The *vav* proto-oncogene product (p95^{vav}) interacts with the Tyk-2 protein tyrosine kinase

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Abstract The *vav* proto-oncogene product participates in the signaling pathways activated by various cell-surface receptors, including the type I IFN receptor. During engagement of the type I IFN receptor, p95^{vav} is phosphorylated on tyrosine residues, but the kinase regulating its phosphorylation has not been identified to date. Our studies demonstrate that p95^{vav} forms a stable complex with the IFN-receptor-associated Tyk-2 kinase in vivo, and strongly suggest that this kinase regulates its phosphorylation on tyrosine. Thus, p95^{vav} is engaged in IFN-signaling by a direct interaction with the functional type I IFN receptor complex to transduce downstream signals.

Key words: *vav* proto-oncogene; Jak kinase; Interferon signaling

1. Introduction

Type I IFNs are pleiotropic cytokines that exert antiproliferative, antiviral, and immunomodulatory activities [1,2] on normal and neoplastic cells. Several of the signaling events that occur during binding of IFNs to the type I IFN receptor (IFNR) have been now identified. IFNα treatment of human cells induces tyrosine phosphorylation of the α and β subunits of the type I IFNR [3,4], and activation of the receptor-associated Tyk-2 and Jak-1 kinases (reviewed in [5]). Engagement of these kinases results in activation of the Stat- [5] and the IRS- [6,7] signaling pathways. The tyrosine phosphorylation of Stat- and IRS-proteins is a common event in the signaling pathways of all type I IFNs [4,6,7]. However, differences in the signaling pathways of different type I IFNs also exist, as demonstrated by the IFNβ-specific interaction of the α and β_L subunits of the type I IFNR [4,8]. There is also evidence that the *c-cbl* proto-oncogene product is involved in type I IFN signaling, as suggested by its constitutive association with the Tyk-2 kinase, and its IFNα-dependent phosphorylation on tyrosine residues [9].

We have previously reported that the *vav* proto-oncogene product is also involved in type I IFN signaling, as evidenced by its rapid and transient tyrosine phosphorylation during treatment of hematopoietic cells with IFNα, IFNβ, and IFNω [10]. This protein is also phosphorylated during engagement of various cell surface receptors [11–19], suggesting its involvement in diverse signaling cascades. In the present study we provide evidence that p95^{vav} associates with Tyk-2 in vivo, suggesting that Tyk-2 is the kinase that regulates its engagement in IFNα signaling.

2. Materials and methods

2.1. Cells and reagents

The U-266 and Daudi human cell lines were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) or 10% (v/v) defined calf serum (Hyclone Laboratories, Logan, UT) and antibiotics. Human recombinant IFNα2 (IFNα) was provided by Hoffmann Laroche and Schering Plough. An antiphosphotyrosine monoclonal antibody (4G-10) was obtained from UBI (Lake Placid, NY). Polyclonal antibodies against Tyk-2 have been previously described [20,21]. The anti-p95^{vav} polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Immunoprecipitations and immunoblotting

Cells were stimulated with IFNα for the indicated times. Cell lysis, immunoprecipitations, and immunoblotting using an enhanced chemiluminescence method were performed as previously described [10,22].

2.3. In vitro kinase assays and phosphoamino acid analysis

These assays were performed essentially as described in previous reports [15,20].

3. Results and discussion

We initially sought to determine whether IFNα-dependent kinase activity can be detected in association with p95^{vav} in in vitro kinase assays. U-266 myeloma cells were incubated at 37°C in the presence or absence of IFNα, cell lysates were immunoprecipitated with an anti-p95^{vav} antibody and the immunoprecipitates were subjected to an in vitro kinase assay. Some baseline kinase activity was detected in association with p95^{vav}, and after IFNα treatment increased significantly (Fig. 1A). Interestingly, a 135 kDa protein which was phosphorylated in an IFNα-dependent manner, was also detectable in the anti-p95^{vav} immunoprecipitates (Fig. 1A). Immunoblotting of the membrane with the anti-p95^{vav} antibody confirmed that equal amounts of the p95^{vav} protein were present prior to and after IFNα treatment (Fig. 1B). When the bands corresponding to p95^{vav} were excised and subjected to phosphoamino acid analysis, we noticed that after IFNα treatment there was induction of both serine and tyrosine phosphorylation,

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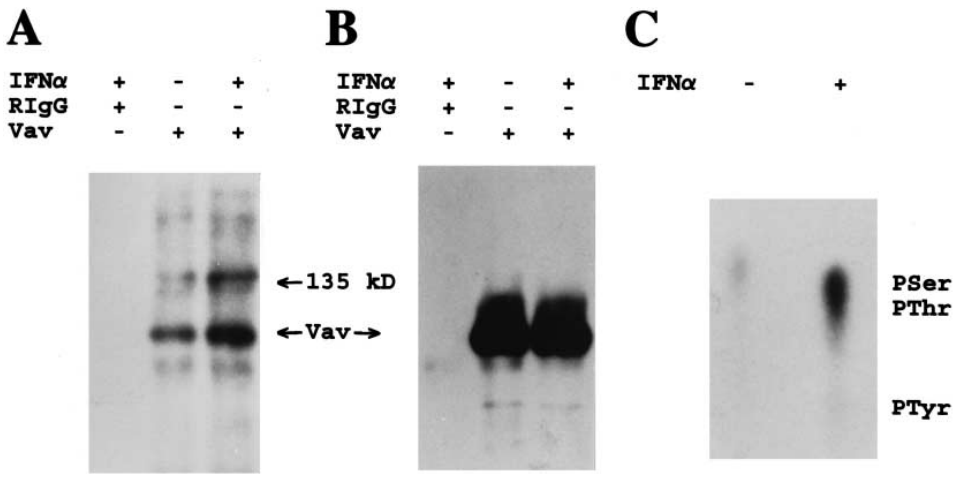

Fig. 1. Detection of IFNα-dependent kinase activity in association with p95^{vav}. (A) U-266 cells were serum starved for 2 h and were subsequently treated with IFNα (10^4 U/ml) for 3 min at 37°C as indicated. Cells were lysed and cell lysates were immunoprecipitated with either non-immune rabbit immunoglobulin (RIgG) or an antibody against p95^{vav} as indicated. Immunoprecipitates were subjected to an in vitro kinase assay, analyzed by SDS-PAGE, and transferred to Immobilon membranes prior to autoradiography. (B) The membrane shown in A was immunoblotted with the anti-p95^{vav} antibody to demonstrate that equal amounts of the protein were present prior to and after IFNα stimulation. (C) Phosphoamino acid analysis of p95^{vav} from the experiment shown in (A,B).

suggesting that the protein associates with IFNα-dependent serine and tyrosine kinases (Fig. 1C).

To determine the interaction of p95^{vav} with other cellular proteins, we performed studies in which ^{35}S-labeled Daudi cells were treated with IFNα, and cell lysates were immunoprecipitated with the anti-p95^{vav} antibody. Proteins with approximate molecular masses of 190 (p190), 135 (p135), and 70–80 (p80) kDa were detected in association with p95^{vav} in these cells, prior to and after IFNα treatment (Fig. 2). These findings raised the possibility that p95^{vav} may be constitutively associated with a tyrosine kinase that regulates its phosphorylation in an IFNα-dependent manner.

Among the proteins detected in association with p95^{vav} in the ^{35}S-labeling and in vitro kinase assay experiments, there was a protein migrating at 135 kDa, which is similar to the molecular mass of the IFNα-dependent Tyk-2 kinase. We therefore sought to determine whether p95^{vav} interacts with Tyk-2. Daudi cells were incubated in the presence or absence of IFNα, and cell lysates were immunoprecipitated with antibodies against p95^{vav} or Tyk-2 and immunoblotted with antiphosphotyrosine. For these studies a high number of cells was used, in order to immunoprecipitate a relatively large amount of the p95^{vav} protein, and avoid missing detection of proteins interacting with it due to low stoichiometry of such associa-

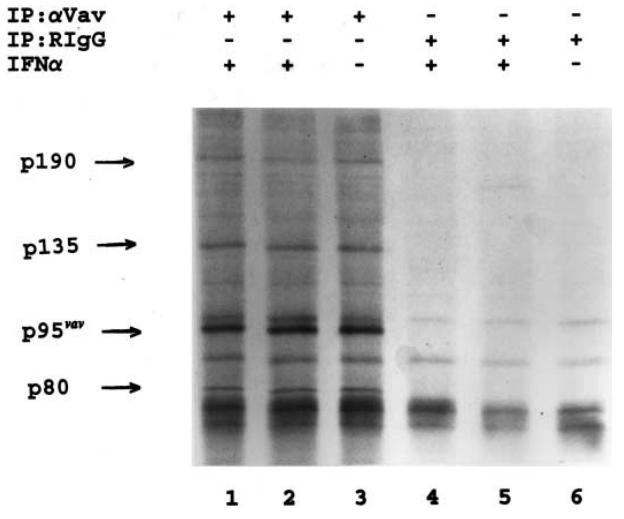

Fig. 2. Association of p95^{vav} with cellular proteins in Daudi cells. ^{35}S-labeled Daudi cells were either not treated with IFNα (lanes 3,6) or treated at 37°C with IFNα (2000 U/ml) for 5 min (lanes 2,5) or 30 min (lanes 1,4). Cells were lysed, and cell lysates were immunoprecipitated with either control normal rabbit immunoglobulin (RIgG) or an antibody against p95^{vav} as indicated. Immunoprecipitates were analyzed by SDS-PAGE, and the gel was dried and subjected to autoradiography.

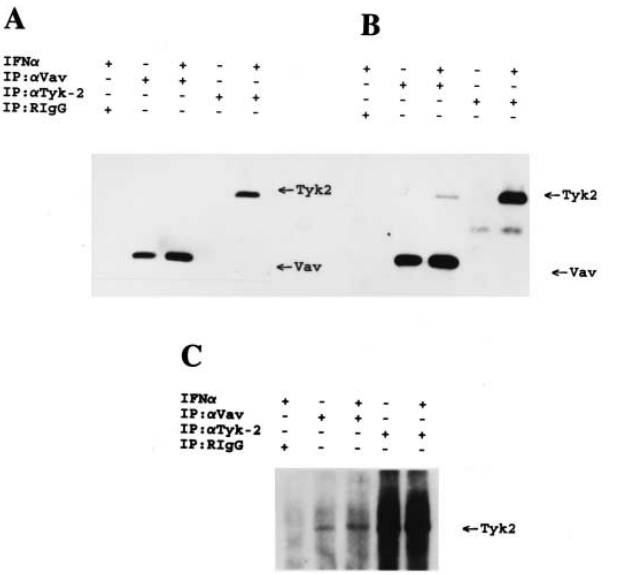

Fig. 3. p95^{vav} is associated with the Tyk-2 protein tyrosine kinase. (A) Daudi cells (2.2×10^7/lane) were treated with IFNα for 5 min at 37°C as indicated, cell lysates were immunoprecipitated with the indicated antibodies, analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. (B) Longer exposure of the blot shown in (A). (C) The blot shown in (A,B) was stripped and re-probed with an antibody against Tyk-2.

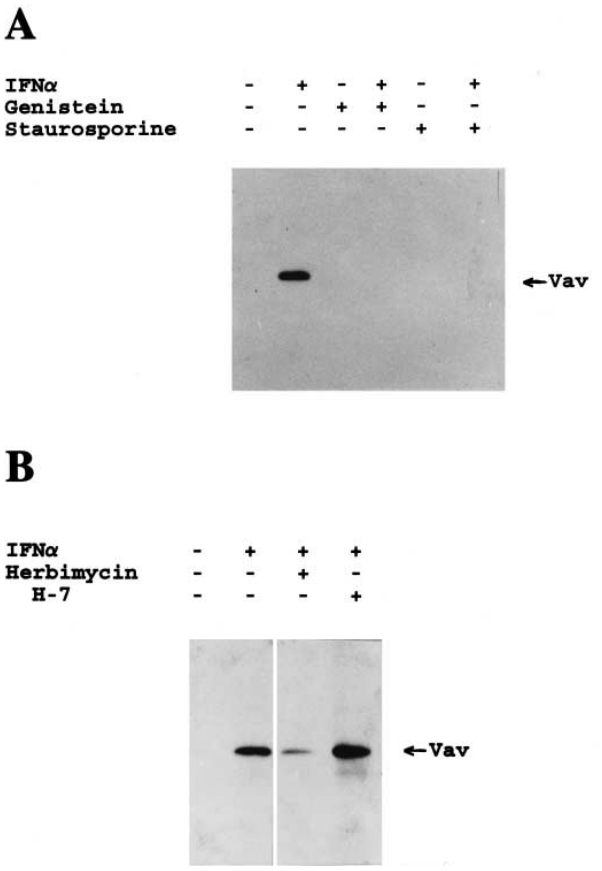

Fig. 4. Effect of kinase inhibitors on the IFNα-induced phosphorylation of p95^{vav}. (A) U-266 cells were incubated for 60 min at 37°C in the presence or absence of genistein (150 μg/ml) or staurosporine (500 μM) as indicated. The cells were subsequently treated for 5 min with IFNα (10^4 U/ml) as indicated. Cell lysates were immunoprecipitated with an antibody against p95^{vav}, analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine. (B) U-266 cells that were either not incubated with kinase inhibitors, or incubated overnight with herbimycin A (1 μM), or incubated for 2 h with H-7 (50 μM) as indicated, were treated with IFNα (1000 U/ml) for 20 min at 37°C. Cell lysates were immunoprecipitated with an anti-p95^{vav} antibody, analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine.

tions. Consistent with our previous findings [10], p95^{vav} exhibited baseline tyrosine phosphorylation in these cells, but IFNα treatment further increased its phosphotyrosine content (Fig. 3A). After longer exposure of the same blot, we noticed that a tyrosyl-phosphoprotein that co-migrated with Tyk-2, was also detectable in the anti-p95^{vav} immunoprecipitates (Fig. 3B). When the same blot was stripped and re-probed with an anti-Tyk-2 antibody, we found that Tyk-2 was present in anti-p95^{vav} immunoprecipitates prior to and after IFNα stimulation (Fig. 3C). Thus, p95^{vav} forms a stable complex with Tyk-2 in vivo, strongly suggesting that Tyk-2 is the kinase that regulates its phosphorylation during IFNα stimulation.

We subsequently sought to determine the effect of tyrosine kinase inhibitors on the IFNα-induced tyrosine phosphorylation of p95^{vav}. Cells were pre-incubated in the presence or absence of various kinase inhibitors, and were subsequently treated with IFNα.

Fig. 4A shows that the IFNα-dependent tyrosine phosphorylation of p95^{vav} is inhibited in the presence of the specific tyrosine kinase inhibitor genistein and the non-specific kinase inhibitor staurosporine, which has been previously shown also to block IFNα-dependent phosphorylation of Stat-proteins [23]. Similarly, the IFNα-induced phosphorylation of p95^{vav} was partially inhibited when cells were treated in the presence of the specific tyrosine kinase inhibitor herbimycin A, but not in the presence of the protein kinase C inhibitor H-7 (Fig. 4B). Thus, the IFNα-dependent tyrosine phosphorylation of p95^{vav} is blocked in the presence of tyrosine kinase inhibitors, further suggesting that its phosphorylation is regulated by the associated Tyk-2 tyrosine kinase.

The *vav* proto-oncogene product participates in signaling cascades for various cytokines, growth factors, and hormones [11–19], and plays a critical role in embryogenesis [24] and T- and B-cell function [25–27]. p95^{vav} interacts with the ENX-1 [28] and Ku-70 [29] proteins, as well as the heterogeneous nuclear ribonucleoprotein K [30,31] and a poly(rC)-specific RNA-binding protein [31], suggesting that it plays an important role in the regulation of gene transcription and RNA biogenesis. The results of the present study provide evidence that p95^{vav} interacts with the functional type I IFN receptor complex, and strongly suggest that its engagement in IFN-signaling is regulated by the Tyk-2 protein tyrosine kinase. Thus, in addition to Stats, p95^{vav} may provide an alternative direct link between the type I IFN receptor and nuclear events that regulate IFN-induced gene expression.

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