Requirement of Tyrosine-phosphorylated Vav for Morphological Differentiation of All-trans-Retinoic Acid-treated HL-60 Cells

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Abstract

Our previous data demonstrated that cellular and nuclear tyrosine-phosphorylated Vav associate with phosphoinositide 3-kinase during all-trans-retinoic acid-dependent granulocytic differentiation of HL-60 cells. In this study, aimed to analyze the mechanism by which Vav is recruited and activated, we report that the Src homology 2 domain of Vav interacts with tyrosine-phosphorylated proteins in a differentiation-dependent manner. Two adaptor proteins, Cbl and SLP-76, were identified, showing a discrete distribution inside the cells, with Cbl absent from the nuclei and SLP-76 particularly abundant in the nuclear compartment. Of note, Vav interacts with the tyrosine kinase Syk, which is also present in the nuclear compartment and may phosphorylate Vav in vitro when cells differentiate. Inhibition of Syk activity by piceatannol prevents both in vitro and in vivo Vav tyrosine phosphorylation, its association with the regulatory subunit of phosphoinositide 3-kinase, and the nuclear modifications typically observed during granulocytic differentiation of this cell line. These findings suggest that tyrosine-phosphorylated Vav and its association with phosphoinositide 3-kinase play a crucial role in all-trans-retinoic acid-induced reorganization of the nucleoskeleton, which is responsible for the changes in nuclear morphology observed during granulocytic differentiation of HL-60 cells.

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Introduction

The Vav family has three known members in mammalian cells expressed in many tissues, all characterized by an array of structural motifs including a GEF for the Rho/Rac/GTPases, a pleckstrin homology domain, and two SH3 that flank on the SH2 domain (reviewed in Ref. 1). By means of these different motifs, Vav is capable of physically associating with a number of proteins, both in the cytoplasm and in the nucleus, playing a role in several distinct cellular functions, such as cell proliferation and maturation, cytoskeletal reorganization, regulation of gene expression, and apoptosis (2–6).

The most relevant feature of the Vav family is the regulation of their GDP-GTP exchange activities by direct phosphorylation. More recently, it has been reported that tyrosine phosphorylation of Vav may also regulate other functions, including the formation of heteromolecular complexes that modulate the signal transmission and the termination of the activity of Vav itself at the end of the agonist stimulation (1). Vav becomes rapidly and transiently tyrosine phosphorylated upon triggering of a variety of surface receptors in almost every hematopoietic cell. It is reported extensively that Vav plays a critical role in lymphocyte development and activation, because it is phosphorylated in response to T-cell receptor and B-cell receptor activation (7, 8). Upon receptor cross-linking, ITAMs become tyrosine phosphorylated by the Src family of tyrosine kinases and recruit the Syk/ZAP-70 family of tyrosine kinases, resulting in their phosphorylation and activation (9). Both Src and Syk families of protein tyrosine kinases then phosphorylate multiple intracellular proteins, including phospholipase C-γ, SLP-76, and Vav (9–11). Tyrosine phosphorylation and/or activation of these substrates ultimately result in downstream cytokine gene induction and other effector functions.

The Syk/ZAP-70 family of tyrosine kinases constitutes an example of proteins that contain two SH2 domains, a tandem sequence that might confer high specificity in tyrosine kinase-mediated signaling (12). Both ZAP-70 and Syk contain a consensus Vav SH2 domain binding sequence that seems to be critical for antigen receptor-mediated signal transduction (13).

Recent data indicate that, despite the interaction with tyrosine kinases, the optimal phosphorylation of Vav requires the association with adaptor molecules that facilitate the spatial proximity between Vav and the upstream kinases.

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3 The abbreviations used are: GEF, guanine nucleotide exchange factor; SH, Src homology; ITAM, immunoreceptor tyrosine-based activation motif; ATRA, All-trans-retinoic acid; DAPI, 4′,6-diamidino-2-phenylindole; PI 3-K, phosphoinositide 3-kinase; FcγR, Fcγ receptor; P-Tyr, phosphorylated tyrosine.
These associations also depend on the tyrosine phosphorylation of the adaptor proteins and the utilization of either the Vav SH3 or SH2 domains as interacting domains (1). SLP-76, an adaptor protein predominantly expressed in T cells and myeloid cells and a substrate for ZAP-70 and Syk tyrosine kinases, was reported to associate via tyrosine-phosphorylated residues in its NH2-terminal domain with the SH2 domain of Vav, after ligation of the T-cell antigen receptor (14). SLP-76 was also described as an important adaptor molecule that is regulated by Syk in C-reactive protein-stimulated platelets (15) and plays a critical role in FcRI-mediated activation of mast cells in vivo and in vitro (16).

Little is known about the regulatory molecules that trigger the down-modulation of Vav signals. A potential negative regulator of Vav is Cbl, a proto-oncoprotein that has emerged recently as a component of tyrosine kinase-mediated signal transduction in a variety of cell types. Biochemical and genetic studies have identified Cbl as a negative regulator of Syk/ZAP-70 as well as other protein tyrosine kinases (reviewed in Ref. 17). Recently, Cbl has been found to associate with Vav upon T-cell receptor stimulation of primary murine lymphocytes and Jurkat T cells. This interaction appears to require the whole SH3-SH2-SH3 COOH-terminal domain of Vav and a proline-rich sequence of Cbl and seems to inhibit the Vav-dependent signal transduction (18).

The biological responses induced by Vav can be placed in three different, although interdependent, groups: stimulation of Rho/Rac effectors; activation of transcriptional factors; and morphological changes associated with the actin reorganization (1). Several reports indicated that the relationship between Vav and cytoskeleton, demonstrated also by partial colocalization of this protein with actin in several cytoskeletal structures, involves its GEF activity, inducing Rac and Rho-dependent cytoskeletal changes, and the induction of specific patterns of gene expression (1).

A functional role of Vav was demonstrated in myeloid cells. Recent data reported the tyrosine phosphorylation of Vav in promyelocytic HL-60 cells upon cross-linking of the human FcγRIIa1 (CD32), which contains an ITAM motif in its intracellular region (19). In addition, communoprecipitation experiments performed on the same cell model demonstrated that Vav is associated with SLP-76 upon FcγRIIa1 activation (19).

We have demonstrated recently that Vav protein increases its amount and its level of tyrosine phosphorylation in HL-60 cells induced to differentiate along the granulocytic lineage with ATRA (20). Although ATRA acts through a nuclear receptor, which directly elicits transcriptional activity, it also induces a specific pattern of expression and activity of enzymes of the phosphoinositide metabolism (21–25). In addition, we have reported that tyrosine-phosphorylated Vav is particularly abundant in nuclei of differentiated cells, in which we demonstrated the presence of Vav/PI 3-K/phospholipase C-γ1 complexes (20).

The aim of this work was to identify the mechanism by which Vav is recruited and phosphorylated in HL-60 cells induced to differentiate with ATRA. We describe here the existence of protein complexes, including the tyrosine kinase Syk and the adapting molecules SLP-76 and Cbl, which can play a role in recruiting Vav. We also report the identification of Syk as a tyrosine kinase able to phosphorylate Vav when HL-60 cells differentiate, and the finding that tyrosine phosphorylation of Vav and its association with the p85 regulatory subunit of PI 3-K are essential for the modifications of nuclear morphology characterizing the granulocytic differentiation of this cell line.

**Results**

**Vav Is Associated with Phosphorylated Proteins in Differentiated HL-60 Cells.** We demonstrated previously that the ATRA-induced granulocytic differentiation of HL-60 cells is accompanied by a progressive increase in amount and tyrosine phosphorylation of the Vav protein, particularly evident at the nuclear level, and by its differentiation-dependent association with nuclear PI 3-K (20). We also have evidence that the ATRA-dependent interaction with PI 3-K was dependent on one or more phosphorylated tyrosines of Vav and on the SH2 domains of the regulatory subunit p85 (20).

To identify the molecules involved in the recruitment and phosphorylation of Vav in HL-60 cells induced to granulocytic differentiation, we analyzed the ability of Vav to associate with tyrosine-phosphorylated proteins. For this purpose, we have used lysates from whole cells and from highly purified membrane-depleted nuclei (Fig. 1A) derived from both control and differentiated conditions. In control conditions, the cells were cultured without ATRA for 96 h, whereas differentiation was obtained by treatment with 1 μM ATRA for 96 h (21, 22). The lysates were adsorbed on GST-fusion NH2-terminal and COOH-terminal Vav-SH3 and GST-fusion Vav-SH3-SH2-SH3 proteins. As expected (Fig. 1B), only the SH3-SH2-SH3 fragment of Vav associated with phosphorylated proteins, the most abundant of which migrated at M, ~75,000 and M, 116,000 and at M, 68,000 and M, 75,000 in differentiated cells and nuclei, respectively. By probing with an anti-Cbl antibody, a protein comigrating with the phosphorylated band present in differentiated cells at M, 116,000 was identified, the amount of which was closely similar in control and ATRA-treated cells. No detectable amount of Cbl associated with the SH3-SH2-SH3 fragment of Vav when purified nuclei were used for the adsorption experiments (Fig. 1B).

Probing the same membrane with an anti-SLP-76 antibody identified the M, 75,000 phosphoprotein, present in both differentiated cells and nuclei. The association of this adaptor protein with the Vav fragment was particularly evident in nuclei, with no differences attributable to ATRA treatment (Fig. 1B).

By reprobing the same membrane with an anti-Syk antibody, we identified the M, 68,000 phosphoprotein shown in nuclei. Syk appeared to associate with the SH3-SH2-SH3 fragment of Vav in both cells and nuclei, without quantitative differences between control and differentiated conditions in whole cells, and increased during ATRA-induced differentiation in nuclei (Fig. 1B).

Communoprecipitation experiments with the anti-Cbl antibody confirmed the presence of this adaptor protein in whole
Fig. 1. Tyrosine phosphorylated proteins associate with the SH2 domain of Vav in ATRA-treated cells and nuclei. A, purity of isolated nuclei was confirmed by immunoblotting total lysates from control (-; 4 days of culture without ATRA) and differentiated (+; 4 days of ATRA treatment) conditions with the antibody against the cytoplasmic protein β-tubulin. B, lysates of cells (C) and nuclei (N) from control (-) or differentiated (+) conditions were adsorbed on GST-fusion SH3-COOH terminal (SH3-CT), SH3-NH$_2$ terminal (SH3-NT), and SH3-SH2-SH3 fragments of Vav. Phosphorylated proteins interacting with the SH3-SH2-SH3 domain were analyzed with the indicated antibodies. Arrows, the phosphorylated bands corresponding to Vav and Syk, respectively. In C, Cbl was immunoprecipitated from cells and nuclei, and the associated proteins were identified by probing with the indicated antibodies, in both control (-) and differentiated (+) conditions. Arrow, the tyrosine phosphorylated band corresponding to Cbl. D, Syk immunoprecipitates from cells and nuclei, in control conditions (-) and after 96 h of ATRA treatment (+), were probed with the indicated antibodies. Arrow, the tyrosine-phosphorylated bands corresponding to Syk. E, Vav immunoprecipitates from cells and nuclei, during the time course of ATRA treatment (0–96 h), were probed with an anti-Syk antibody. The data are representative of four separate experiments. ip, immunoprecipitation; WB, Western blot.

cells and showed only a weak increase in its amount and level of tyrosine phosphorylation during the differentiation process. In addition, Cbl associated with nonphosphorylated Vav and Syk, particularly in differentiated conditions (Fig. 1C). Incidentally, this kind of experiment confirmed that the preparations of nuclei used were purified rigorously. In fact, although Cbl is a major cytoplasmic protein in our cell model, no transfer of it from cytosplasms to nuclei occurred. The complete absence of any cytoskeletal contamination of the nuclei was demonstrated by probing nuclear lysates with an anti-β-tubulin antibody, as reported in Fig. 1A.

Immunoprecipitation with an anti-SLP-76 antibody failed to show any protein, probably because of the inadequacy of the antibody used (data not shown), although immunoprecipitations performed with an anti-Syk antibody confirmed the presence of this protein at the nuclear level, and showed after treatment with ATRA, an increase in its amount and tyrosine phosphorylation, particularly in purified nuclei (Fig. 1D). No significant amount of Vav protein was detected in anti-Syk immunoprecipitates (data not shown).

The Vav/Syk interaction was analyzed along the differentiation process, demonstrating that the association between these two molecules increases during ATRA treatment, particularly at the nuclear level, reaching a maximum after 4 days, in correspondence with a fully differentiated phenotype (Fig. 1E).

Syk Phosphorylates Vav in Vitro. Because we have observed that Vav interacts with Syk, the tyrosine phosphorylation level of which increased after ATRA treatment, a possible role of Syk in phosphorylating Vav was analyzed. To determine the capability of Syk to phosphorylate Vav in our cell model, we performed an in vitro Syk kinase assay in which Vav, immunoprecipitated from control cells, was incubated with Syk immunoprecipitated from control or ATRA-treated cells. As shown in Fig. 2, Vav became tyrosine phosphorylated when Syk that had immunoprecipitated from treated cells was used as a source of tyrosine kinase activity. Inhibition of Syk Kinase Activity Prevents Tyrosine Phosphorylation of Vav and Its Interaction with PI 3-K. To elucidate the role of Syk in the ATRA-dependent tyrosine phosphorylation of Vav, a pharmacological model of Syk inhibition was used, in which HL-60 cells were treated with piceatannol, a tyrosine kinase inhibitor with a reported selectivity for Syk (26). The effects of different concentrations of piceatannol on HL-60 viability were analyzed for a time course corresponding to the days of ATRA treatment (Fig. 3A). Cells were then cultured in the presence of 1 µg/ml
piceatannol, the concentration at which cell viability was not affected at any of the examined times, either alone or in association with ATRA, and the ability of Syk to phosphorylate Vav was analyzed. As reported in Fig. 2, Syk immunoprecipitated from cells treated with piceatannol simultaneously with ATRA failed to phosphorylate Vav in vitro; its own tyrosine phosphorylation was highly reduced.

In the presence of piceatannol, the level of tyrosine phosphorylated Syk was significantly lower, in both cells and nuclei, suggesting a reduced level of activated kinase. The amount of nuclear Syk was not modified by piceatannol, indicating that tyrosine phosphorylation is not essential for its nuclear accumulation (Fig. 3B).

The level of tyrosine-phosphorylated Cbl was not affected by the simultaneous treatment of HL-60 with ATRA and piceatannol (Fig. 3C), suggesting that it is independent of Syk kinase activity. On the contrary, in the presence of piceatannol, the amount of tyrosine-phosphorylated Vav was reduced, after ATRA treatment, in both cells and nuclei (Fig. 3D), confirming a role of activated Syk in the tyrosine-phosphorylation of this molecule. The amount of Vav in samples treated with both ATRA and piceatannol was similar to the ATRA-treated conditions, indicating that tyrosine phosphorylation is not required for nuclear localization of this protein (Fig. 3D).

To clarify the role of the ATRA-dependent tyrosine phosphorylation of Vav, we also evaluated its ability to interact with the different molecules when Syk activity was reduced by piceatannol. As demonstrated in Fig. 3D, the association of Vav with Syk is not modified in the presence of piceatannol, in both cells and nuclei. Interestingly, the interaction with the regulatory subunit of PI 3-K we described previously (20) is impaired when piceatannol is administered simultaneously with ATRA (Fig. 3D), indicating that the Syk kinase inhibitor reduces the phosphorylation of a tyrosine residue involved in the Vav/p85 association.

Inhibition of Syk Kinase Activity Prevents the Nuclear Changes Typical of Granulocytic Differentiation. The effects of the reduced Syk-dependent tyrosine phosphorylation of Vav on granulocytic differentiation were analyzed. As reported in Table 1, the accumulation of cells in the G0-G1 phases of the cell cycle observed during the ATRA treatment of HL-60 was not modified by piceatannol. Likewise, the number of cells expressing the surface antigen CD11b, commonly used to evaluate the level of ATRA-induced granulocytic differentiation of HL-60 (21, 27), after ATRA administration, was not affected by simultaneous treatment with piceatannol (Fig. 4A).

On the contrary, treatment with piceatannol and ATRA impaired the occurrence of the nuclear modifications typical
of granulocytic differentiation of this cell line (Fig. 4B). In particular, the percentage of cells that reached the multilobated granulocytic-like nuclear morphology, compared with the treatment with ATRA alone, was reduced to ~20%, as reported in Fig. 4C, which also shows the reduced level of tyrosine-phosphorylated Vav under the same experimental conditions.

Discussion

The purpose of this study was to identify the molecules involved in the recruitment and phosphorylation of Vav during the ATRA-dependent granulocytic differentiation of HL-60 cells. Because the interaction between the SH2 domain of Vav and phosphorylated proteins is thought to serve for recruitment of activated kinases, which in turn can phosphorylate Vav (1), we performed experiments in which the SH2 domain of Vav was analyzed for its capability of interacting with phosphorylated proteins. One of these, identified as the adaptor molecule Cbl, was absent in nuclei and became phosphorylated in differentiated cells. A constitutive association between the fragment of Vav and Cbl, not modified during the differentiation process, indicates that an association between these two molecules is independent from phosphorylative events. In this case, the interaction between Vav and Cbl may be partially explained by the fact that, although in vitro experiments suggest the interaction is mediated via the Vav-SH2 domain binding to Cbl phosphotyrosine (28), it seems to require the entire SH3-SH2-SH3 COOH-terminal domain of Vav and a long stretch of proline-rich sequences present in the central region of Cbl (17).

The adaptor protein SLP-76 was identified here as well as a phosphorylated protein interacting with the SH3-SH2-SH3 fragment of Vav in both cells and nuclei after ATRA treatment. Similar to Cbl, also in control conditions SLP-76 was found to be associated with the Vav fragment, without quantitative changes attributable to differentiation. Vav-associated SLP-76 was more abundant in nuclei than in whole-cell lysates, indicating a preferential association into the nucleus of these two molecules, at variance with an exclusive cytoplasmic distribution of Vav/Cbl complexes.

An interaction between Vav, Cbl, and SLP-76 was described previously. In particular, a role for Cbl was reported in HL-60 cells in which stimulation of ATRA-differentiated cells with anti-CD-38 monoclonal antibody induced its rapid tyrosine phosphorylation, suggesting the involvement of this adaptor protein in the transmembrane signaling mediated by CD-38 (29). An association between Vav and SLP-76 was demonstrated after treatment of HL-60 cells with the human Fc receptor for IgG, FcγRII (CD32), which contains an ITAM motif in its intracellular region. A strong phosphorylation of Vav, SLP-76, and Cbl was also observed, suggesting that CD32 cross-linking induces the recruitment of molecules involved in gene activation or cytoskeletal rearrangement (19). A critical role for the interaction between SLP-76 and Vav was also shown in T-cell development and activation, in which these molecules may have a synergistic effect on interleukin-2 promoter activity (30).

Our report describes, for the first time, a compartmentalized association between these molecules during ATRA treatment (Cbl/Vav in the cytoplasm and SLP-76/Vav in the inner nuclear compartment), suggesting the activation of one
or more Vav-related mechanisms of signal transduction from the cell membrane to the nuclear compartment.

A tyrosine-phosphorylated Syk was also found to be associated with the SH2 domain of Vav, according to the known presence of a consensus Vav-SH2 domain binding sequence in the Syk molecule (13). Syk protein was also shown in control conditions, in both cells and nuclei, indicating that the interaction between these two proteins may also occur independently from Syk phosphorylation.

Cell fractionation studies confirmed that Syk is present in the nucleus of HL-60 cells and that Vav/Syk complexes are also present in the nuclear compartment, in which Vav-associated Syk increased during differentiation and was particularly evident after 4 days of ATRA treatment. These data, which report for the first time the presence of Syk and its association with Vav in the nuclear compartment, suggest a specific role for this tyrosine kinase in the nucleus of HL-60 cells induced to differentiate with ATRA, contributing to the modulation of tyrosine phosphorylation of Vav.

An increase of Syk activity and of its tyrosine phosphorylation levels was observed previously during HL-60 differentiation along the granulocytic lineage induced by ATRA (31). It has been also reported that Syk plays a specific physiological role in signaling from FcγRs in neutrophils and macrophages (32), as shown by experiments performed with Syk-deficient cells.

In this paper we report that Vav is a potential target of Syk during ATRA-dependent maturation of HL-60 cells. We have demonstrated that Syk phosphorylates Vav in vitro, when immunoprecipitated from differentiated cells, and that this phosphorylation was abrogated when a specific Syk kinase inhibitor was administrated simultaneously with the differentiating agent. In particular, when we used piceatannol to inhibit Syk kinase activity, a strong decrease of the tyrosine phosphorylation of both Syk and Vav occurred. This confirms the capability of Syk to autophosphorylate and supports its role in phosphorylating Vav in an ATRA-dependent manner.

The reduced level of tyrosine phosphorylation of both Vav and Syk had no effect on their reciprocal association and on their accumulation inside the nuclear compartment. On the contrary, the reduced tyrosine phosphorylation of Vav is accompanied by the decrease of its ability to interact with the p85 regulatory subunit of PI 3-K. This indicates that the ATRA-induced association of Vav with PI 3-K involves one or more tyrosines, the phosphorylation of which is prevented by the Syk inhibitor piceatannol.

Analysis of the cellular morphology revealed that the reduction of tyrosine phosphorylation of Vav corresponds to a strong reduction of the modifications of nuclear morphology, typically observed during granulocytic differentiation. This suggests that the ATRA-dependent tyrosine phosphorylation of Vav plays a crucial role in regulating the nucleoskeleton reorganization. Vav phosphorylation does not appear to be relevant for the expression of CD11b, widely used as a differentiation marker of HL-60 cells after ATRA treatment. However, it is essential for the changes in the nucleoskeleton assembly required to reach the morphological differentiated phenotype, according to the notion that Vav is involved in regulating the architecture of the cytoskeleton (1).

We reported previously that reduced expression and activity of PI 3-K in HL-60 cells prevents ATRA-induced nuclear modifications, similarly to what we observed when we inhibited Vav phosphorylation and its association with p85. This evidence suggests that the Vav/PI 3-K complexes may regulate the rearrangement of nucleoskeleton of differentiating HL-60 cells in two different ways: (a) by Vav activity. It is known that GEF activity of Vav, regulated by second messengers derived from PI 3-K activity, induces GTPase-related cytoskeletal changes (1); and (b) by PI 3-K activity. The interaction between Vav and PI 3-K may be important for targeting this key enzyme of the lipid metabolism to its nuclear substrates. PI 3-K products may then be responsible for the nucleoskeletal reorganization by affecting actin polymerization (33, 34). In addition, the presence and the accumulation of a number of signaling molecules inside the nuclear compartment during granulocytic differentiation may indicate a cooperative role of different signaling pathways in the nuclear events leading to ATRA-induced differentiation.

The presence in our cell model of molecules with known functions of adaptor proteins downstream to a number of membrane receptor-induced signals, differently distributed in the cytoplasm and nucleus (Cbl, completely absent, and SLP-76, particularly abundant in the nuclear compartment), suggests that some steps of HL-60 differentiation depend on signals directed to the nuclear compartment that originated from specific membrane receptors. It is reported that in myeloid cells, ATRA induces modulation of expression and release of different cytokines (35–37), some of which activate transduction pathways involving molecules that we have identified in ATRA-induced differentiation (38, 39). It is then possible that the ATRA-dependent transcriptional activity may modulate the expression of cytokines and/or their receptors and regulate the middle to late events of granulocytic differentiation by autocrine-paracrine mechanisms.

**Materials and Methods**

**Cells and Antibodies.** HL-60 cells were obtained from the American Type Culture Collection (ATCC CCL-240; Rockville, MD) and cultured in liquid suspension in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies) in a 94% air/6% CO₂ atmosphere. Cells were maintained at an optimal cell density between 5 × 10⁶/ml and 1.5 × 10⁷/ml and were treated with 1 μM ATRA (Sigma Chemical Co., St Louis, MO) for the indicated times. Cell differentiation was evaluated by means of analysis of CD11b expression, and by specific nuclear staining with DAPI, as reported previously (21, 22). Concerning the nuclear staining with DAPI, we considered the cells showing a multilobated nucleus (≥2 clearly defined lobes) after 96 h of ATRA treatment as differentiated. The evidence obtained with DAPI exactly overlapped the results obtained with histological staining of the cells (May–Grunwald Giemsa).

Monoclonal anti-β-tubulin was from Sigma, and monoclonal antibodies against Vav and Syk and polyclonal antibody against SLP-76 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-P-Tyr was obtained from Transduction Laboratories (PY20; Lexington,
KY), and polyclonal anti-Cbl antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-conjugated antirabbit, antimouse, and antineph IgG (Sigma) were used as secondary antibodies.

Agarose-conjugated GST-fusion Vav SH3-COOH-terminal, SH3-NH2-terminal, and SH3-SH2-SH3 proteins were kindly provided by Andy Chan (Washington University School of Medicine, St. Louis, MO).

Preparation of Nuclei, Lysates, and Immunoprecipitates. Nuclei depleted of nuclear membranes were isolated essentially following a procedure described previously (21). Briefly, the cell fractionation buffer contained 10 mM Tris-HCl (pH 7.4), 2 mM MgCl2, and 10 mM NaCl. Triton X-100 (0.5%) was added, and after passage through a syringe with a 22-gauge needle, nuclei were stabilized by adding 3 mM MgCl2. All of the purification buffers contained 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 0.5 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM DTT, and 1 mM Na3VO4 (all from Calbiochem, La Jolla, CA). Nuclear purity was assessed by ultrastructural analysis and marker enzyme assays, as reported previously (21), and by probing nuclear lysates with an anti-β-tubulin antibody.

Cells (10 × 106) and nuclei (100 × 106) were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each a protein and leupeptin, 1 mM Na3VO4, and 1 mM NaF. After 20 min of incubation at 4°C, the insoluble materials were removed by centrifugation for 15 min at 14,000 rpm. Supematant, which constituted the total lysate, was incubated with the indicated antibodies overnight at 4°C and immunoprecipitated with protein A-Sepharose (Pharmacia, Uppsala, Sweden). The immunoprecipitates were washed four times with lysis buffer and resuspended in Laemmli’s SDS sample buffer. The adsorption experiments with the GST-fusion proteins were performed, incubating lysates from cells and nuclei, overnight at 4°C with the different Vav fragments.

Immunoochemical Analysis. Total lysates (50 μg of protein) from cells (1 × 10⁸) and nuclei (10 × 10⁶), immunoprecipitates, and absorbed samples (from 1 mg protein) were separated on 7.5% polyacrylamide denaturing gels (40) and blotted to nitrocellulose membrane (Amersham Life Science, Little Chalfont, United Kingdom). The blots were then incubated with the antibodies, and the final detection was performed using the ECL system (DuPont, NEN Research Products, Boston, MA), according to the manufacturer’s instructions. Densitometric analysis was performed on the Molecular Analyst GS670 (Bio-Rad).

Assay of Syk Kinase Activity. Vav immunoprecipitated from total cells was subjected to in vitro phosphorylation with Syk immunoprecipitated from control and differentiated cells in the presence of 20 mM HEPES (pH 7.4), 10 mM MgCl2, 3 mM MnCl2, and 0.5 mM ATP. The reaction was allowed to continue for 15 min at 30°C and stopped by the addition of Laemmli’s SDS sample buffer. The samples were then separated by electrophoresis, blotted, and analyzed for the amount of phosphorylated proteins, as reported above.

Inhibition of Syk Kinase Activity. To inhibit Syk kinase activity, cells were treated with different concentrations of piceatannol (0.5 mg/ml in PBS), alone or simultaneously with 1 μM ATRA for 4 days; then lysates from total cells and purified nuclei were analyzed for their amount of phosphorylated proteins, as described above. The same samples were subjected to morphological examination of the nuclear shape and analysis of CD11b expression for evaluating their level of granulocytic differentiation, as described previously (21, 27).

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References

Tyrosine Phosphorylated Vav in Granulocytic Differentiation


