# Vav is associated with signal transducing molecules gp130, Grb2 and Erk2, and is tyrosine phosphorylated in response to interleukin-6

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**Abstract Vav is a hematopoietic cell-specific proto-oncogene. We show that interleukin-6 (IL-6) induces transient tyrosine phosphorylation of Vav in a human myeloma cell line, U266. A membrane-distal part of the cytoplasmic region of gp130 is critical for association between Vav and gp130, and the IL-6-induced tyrosine phosphorylation of Vav. Mitogen-activated protein kinase (MAPK) ($p42^{MAPK}$ or extracellular signal-regulated kinase 2 (Erk2)) is coprecipitated with Vav. MAPK activity in the anti-Vav immunoprecipitates is upregulated by IL-6 stimulation. Furthermore Vav is associated with Grb2 which is known as an adapter protein leading to Ras activation. The results imply that Vav may link gp130 activation to downstream MAPK activation in hematopoietic cells.**

*Key words:* Signal transduction; Tyrosine phosphorylation; Vav; gp130

## 1. Introduction

Cytokines function initially through interaction with cell surface receptors, leading to activation of associated tyrosine kinases. These activated tyrosine kinases phosphorylate receptors on their tyrosine residues, providing binding sites for the downstream intracellular molecules and inducing their activation. IL-6 is a pleiotropic cytokine and exerts its effects first by binding to its receptor (IL-6R) [1]. The IL-6/IL-6R complex subsequently interacts with the transmembrane signal transducing receptor component gp130 [2], which results in homodimerization and tyrosine phosphorylation of gp130 [3]. gp130 is a common receptor component for IL-6-related cytokines like leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, IL-11 and cardiotrophin-1 ([4] and references therein). We and others have observed that gp130 recruits multiple kinases and adapter proteins. For example, cytoplasmic tyrosine kinases in the Jak family are associated with gp130 and are activated in response to IL-6 [5–7]. Moreover, IL-6 induces activation of MAPK [8], and gp130 is known to be associated with Grb2, an adapter protein in the MAPK pathway [9]. Grb2 has been shown to bind constitutively to a guanine nucleotide exchange factor, Sos, which activates Ras by replacing GDP with GTP [10–12].

Vav has been observed as a substrate for receptor- and non-receptor-tyrosine kinases and known to link activated cytokine receptors to downstream effectors such as Shc, Grb2 and MAPK. Vav is a 95-kDa proto-oncogene product expressed exclusively in hematopoietic cells and trophoblasts [13,14]. Vav deficient mice showed that Vav is critical for proliferative response of T- and B-cells [15–17] and embryo development [14]. Vav undergoes tyrosine phosphorylation in response to ligation of antigen receptors on B- and T-cells [18–20], and stimulation by epidermal growth factor [20,21], IL-2 [22], steel factor [23], IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [24,25], G-CSF [26], erythropoietin (EPO) [27], interferon type I [28], and insulin [29]. Vav encodes an array of structural motifs including two Src homology (SH) 3 domains, and one each of the SH2, Dbl-Cdc 24 homology (DH) and pleckstrin homology domains [20,21,30,31]. The SH2 and SH3 domains of Vav have been reported to interact with Shc, Grb2, PI3K, GAP and tyrosine kinases including the Jak family and ZAP-70, which are all known to take part in cytokine signal transduction pathways [25,32–34].

In the current study using a human myeloma B-cell line U266, we demonstrate that IL-6 induces tyrosine phosphorylation of Vav, which requires the cytoplasmic region of gp130. We further show that downstream signaling molecules such as Grb2 and Erk2 are associated with Vav, suggesting possible involvement of Vav in MAPK cascade in IL-6 signal transduction.

## 2. Materials and methods

### 2.1. Cell culture

The human myeloma B cell line U266 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), penicillin (100 units/ml), streptomycin (100 μg/ml), and $5\times10^{-5}$ M β-mercaptoethanol. Vaccinia virus infection of U266 cells was done as described except that a poly-L-lysine (Sigma) coated dish was used to enhance the infection efficiency [3].

### 2.2. Antibodies and reagents

Recombinant human IL-6 was kindly provided by Dr. K. Yasukawa, Tosoh Corp. (Japan). Rabbit polyclonal antibodies to Vav and Erk2 as well as respective immunizing peptides were purchased from

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*Abbreviations:* IL, interleukin; MAPK, mitogen-activated protein kinase; Erk2, extracellular signal-regulated kinase 2; GST, glutathione *S*-transferase


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Santa Cruz Biotechnology (USA). Mouse monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. (USA), and mouse monoclonal anti-Erk2 antibody from Seikagaku Kogyo (Japan). Rabbit polyclonal anti-Grb2 antibody was prepared against a peptide corresponding to DPQEDGELGFRRGD. Mouse monoclonal anti-Grb2 antibody was kindly provided by Dr. J. Schlessinger. Myelin basic protein (MBP) was purchased from Sigma, and [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol), BIOTRAK MAPK assay kit and enhanced chemiluminescence (ECL) kit from Amersham (USA).

*2.3. Immunoprecipitation and immunoblotting*

Cells were starved for 9–12 h in FCS-free RPMI 1640 and stimulated with or without recombinant human IL-6 (0.5 μg/ml) at 37°C. The reaction was quenched by ice-cold lysis buffer (20 mM HEPES, 0.5% (v/v) Brij 96, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 10 mM NaF, 0.5 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, pH 7.4). Cell lysates were collected after spinning at 15000 rpm for 15 min. Immune complexes with anti-Vav, -Erk2, -Grb2 and -gp130 antibodies were precipitated with protein A-Sepharose (Pharmacia), and washed with lysis buffer. Samples were subjected to SDS-PAGE and electrotransferred to ECL-nitrocellulose membrane (Amersham). The immobilized proteins on the membrane were detected with the specific primary and secondary antibodies, and developed with the ECL kit according to the manufacturer's recommended procedure (Amersham).

*2.4. Preparation of glutathione S-transferase (GST) fusion proteins*

The pGEX-2T vector (Pharmacia) was used for overexpression of GST fusion proteins in *Escherichia coli*. GST-gp130 fusion proteins were prepared as described [49]. Briefly, each fragment of gp130 cDNA encoding amino acids 620–692, amino acids 620–706 or amino acids 620–918 [35] was cloned into the *Eco*RI site of the pGEX-2T vector. GST fusion proteins were induced with 0.3 mM isopropyl β-D-thiogalactoside and purified on glutathione-Sepharose (Pharmacia).

*2.5. In vitro kinase assay*

The immunoprecipitates prepared as described above were further washed with kinase buffer (20 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4), and incubated with MBP or synthetic peptide (MAPK assay kit, Amersham). Kinase reaction was performed in 25 μl of kinase buffer containing 5 μCi of [$\gamma$-$^{32}$P]ATP at 30°C for 30 min. The $^{32}$P-labeled MBP was resolved by SDS-PAGE and visualized by autoradiography. The labeled peptide was trapped on a binding paper (MAPK assay kit, Amersham) and the radioactivity was counted according to the manufacturer's recommended procedure.

## 3. Results

*3.1. IL-6 stimulation induces rapid and transient tyrosine phosphorylation of Vav in U266 cells*

U266 is a human myeloma cell line and exhibits enhanced proliferation in the presence of IL-6 [36]. To examine involvement of Vav in IL-6 signal transduction, U266 cells were stimulated with IL-6 for varying periods. Cell lysates were subjected to precipitation with anti-Vav antibody and subsequent immunoblot with anti-phosphotyrosine antibody. Fig. 1 (upper panel) shows that tyrosine phosphorylation of Vav occurred rapidly within 5 min after IL-6 stimulation and reached the maximum level at 10 min. It declined significantly after 30 min. Reblotting the same membrane with anti-Vav antibody confirmed that a comparable amount of Vav was precipitated from IL-6-stimulated and non-stimulated cell lysates (Fig. 1, lower panel).

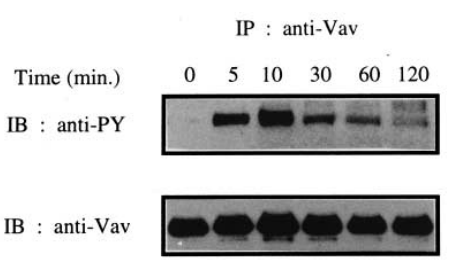


Fig. 1. Identification of Vav phosphorylation on tyrosine. U266 ($6\times10^7$/lane) were stimulated without or with IL-6 (0.5 μg/ml) for indicated periods. Cell lysates were immunoprecipitated (IP) with rabbit polyclonal anti-Vav antibody. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (IB) with mouse monoclonal anti-phosphotyrosine antibody (anti-PY, upper panel). This blot was stripped and reprobed with mouse monoclonal anti-Vav antibody (lower panel).

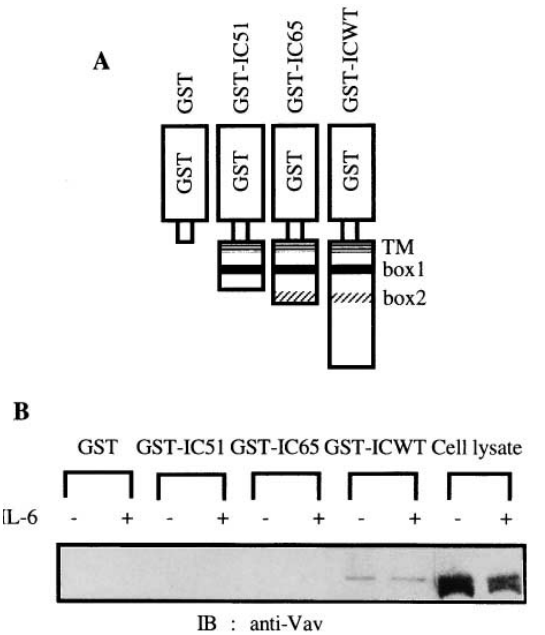


Fig. 2. Association between Vav and gp130. A: Schematic diagram of gp130 constructs. GST was fused to the transmembrane domain (TM) and different numbers of amino acids in the cytoplasmic domain of gp130. B: U266 ($7\times10^7$/lane) were stimulated without (−) or with (+) IL-6 (0.5 μg/ml) for 10 min and cell lysates were incubated with GST or various GST-gp130 fusion proteins. The purified GST fusion proteins with glutathione-Sepharose beads were separated on SDS-PAGE and immunoblotted with monoclonal anti-Vav antibody. In the last two lanes, cell lysates were directly loaded onto SDS-PAGE.

*3.2. Vav is associated with the membrane-distal region of gp130*

To examine whether Vav is associated with the cytoplasmic region of gp130, we utilized various GST-human gp130 fusion proteins designed according to its structural features [35]. These GST-gp130 fusion proteins have either 51, 65 or 277 cytoplasmic amino acids (GST-IC51, GST-IC65 and GST-ICWT, respectively; see Fig. 2A). Lysates from cells stimulated with or without IL-6 were incubated with these fusion proteins, and then the absorbents with glutathione beads were analyzed by SDS-PAGE and immunoblotted with anti-Vav antibody. As seen in Fig. 2B, GST-ICWT, which contains the entire cytoplasmic region of gp130, showed constitutive binding with Vav. However, GST-IC65, which contains box1 and box2 but no further membrane-distal region, did not. This blot was reprobed with anti-GST antibody and the amounts of GST and GST-gp130 fusion proteins were found to be compatible (data not shown).

Based on these results, IL-6-induced tyrosine phosphorylation of Vav was then examined in U266 cells expressing wild

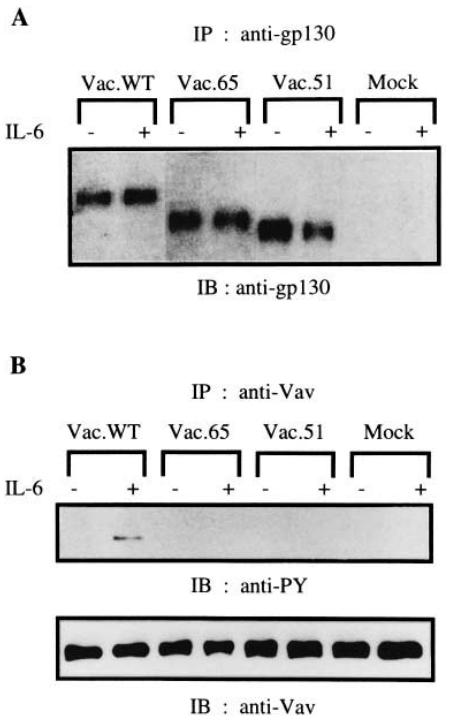


Fig. 3. Requirement of a membrane-distal part of gp130 for tyrosine phosphorylation of Vav. U266 cells ($2\times10^6$/lane) were infected with vaccinia virus ($2\times10^7$) encoding entire or truncated gp130 cDNAs. After incubation with virus for 16 h and following starvation for 2 h, cells were stimulated without (−) or with (+) a combination of IL-6 (5 ng/ml) and soluble IL-6R (10 ng/ml) for 10 min. A: Cell lysates were immunoprecipitated and immunoblotted with monoclonal anti-gp130. B: The cell lysates were immunoprecipitated with polyclonal anti-Vav antibody and blotted with monoclonal anti-PY antibody (upper panel). After stripping, this blot was reprobed with monoclonal anti-Vav antibody (lower panel).

type or truncated gp130. U266 cells were transfected with vaccinia virus which encodes various human gp130 constructs: Vac.WT expresses intact human gp130. Vac.65 and Vac.51 are deletion mutants of gp130, truncated just below box2 or box1, respectively, just as GST-IC65 and GST-IC51 (see Fig. 2A). All of the viral gp130 proteins contain the entire extracellular and transmembrane regions of gp130. The amount of viral gp130 proteins was examined by precipitation and blotting with monoclonal anti-gp130 antibody (Fig. 3A). Under this experimental condition, the expression of the endogenous gp130 was below the detection limit (see Mock lanes). In this system, tyrosine phosphorylation of Vav was only observed following IL-6 treatment of cells expressing Vac.WT, but not cells expressing the truncated mutants of gp130. The immunoblotting with anti-Vav antibody showed a comparable amount of Vav protein present in all these U266 transfectants (Fig. 3B). Taken together, the membrane-distal region of gp130 is likely to be necessary for tyrosine phosphorylation of Vav in response to IL-6.

### 3.3. *Erk2 is associated with Vav*

Phospho-amino acid assays of $^{32}$P-labeled Vav showed phosphorylation not only on tyrosine but also on serine residues [20,25]. Recently, experiments with prolactin-induced Vav revealed the increased phosphorylation of Vav on serine/threonine residues in Nb2, a rat T-cell lymphoma cell line [37]. These previous findings led us to consider a possible association of serine/threonine kinase with Vav. We examined whether Erk2 is associated with Vav. As shown in Fig. 4A, Erk2 was detected in anti-Vav immunoprecipitates from U266. The electrophoretic mobility of the Vav-associated Erk2 protein was shifted upwards when cells were stimulated with IL-6. The Erk2 with a slower migration was suggested to be phosphorylated Erk2. The inclusion of the immunizing peptide during the immunoprecipitation step resulted in the complete disappearance of Erk2, indicating that Erk2 was present specifically in the anti-Vav immunoprecipitates.

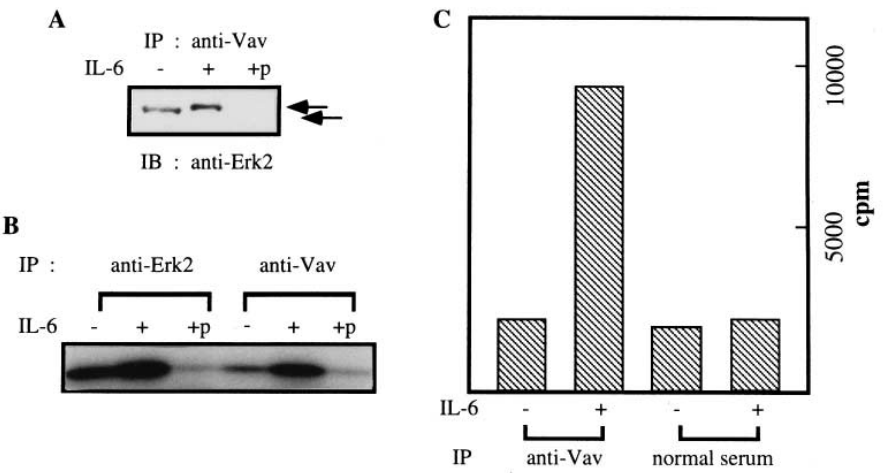


Fig. 4. Coprecipitation of Vav with Erk2. U266 ($7\times10^7$/lane) were stimulated without (−) or with (+) IL-6 (0.5 μg/ml) for 10 min. A: Cell lysates were immunoprecipitated with polyclonal anti-Vav antibody in the absence or presence of immunizing peptide (p) and were immunoblotted with monoclonal anti-Erk2 antibody. The upper and lower arrows indicate activated and ordinary Erk2, respectively [46]. B: The cell lysates were immuoprecipitated with polyclonal anti-Vav antibody or polyclonal anti-Erk2 antibody. The immunoprecipitates were subjected to immune-complex kinase assay with MBP. Phosphorylated MBP was visualized by autoradiography. To equalize radioactivity loaded onto SDS-PAGE, 1/20 of the amount of phosphorylated MBP from anti-Erk2 antibody was loaded. C: The cell lysates were immuoprecipitated with polyclonal anti-Vav antibody or rabbit normal serum. The immunoprecipitates were incubated with MAPK-specific synthetic peptide (see Section 2) and incorporated $^{32}$P was counted.

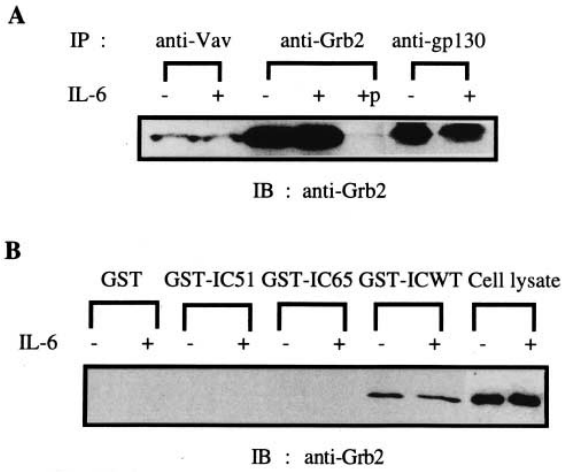


Fig. 5. Association of Grb2 with Vav and gp130. U266 ($7\times10^7$/lane) were stimulated without (−) or with (+) IL-6 (0.5 μg/ml) for 10 min. A: Cell lysates were immunoprecipitated with polyclonal anti-Vav antibody, polyclonal anti-Grb2 antibody or monoclonal anti-gp130 antibody, as indicated. In the fifth lane, a Grb2 peptide (p) was included during the immunoprecipitation step. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with monoclonal anti-Grb2 antibody. B: The cell lysates were incubated with GST or various GST-gp130 fusion proteins. Proteins bound to the fusion proteins were purified with glutathione-Sepharose beads, and analyzed by SDS-PAGE and immunoblotting with monoclonal anti-Grb2 antibody. In the last two lanes, cell lysates were directly loaded onto SDS-PAGE.

Furthermore, we examined whether these anti-Vav immunoprecipitates contain an activity which phosphorylates MAPK-specific substrates. Following preparation of immunoprecipitates of anti-Vav and anti-Erk2 from U266 cell lysates, these immunoprecipitates were incubated with MBP. As seen in Fig. 4B, anti-Vav and anti-Erk2 immunoprecipitates from IL-6-stimulated cells phosphorylated MBP to a greater extent than those from unstimulated cells. MBP phosphorylation was not observed when immunoprecipitation was done in the presence of respective immunizing peptide. Additionally we performed a kinase reaction with a synthetic peptide containing the consensus phosphorylation sequence for MAPK. The immunoprecipitates of anti-Vav antibody and control rabbit normal serum were incubated with the synthetic peptide. As shown in Fig. 4C, anti-Vav immunoprecipitates from the IL-6-stimulated cells contained significant MAPK activity. The activity in the anti-Vav immunoprecipitates from the non-stimulated cells was as low as that of the normal serum immunoprecipitates. These results suggest that coprecipitation of Vav with Erk2 is specific.

### *3.4. Vav is coprecipitated with Grb2*

SH2/SH3 domains of Vav have affinity for specific tyrosine-containing motifs, and are also found to bind with other SH2/SH3 containing molecules including Shc, p85-PI3K, GAP and Grb2 [32,33,38]. Grb2 is known as an adapter protein to link stimulation of receptor/non-receptor tyrosine kinases to activation of the Ras/MAPK cascade [10–12], and Grb2 is suggested to be involved in IL-6 signal transduction [9]. We thus examined whether Vav is associated with Grb2. The anti-Vav immunoprecipitates from cell lysates were separated on SDS-PAGE and blotted with anti-Grb2 antibody. Fig. 5A shows that Grb2 was associated with Vav regardless of the stimulation by IL-6. Grb2 was detected in the immunoprecipitates of anti-gp130, which is in agreement with recent data by Kumar et al. [9] who showed that Grb2 associates with gp130. Subsequently we tested GST-gp130 fusion proteins to determine which part of the cytoplasmic domain of gp130 is responsible for Grb2 binding. Various GST-gp130 fusion proteins as in Fig. 2A were incubated with stimulated or non-stimulated U266 cell lysates. The eluates from glutathione beads were analyzed by SDS-PAGE and immunoblotted with anti-Grb2 antibody. Corresponding with the result in Fig. 2B, Grb2 was bound with GST-ICWT, but neither with GST-IC65 nor with GST-IC51.

## 4. Discussion

In the current study we determined that Vav was activated in response to IL-6 and transiently phosphorylated on tyrosine residues in U266 cells. Recent studies have demonstrated that tyrosine motifs in gp130 and cytoplasmic subdomains like box1, box2 and box3 of gp130 are responsible for recruitment and/or activation of different cytoplasmic signaling components. For example, association of Jak1, Jak2 and Tyk2 requires the membrane-proximally located box1 in gp130, and APRF/STAT3 requires the YXXQ motif located in the membrane-distal region of gp130, including box3 [39,40]. From the studies using mutants of gp130, we suggest that the cytoplasmic subdomain of gp130 required for tyrosine phosphorylation of Jak family kinase (i.e. box1 and box2) [7] may not be sufficient for tyrosine phosphorylation of Vav (Fig. 3B). It will be important to determine which subdomain in the membrane-distal part (including box3) of gp130 is related to tyrosine phosphorylation of Vav and what mechanisms are responsible for this, since most of the phosphorylated tyrosine residues in response to IL-6 are located in the membrane-distal part of gp130. In the case of EPO receptor (although this molecule does not show box3 homology), the truncated form of EPO receptor retaining box1 and box2 as well as membrane-distal extra ~70 amino acids in the cytoplasmic region is capable of mediating tyrosine phosphorylation of Vav in response to EPO [27].

Grb2 is an adapter protein to link receptors to the Ras/MAPK pathway via association with Sos or Cdc25. By using a yeast two-hybrid system it was detected that a C-terminal SH3 domain of Grb2 binds to the N-terminal SH3 domain of Vav [33]. The linkage between tyrosine-phosphorylated receptor and Grb2 was explained by the formation of a triple complex, Shc-Grb2-Vav [32]. The results in our present study and also of others showed that Grb2 is associated with gp130 [9]. Consequently our finding that Vav binds to Grb2 in U266 predicts that Vav may lie between gp130 and Ras/MAPK pathway. As seen in Fig. 5, the association of Vav with Grb2 existed regardless of IL-6 stimulation. Thus, IL-6 stimulation may further modify or activate a component in this Ras/MAPK pathway for its full activation.

Several reports have shown that Vav activates GDP/GTP exchange of Ras [19,37,41–43]. Although it is still controversial whether Vav functions to accelerate directly the GDP/GTP exchange in the Ras protein [44], Vav and Ras transformations activate common effectors like c-Myc or Erk2 [45,46]. Interestingly the GDP/GTP exchange factors Dbl and Sos show their serine phosphorylation. For example, the oncogene Dbl, whose DH domain is distantly related to that of Vav,

shows serine phosphorylation [47]. Sos was reported to be phosphorylated by downstream kinase of MEK [48]. In our results Erk2 is coprecipitated with anti-Vav immunoprecipitates, but it remains to be tested using recombinant molecules whether Vav is directly phosphorylated by Erk2 in response to IL-6 stimulation.

Proto-oncogenes which lie upstream of Raf/MAPK are known to be critical regulators in cell growth and differentiation. In this sense Vav is an interesting molecule because it might link to the Raf/MAPK pathway. Further studies should focus on how Vav exactly contributes to Erk2 activation and how Vav plays a role in exerting the biological function of IL-6.

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