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Tyrosine Phosphorylation of the Proto-oncogene Product Vav and Its Association with the Adapter Grb2/Ash in a Human Leukemia Cell Line UT-7

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The *vav* proto-oncogene product (Vav) is expressed exclusively in hematopoietic cells and is reported to have guanine nucleotide exchange activity. Here we report that granulocyte-macrophage colony-stimulating factor, interleukin-3, and erythropoietin induce tyrosine phosphorylation of Vav in a human leukemia cell line UT-7. Tyrosine phosphorylation of Vav is rapid and transient; it occurs within 1 min of the stimulation and at physiological concentrations of the factors. Furthermore, we show that Vav is constitutively associated with the adapter molecule Grb2/Ash in UT-7. These data suggest that tryosine kinases, the adapter Grb2/Ash, and the guanine nucleotide exchange factor Vav are members of a signaling pathway leading to Ras activation in hematopoietic cells.

Key words: Vav — Grb2/Ash — Hematopoietic growth factor — Tyrosine phosphorylation — Signal transduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin (EPO) are hematopoietic growth factors. Their receptors are members of the cytokine receptor superfamily and lack protein-tyrosine kinase activity. However, GM-CSF, IL-3, and EPO activate certain protein-tyrosine kinases and induce tyrosine phosphorylation in some hematopoietic cells. This is the first event when these hematopoietic growth factors transduce mitogenic signals into the cells.[1–4] Ras is then activated in the cells,[5, 6] but the pathway through tyrosine phosphorylation events to Ras activation is still unknown.

The primary role of the protein-tyrosine kinases is now considered to be the generation of tyrosine-phosphorylated recognition sites for the binding of proteins that have Src-homology (SH) 2 domains.[7, 8] These proteins with SH2 domains have enzymatic or regulatory activities, for example, phospholipase Cγ or GTPase-activating protein for Ras. Alternatively, they may serve simply as adapters to complex signaling molecules. The latter group includes p85, which binds to the catalytic p110 subunit of the phosphatidylinositol 3-kinase,[9] and the Grb2/Ash, which links protein-tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor to the guanine nucleotide exchange factor SOS, leading to the activation of Ras.[10–17]

The proto-oncogene *vav* product (Vav) is a 95-kD protein in humans.[18] Vav is exclusively expressed in hematopoietic cells. Vav contains a single SH2 domain and two SH3 domains but does not contain any kinase domains.[19, 20] It has also been reported that Vav contains the Dbl/Cdc25-like sequences that encode regions responsible for the guanine nucleotide exchange activity.[21] Recently, Vav was reported to be an *in vivo* and *in vitro* Ras activator.[22, 23] Tyrosine phosphorylation of Vav is observed in T cells after activation of the T-cell antigen receptor,[19, 20] in mast cells after engagement of the immunoglobulin E antigen receptor,[20] in B cells after engagement of the immunoglobulin M antigen receptor,[24] and in myeloid cells after stimulation with stem cell factor.[25] Wulf *et al.* have reported that, in embryonic stem cell lines constitutively expressing high levels of antisense molecules to *vav* transcripts, differentiation into hematopoietic cells is disrupted.[26] These observations suggest that Vav is implicated in signal transduction in hematopoietic cells. In this study, we investigated the possible involvement of Vav in signaling pathways triggered by GM-CSF, IL-3, and EPO.

UT-7 cells were maintained in RPMI medium 1640 containing 8% bovine serum and 10 ng/ml GM-CSF. Polyclonal anti-Vav antibody was prepared from serum of a rabbit immunized against a synthetic peptide. The peptide sequence was KKDKLHRRAQDKKRNELG-LP, corresponding to the region downstream of the nuclear localization signal sequence.[18] This antibody was used for the immunoprecipitation of Vav. The monoclonal antibody to Vav which was purchased from Upstate Biotechnology (Lake Placid, NY) was used for the immunoblotting of Vav. The polyclonal anti-Grb2/Ash antibody was generated from serum of a rabbit im-

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munized against the glutathione S-transferase (GST)-Grb2/Ash (amino acids 15–217) fusion protein.[27] This antibody was used for the immunoprecipitation of Grb2/Ash. The monoclonal antibody to Grb2/Ash was purchased from Transduction Laboratories (Lexington, KY) and used for the immunoblotting of Grb2/Ash. 4G10, a mouse monoclonal anti-phosphotyrosine antibody (anti-Ptyr), was used for the immunoblotting of phosphotyrosine-containing proteins.

To prepare cell lysates, cells were incubated in RPMI medium 1640 containing 0.1% bovine serum albumin without serum or growth factors for 8–15 h prior to stimulation with growth factors and then resuspended in RPMI medium 1640 containing 100 μM Na_3VO_4. The cells were treated with 10 ng/ml GM-CSF, 10 ng/ml IL-3, or 20 U/ml EPO for 5 min at 37°C unless otherwise specified and then lysed at 4°C in the lysis buffer containing 20 m*M* Tris-HCl pH 8.0, 1% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 500 U/ml aprotinin, 2 m*M* ethylenediaminetetraacetic acid (EDTA), 50 m*M* NaF, and 1 m*M* Na_3VO_4. Unsolubilized materials were removed by centrifugation for 10 min at 15,000*g* at 4°C. To immunoprecipitate Vav or Grb2/Ash, cell lysates were mixed with the polyclonal anti-Vav antibody or with the polyclonal anti-Grb2/Ash antibody, respectively. The immunoprecipitates were collected with protein A-Sepharose (Sigma, St. Louis, MO). All the immunoprecipitates were intensively washed with the lysis buffer before resuspension in Laemmli's sample buffer. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) filters (Nihon Millipore, Yonezawa). The filters were blocked with the buffer containing 10 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, 10% skimmed milk, and 0.05% Triton X-100. For the immunoblotting of phosphotyrosine-containing proteins, filters were incubated with 4G10 and then with the goat alkaline phosphatase-conjugated anti-mouse IgG (Fc) antibody (Promega, Madison, WI). For the immunoblotting of Vav, filters were sequentially incubated with the monoclonal anti-Vav antibody and with the goat alkaline phosphatase-conjugated anti-mouse IgG (Fc) antibody (Promega). For the immunoblotting of Grb2/Ash, filters were sequentially incubated with the monoclonal anti-Grb2/Ash antibody and with the goat alkaline phosphatase-conjugated anti-mouse IgG (Fc) antibody (Promega). After each incubation, the filters were washed four times in the buffer containing 10 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, and 0.05% Triton X-100. Color reaction was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega).

UT-7 is a human leukemia cell line which requires GM-CSF, IL-3, or EPO for growth, and the cells die within several days when the factors are depleted even in medium supplemented with fetal calf serum.[28] To examine whether Vav is tyrosine-phosphorylated by stimulation with GM-CSF, IL-3, and EPO in UT-7 cells, we immunoprecipitated Vav from UT-7 cells which were unstimulated or stimulated with the factors and checked the phosphorylation level of the protein by immunoblotting with 4G10 (Fig. 1A). Vav was revealed to be tyrosine-phosphorylated when the cells were stimulated with GM-CSF, IL-3, and EPO, while little phosphorylation of Vav on tyrosine residues was observed in unstimulated cells. The level of tyrosine phosphorylation of Vav induced by stimulation with EPO was higher than that by stimulation with GM-CSF or IL-3 in UT-7. To determine the amount of Vav during the stimulation, the anti-Vav immunoprecipitates were then immunoblotted

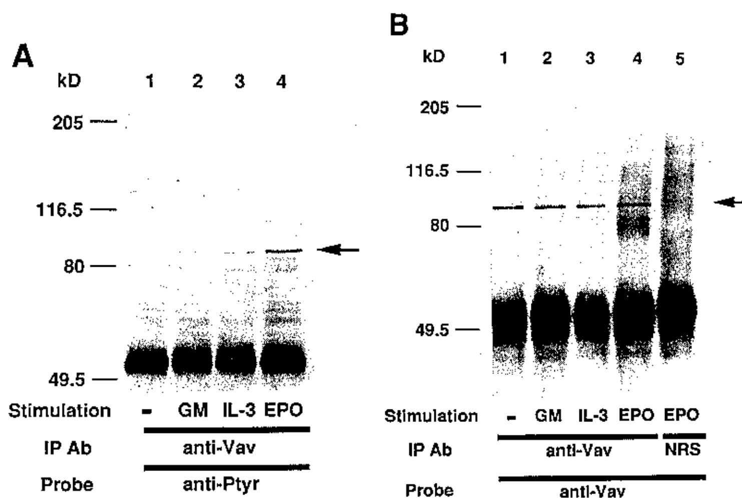

Fig. 1. GM-CSF, IL-3, and EPO induce tyrosine phosphorylation of Vav in UT-7 cells. The lysates from 1×10^7 UT-7 cells unstimulated (lane 1) or stimulated with GM-CSF (lane 2), IL-3 (lane 3), and EPO (lanes 4 and 5) were mixed with the polyclonal anti-Vav antibody (lanes 1–4) or with normal rabbit serum (NRS, lane 5). The immunoprecipitates were collected with protein A-Sepharose, subjected to 7% SDS-PAGE, and immunoblotted with the anti-phosphotyrosine antibody 4G10 (A) and with the monoclonal anti-Vav antibody (B). The arrows indicate the position of Vav.

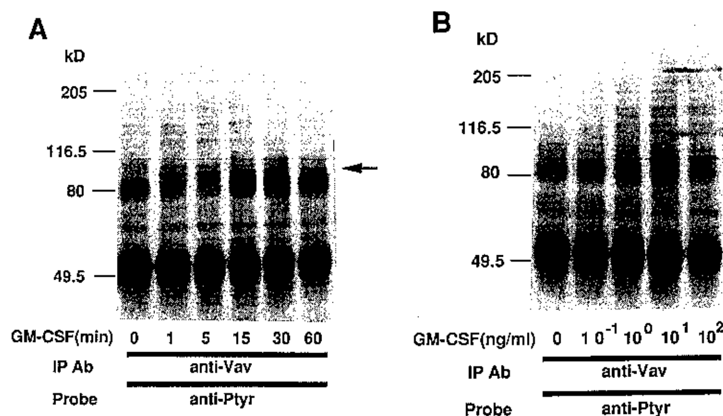

Fig. 2. Time and dose dependency of tyrosine phosphorylation of Vav by stimulation with GM-CSF. UT-7 cells (1×10^7) were stimulated with GM-CSF for the indicated times (A) and at the indicated concentrations (B) at 37°C. The cell lysates were mixed with the polyclonal anti-Vav antibody. The immunoprecipitates were collected with protein A-Sepharose, subjected to 7% SDS-PAGE, and immunoblotted with the anti-phosphotyrosine antibody 4G10. The arrows indicate the position of Vav.

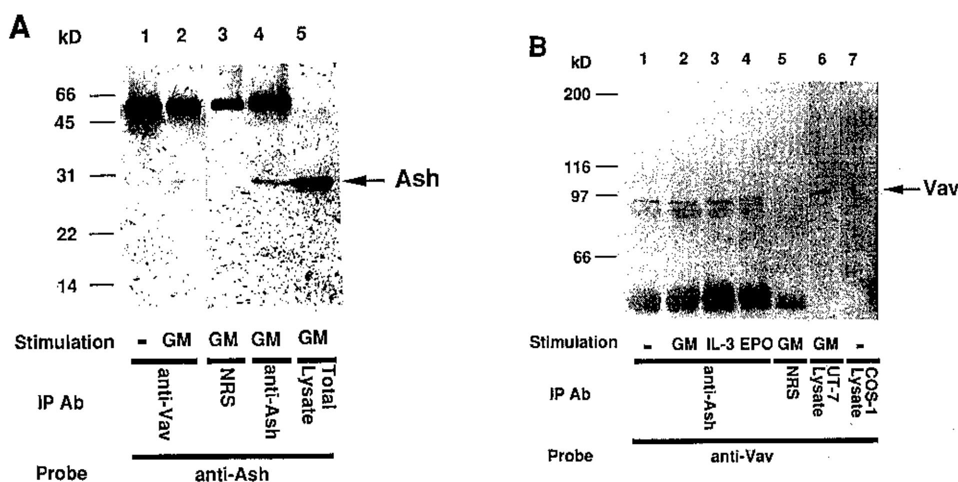

Fig. 3. (A) Anti-Vav immunoprecipitates contain Grb2/Ash in UT-7. The lysates from 1×10^7 UT-7 cells unstimulated (lane 1) or stimulated with GM-CSF (lanes 2–4) were mixed with the polyclonal anti-Vav antibody (lanes 1 and 2), normal rabbit serum (NRS, lane 3), or the polyclonal anti-Grb2/Ash antibody (lane 4). The immunoprecipitates were collected with protein A-Sepharose, subjected to 12% SDS-PAGE, and immunoblotted with the monoclonal anti-Grb2/Ash antibody. The total lysate (100 μg protein per lane) from UT-7 cells was also applied as a reference (lane 5). The arrow indicates the position of Grb2/Ash. (B) Anti-Grb2/Ash immunoprecipitates contain Vav in UT-7. The lysates from 1×10^7 UT-7 cells unstimulated (lane 1) or stimulated with GM-CSF (lanes 2 and 5), IL-3 (lane 3), and EPO (lane 4) were mixed with the polyclonal anti-Grb2/Ash antibody (lanes 1–4) or with normal rabbit serum (NRS, lane 5). The immunoprecipitates were collected with protein A-Sepharose, subjected to 7% SDS-PAGE, and immunoblotted with the monoclonal anti-Vav antibody. The total lysates (100 μg protein per lane) from UT-7 cells (lane 6) and COS-1 cells (lane 7) were also applied as a reference. COS-1 cells do not express Vav and they were used as the negative contol. The arrow indicates the position of Vav.

with the anti-Vav antibody (Fig. 1B). The amount of Vav was not affected by stimulation with the factors.

We then investigated the time- and dose-dependency of tyrosine phosphorylation of Vav by stimulation with GM-CSF. UT-7 cells were treated with GM-CSF for various times and at various concentrations at 37°C. The anti-Vav immunoprecipitates from the cell lysates were immunoblotted with 4G10. As shown in Fig. 2A, Vav was tyrosine-phosphorylated within 1 min during GM-CSF treatment of the cells at 37°C; the maximum level of phosphorylation was attained at 5 min, and Vav was dephosphorylated after 60 min. Tyrosine phosphorylation of Vav was also dose-dependent and the phosphorylation occurred at physiological concentrations of GM-CSF (Fig. 2B). We obtained similar results when the cells were treated with EPO (data not shown).

Vav is now considered to be a guanine nucleotide exchange factor (GEF) for Ras.[22,23] Among GEFs, SOS is a well-known molecule which binds to Grb2/Ash and activates Ras in response to EGF in fibroblasts.[12-17] SOS is associated with Grb2/Ash in UT-7 as well (data not shown). We have examined whether Vav is also associated with Grb2/Ash in UT-7 cells. The anti-Vav immunoprecipitates were immunoblotted with the anti-Grb2/Ash antibody (Fig. 3A). Grb2/Ash was shown to be co-precipitated with Vav in UT-7 cells. Conversely, the anti-Grb2/Ash immunoprecipitates were immunoblotted with the anti-Vav antibody (Fig. 3B). Vav was shown to be co-precipitated with Grb2/Ash in UT-7 cells. The amount of Grb2/Ash was unchanged during the stimulation (data not shown). The association between Vav and Grb2/Ash was observed both in unstimulated cells and in cells stimulated with GM-CSF.

We have shown that GM-CSF, IL-3, and EPO induce tyrosine phosphorylation of Vav in UT-7. Alai *et al.* have reported that stem cell factor stimulates tyrosine phosphorylation of Vav in two human leukemia cell lines, MO7E and TF-1.[25] Therefore, Vav may be implicated in the signal transduction pathways activated by both the cytokine receptors and the tyrosine kinase receptors on hematopoietic cells.

Tyrosine phosphorylation of Vav is rapid (within 1 min after the start of the stimulation) and transient (dephosphorylated after 60 min). Therefore, Vav seems to be located downstream of tyrosine kinase(s) but very near to the kinase(s). However, it is unknown which protein-tyrosine kinase is responsible for the phosphorylation of Vav. One candidate in T cells is Lck protein-tyrosine kinase.[22] We have reported that GM-CSF, IL-3, and EPO activate c-Fes in TF-1.[29,30] However, we could not find an apparent association between c-Fes and Vav. Lyn protein-tyrosine kinase was also reported to be activated by stimulation with IL-3 in some myeloid-committed leukemia cell lines.[31] Recently, JAK2 was shown to be activated by stimulation with GM-CSF, IL-3, and EPO.[32-34] However, Lyn or JAK2 was not co-precipitated with Vav in UT-7 (data not shown).

Grb2/Ash is an adapter molecule containing one SH2 and two SH3 domains.[35,36] In fibroblasts, the SH2 domain of Grb2/Ash binds to phosphorylated tyrosines in a number of proteins such as the EGF receptor,[12,14] Shc,[37] insulin receptor substrate (IRS)-1,[38] and Syp.[39] The SH3 domain of Grb2/Ash binds to the carboxyl-terminal proline-rich domain of SOS that activates Ras.[13-15,17] The interactions between the Grb2/Ash SH2 domain and these tyrosine-phosphorylated proteins are proposed to be important in determining the intracellular localization and function of SOS.[40] However, recent studies of the *in vitro* binding of Grb2/Ash have shown that the Grb2/Ash SH3 domain binds to other molecules, dynamin and C3G, which also regulate the small G-proteins.[41,42] These data suggest that the Grb2/Ash SH3 domain can couple to various effector molecules, especially to the small G-protein regulators. In this paper, we have demonstrated that Grb2/Ash also binds to Vav in a human leukemia cell line. It is likely that Vav is one of the downstream effector molecules for the adapter Grb2/Ash and that it functions as a regulator for Ras or other small G-proteins in the hematopoietic cells. It would be interesting to determine the relative contribution of these molecules including SOS and Vav to the receptor signaling to Ras in hematopoietic cells.

By analogy with SOS, dynamin, and C3G, Vav may also bind to the SH3 domain of Grb2/Ash. This hypothesis is supported by the fact that Vav has proline-rich sequences which are proposed to be binding site for SH3 domains.[18] Very recently, Ramos-Morales *et al.* have reported that Vav binds to the SH3 domain of Grb2/Ash in a manner independent of tyrosine phosphorylation of Vav.[43] Our results also show that the binding of Vav to Grb2/Ash is constitutive. The other SH3-binding proteins such as SOS also constitutively bind to the SH3 domain of Grb2/Ash. However, it is unique that Vav is tyrosine-phosphorylated by stimulation with GM-CSF, IL-3 and EPO, because other SH3-binding proteins including SOS, C3G, and dynamin are not tyrosine-phosphorylated. Therefore, it is possible that Vav also binds not only to the SH3 domain of Grb2/Ash but also to certain proteins with SH2 domain. It has been reported that Vav binds to several SH2 domains expressed as GST fusion proteins, including the Grb2/Ash SH2, the p85 C-terminal SH2, and the Shc SH2 domains.[43] It remains to be investigated which molecules bind to the phosphorylated tyrosines of Vav *in vivo*.

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