receptor and non-receptor tyrosine kinases. Immunoblotting experiments using an anti-phosphotyrosine monoclonal antibody showed that interferon-α (IFNα) induces rapid tyrosine phosphorylation of p95\textsuperscript{vav} after binding to its cell surface receptor in the U-266 human myeloma cell line. The IFNα-induced tyrosine phosphorylation of p95\textsuperscript{vav} was time- and dose-dependent, confirming the specificity of the process. IFNα-dependent tyrosine phosphorylation of p95\textsuperscript{vav} was also observed in other hematopoietic cell lines of B-cell origin (Daudi), T-cell origin (MOLT-4), and promyelocytic origin (HL-60). Immunoprecipitation experiments performed with \textsuperscript{32P}-labeled U-266 cells and phosphoaminoacid analysis of the bands corresponding to p95\textsuperscript{vav} showed that p95\textsuperscript{vav} is phosphorylated on serine residues prior to IFNα stimulation of the cells. After IFNα stimulation significant amounts of phosphorylation of p95\textsuperscript{vav} on tyrosine residues were detectable. Tyrosine phosphorylation of p95\textsuperscript{vav} in U-266 and HL-60 cells was also induced by two other Type I IFNs, IFNβ and IFNγ. Altogether these data suggest that the vav proto-oncogene product is a substrate for a Type I IFN-regulated tyrosine kinase(s) and may be involved in the signal transduction pathway of Type I IFNs in hematopoietic cells.

The vav proto-oncogene product is a tyrosine kinase substrate containing one Src homology 2 and two Src homology 3 domains. p95\textsuperscript{vav} is specifically expressed in cells of hematopoietic origin, and the protein has several other interesting structural motifs, including helix-loop-helix and leucine zipper-like domains in its NH\textsubscript{2}-terminal region, zinc finger-like domains, and regions of homology with the dbl proto-oncogene, the yeast CDC24 GDP-GTP exchange factor, and the bcr gene (1-6, 23, 26).

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p95\textsuperscript{vav} has been shown to undergo transient tyrosine phosphorylation in T-cells during activation of the T-cell receptor-CD4 complex (5, 6), in activated mast cells (6), in cells transiently expressing epidermal or platelet-derived growth factor receptors in response to receptor activation (5, 6), in B-cells activated by engagement of their immunoglobulin M receptors (7), and in stem cell factor and interleukin-2-dependent human cells in response to stem cell factor activation (8).

IFNα\textsuperscript{1} has been shown to transduce signals by inducing tyrosine phosphorylation of three components of the interferon-stimulated gene factor 3 (ISGF3\textalpha) leading to the formation of the ISGF3\textbeta complex (9-11). ISGF3\textbeta translocates to the cell nucleus and in association with ISGF3\textgamma binds to a DNA sequence, the interferon-stimulated response element, to initiate IFNα-dependent gene transcription (9-11). It has also been shown that an early event in the IFNα signal transduction pathway is tyrosine phosphorylation of the α-subunit of its receptor (12). However, in some cells expressing the variant form of the IFNα receptor, IFNα response can be elicited in the absence of phosphorylation of the α-subunit (13). DNA complementation studies have shown that a tyrosine kinase involved in the IFNα signal transduction pathway is the tyrosine kinase p135\textsuperscript{src} (14). We have recently observed that IFNα induces tyrosine phosphorylation of multiple proteins in addition to the p135\textsuperscript{src} kinase and the ISGF3\textalpha components in hematopoietic cells.\textsuperscript{2} As the proto-oncogene product p95\textsuperscript{vav} has been shown to be a substrate for receptor and non-receptor tyrosine kinases, and possibly link surface signals with downstream cellular events, we sought to determine whether p95\textsuperscript{vav} is among the substrates phosphorylated in response to IFNα. Our findings suggest that p95\textsuperscript{vav} may be part of an IFNα-signaling pathway in cells of hematopoietic origin.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—The human myeloma U-266 cell line and the HL-60 acute promyelocytic leukemia cell line were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) and antibiotics. The human lymphocytic cell lines Daudi (Burkitt's lymphoma) and MOLT-4 (acute T-cell lymphocytic leukemia) were grown in RPMI 1640 supplemented with 10% (v/v) defined calf serum (HyClone Laboratories, Logan, UT) and antibiotics. Reagents—Human recombinant IFNα2 (specific activity 2.2 × 10\textsuperscript{8} units/mg) was provided by Schering Plough (Kenilworth, NJ). Human recombinant IFNβ (specific activity 3.2 × 10\textsuperscript{8} units/mg using the WHO IFNβ standard) was supplied by Berlex Laboratories (Richmond, CA). Human recombinant IFNγ (specific activity 2 × 10\textsuperscript{8} units/mg) was a gift (to Dr. M. O. Diaz) from Dr. G. Addolf (Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria). The anti-phosphotyrosine monoclonal antibody (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Sodium orthovanadate and protease inhibitors were obtained from Sigma. A rabbit polyclonal antibody against a peptide corresponding to residues 578-588 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). A rabbit polyclonal antibody against residues 528-541 of the human Vav protein obtained from Upstate Biotechnology, Inc. was also used in some experiments. Immunoblotting—Cells were stimulated with the indicated amounts of IFNα for different periods of time. After IFNα stimulation, the cells were rapidly centrifuged and lysed in phosphorylation lysis buffer

\textsuperscript{1} The abbreviations used are: IFNα, interferon-α; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ISGF3\textbeta, interferon-stimulated gene factor 3.

\textsuperscript{2} L. C. Platanias, M. E. Sweet, J. J. Krolewski, and O. R. Colamonici, unpublished data.
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(0.5–1% Triton X-100, 150 mm NaCl, 1 mm EDTA, 200 mm sodium orthovanadate, 50 mm Heps, 10 mm sodium pyrophosphate, 100 mm sodium fluoride, 1.5 mm magnesium chloride, 1 mm phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Lysates obtained from 1–4 x 10\textsuperscript{7} cells were immunoprecipitated with either a polyclonal antibody against p95\textsuperscript{\textalpha\nu} (1–5 μg) or an equivalent amount of control purified rabbit immunoglobulin (Sigma), or with control rabbit serum (Oncogene Science), followed by analysis by SDS-PAGE. The proteins were transferred onto nitrocellulose filters (Schleicher & Schuell), 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), 7% bovine serum albumin for 1–3 h at room temperature or overnight at 4 °C. The nitrocellulose filters were subsequently incubated with anti-phosphotyrosine, washed with TBST and developed using an enhanced chemiluminescence (ECL) kit following the manufacturer's recommended procedure (Amersham Corp.).

Labeling of Cells with [\textsuperscript{\textalpha}\textsuperscript{P}]Orthophosphoric Acid—Cells (0.5–1 x 10\textsuperscript{8}) were washed three times with phosphate-free RPMI 1640 and cultured for 30 min at 37 °C in phosphate-free medium. The cells were subsequently incubated for 3 h in phosphate-free medium with carrier-free [\textsuperscript{\textalpha}\textsuperscript{P}]orthophosphoric acid (Du Pont NEN) at a concentration of 0.1 mCi/ml. The labeled cells were subsequently stimulated with 10\textsuperscript{4} units/ml IFNa for the indicated times and lysed in phosphorylation lysis buffer. The lysates were immunoprecipitated with the indicated antibodies, washed five times in phosphorylation lysis buffer, and analyzed by SDS-PAGE.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as previously described (12, 15). Briefly, [\textsuperscript{\textalpha}\textsuperscript{32}\textsuperscript{P}]labeled proteins were transferred to polyvinylidene fluoride 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 rewedeted sequentially with methanol and deionized water. The peptides were subsequently hydrolyzed in 200 μl of 6 N HCl at 110 °C for 1 h. 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, v/v/v), 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 phosphoamino acids were detected by autoradiography.

RESULTS

Tyrosine Phosphorylation of p95\textsuperscript{\textalpha\nu} by Type I IFNs—To determine whether p95\textsuperscript{\textalpha\nu} is a substrate for IFNa-dependent tyrosine phosphorylation, U-266 cells were treated with IFNa2 for different periods of time, the cells were lysed, and the lysates were immunoprecipitated with either control normal rabbit serum or a polyclonal antibody against p95\textsuperscript{\textalpha\nu}, prior to SDS-PAGE analysis and anti-phosphotyrosine immunoblotting. Fig. 1A shows that IFNa induced tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} within 1 min after binding to its receptor, and the signal gradually declined after 60 min of IFNa treatment. Weak base-line tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} (prior to IFNa stimulation) was detectable after longer exposure of this and other blots. Reprobing of the same blot with the anti-p95\textsuperscript{\textalpha\nu} polyclonal antibody confirmed that equal amounts of p95\textsuperscript{\textalpha\nu} were present in all lanes.

We subsequently studied whether IFNa induces tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} in other cell lines of hematopoietic origin. IFNa-dependent tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} was also detected in cells of promyelocytic origin (HL-60), T-cell origin (MOLT-4), and B-cell origin (Daudi) (Fig. 3). Significant amounts of base-line tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} were detected in Daudi and MOLT-4 cells, but IFNa significantly increased the tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} (Fig. 3). Tyrosine phosphorylation of p95\textsuperscript{\textalpha\nu} in U-266 and HL-60 cells was also observed when cells were treated with IFNβ or IFNα, suggesting that p95\textsuperscript{\textalpha\nu} may be involved in the signaling pathway of all Type I IFNs (Fig. 4).

32P Labeling and Phosphoamino acid Analysis—We next sought to determine whether p95\textsuperscript{\textalpha\nu} is also phosphorylated on serine and/or threonine residues in the human U-266 myeloma cell line. Cells were labeled with [\textsuperscript{\textalpha}\textsuperscript{32}\textsuperscript{P}]orthophosphate, and the phosphoaminoacid content of p95\textsuperscript{\textalpha\nu} was examined before and
tyrosine phosphorylation can directly activate an IFNα-depen-
dent latent transcription factor (9−11), suggesting that sec-
ond messengers may not be necessary for the initiation of tran-
scription of known interferon-stimulated genes (9−11). The
tyrosine kinase p135\(^{\text{cyt}}\) has been shown to associate with the α-subunit of the IFNα receptor and to be activated by IFNα
(17), suggesting that it may be the kinase regulating the phos-
phorylation of the ISGF3 components. In the current study we
sought to determine whether p95\(^{\text{cyt}}\) is a substrate for IFNα-depen-
dent tyrosine kinase activity in the U-266 human my-
eloma cell line. Immunoblotting experiments disclosed that p95\(^{\text{cyt}}\) is phosphorylated on tyrosine in a time- and dose-de-
dependent manner in response to IFNα. The immunoblotting
experiments were subsequently confirmed by \(^{32}\)P labeling
experiments and phosphoaminoacid analysis of the immuno-
precipitated p95\(^{\text{cyt}}\) protein. Tyrosine phosphorylation of p95\(^{\text{cyt}}\) was also inducible by two other Type I IFNs, IFNβ and IFNα.

Taken together these data suggest that p95\(^{\text{cyt}}\) may be involved in a Type I IFN-dependent tyrosine kinase signaling pathway.
The biological significance of the IFNα-dependent tyrosine
phosphorylation of p95\(^{\text{cyt}}\), however, remains to be determined.
Three of the cell lines studied here (U-266, Daudi, and MOLT-4)
have been shown previously to be sensitive to the anti-prolif-
eerative effects of IFNs (13, 18, 19). HL-60 cells, in which we
also demonstrated phosphorylation of p95\(^{\text{cyt}}\) by IFNα and
IFNβ, do not respond directly to the anti-proliferative effect of
IFNα (19, 20). However, it has been shown that in these cells
IFNα and IFNβ act synergistically with phorbol esters and
retinoic acid to induce differentiation (20, 21). Interestingly, in
these cells tyrosine phosphorylation of p95\(^{\text{cyt}}\) increases dra-
matically when cells differentiate to neutrophils in response to
retinoic acid (22). Based on these observations and our find-
ings, it is tempting to hypothesize that p95\(^{\text{cyt}}\) may be involved in a Type I IFN-signaling pathway regulating hematopoietic
cell differentiation. Such a hypothesis is further supported by
the fact that p95\(^{\text{cyt}}\) is specifically expressed in cells of hemato-
opoeitic origin; therefore, the pathway in which it may be
involved should mediate signals specific for the effects of IFNα
in these cells.

p95\(^{\text{cyt}}\) contains regions of homology with the bcr and dbl onco-
genesis and the yeast CDC24 proteins (4, 26). It also con-
ains zinc finger-like, helix-loop-helix-like, and leucine zipper-
like domains (1−3). Because of these later motifs, it has been
suggested that p95\(^{\text{cyt}}\) may either act as a transcriptional activ-
or regulate the activity of transcription factors in a simi-
lar manner with other proteins containing these motifs (5, 6).
Although there is not evidence at this time that p95\(^{\text{cyt}}\) binds
DNA, it is conceivable that p95\(^{\text{cyt}}\) may function as a transcrip-
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