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Tyrosine phosphorylation of p95^{Vav} in myeloid cells is regulated by GM-CSF, IL-3 and Steel factor and is constitutively increased by p210$^{BCR/ABL}$

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***Vav* is a recently described proto-oncogene expressed only in hematopoietic cells which contains an SH2 and two SH3 domains and shares homology with the Dbl GDP–GTP exchange factor and BCR. p95^{Vav} is phosphorylated on tyrosine residues in response to stimulation of the T cell antigen receptor, cross-linking of IgE or IgM receptors and stimulation of immature hematopoietic cells by Steel factor. Monoclonal antibodies to human Vav were generated and used to examine the events which regulate tyrosine phosphorylation of p95^{Vav} in myeloid cells. In the factor-dependent MO7e cell line, p95^{Vav} was rapidly phosphorylated on tyrosine residues in a dose- and time-dependent manner by GM-CSF, IL-3 and Steel factor. Introduction of the *BCR/ABL* oncogene into this cell line resulted in factor-independent proliferation and constitutive phosphorylation of p95^{Vav}. Tyrosine phosphorylation of p95^{Vav} was also substantially increased by treatment of cytokine-deprived cells with the tyrosine phosphatase inhibitor sodium vanadate. Since many of the cytokines known to induce tyrosine phosphorylation of p95^{Vav} are also known to activate JAK family tyrosine kinases, we looked for an interaction of p95^{Vav} with JAK kinases. p95^{Vav} co-precipitated with JAK2 in MO7e cells stimulated with GM-CSF, but not in unstimulated cells. Also, JAK2 was found to be constitutively associated with p95^{Vav} *in vivo* when expressed at high levels in insect cells using baculovirus vectors. A fusion protein consisting of glutathione-*S*-transferase and the SH2 domain of p95^{Vav} (GST–Vav-SH2) precipitated JAK2, suggesting that this interaction is mediated by the SH2 domain of p95^{Vav}. GST–Vav-SH2, but not GST, also precipitated JAK1, JAK3 and Tyk2, suggesting that other JAK family kinases might interact with p95^{Vav}. These results suggest that tyrosine phosphorylation of p95^{Vav} is potentially directly regulated by JAK kinases, and further suggest that Vav is broadly involved in signal transduction in myeloid cells initiated by many cytokines and the oncogene *BCR/ABL*.**

Key words: BCR/ABL/GM-CSF/IL-3/*Vav*

Introduction

The *Vav* proto-oncogene is selectively and universally expressed in hematopoietic cells (Katzav *et al.*, 1989; Bustelo *et al.*, 1992) and encodes a 95 kDa protein with structural features of both a transcription factor and a signal transduction molecule, including a helix–loop–helix (HLH) domain, a leucine-zipper (LZ)-like domain, two SH3 and one SH2 domain in the C-terminus and an internal Dbl-homologous sequence (Adams *et al.*, 1992; Cen *et al.*, 1992; Galland *et al.*, 1992). p95^{Vav} has been shown to be phosphorylated on tyrosine residues in response to cross-linking of the T cell antigen receptor and the IgE receptor, and by activation of the flt2/flk3 or c-kit tyrosine kinase receptors in hematopoietic cells (Alai *et al.*, 1992; Bustelo and Barbacid, 1992; Margolis *et al.*, 1992; Dosil *et al.*, 1993; Evans *et al.*, 1993). When introduced into fibroblasts, p95^{Vav} is also phosphorylated in response to epidermal growth factor and platelet-derived growth factor (Bustelo and Barbacid, 1992; Margolis *et al.*, 1992). *Vav* oncogene has a deletion of the helix–loop–helix (HLH) domain and was originally generated by Katzav and colleagues as a genetic rearrangement during a gene transfer assay (Katzav *et al.*, 1989; Coppola *et al.*, 1991). Although the function of Vav in hematopoiesis is unknown, two lines of evidence suggest that it is an important molecule. First, Vav has been shown to be expressed at an early point in the development of hematopoietic cells from embryonic stem cells *in vitro*, and inhibition of Vav synthesis by constitutive expression of antisense Vav transcripts inhibits hematopoietic differentiation (Wulf *et al.*, 1993). Second, p95^{Vav} has been reported to function as a guanine nucleotide exchange factor (GEF) for p21ras in T cells and NIH 3T3 cells (Gulbins *et al.*, 1993, 1994). However, this Ras GEF activity of p95^{Vav} was not observed in a recent report (Bustelo *et al.*, 1994). The ability of p95^{Vav} oncogene to transform NIH 3T3 cells also suggests that it could play a role in diseases of hematopoietic tissue.

One hematopoietic disorder known to be associated with aberrant signal transduction is chronic myelogenous leukemia (CML) (Sawyers *et al.*, 1991). CML is caused by the *BCR/ABL* oncogene which produces a 210 kDa protein in which the N-terminal segment is derived from the BCR gene on chromosome 22 and the C-terminal segment is derived from the ABL gene on chromosome 9 (Heisterkamp and Groffen, 1991). The resulting fusion protein, p210$^{BCR/ABL}$, has increased ABL tyrosine kinase activity, and we and others have previously shown that it interacts with several proteins involved in controlling ras activity, including rasGAP, Shc and Grb2 (Druker *et al.*, 1992; Matsuguchi *et al.*, 1994; Pendergast *et al.*, 1993). Also, recent studies indicate that aberrant activation of the ras and myc pathways may be important for the

transformation by BCR/ABL (Sawyers *et al.*, 1992; Afar *et al.*, 1994).

A prominent biological effect of BCR/ABL is to reduce or eliminate the requirement of hematopoietic cells for interleukin-3 (IL-3) for proliferation or viability (Laneuville *et al.*, 1991; Carlesso *et al.*, 1994). We have previously compared the pattern of proteins phosphorylated on tyrosine residues in factor-dependent myeloid cells lines stimulated with IL-3 or transformed by BCR/ABL, and noted that both IL-3 and BCR/ABL consistently induce tyrosyl phosphorylation of several proteins of molecular weights 90–95 kDa, which on 2-D gels were found to be partially overlapping (Matulonis *et al.*, 1993). In this report, we show that $p95^{Vav}$ is phosphorylated in response to IL-3, GM-CSF, Steel factor (SF) and $p210^{BCR/ABL}$ in myeloid cells. The kinases responsible for $p95^{Vav}$ phosphorylation were also investigated and an association between $p95^{Vav}$ and JAK kinases was identified. In cells expressing $p210^{BCR/ABL}$, tyrosine phosphorylation of $p95^{Vav}$ is directly correlated with the level of BCR/ABL tyrosine kinase activity.

Results

$p95^{Vav}$ protein expression in hematopoietic cell lines

Cell lysates from HeLa cells; four different human myeloid cell lines (MO7e, HL-60, TF-1 and K562), a human B cell line (Raji) and a human T cell line (Jurkat) were immunoprecipitated with anti-Vav 8 MoAb and blotted with the same antibody. As shown in Figure 1, $p95^{Vav}$ was visualized in every hematopoietic cell line examined but not in HeLa cells, in agreement with previous a previous study (Katzav *et al.*, 1989). The molecular size of Vav was the same in each cell line examined except in K562 cells, where a second anti-Vav reactive protein of 105 kDa was observed. In studies not shown, Vav expression was also detected in mature neutrophils, a non-proliferative cell, suggesting that Vav may function in biological events not related to proliferation.

Tyrosine phosphorylation of $p95^{Vav}$ is induced in the MO7e cell line with GM-CSF, IL-3 and Steel factor stimulation and following the transfection of $p210^{BCR/ABL}$

Since $p95^{Vav}$ is known to be tyrosine phosphorylated by growth factors when transfected into fibroblasts (Bustelo and Barbacid, 1992; Margolis *et al.*, 1992), tyrosine phosphorylation of $p95^{Vav}$ was examined in the factor-dependent human myeloid cell line MO7e, before and after stimulation with GM-CSF, IL-3 and SF and in MO7e cells transfected with $p210^{BCR/ABL}$ (MO7p210). The tyrosine phosphorylation of $p95^{Vav}$ was increased with 5 min incubation with GM-CSF, IL-3 and SF (Figure 2A). The tyrosine phosphorylation of $p95^{Vav}$ was higher in MO7p210 cells than after cytokine stimulation of MO7e cells. Tyrosine phosphorylation of $p95^{Vav}$ was also observed in two other separately isolated $p210^{BCR/ABL}$ transfected MO7e cell lines (data not shown). The same immunoprecipitates were blotted with anti-Vav 8 MoAb, showing that these cells contain approximately the same amount of $p95^{Vav}$ (lower panel of Figure 2A). This inducible tyrosine phosphorylation of $p95^{Vav}$ in MO7e cells was also observed using the other monoclonal antibody, Vav3, for immunoprecipitation (data not shown).

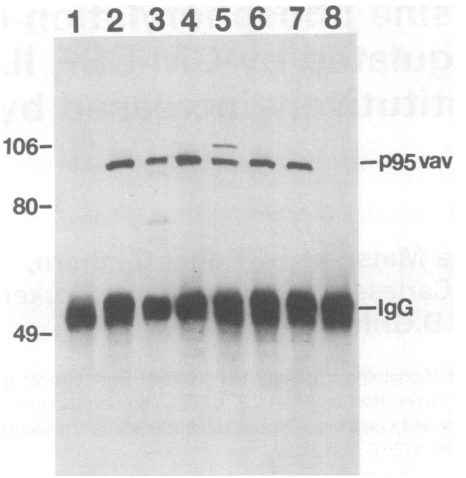


Fig. 1. Identification of $p95^{Vav}$ in various cell lines. Cell lysates prepared from 1×10^7 cells of (1) HeLa, (2) MO7e, (3) HL-60, (4) TF-1, (5) K562, (6) Raji, (7) Jurkat cells were immunoprecipitated with 2 μg of anti-$p95^{Vav}$ antibody (Vav 8), separated by 7.5% SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-Vav 8 MoAb. The same amount of MO7e cell lysate was also immunoprecipitated with preimmune antibody as a control (8).

The tyrosine phosphorylation of $p95^{Vav}$ by GM-CSF was studied in more detail. Tyrosine phosphorylation of $p95^{Vav}$ was dose dependent between 0.2 and 20 ng/ml of GM-CSF, approximately the physiological range of GM-CSF concentration required to support proliferation (Figure 2B). We also examined the time course of $p95^{Vav}$ phosphorylation after stimulation (Figure 2C). MO7e cells were stimulated with 20 ng/ml of GM-CSF, and cell lysates were prepared at various times for immunoprecipitation with anti-$p95^{Vav}$ antibody. As a comparison, we also stimulated the cells with SF at 40 ng/ml. Tyrosine phosphorylation was induced by GM-CSF within 2 min, reached a maximum level by 5 min, and declined slowly after 30 min. Interestingly, tyrosyl phosphorylated $p95^{Vav}$ accumulated more slowly after GM-CSF than after SF, where maximum phosphorylation occurred in 2 min. $p95^{Vav}$ was also tyrosine phosphorylated by GM-CSF in another factor-dependent myeloid cell line, TF-1 (data not shown), suggesting that this is a general effect of GM-CSF. In contrast to $p95^{Vav}$ phosphorylation in transfected fibroblasts, $p95^{Vav}$ was not tyrosyl phosphorylated by stimulation with fetal calf serum (FCS) or phorbol ester (data not shown).

The increase in tyrosine phosphorylation of $p95^{Vav}$ in cytokine-stimulated MO7e cells and MO7p210 cells detected by immunoblotting was confirmed by studies in which these cells were labeled *in vivo* with [^{32}P]orthophosphate (Figure 3A). Overall phosphorylation of $p95^{Vav}$ was not significantly increased with cytokine stimulation, but was markedly increased in MO7p210 cells. Before GM-CSF stimulation only serine phosphorylation was detected (Figure 3B). Tyrosine phosphorylation was induced by GM-CSF stimulation, without any significant change in the amount of serine phosphorylation. Both tyrosine and serine residues were phosphorylated in MO7p210 cells, but the ratio of ^{32}P incorporated into

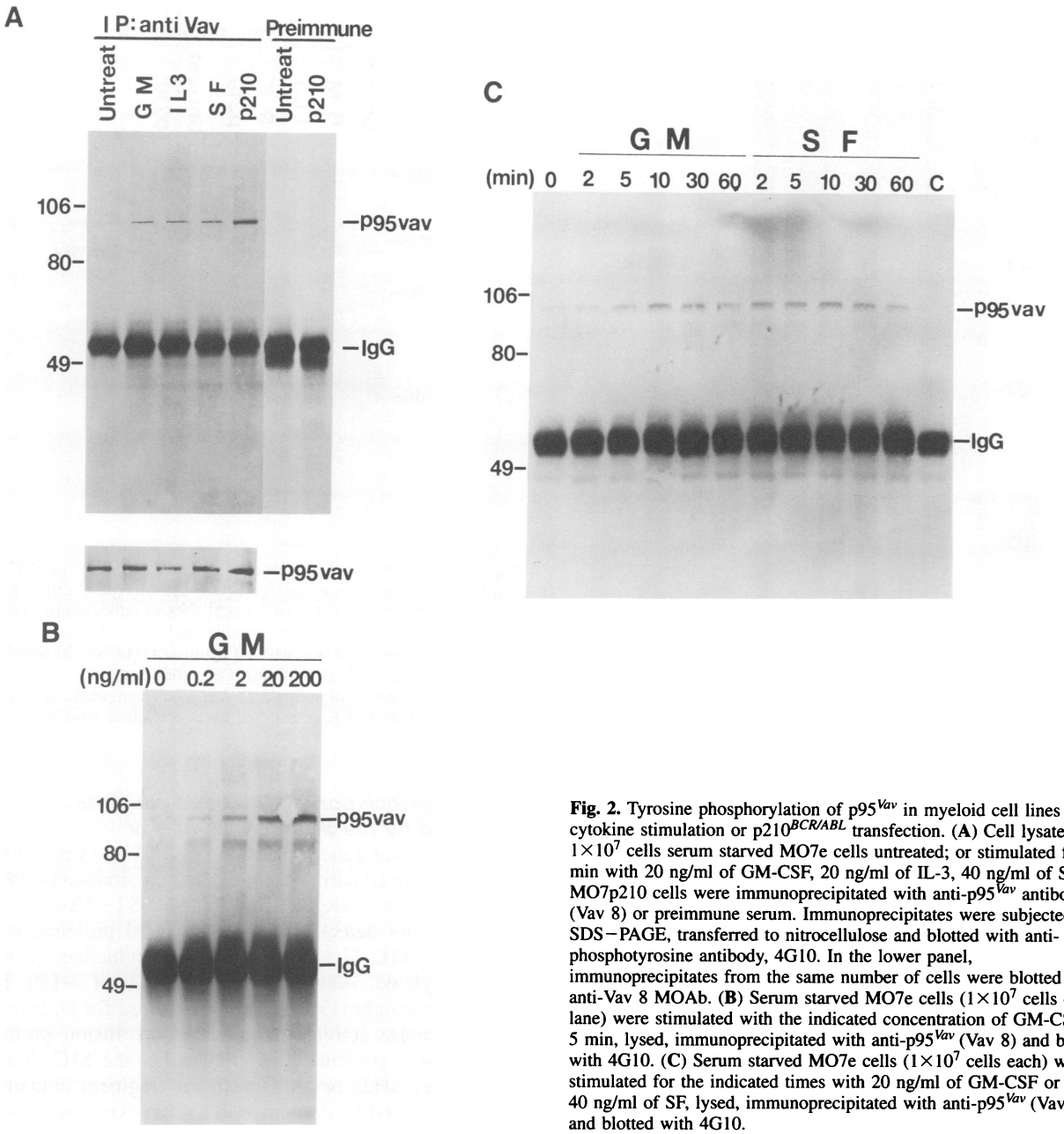


Fig. 2. Tyrosine phosphorylation of p95^{Vav} in myeloid cell lines with cytokine stimulation or p210$^{BCR/ABL}$ transfection. (**A**) Cell lysates from 1×10^7 cells serum starved MO7e cells untreated; or stimulated for 5 min with 20 ng/ml of GM-CSF, 20 ng/ml of IL-3, 40 ng/ml of SF and MO7p210 cells were immunoprecipitated with anti-p95^{Vav} antibody (Vav 8) or preimmune serum. Immunoprecipitates were subjected to SDS−PAGE, transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody, 4G10. In the lower panel, immunoprecipitates from the same number of cells were blotted with anti-Vav 8 MOAb. (**B**) Serum starved MO7e cells (1×10^7 cells each lane) were stimulated with the indicated concentration of GM-CSF for 5 min, lysed, immunoprecipitated with anti-p95^{Vav} (Vav 8) and blotted with 4G10. (**C**) Serum starved MO7e cells (1×10^7 cells each) were stimulated for the indicated times with 20 ng/ml of GM-CSF or 40 ng/ml of SF, lysed, immunoprecipitated with anti-p95^{Vav} (Vav 8) and blotted with 4G10.

serine and tyrosine was reversed compared with cytokine stimulated cells.

Phosphatase inhibitor treatment increases tyrosine phosphorylation of p95^{Vav}

It has recently been shown that some tyrosine phosphatases play an important role in signal transduction pathways regulating proliferation (Schultz *et al.*, 1993). MO7e cells and MO7p210 cells were treated with the protein tyrosine phosphatase inhibitor pervanadate and p95^{Vav} phosphorylation was determined. As shown in Figure 4, when MO7e cells were treated with pervanadate, p95^{Vav} was significantly tyrosine phosphorylated even without cytokine stimulation. Stimulation with GM-CSF, IL-3 or SF further increased the tyrosine phosphorylation of p95^{Vav}, but the previously noted dramatic difference in the extent of tyrosine phosphorylation of p95^{Vav} between cytokine stimulated MO7e cells and MO7p210 cells (Figures 2A and 3A) was abrogated. Also, pervanadate pretreatment allowed visualization of several cytokine- and p210$^{BCR/ABL}$-inducible tyrosine phosphorylated proteins which co-precipitated with Vav, notably proteins of ~140–150 kDa (Figure 4).

Association of p95^{Vav} with JAK2 tyrosine kinase

Since p95^{Vav} has been shown to be physically associated with the EGF receptor kinase after EGF stimulation when transfected in fibroblasts (Bustelo and Barbacid, 1992; Margolis *et al.*, 1992), we asked if p95^{Vav} is associated with JAK2, the major tyrosine kinase activated by the GM-CSF receptor in myeloid cells (Quelle *et al.*, 1994). Anti-JAK2 immunoprecipitates from MO7e cells were immunoblotted with anti-Vav3 MoAb. Using an alkaline phosphatase detection system, JAK2 was not reproducibly observed, However, using a more sensitive chemiluminescence detection system, we could readily visualize the co-

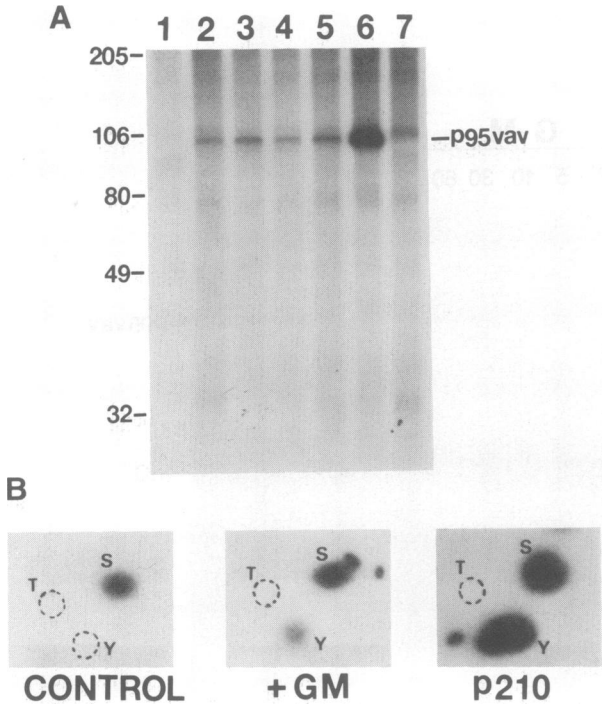


Fig. 3. *In vivo* labeling studies of $p95^{Vav}$. (**A**) *In vivo* ^{32}P-labeling of $p95^{Vav}$. 1×10^7 cells of serum starved MO7e or MO7p210 cells were labeled with 1 mCi/ml of [^{32}P]orthophosphate for 2 h, lysed and immunoprecipitated with anti-$p95^{Vav}$ antibody (Vav 8) (lanes 2–6) or pre-immune serum (lanes 1 and 7). The precipitates in each lane are from: (1 and 2) unstimulated MO7e cells; MO7e cells treated for 5 min with (3) 20 ng/ml of GM-CSF; (4) 20 ng/ml of IL-3 or (5) 40 ng/ml of SF; (6 and 7) unstimulated MO7p210 cells. Immunoprecipitates were resolved by SDS−PAGE and autoradiographed. (**B**) Phosphoamino acid analysis of $p95^{Vav}$. ^{32}P-labeled $p95^{Vav}$ from 1×10^7 cells of MO7e untreated; stimulated with 20 ng/ml of GM-CSF; or MO7p210 were hydrolyzed, resolved on TLC paper and autoradiographed. Positions of phosphoserine (S), phosphothreonine (T), phosphotyrosine (Y) markers are indicated.

precipitation of $p95^{Vav}$ and JAK2 (Figure 5). Interestingly, the co-precipitation was significantly increased after GM-CSF stimulation which would induce tyrosine phosphorylation of both $p95^{Vav}$ and JAK2.

$p95^{Vav}$ is tyrosine phosphorylated and associates with JAK2 protein tyrosine kinase when expressed in insect cells

In order to confirm the *in vivo* association we observed between $p95^{Vav}$ and JAK2 in MO7e cells, we repeated the experiments after overexpressing both proteins in insect cells infected with baculoviruses encoding murine JAK2, human $p95^{Vav}$, or a combination of the two baculoviruses. As shown in Figure 6A, $p95^{Vav}$ was tyrosine phosphorylated when infected alone, presumably due to endogenous tyrosine kinases in Sf21 cells. When JAK2 was co-infected with $p95^{Vav}$, tyrosine phosphorylation of $p95^{Vav}$ was significantly increased and a tyrosine phosphorylated 130 kDa protein was co-immunoprecipitated, which was identified as JAK2 by co-precipitation (Figure 6B). Using serial dilution studies and immunoblotting, we found that ~25% of the total JAK2 in infected Sf21 cells associates with $p95^{Vav}$ in double-infected cells. These results indicate that co-precipitation of JAK2 and $p95^{Vav}$ in insect cells is growth factor independent.

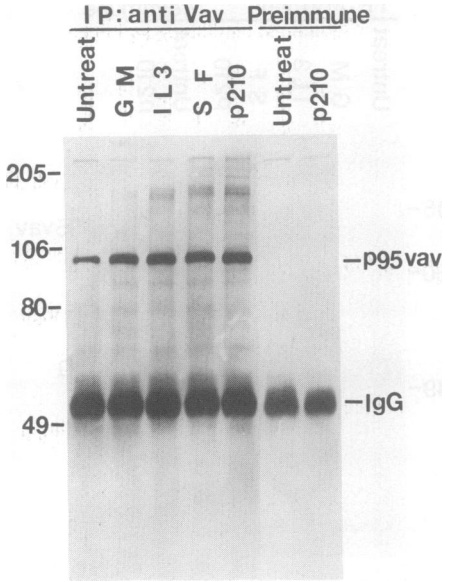


Fig. 4. Effect of tyrosine phosphatase inhibition on $p95^{Vav}$ in MO7e and MO7p210 cells. Serum starved MO7e and MO7p210 cells were incubated with 2 mM H_2O_2 and 0.1 mM sodium orthovanadate for 15 min, then were incubated for an additional 5 min without cytokines or were stimulated for 5 min with 20 ng/ml of GM-CSF; 20 ng/ml of IL-3 or 40 ng/ml of SF as indicated. Cells were lysed, immunoprecipitated with anti-$p95^{Vav}$ (Vav 8) or preimmune serum, resolved by SDS−PAGE, transferred to nitrocellulose filter, and blotted with 4G10.

In vitro association of JAK2 and $p95^{Vav}$ is mediated by the SH2 domain of Vav

Several glutathione-*S*-transferase (GST)−Vav fusion proteins were constructed to localize the domain of $p95^{Vav}$ which binds to JAK2. GST−Vav-N, GST−Vav, or GST alone did not detect any Vav-associated proteins, while GST−Vav-SH2 identified a GM-CSF-inducible, tyrosine phosphorylated, Vav-associated protein of ~130 kDa molecular weight (Figure 7A). We looked for an association of kinase activity with GST−Vav fusion proteins. These fusion proteins containing either the SH2 domain (GST−Vav-SH2) or an N-terminal fragment containing the putative HLH domain (GST−Vav-N) were used in *in vitro* kinase assays. GST−Vav-SH2 precipitates, but not GST alone or GST−Vav-N precipitates, resulted in prominent phosphorylation of a 130 kDa protein (Figure 7B). Phosphoamino acid analysis of the phosphorylated fusion protein showed that it was exclusively phosphorylated on tyrosine residues (Figure 7C). The 130 kDa protein was reactive with anti-JAK2 antibody, and co-migrated with JAK2 produced in insect cells (Figure 7D). GST−Vav-N, GST−Vav and GST did not precipitate the anti-JAK2 reactive protein. Moreover, GST−SH2 fusions of another signal transduction protein (Shc) did not precipitate the protein, suggesting that this association is specific to the Vav SH2 domain. Interestingly, GST−Vav-SH2 precipitation of JAK2 was not dependent on GM-CSF stimulation. It did, however, require phosphorylation since treatment of lysates with potato acid phosphatase reduced precipitation of JAK2 by GST−Vav-SH2 (Figure 7E). In MO7e cells, without cytokine stimulation, JAK2 was weakly phosphorylated on serine residues (Figure 7F).

We asked if other members of the JAK family kinases

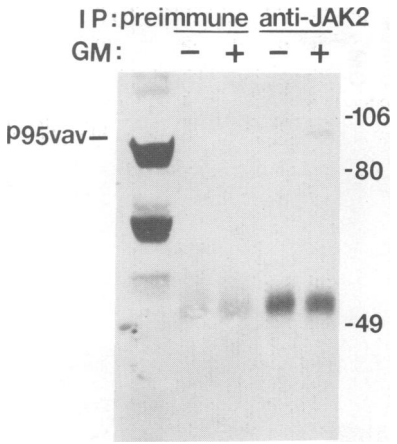


Fig. 5. *In vivo* association of p95^{Vav} and JAK2. Cell lysates from 1×10^7 cells of MO7e untreated; and stimulated with 20 ng/ml of GM-CSF were immunoprecipitated with preimmune serum or a mono-specific anti-JAK2 antiserum. Immunoprecipitates were blotted with Vav 3 MoAb, and detected with a chemiluminescence kit.

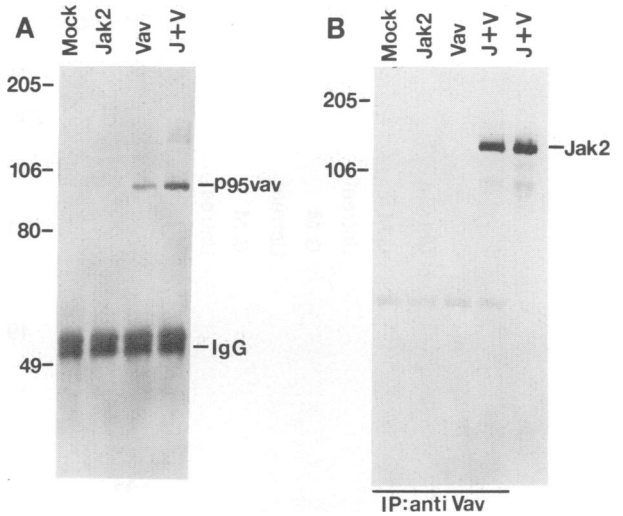


Fig. 6. p95^{Vav} is tyrosine phosphorylated and associated with JAK2 in baculovirus-Sf21 system. (**A**) p95^{Vav} is immunoprecipitated with anti-p95^{Vav} antibody (Vav 8) from Sf21 cells infected with baculoviruses expressing either vector alone, JAK2, p95^{Vav}, or doubly infected with baculoviruses expressing JAK2 and p95^{Vav} (J+V). The immunoprecipitates were resolved by SDS−PAGE, transferred to nitrocellulose and blotted with 4G10. (**B**) The same immunoprecipitates as in (A) were blotted with anti-JAK2 antibody. In the right lane, 25% of the doubly transfected lysate used for immunoprecipitation was blotted with anti-JAK2 as a control.

could associate with the GST−Vav-SH2 fusion protein. As shown in Figure 7G, JAK1, JAK3 and Tyk2 were precipitated by GST−Vav-SH2, suggesting that this interaction may involve a motif common to all the JAK family members. In Figure 7G, we used Jurkat cells to study JAK3, since JAK3 protein expression was barely detectable in MO7e cells (data not shown).

Tyrosine phosphorylation of p95^{Vav} is directed by kinase activity of p210$^{BCR/ABL}$ in p210$^{BCR/ABL}$ transfected cells

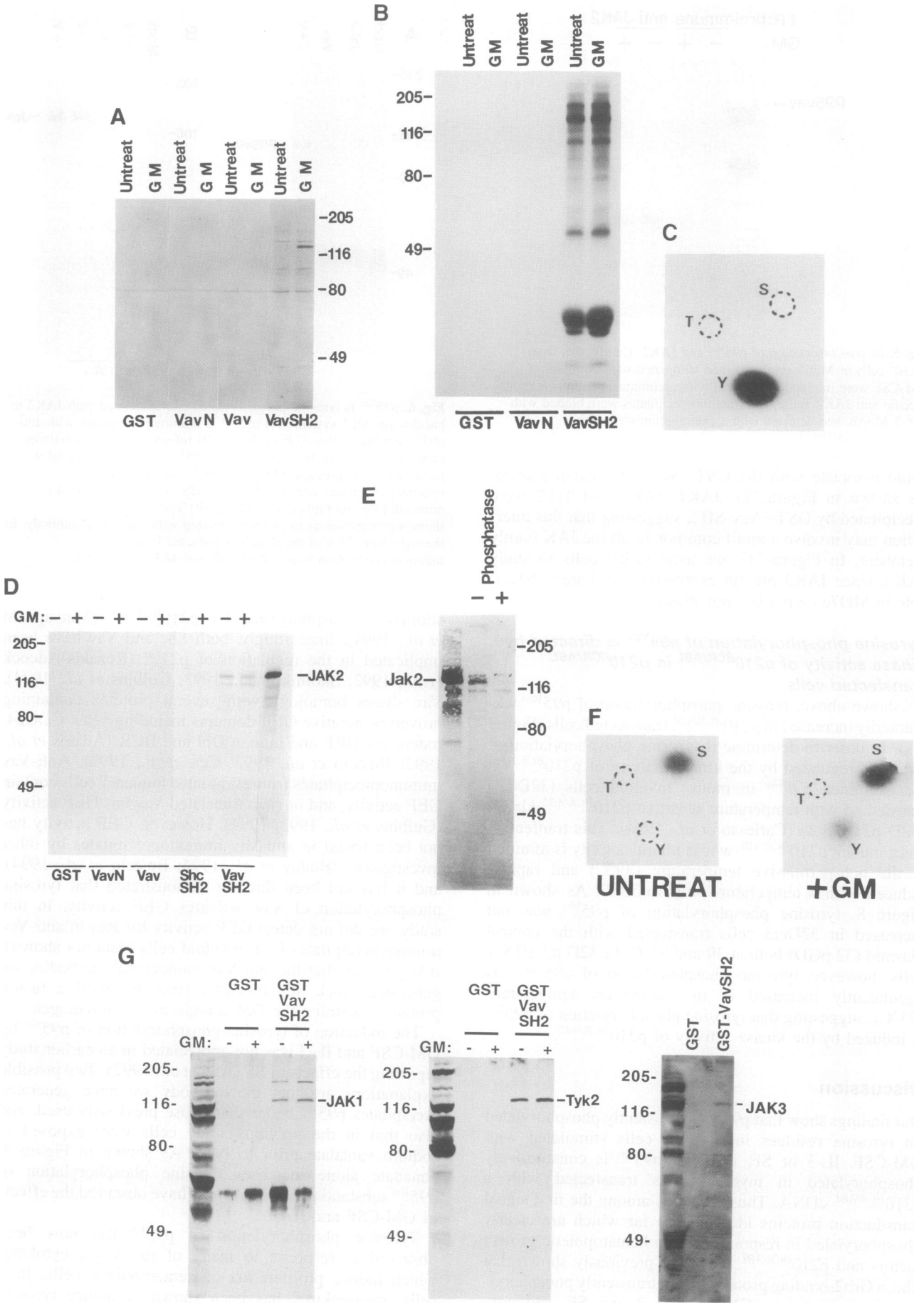
As shown above, tyrosine phosphorylation of p95^{Vav} was markedly increased in p210$^{BCR/ABL}$ transfected cells (Figure 2A). In order to determine if tyrosine phosphorylation of p95^{Vav} is regulated by the kinase activity of p210$^{BCR/ABL}$, we examined p95^{Vav} in mouse myeloid cells (32Dcl3) transfected with temperature sensitive p210$^{BCR/ABL}$ kinase (32D p210TS-1) (Carlesso *et al.*, 1994). This tranfectant has a mutant p210$^{BCR/ABL}$ whose kinase activity is minimal at the non-permissive temperature (39°C) and rapidly induced with a temperature shift to 33°C. As shown in Figure 8, tyrosine phosphorylation of p95^{Vav} was not increased in 32Dcl3 cells transfected with the control plasmid (32 pGD) both at 39 and 33°C. In 32D p210TS-1 cells, however, tyrosine phosphorylation of p95^{Vav} was significantly increased at the permissive temperature (33°C), suggesting that tyrosine phosphorylation of p95^{Vav} is induced by the kinase activity of p210$^{BCR/ABL}$.

Discussion

Our findings show that p95^{Vav} is transiently phosphorylated on tyrosine residues in myeloid cells stimulated with GM-CSF, IL-3 or SF, and that p95^{Vav} is constitutively phosphorylated in myeloid cells transfected with a p210$^{BCR/ABL}$ cDNA. Thus, p95^{Vav} is among the first signal transduction proteins identified so far which are clearly phosphorylated in response to both hematopoietic growth factors and p210$^{BCR/ABL}$. We have previously shown that Shc, a Grb2-binding protein, is also transiently phosphorylated in response to GM-CSF, IL-3 and SF, and constitutively phosphorylated by p210$^{BCR/ABL}$ (Matsuguchi *et al.*, 1994). Interestingly, both Shc and Vav have been implicated in the regulation of p21^{ras} (Rozakis-Adcock *et al.*, 1992; Skolnik *et al.*, 1993; Gulbins *et al.*, 1994). Vav shares homology with several proteins containing proven or putative GEF domains including yeast CDC24, rodent ras-GRF and human Dbl and BCR (Adams *et al.*, 1992; Bustelo *et al.*, 1992; Cen *et al.*, 1992). Anti-Vav immunoprecipitates from stimulated human T cells contain GEF activity, and *in vitro* translated Vav has GEF activity (Gulbins *et al.*, 1993, 1994). However, GEF activity has not been found in anti-Vav immunoprecipitates by other investigators (Buday *et al.*, 1994; Bustelo *et al.*, 1994), and it has not been directly demonstrated that tyrosine phosphorylation of Vav activates GEF activity. In this study, we did not detect GEF activity for Ras in anti-Vav immunoprecipitates from myeloid cells (data not shown). It is possible that the anti-Vav monoclonal antibodies we generated block this function, since we used a fusion protein containing the Dbl domain as an immunogen.

The induction of tyrosine phosphorylation of p95^{Vav} by GM-CSF and IL-3 was not appreciated in an earlier study reporting the effects of SF (Alai *et al.*, 1992). Two possible explanations are that the antibody we have generated precipitates p95^{Vav} better than those previously used, and also that in the previous study cells were exposed to sodium vanadate prior to lysis. As shown in Figure 4, vanadate alone increases tyrosine phosphorylation of p95^{Vav} substantially, and this may have obscured the effects of GM-CSF and IL-3.

Tyrosine phosphorylation of p95^{Vav} has now been observed in response to many of the major cytokines which induce proliferation of hematopoietic cells. In T cells, interleukin-2 has been shown to induce tyrosine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Tyk2
GST-VavSH2
JAK3

phosphorylation of p95^{Vav} in lck$^-$ cells, indicating that a kinase other than lck is responsible (Evans *et al.*, 1993), although Gulbins *et al.* (1994) have shown that lck can phosphorylate p95^{Vav} *in vitro*. Since IL-2, GM-CSF and IL-3 each activate one or more members of the JAK family of tyrosine kinases, we looked for an association between p95^{Vav} and JAK2 in MO7e cells stimulated with GM-CSF. GM-CSF dependent co-precipitation of a small fraction of p95^{Vav} and JAK2 was detected, although this required use of a sensitive chemiluminescent detection system. The apparent *in vivo* interaction of these proteins was confirmed by overexpressing JAK2 and p95^{Vav} in insect cells using baculovirus vectors; ~25% of the total JAK2 in infected Sf21 cells associated with p95^{Vav}. p95^{Vav} was slightly phosphorylated on tyrosine residues in the absence of JAK2 in insect cells, but this measurably increased with co-infection, suggesting that p95^{Vav} can be tyrosine phosphorylated by JAK2 *in vivo* at least when overexpressed in insect cells.

The domain of Vav involved in JAK2 interactions was localized using GST−Vav fusion proteins. GST−Vav-SH2, but not other GST−Vav proteins containing the N-terminal putative HLH-LZ domain or the Dbl domain, precipitated a GM-CSF inducible, tyrosine phosphorylated 130 kDa protein immunoreactive with anti-JAK2 antibody. Overall, these data show that JAK2 can associate with GST−Vav-SH2 *in vitro*, with p95^{Vav} *in vivo* and can induce tyrosine phosphorylation of p95^{Vav} in insect cells. Given the complexity of p95^{Vav} structure, however, Vav could be the target of more than one tyrosine kinase. Interestingly, GST−Vav-SH2 also precipitated other members of the JAK family of tyrosine kinases, JAK1 and Tyk2 from MO7e cells, and JAK3 from Jurkat T cells. Although not tested here, these results suggest that p95^{Vav} may be a common target for JAK tyrosine kinases in hematopoietic cells, and *in vivo* association of p95^{Vav} with other JAK kinases should be examined. The presence of multiple potentially functional domains in Vav suggests that its functions may be complex, and it is possible that it is involved in signaling in more than one pathway (Wulf *et al.*, 1993), with some variability among different lineages.

We found that GM-CSF induces increased phosphorylation of JAK2 on tyrosine residues in MO7e cells, and this suggests that the inducible association of JAK2 and Vav we found *in vivo* is due to binding of the Vav-SH2 to a newly phosphorylated tyrosine residue on JAK2. However, the GST−Vav-SH2 fusion protein precipitated JAK2 from cell lysates whether or not they were stimulated with GM-CSF, and a further enhancement was not seen using lysates from GM-CSF stimulated cells. It is possible that JAK2 is tyrosine phosphorylated at a low level without stimulation, and that this is sufficient to allow binding of GST−Vav-SH2, but not sufficient for Vav binding *in vivo*. It is also possible that GST−Vav-SH2 binding activates JAK2 kinase activity or reduces binding of a phosphatase, or that GM-CSF induces an *in vivo* change in the subcellular localization of p95^{Vav}, JAK2 or both, to facilitate interaction. Interestingly, JAK2 does not contain the reported optimal binding motif for Vav-SH2 domain, Y-M-E-P (Silvennoinen *et al.*, 1993; Songyang *et al.*, 1994). Since we observed that the binding of GST−Vav-SH2 to pp130 was clearly phosphorylation dependent (Figure 7E), the possibility should be considered that GST−Vav-SH2 binds to JAK2 in a phosphoserine-dependent manner, as has recently been observed for the binding of BCR to the SH2 domain of ABL (Pendergast *et al.*, 1991; Muller *et al.*, 1992). JAK2 isolated from resting MO7e cells is phosphorylated on serine residues as shown in Figure 7F. Additional studies will be needed to determine the exact mechanism of interaction of Vav-SH2 with JAK2, which residues are involved and whether or not JAK2 is involved in phosphorylation of p95^{Vav} *in vivo*.

Finally, the mechanisms whereby p210$^{BCR/ABL}$ reduces or eliminates growth factor dependence in hematopoietic cells have not yet been identified, and our results suggest that Vav could play a role. p210$^{BCR/ABL}$ transforms hematopoietic cells in culture more readily than non-hematopoietic cells for unknown reasons, although overexpression of myc has been shown to facilitate p210$^{BCR/ABL}$-transformation of rodent fibroblasts (Afar *et al.*, 1994). Our results demonstrate that p95^{Vav} is dramatically phosphorylated on tyrosine residues in myeloid cell lines made to express p210$^{BCR/ABL}$. The studies presented with a temperature sensitive p210$^{BCR/ABL}$ indicate that increased tyrosine phosphorylation of p95^{Vav} is directly due to p210$^{BCR/ABL}$, but do not show that p95^{Vav} is necessarily a direct substrate of p210$^{BCR/ABL}$. We did not detect co-precipitation of p95^{Vav} with p210$^{BCR/ABL}$, and an indirect effect is therefore possible. However, we have not detected activation of JAK2 by BCR/ABL in myeloid cells, and it is intriguing to speculate that p95^{Vav} is phosphorylated by

Fig. 7. Association of JAK family tyrosine kinases with the Vav-SH2 domain *in vitro*. (**A**) Binding of tyrosine phosphorylated proteins with Vav−GST fusion proteins. Serum starved MO7e cells untreated or stimulated with 20 ng/ml of GM-CSF were lysed and incubated with immobilized GST, GST−Vav-N, GST−Vav or GST−Vav-SH2. After incubation for 2 h at 4°C, the beads were washed three times with lysis buffer and the bound proteins were analyzed by SDS−PAGE, transferred to nitrocellulose and blotted with 4G10. (**B**) *In vitro* kinase assay of GST fusion protein precipitates. Lysates from starved or GM-CSF stimulated MO7e cells (1×10^7 cells each lane) were incubated with GST, GST−Vav-N or GST−Vav-SH2 bound to beads. Precipitates were incubated with [γ-^{32}P]ATP in kinase buffer, washed with lysis buffer three times, separated by SDS−PAGE and autoradiographed. (**C**) Phosphoamino acid analysis of *in vitro* kinase reaction. ^{32}P-labeled GST−Vav-SH2 fusion protein from *in vitro* kinase reaction was hydrolyzed, resolved on TLC paper and autoradiographed. The markers are the same as in Figure 3B. (**D**) Association of JAK2 with Vav GST fusion proteins. Cell lysates were prepared as described in (A) and precipitated with GST, GST−Vav-N, GST−Vav, GST−Shc-SH2 or GST−Vav-SH2. Precipitates were blotted with anti-JAK2 antibody. The lysate from Sf21 insect cells infected with JAK2 was used as a control. (**E**) Dephosphorylation of JAK2 abolishes binding to the Vav SH2 domain. Starved MO7e cells (1×10^7 cells) were lysed in the presence of phosphatase inhibitors (lane 2). The same number of cells were lysed in the absence of phosphatase inhibitors, and the lysate was incubated with PAP (lane 3). Precipitates with GST−Vav-SH2 beads were blotted with anti-JAK2 antibody. The lysate from Sf21 insect cells infected with JAK2 was used as a control (lane 1). (**F**) Phosphoamino acid analysis of JAK2. ^{32}P-labeled JAK2 immunoprecipitates from 1×10^7 cells of MO7e untreated or stimulated with 20 ng/ml of GM-CSF were hydrolyzed, resolved and autoradiographed. The markers are the same as in Figure 3B. (**G**) Binding of other JAK family kinases with GST−Vav-SH2 fusion protein. Left; cell lysates from untreated and GM-CSF-stimulated MO7e cells were incubated with immobilized GST, or GST−Vav-SH2, washed, and the bound proteins were blotted with anti-JAK1 antibody. Middle; the same filter was stripped and reblotted with anti-Tyk2 antibody. Right; cell lysates from Jurkat cells were incubated with GST fusion proteins and the bound proteins were blotted with anti-JAK3 antibody.

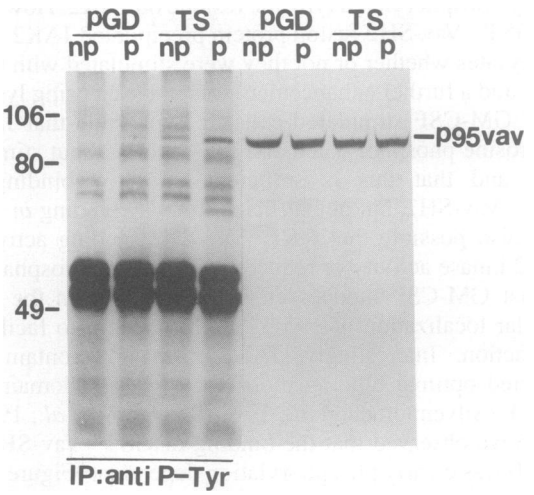

Fig. 8. Inducible tyrosine phosphorylation of $p95^{Vav}$ by temperature sensitive $p210^{BCR/ABL}$. 32D pGD and 32D p210TS-1 cells were incubated either at 39°C (np) or 33°C (p) and lysed. In the left lanes, cell lysates (1×10^7 cells) were immunoprecipitated with 4G10, resolved by SDS−PAGE, transferred to nitrocellulose filter and blotted with anti-$p95^{Vav}$ (Vav 3). In the right lanes the same amounts of cell lysates were immunoprecipitated with anti-$p95^{Vav}$ (Vav 8) and blotted with anti-Vav 3 MoAb.

JAK2 or a related kinase during normal signaling, and by $p210^{BCR/ABL}$ in CML. Further studies of phosphorylation sites and their biological significance are warranted in both situations. It is possible that $p95^{Vav}$, which is unique to hematopoietic cells, contributes to the specificity of transformation of this lineage exhibited by $p210^{BCR/ABL}$. However, other hematopoietic restricted proteins such as $p93^{fes}$ are also targets of $p210^{BCR/ABL}$ (Ernst *et al.*, 1994). Overall, since $p95^{Vav}$ is phosphorylated in response to both hematopoietic cytokines and by $p210^{BCR/ABL}$, our results suggest that Vav is potentially involved in mediating some of the transforming effects of this oncogene.

Materials and methods

Cells and cell culture

The human GM-CSF, IL-3 and SF-dependent cell line, MO7e, was obtained from Dr Steve Clark (Genetics Institute, Cambridge, MA) and was originally derived by Avanzi and colleagues from the peripheral blood cells of an infant with acute megakaryocytic leukemia (Avanzi *et al.*, 1987). The cell line was cultured in DMEM medium (Gibco) supplemented with 20% FCS, rhGM-CSF, 10 ng/ml and rhIL-3, 10 ng/ml (Genetics Institute, Cambridge, MA). The 32Dcl3 cell line was obtained from Joel Greenberger (University of Massachusetts Medical Center, Worcester, MA) and was cultured in RPMI 1640, 10% FCS and 15% medium conditioned by the WEHI-3B cell line as a source of murine IL3. Other cell lines were obtained from American Type Culture Collection.

MO7e sublines expressing $p210^{BCR/ABL}$ were generated by transfection of plasmid pGD210 (Daley *et al.*, 1990) (a gift from Richard van Etten and George Daley, MIT) into MO7e cells by electroporation as previously described (Matsuguchi *et al.*, 1994) using a Bio-Rad Gene Pulser (Richmond, CA) and selecting for G418 resistant sublines. Three independently isolated cell lines were generated. Each MO7p210 cell line was factor independent and was shown to express $p210^{BCR/ABL}$ by immunoblotting as described below. No autocrine factors were detected in media conditioned by these cell lines. The same subline MO7p210-1 was used for all experiments described.

Antibodies

A cDNA encoding N-terminal truncated Vav (Katzav *et al.*, 1989) was kindly provided by Dr Mariano Barbacid (Bristol Myers Squibb Pharmaceutical Research Institute). The remaining 5′ portion of the cDNA was prepared from HL-60 total RNA by reverse transcriptase PCR, and was ligated to the 5′ truncated cDNA to give a cDNA encoding the complete $p95^{Vav}$ protein. A 1023 bp *Pvu*II fragment (nucleotides 504–1527) was cloned into the *Sma*I site of the pGEX-3X vector. This fragment encodes amino acids 46–385 of $p95^{Vav}$ including the putative LZ domain and the Dbl domain. Bacterial cultures containing the pGEX-3X-Vav plasmid were induced with 0.04 mM IPTG and lysed by sonication. The resulting GST fusion protein was purified on glutathione agarose as described (Kaelin *et al.*, 1991). A Balb/c mouse was immunized with a series of five bi-weekly subcutaneous injections of purified fusion protein. Hybridoma cell lines producing antibodies against the injected protein were prepared as described (Griffin *et al.*, 1981). Two of the positive hybridoma cell lines (clones 3 and 8) were injected intraperitoneally into Balb/c mice and monoclonal antibody from the resulting ascites was purified on a protein A−Sepharose 4B (Pharmacia) column. Vav immunoblots were done with the monoclonal antibody dissolved in TBST (10 mM Tris−HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) at 1 μg/ml. Immunoblots using anti-phosphotyrosine monoclonal antibody 4G10 were performed as described (Druker *et al.*, 1992). Anti-JAK2 rabbit antiserum and anti-JAK3 antiserum were generous gifts from Dr James N.Ihle (St Jude Children's Hospital) and used at 1:5000 for immunoblotting. Anti-JAK1 antibody was purchased from UBI (Lake Placid, NY). Anti-Tyk2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These anti-JAK family kinase antibodies were raised against their specific peptide sequences and do not cross-react with the other JAK family kinases.

Preparation of GST fusion proteins

The pGEX-3X vector was used for overexpression of GST fusion proteins in *Escherichia coli*. GST−Vav was prepared as described above. pGEX−Vav-SH2 contains a 285 bp fragment of Vav SH2 domain (encoding amino acids 671–765) prepared by PCR from Vav cDNA. GST−Vav-N contains a 240 bp PCR fragment of Vav N-terminal portion (amino acids 14–93). This fragment contains both the putative HLH domain and the LZ domain. GST−Shc-SH2 contains a 285 bp fragment of Shc SH2 domain (amino acids 377–471) as previously described (Matsuguchi *et al.*, 1994). GST fusion proteins (GST−Vav-SH2, GST−Vav-N, GST−Shc-SH2 and GST protein alone) were induced in *E.coli* with 0.1 mM IPTG, purified on glutathione−agarose as described (Kaelin *et al.*, 1991) and used for precipitation and other assays.

Immunoprecipitation and immunoblotting

Cells were incubated with cytokines as indicated, rinsed with ice cold phosphate buffered saline and lysed in PLC buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin) at 10^8 cells/ml. Cell lysates were centrifuged at 12 000 *g* for 5 min and the resulting supernatants incubated with either anti-Vav antibody or pre-immune mouse serum for 2 h at 4°C. Immune complexes were collected on protein A–Sepharose during a 1 h incubation at 4°C, washed three times with lysis buffer and boiled for 5 min in sample buffer (62.5 mM Tris−HCl, pH 6.8, 2% SDS, 5% glycerol, 2% β-mercaptoethanol, 0.001% bromophenol blue). Immunoprecipitates and lysates were subjected to SDS−PAGE, then blotted onto nitrocellulose membranes (Schleicher and Schuell). The membranes were then blocked with 1% gelatin in TBS buffer (10 mM Tris−HCl, pH 8.0, 150 mM NaCl) for 2 h, rinsed in TBST, incubated with the indicated primary antibodies for 2 h, rinsed in TBST and incubated for 1 h in alkaline phosphatase conjugated anti-mouse or anti-rabbit antibodies (Promega). The membranes were washed in TBST and exposed to 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium detection (Promega). For some blots, filters were blocked with 3% gelatin in TBST buffer for 2 h, rinsed, incubated with primary antibodies for 2 h, rinsed, incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (Amersham) for 1 h, rinsed and exposed to enhanced chemiluminescence detection (Amersham).

In some experiments, MO7e cell lysates were treated with potato acid phosphatase (PAP) (Sigma) to non-specifically dephosphorylate phosphoproteins. Lysates from 1×10^7 cells were diluted 6-fold to 600 μl with dephosphorylation buffer (40 mM PIPES, pH 6.0, 1 mM DTT, 1 mM $MgCl_2$, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Dephosphorylation was initiated by addition of 10 μg of PAP. After incubation at 30°C for 15 min, lysates were mixed with GST−Vav-SH2 for precipitation.

In vitro kinase assay

The GST fusion protein precipitates were washed three times with lysis buffer, one time with kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM ATP), suspended in kinase buffer containing [γ-^{32}P]ATP (24 nmol/ml, 7000 Ci/mmol, New England Nuclear (NEN), Boston, MA) for 20 min at 30°C, washed three times with lysis buffer, suspended in sample buffer, heated at 95°C for 5 min, resolved by SDS–PAGE and autoradiographed on Kodak XAR-5 film.

Cell metabolic labeling

For labeling with [^{32}P]orthophosphate, 1×10^7 cells were washed with phosphate-free DMEM containing 0.5% BSA and cultured in the phosphate-free medium with the addition of 1 mCi of [^{32}P]orthophosphate (NEN) for 2 h, incubated with or without cytokine for 5 min and lysed.

Phosphoamino acid analysis

The ^{32}P-labeled immunoprecipitates or GST fusion protein precipitates were resolved by SDS–PAGE, transferred to polyvinylidene difluoride membrane and autoradiographed. The excised bands from the membrane were directly subjected to 6 N HCl hydrolysis at 110°C for 60 min. The hydrolysates were dried and washed with ddH_2O. Samples were then subjected to 2-D electrophoresis on TLC plates with pH 1.9 running buffer (90% formic acid:acetic acid:ddH_2O, 25:78:897 by vol.) and then with pH 3.5 running buffer (pyridine:acetic acid:ddH_2O, 5:50:945 by vol.). Unlabeled phosphoamino acid markers were visualized by spraying with 0.5% ninhydrin in acetone. ^{32}P-labeled amino acids were visualized by autoradiography on Kodak XAR-5 film.

Tyrosine phosphatase inhibition treatment

Tyrosine phosphatase inhibition treatment with H_2O_2 and sodium vanadate (pervanadate) was done as previously described (Okuda *et al.*, 1991). MO7e cells were incubated in serum-free medium at 37°C for 16 h. H_2O_2 (2 mM) and 0.1 mM sodium vanadate were added to the cells for a period of 15 min, followed by cytokine treatment of some cells and further incubation of 5 min before cell lysis.

Baculovirus expression

Baculovirus containing human Vav cDNA was prepared by cloning a Vav cDNA containing the whole coding region into the transfer vector pVL1393. Plasmid constructs were then co-transfected with linearized Baculogold baculovirus DNA (Pharmingen) into Sf21 cells as recommended by the manufacturer. Baculovirus containing murine JAK2 cDNA was a generous gift from Dr James N.Ihle (St Jude Children's Research Hospital). Expression of proteins in Sf21 was achieved by incubating cells in the presence of one or more high titer recombinant viruses (m.o.i. of 5–10). Cells were lysed 48 to 72 h after infection in RIPA buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 20 µg/ml leupeptin and 1 mM sodium orthovanadate) and analyzed by immunoprecipitation and Western blotting as described above.

Temperature sensitive mutant p210$^{BCR/ABL}$ transfected cells

A mutant of BCR/ABL in which the ABL kinase activity was temperature sensitive was created by site directed mutagenesis of Arg to His at amino acid 457 and Tyr to His at amino acid 469 of c-abl as previously described (Carlesso *et al.*, 1994). The temperature sensitive mutant (p210TS-1) was introduced into the IL-3-dependent murine myeloid cell line 32Dc13 by electroporation generating a cell line (32D p210TS-1) which showed a significant increase of kinase activity on temperature shift from 39 to 33°C. 32Dc13 cell line transfected with the control plasmid (32D pGD) was used as a control.

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