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Nuclear association of tyrosine-phosphorylated Vav to phospholipase C-γ1 and phosphoinositide 3-kinase during granulocytic differentiation of HL-60 cells

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Abstract The granulocytic differentiation of HL-60 cells induced by all-*trans* retinoic acid was accompanied by a progressive tyrosine phosphorylation of specific proteins in either cells or isolated nuclei. Among these phosphoproteins, we identified the Vav adaptor in whole cells as well as in the inner nuclear compartment, where the increase in its tyrosine phosphorylation level was more conspicuous. We also demonstrated the differentiation-dependent association of nuclear phosphorylated Vav to phospholipase C-γ1 and to the p85 regulatory subunit of phosphoinositide 3-kinase. The role of the Vav/phospholipase C-γ1/phosphoinositide 3-kinase phosphoprotein complexes in the nuclei of HL-60 induced to differentiate along the granulocytic lineage is discussed.

Key words: Nucleus; Phosphorylated protein; Vav; All-*trans* retinoic acid; HL-60 cell

1. Introduction

Growth factor receptors with intrinsic tyrosine kinase activity, upon activation, autophosphorylate their cytoplasmic moiety on several tyrosine residues, which form distinct binding sites for SH2 domain-containing proteins. These activated receptors can directly recruit a number of enzymes involved in various signal transduction pathways, including components of the lipid-dependent signaling pathways [1–3]. Other agonists, which do not possess an intrinsic tyrosine kinase activity, are still able to activate non-receptor protein tyrosine kinases (PTKs), inducing the tyrosine phosphorylation of several substrates. A number of phosphotyrosine-containing proteins, defined as adaptor proteins, are recognized to play an important role in linking the signal transduction emanating from both activated receptors and non-receptor PTKs to lipid-dependent enzymes, such as phosphoinositide 3-kinase (PI 3-K) and phospholipase C-γ1 (PLC-γ1).

An adaptor protein known to bind to non-receptor PTKs is the 95 kDa *vav* proto-oncogene product (Vav), which is highly expressed in hematopoietic cells, and contains multiple structural motifs commonly found in intracellular signaling molecules: SH2, SH3 and pleckstrin homology domains. It also contains a region, highly homologous to the guanine nucleotide exchange protein Dbl, termed Dbl homology or DH domain (for review, see [4]). The functional properties of the DH domain of Vav in stimulating guanine nucleotide exchange on Rac (GEF activity) are regulated by Lck-dependent phosphorylation on a specific tyrosine residue [5,6].

The binding of the Vav SH2 domain to phosphorylated PTKs is thought to serve for the recruitment of activated kinases, which in turn can phosphorylate Vav [4]. Human Vav protein contains 31 tyrosine residues with unknown functional significance, except for Tyr-174, which is phosphorylated by Lck [4].

Other kinases appear to be responsible for tyrosine phosphorylation of Vav in different cell systems. The *vav* proto-oncogene product participates in the signaling pathways activated by type I IFN receptor in U-266 and Daudi cells, by interacting with the IFN receptor-associated Tyk-2 in vivo [7]. Vav might be a substrate for the kinase ZAP-70 in Jurkat T cells stimulated by TCR, suggesting a role for the microtubule in the signaling functions of these two proteins [8]. Vav was then found to associate with and be tyrosine-phosphorylated by activated JAK2 in vitro, in Epo-induced F-36P cells [9].

In vitro, tyrosine-phosphorylated Vav binds to a host of SH2 domains, including those present in PLC-γ1 and in the p85 regulatory subunit of PI 3-K.

In a recent work it was reported that both PtdIns(4,5)P2 and PtdIns(3,4,5)P3 interact with Vav, modulating the ability of Lck to phosphorylate and activate Vav in response to mitogens. It has been proposed that the PH domain of Vav, when complexed to PtdIns(4,5)P2, inhibits Vav GEF activity. On the other hand, phosphorylation of Vav by Lck was enhanced in the presence of 3-phosphorylated lipids, suggesting that activation of PI 3-K could serve to eliminate a Vav inhibitor and simultaneously produce activators of Vav activity [10].

GAB1 represents a second example of docking protein, which acquires the ability to interact with the SH2 domains of Grb2, PLC-γ1 and PI 3-K upon tyrosine phosphorylation [11]. A constitutive association between GAB1 and PI 3-K was shown in cell lines overexpressing the epidermal growth factor receptor [12]. GAB1 phosphorylation, followed by its specific interaction with several SH2 domain-containing proteins, was also present as a consequence of treatment

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Abbreviations: PTK, protein tyrosine kinase; PI 3-K, phosphoinositide 3-kinase; PLC-γ1, phospholipase C-γ1; ATRA, all-*trans* retinoic acid

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with nerve growth factor [13] and hepatocyte growth factor [14].

Although all-*trans* retinoic acid (ATRA) is known to act through the RAR/RXR nuclear receptors, members of the thyroid/steroid receptor superfamily of transcription factors [15], we have previously demonstrated that the ATRA-induced granulocytic differentiation of the HL-60 cell line is accompanied by a specific nuclear pattern of expression and activity of enzymes related to the phosphoinositide signaling pathways [16]. We report here the presence of Vav adaptor protein in nuclei from HL-60 cells and the association of tyrosine-phosphorylated Vav with PI 3-K and PLC-γ1 in nuclei from differentiated conditions. This suggests the existence of a mechanism for recruiting and activating PI 3-K and PLC-γ1 into the nucleus of cells differentiating along the granulocytic lineage.

2. Materials and methods

2.1. 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 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) in a 94/6% air/CO_2 atmosphere. Cells were maintained at an optimal cell density between 3×10^5/ml and 10^6/ml and treated with 1 μM ATRA (Sigma Chemicals, St. Louis, MO) for the indicated times. Cell differentiation was examined as previously reported [16,17].

Monoclonal anti-phosphotyrosine (P-Tyr) antibody was obtained from Transduction Laboratories (PY20, Lexington, KY). Monoclonal antibodies against p85 α and β subunits of PI 3-K, PLC-γ1 and Vav and polyclonal antibody against GAB1 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-β-tubulin antibody was from Sigma. Agarose-conjugated GST-p85 SH2-carboxyl (C)-terminal and SH2-amino (N)-terminal domains were from UBI.

Peroxidase-conjugated anti-rabbit and anti-mouse IgG (Sigma) were used as secondary antibody, and the final detection was performed using the ECL system (DuPont, NEN Research Products, Boston, MA), according to the manufacturer's instructions.

2.2. Preparation of cell lysates, nuclei and immunoprecipitates

Nuclei depleted of nuclear membranes were isolated following a previously described procedure [16], in the presence of 0.5% Triton X-100. All the purification buffers contained 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 μg/ml STI, 0.5 mM PMSF, 0.5 mM DTT and 1 mM Na_3VO_4 (all from Calbiochem, La Jolla, CA). Nuclear purity was assessed by ultrastructural analysis and marker enzyme assays, as previously reported [16,18]. As to the yield of the nuclear isolation, from 1×10^6 cells were obtained, on average, 0.5×10^6 nuclei. In terms of proteins, the content of 1×10^6 cells was 50 μg, and 1×10^6 nuclei was 10 μg.

Cells and nuclei were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA, 1 mM PMSF; 1 μg/ml each aprotinin, leupeptin; 1 mM Na_3VO_4; 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. Supernatant 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.

2.3. Immunochemical analysis

Total lysates and immunoprecipitates were separated on 7.5% polyacrylamide denaturing gels [19] and blotted to Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). The blots were then incubated with the antibodies, and revealed by the ECL system (DuPont).

Each lane contained 50 μg protein, obtained from 1×10^6 cells in the case of total cell lysate, or from 5×10^6 membrane-depleted nuclei in the case of nuclear lysate. For immunoprecipitation studies, 1 mg of protein was employed in all cases, corresponding to 20×10^6 cells or to 100×10^6 nuclei.

2.4. PI 3-kinase activity assay

The assays of PI 3-K catalytic activity on immunoprecipitates obtained from total cells and nuclei were performed in the presence of phosphatidylinositol (PI, in 10 mM HEPES, 1 mM EDTA, pH 7.5; 0.5 mg/ml final concentration). [γ-^{32}P]ATP (10 μCi/sample) incorporation was achieved for 15 min at room temperature and the reaction was stopped by addition of chloroform/methanol/HCl (200:100:0.75 v/v), followed by two washes with chloroform/methanol/HCl 0.6 N (3:48:47 v/v). The lipid containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica Gel 60, Merck, Darmstadt, Germany) developed in isopropanol/acetic acid/H_2O (65:1:34 v/v). After autoradiography on Kodak X-Omat S films, the spots were quantified by scintillation counting.

2.5. Statistical analysis

The results were expressed as mean ± S.D. of three or more experiments performed in duplicate. Statistical analysis was performed using the two-tailed Student's *t*-test.

3. Results and discussion

For comparative analysis between cells and nuclei, Western blots were performed by loading the same amount of protein (50 μg), obtained from 1×10^6 cells and 5×10^6 nuclei, respectively.

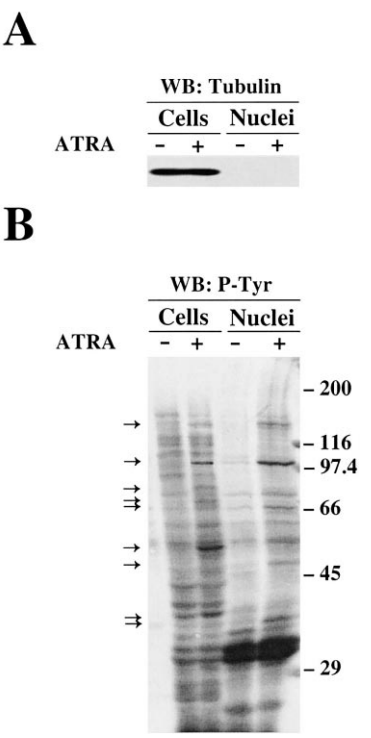

Fig. 1. Pattern of tyrosine-phosphorylated proteins in HL-60 cells treated with ATRA. A: Purity of isolated nuclei was confirmed by immunoblotting total lysates from control (−) and differentiated (+) conditions with the antibody against the cytoplasmic protein tubulin. B: HL-60 cells and nuclei, stimulated (+) or not (−) with ATRA for 96 h, were lysed and subjected to Western blot (WB) analysis with anti-P-Tyr antibody. The positions of the proteins showing an increase in the tyrosine phosphorylation levels upon ATRA treatment are indicated with arrows, while the molecular weights are indicated on the right. A representative of seven separate experiments is shown.

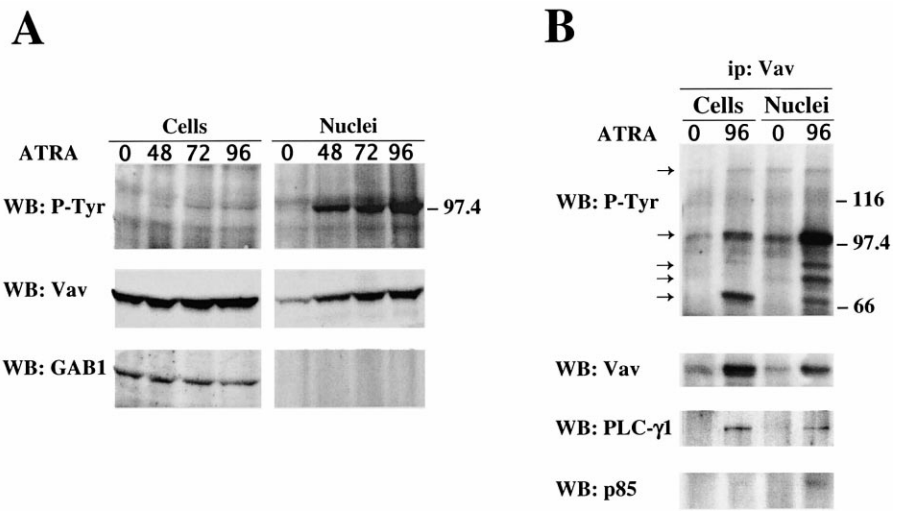

Fig. 2. Modulation of Vav protein and of its association to PLC-γ1 and p85 during ATRA-induced differentiation. HL-60 cells were treated with ATRA for the indicated times (hours). A: Whole lysates from cells and nuclei were subjected to immunoblot analysis with the anti-P-Tyr, anti-Vav and anti-GAB1 antibodies, respectively. B: Cells and nuclei were immunoprecipitated (ip) with anti-Vav antibody and subjected to WB analysis with anti-P-tyr antibody. Appropriate sections of the same membrane were then reprobed with the indicated antibodies. The positions of the tyrosine-phosphorylated proteins immunoprecipitated by anti-Vav antibody are indicated with arrows. In both panels, molecular weights are indicated on the right. A representative of four to five separate experiments is shown.

A 96 h treatment of HL-60 cells with 10^{-6} M ATRA, which induced terminal granulocytic differentiation of most cells [16,17], was accompanied by the tyrosine phosphorylation of a number of proteins, in lysates obtained from either whole cells or highly purified nuclei (Fig. 1). The proteins, whose levels of tyrosine phosphorylation mostly increased after granulocytic differentiation, migrated at about 38, 52, 70, 80, 100 and 150 kDa and 35, 48, 68, 100 and 150 kDa in cells and isolated nuclei, respectively (Fig. 1). Of note, the only bands showing a contemporaneous increase of tyrosine phosphorylation in both cells and nuclei were those at 100 and 150 kDa, the increase of the former band being more prominent in isolated nuclei.

To identify the nature of the 100 kDa phosphoprotein, total lysates obtained from cells and nuclei were examined at different time points of ATRA treatment, using antibodies against two adaptor molecules, the 95 kDa Vav and GAB1. This latter protein, despite the originally described molecular weight of 116 kDa [11], is reported to migrate in the 100 kDa region (Upstate Biotechnology Catalog, Quality Control Testing). As shown in Fig. 2A, the amount of the 100 kDa phosphoprotein progressively increased during granulocytic differentiation in both cells and nuclei, starting from 48 h of ATRA treatment onwards. However, the intensity of this band reached higher levels in isolated nuclei than in total cell lysates. When the membranes were probed with an anti-Vav antibody, Vav protein was revealed in all samples, but it showed an evident increase in nuclei obtained from differentiated cells (Fig. 2A). In contrast, when the membranes were probed with an anti-GAB1 antibody, the presence of GAB1 was revealed in total cell lysates, but not in nuclei, and it did not show significant variations during ATRA treatment.

In the next experiments, Vav protein was immunoprecipitated from both cells (20×10^6) and nuclei (100×10^6) before and after 96 h of ATRA treatment and initially revealed with anti-P-Tyr antibody (Fig. 2B). A high level of tyrosine phosphorylation was observed in various bands (68, 75, 80, 100, 150 kDa), which were prominent in nuclei isolated from differentiated cells. The anti-Vav antibody recognized a band, comigrating with the 100 kDa phosphoprotein, which showed a striking increase after ATRA treatment in both cells and nuclei (Fig. 2B). It is remarkable that while the amount of Vav protein, shown by Western blotting performed on either whole lysates (Fig. 2A) or immunoprecipitates (Fig. 2B), was lower in nuclei than in cells, its tyrosine phosphorylation level was markedly higher in nuclei than in cells. These findings highlight a preferential nuclear localization of tyrosine-phosphorylated Vav during the course of ATRA-induced granulocytic differentiation of HL-60 cells.

Since phosphorylated Vav is known to binds SH2-containing proteins [4] and we have previously demonstrated that HL-60 granulocytic differentiation is accompanied by a selective nuclear increase in both PLC-γ1 [16] and PI 3-K (Bertagnolo et al., in preparation), the Vav immunoprecipitates were also probed with either anti-PLC-γ1 or anti-p85 antibodies (Fig. 2B). Neither of these enzymes coimmunoprecipitated with Vav in untreated cells and nuclei. On the other hand, a significant amount of both enzymes coimmunoprecipitated with Vav after granulocytic differentiation (Fig. 2B). It is also noteworthy that while the anti-PLC-γ1 recognized a band comigrating with the 150 kDa phosphoprotein, the anti-p85 did not recognize any of the phosphorylated bands immunoprecipitated by the anti-Vav antibody. Work is in progress to characterize the other tyrosine-phosphorylated (75 and 80 kDA) bands, observed in isolated nuclei.

To further confirm the existence of protein-protein association between Vav, PLC-γ1 and p85 we next performed crossed immunoprecipitations with anti-PLC-γ1 (Fig. 3A) and anti-p85 (Fig. 3B) antibodies. Probing the anti-PLC-γ1 immunoprecipitates with anti-P-Tyr revealed tyrosine phosphorylated bands of 70, 80, 100, 150 kDa, which increased after ATRA treatment (Fig. 3A). Out of these four bands, three (80, 100, 150 kDa) were detected in nuclei only upon ATRA treatment. When probed with specific antibodies, the 150 kDa band comigrated with PLC-γ1 (Fig. 3A), which increased in the nucleus following treatment with ATRA.

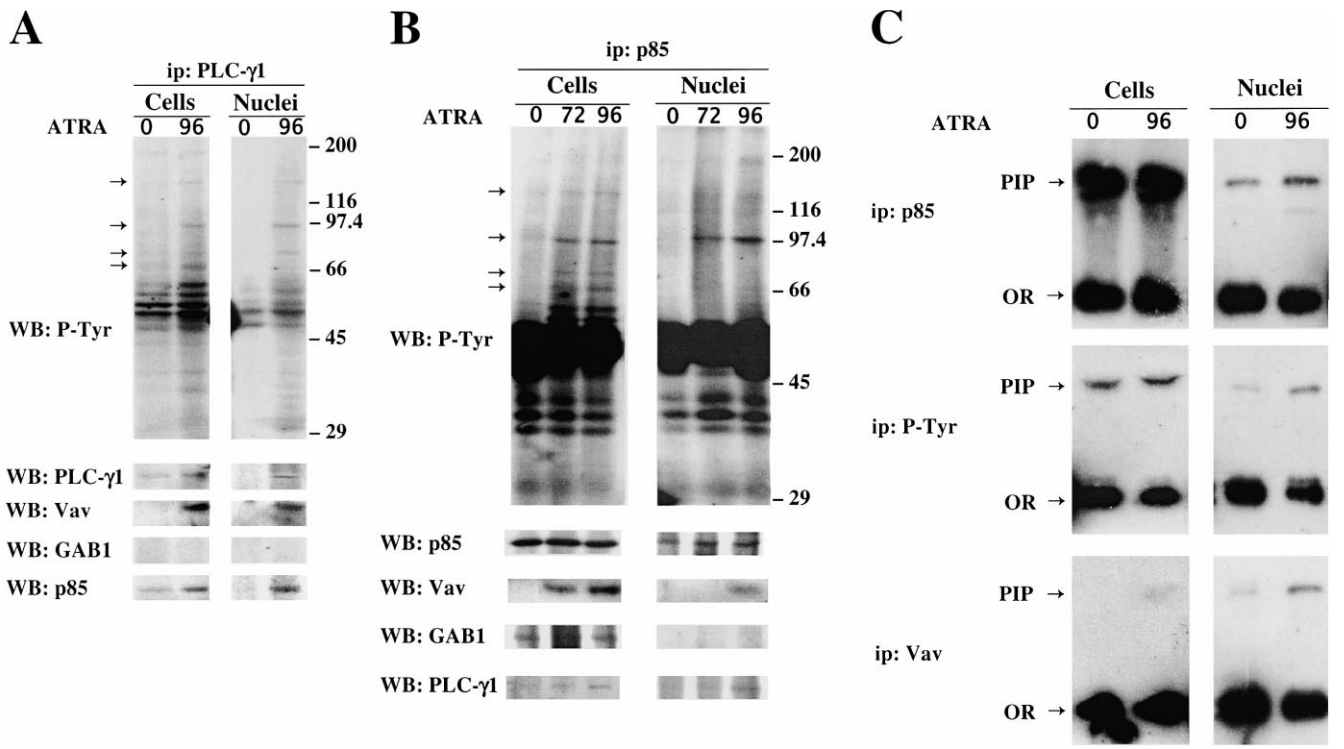

Fig. 3. Association of PLC-γ1 and p85 with Vav. Lysates from cells and nuclei at different times (hours) of ATRA treatment were immunoprecipitated (ip) with (A) anti-PLC γ1 and (B) anti-p85 antibodies and immunoblotted (WB) with anti-P-Tyr antibody. Appropriate sections of the same membrane were then reprobed with the indicated antibodies. The positions of the relevant tyrosine-phosphorylated proteins associated to PLC γ1 and p85 are indicated with arrows. In both panels, molecular weights are indicated on the right. C: Immunoprecipitates (ip) with the indicated antibodies from cells and nuclei were subjected to an in vitro PI 3-K assay, using PI as substrate. OR = origin, PIP = phophatidylinositol 3-phosphate. A representative of three to six separate experiments is shown.

Vav protein was also clearly detectable in anti-PLC-γ1 immunoprecipitates of differentiated cells and nuclei, further indicating that the PLC-γ1/Vav association took place only upon granulocytic differentiation. On the other hand, no detectable levels of GAB1 were present in anti-PLC-γ1 immunoprecipitates, suggesting that Vav, but not GAB1, played a prominent role as a docking molecule for PLC-γ1 in the different subcellular compartments of HL-60. Additionally, the anti-PLC-γ1 antibody coimmunoprecipitated p85 in both untreated and ATRA-treated cells, but the association of the two enzymes increased significantly after ATRA treatment (Fig. 3A). In contrast, the association between PLC-γ1 and p85 in the nuclear compartment occurred only in differentiated conditions (Fig. 3A).

As shown in Fig. 3B, anti-p85 immunoprecipitated phosphoproteins of 70, 80, 100 and 150 kDa, and 100 and 150 kDa in HL-60 cells and isolated nuclei, respectively. Once again, these bands became more prominent (cells) or appear ex novo (nuclei) only after ATRA-induced differentiation. Of note, also in this group of experiments, no phosphoproteins of 85 kDa could be shown, clearly indicating that phosphorylation of p85 was not induced by ATRA. The amount of immunoprecipitated p85 was constant in cells, while increasing in isolated nuclei during granulocytic differentiation. The 100 and 150 kDa phosphoproteins, present in both cells and nuclei, comigrated with Vav and PLC-γ1, respectively (Fig. 3B). In particular, immunoblotting with anti-Vav antibody demonstrated that Vav copurifies in anti-p85 immunoprecipitates, in both cells and nuclei, in a manner that correlated well with ATRA treatment. In fact, no Vav protein could be detected without differentiative stimulus, clearly indicating that also the interaction between p85 and Vav was induced by the differentiative process.

Probing the p85 immunoprecipitates against GAB1 showed the presence of this adaptor in cells, but not in nuclei, confirming the absence of GAB1 from nuclei as shown in Fig. 2A. Moreover, the amount of GAB1 coimmunoprecipitated by p85 was constant and, therefore, independent of granulocytic differentiation (Fig. 3B).

As PI 3-K holoenzyme is composed by a regulatory (p85) and a catalytic (p110) subunit [20], a further set of experiments was directed to evaluate the catalytic activity of PI 3-K in different immunoprecipitation experiments. The in

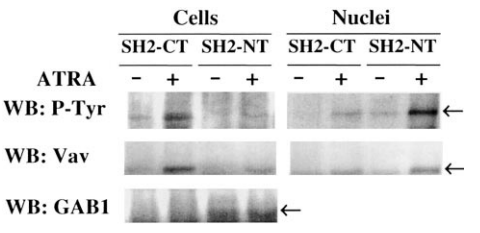

Fig. 4. In vitro association of Vav and GAB1 with the N- and C-terminal SH2 domains of p85. Lysates from cells and nuclei treated (+) or not (−) with ATRA for 96 h were incubated with agarose-conjugated GST-p85 SH2 N-terminal or SH2 C-terminal. The samples were immunoblotted (WB) with the indicated antibodies. The positions of 97.4 phosphoprotein, Vav and GAB1 are indicated by arrows. A representative of three separate experiments is shown.

vitro PI 3-K activity in the anti-p85 immunoprecipitates showed a significant ($P < 0.01$) increase in isolated nuclei, but not in cells, upon granulocytic differentiation (Fig. 3C). These data correlated well with the modifications in the amount of p85 immunoprecipitated from untreated and ATRA-treated HL-60 cells and nuclei (Fig. 3B). According to previous results, PI 3-K activity in anti-P-Tyr and anti-Vav immunoprecipitates was also significantly ($P < 0.01$) higher in nuclei isolated after ATRA treatment than in nuclei isolated from untreated cells (Fig. 3C).

The association of p85 with the tyrosine-phosphorylated Vav suggests that this binding might be mediated through the SH2 domains of p85 [4]. To evaluate whether the two SH2 domains of p85 [20] were responsible for its interaction with Vav, in the next experiments cells and nuclei were adsorbed on GST-fusion N-terminal and C-terminal p85-SH2 proteins. In control cells, ATRA treatment increased the Vav binding to both N- and C-terminal SH2s, with most of Vav being copurified on C-terminal p85 SH2 (Fig. 4). In contrast, in isolated nuclei, it was the SH2 N-terminal that increased its association with tyrosine-phosphorylated proteins in differentiated conditions (Fig. 4). We also probed the binding of the other 100 kDa phosphoprotein GAB1 and we showed that, in total cell lysates, the GAB1 protein was prevalently associated with SH2 N-terminal, without significant changes due to differentiation (Fig. 4). Although we have not addressed the reason for the differential ability of Vav to bind the SH2 domains of p85 in whole cells and nuclei, this might be due to a distinct pattern of tyrosine phosphorylation of cytosolic vs. nuclear Vav, or it may depend on steric hindrance in cytosolic Vav due to the binding of other proteins to its phosphotyrosines.

In this study, we have demonstrated for the first time the presence of Vav/PLC-γ1/PI 3-K proteins including complexes in the nucleus of differentiating HL-60 cells. Since PLC-γ1 antibody immunoprecipitates p85, the presence of a complex including at least these three proteins is likely. Although we have previously shown that PLC-γ1 can strongly associate with the nuclear matrix (nucleoskeleton) of the HL-60 cell line [16], and a study is in progress to characterize the presence and function of PI 3-K in the inner nuclear compartment, the demonstration that Vav functions as an adaptor protein for these enzymes also at the nuclear level was unexpected and represents a novel finding. Since Vav is the only one of the Vav/PLC-γ1/PI 3-K proteins to possess a nuclear localization sequence [4], it is possible that Vav is directly involved in the nuclear transportation of PLC-γ and PI 3-K.

Several possibilities can be envisioned to explain the progressive accumulation of Vav adaptor protein in the nucleus of the HL-60 cell line induced to differentiate by ATRA. Some studies have already reported the presence of Vav in the nuclear compartment [21,22], and recent observations may revive interest in the role of Vav for the regulation of gene expression and other nuclear functions [23–25]. It has also been demonstrated that Vav can associate with several cytoskeletal proteins, including tubulin, zyxin and polymerized actin [4]. Although the meaning of the Vav association with proteins of the cytoskeleton is not known, it is possible that Vav translocates into the nucleus via components of the cytoskeleton. Since we and other groups of investigators have previously shown that the nuclear matrix contains all the inositol lipid substrates for PLC-γ1 and PI 3-K [26–28], the presence of Vav inside the nucleus might suggest an essential role in targeting PLC-γ1 and PI 3-K enzymes to their relevant nuclear substrates.

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