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REGULAR ARTICLE

Vav1 is a crucial molecule in monocytic/macrophagic differentiation of myeloid leukemia-derived cells

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Abstract Vav1 is a critical signal transducer for both the development and function of normal hematopoietic cells, in which it regulates the acquisition of maturation-related properties, including adhesion, motility, and phagocytosis. Vav1 is also important for the agonist-induced maturation of acute promyelocytic leukemia (APL)-derived promyelocytes, in which it promotes the acquisition of a mature phenotype by playing multiple functions at both cytoplasmic and nuclear levels. We investigated the possible role of Vav1 in the differentiation of leukemic precursors to monocytes/macrophages. Tumoral promyelocytes in which Vav1 was negatively modulated were induced to differentiate into monocytes/macrophages with phorbol-12-myristate-13-acetate (PMA) and monitored for their maturation-related properties. We found that Vav1 was crucial for the phenotypical differentiation of tumoral myeloid precursors to monocytes/macrophages, in terms of CD11b expression, adhesion capability and cell morphology. Confocal analysis revealed that Vav1 may synergize with actin in modulating nuclear morphology of PMA-treated adherent cells. Our data indicate that, in tumoral promyelocytes, Vav1 is a component of lineage-specific transduction machineries that can be recruited by various differentiating agents. Since Vav1 plays a central role in the completion of the differentiation program of leukemic promyelocytes along diverse hematopoietic lineages, it can be considered a common target for developing new therapeutic strategies for the various subtypes of myeloid leukemias.

Keywords Myeloid leukemia · Vav1 · Monocytic differentiation · Cytoskeleton · Actin · Cell culture

Abbreviations

ATRA	All *trans*-retinoic acid
APL	Acute promyelocytic leukemia
BSA	Bovine serum albumin
DAPI	4,6-Diamino-2-phenylindole
DABCO	1,4-Diazabicyclo [2.2.2] octane
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GEF	Guanine Nucleotide exchange factor
PBS	Phosphate-buffered saline
PIC	Piceatannol
PMA	Phorbol-12-myristate-13-acetate

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siRNA	Small interfering RNA
TRITC	Tetramethylrhodamine isothiocyanate
TBS	TRIS-buffered saline

Introduction

Vav1 is the sole member of the Vav family of proteins physiologically expressed exclusively in hematopoietic cells, in which, by means of a number of different structural motifs, it works as an important signal transducer in the immune response (Katzav 2009). Vav1 acts mainly as a GDP/GTP nucleotide exchange factor for Rho/Rac small GTPases, a function strictly regulated by tyrosine phosphorylation and mainly devoted to the rearrangement of the cytoskeleton. Vav1 also works as an adapter molecule, facilitating the interaction between other signaling proteins (Bustelo 2001; Tybulewicz 2005). Recently, we and others have demonstrated that Vav1 is involved in the control of gene transcription by modulating the formation of DNA-associated complexes (Brugnoli et al. 2010; Houlard et al. 2002) and in the regulation of protein expression (Bertagnolo et al. 2008).

Studies with knockout mice have provided important insights into the function of Vav1 in the development of hematopoietic cells. The targeted down-modulation of Vav1 has been reported to result in the loss of mature T and B cells and in a severe impairment of their interleukin-2 production and calcium mobilization in response to external stimuli (Fujikawa et al. 2003; Haubert and Weckbecker 2010). The crawling defect observed in $Vav1^{-/-}$ neutrophils, concomitant with the decrease in migration, has been demonstrated to reduce the capacity for an innate immune response (Phillipson et al. 2009). A critical role for Vav1 has also been described in the regulation of the acquisition by macrophages of maturation-related competence, including phagocytosis (Hall et al. 2006). In particular, macrophages from Vav1-deficient mice have a smaller adhesive area, reduced motility and lower migration speed (Wells et al. 2005).

In addition to the crucial role played in the acquisition of a mature phenotype by normal hematopoietic cells (Denkinger et al. 2002), Vav1 has been found to promote the agonist-induced completion of the differentiation program of tumoral myeloid precursors. In particular, we have demonstrated that the sole over-expression of Vav1 promotes the overcoming of the differentiation blockade and enhances the all *trans*-retinoic acid (ATRA)-induced maturation of cells derived from acute promyelocytic leukemia (APL; Bertagnolo et al. 2005).

The down-modulation of Vav1 during the differentiation treatment of APL-derived cells has revealed a number of interconnected roles played by this protein in tumoral promyelocytes. By using proteomic approaches with both HL-60 and NB4 cells, we have demonstrated that Vav1 takes part in the maturation of tumoral promyelocytes by modulating the expression level of the protein tool by means of which ATRA promotes their differentiation program (Bertagnolo et al. 2008). A more specific role for Vav1 in the differentiation of tumoral promyelocytes concerns its ability to affect their cellular and nuclear morphology, possibly by regulating actin polymerization (Bertagnolo et al. 2004). All the roles that we have described for Vav1 in APL-derived cells induced to differentiate along the neutrophilic lineage are regulated by its tyrosine phosphorylation level (Bertagnolo et al. 2004, 2008, 2010; Brugnoli et al. 2010), which is, at least in part, dependent on spleen tyrosine kinase (Syk; Bertagnolo et al. 2001).

Although consolidated evidence has been gained concerning the multiple functions of Vav1 in the maturation program of tumoral promyelocytes to neutrophils, the role played by Vav1 in the monocytic/macrophagic differentiation of leukemic precursors has not been investigated so far. To address this issue, HL-60 and NB4 cells in which Vav1 was negatively modulated were induced to differentiate into monocytes/macrophages with phorbol-12-myristate-13-acetate (PMA) and monitored for their maturation level. The obtained results indicate that, in the same tumoral myeloid precursors, Vav1 can be recruited by different agonists as part of lineage-specific transduction machineries, highlighting the key role of this protein in the completion of the differentiation program of leukemic promyelocytes.

Materials and methods

Cell culture and treatments

All reagents were from Sigma (St Louis, Mo., USA) unless otherwise indicated. The HL-60 promyelocytic cell line (American Type Culture Collection, ATCC CCL-240, Rockville, MD., USA) and the APL-derived NB4 cell line (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories) in a 94%/6% air/CO_2 atmosphere. Cell density was maintained between 5×10^5/ml and 1.5×10^6/ml.

To induce monocytic/macrophagic differentiation, both cell lines were treated for 48 h with 100 nM PMA. Alternatively, neutrophil-like differentiation was obtained by cultivating the cells in the presence of 1 μM ATRA for 96 h.

To inhibit Syk activity, cells were cultured in the presence of 1 μg/ml piceatannol (PIC), as previously reported (Bertagnolo et al. 2010).

Estimation of differentiation levels

Cell adherence and morphology To establish the percentage of PMA-treated adherent cells, cells in suspension were collected, and the cells that had adhered to flask were detached with a trypsin-EDTA solution. Both suspended and adherent cells were counted by using a hemocytometer, and adherence was expressed as the percentage of adherent cells on the total cell number. The morphology of both HL-60 and NB4 cells growing under the various experimental conditions was analyzed with an inverted phase-contrast microscope (Nikon Eclipse TE2000-E; Nikon, Florence, Italy). Cell images were acquired by using the ACT-1 software for a DXM1200F digital camera (Nikon). To analyze cell adhesive area and elongation of adherent cells, digitized images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/; Papadopulos et al. 2007). For each experimental condition, three different areas containing at least 30 cells were analyzed. Treated adherent cells were defined as "flattened" when their cytoplasm area was equivalent to or larger than the nucleus area. Cells were defined as "elongated" when their longest axis was at least two times larger than their shortest axis. Cell with a morphology similar to untreated cells were defined as "compacted".

CD11b expression The expression of the CD11b myeloid surface antigen was examined by direct staining with a phycoerythrin-conjugated anti-CD11b antibody (Immunotech, Coulter Company, Marseille, France), as previously reported (Bertagnolo et al. 2010). After being stained, samples were analyzed by flow cytometry (FACScan, Becton-Dickinson, San José, CA, USA) with Lysis II software (Becton-Dickinson). Data collected from 10,000 cells are presented as mean fluorescence intensity values.

RNA interference assays

Three small interfering RNA (siRNA) sequences targeting the mRNA for Vav1 were synthesized by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Exponentially growing NB4 and HL-60 cells were transfected with a mixture of Vav1 siRNAs and with a non-silencing control siRNA (Santa Cruz Biotechnology) by using the electroporation procedure, as previously described (Bertagnolo et al. 2005). As a control of transfection efficiency, which was always higher than 60%, a non-silencing fluorescein-labeled duplex RNA, purchased from Qiagen (Milan, Italy), was used. Following electroporation, cells were recovered in 600 μl RPMI culture medium with 20% FBS and, after 5 h, centrifuged, resuspended in RPMI plus 10% FBS at a density of 5×10^5 cells/ml, and treated with PMA. After a 48 h treatment, transfected cells were subjected to an evaluation of their differentiation level and to immunochemical and immunocytochemical analysis.

Immunoprecipitation and immunochemical analysis

Vav1 was immunoprecipitated from both HL-60 and NB4 cells (10×10^6) by using an anti-Vav1 antibody (Santa Cruz Biotechnology), following a previously reported procedure (Bertagnolo et al. 2010).

Total lysates (50 μg protein) and immunoprecipitates (from 1 mg protein) were separated on 7.5% polyacrylamide denaturing gels and blotted onto nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK).

For analysis of tyrosine-phosphorylated proteins, membranes were saturated for 1 h in TRIS-buffered saline (TBS) containing 3% bovine serum albumin (BSA) and incubated with a monoclonal anti-phosphotyrosine antibody (PY20; Transduction Laboratories, Lexington, KY., USA), as previously reported (Bertagnolo et al. 2010).

For analysis of the total Vav1 expression and for Vav1 phosphorylated at the Y174 residue, membranes were saturated for 1 h in TBS containing 0.05% Tween-20 and 5% milk and incubated overnight, respectively, with an anti-Vav1 and with an anti-PY174 Vav1 polyclonal antibody (Santa Cruz Biotechnology), as previously reported (Bertagnolo et al. 2010).

For the analysis of α- and β-tubulin content, the nitrocellulose membranes were saturated for 1 h in phosphate-buffered saline (PBS) with 3% BSA and then hybridized with the specific monoclonal antibodies, as previously reported (Bertagnolo et al. 2008).

After reaction with the specific primary antibodies, all membranes were incubated with IgG peroxidase-conjugated secondary antibodies and revealed by chemiluminescence by using the ECL system (PerkinElmer, Boston, MA, USA), according to the manufacturer's instructions. Densitometric analysis of autoradiograms was performed with Image Quant TL software (Amersham Bioscience, Uppsala, Sweden), as previously reported (Bertagnolo et al. 2008).

Immunocytochemical analysis

Differentiating NB4 and HL-60 cells were allowed to grow onto round 12-mm glass coverslips, and untreated cells were placed on coverslips by means of cytocentrifugation (Cytospin 3, Shandon Scientific, Astmoor, UK). Cells on the coverslips were then washed in PBS and fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature, as previously reported (Bertagnolo et al. 2004).

For immunocytochemical analysis of Vav1 and α-tubulin, cells on coverslips were incubated with a Net Gel solution (150 mM NaCl, 5 mM EDTA, 50 mM TRIS-HCl

pH 7.4, 0.05% NP40, 0.25% Carrageenan Lambda gelatine, and 0.02% Na azide) for 1 h at room temperature to block non-specific binding and to allow the entry of the antibody into the cell and then incubated with the specific primary antibodies for 3 h in NET gel at room temperature. Samples were subsequently reacted with the specific secondary fluorescein isothiocyanate (FITC)-conjugated antibodies in NET gel for 45 min at room temperature. After two washes with NET gel and PBS, coverslips were dried with ethanol and mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane (DABCO). Fluorescent samples were analyzed with a Nikon Eclipse TE2000-E microscope or with a Zeiss 510 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Analysis of filamentous actin and F-actin/Vav1 co-localization in adherent cells

To analyze F-actin, fixed cells were washed with PBS, permeabilized with 1% Triton X-100 in PBS for 5 min at room temperature, incubated with 1% BSA for 30 min, and then stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin in PBS for 30 min at room temperature in the dark. After three washes with PBS, cells on coverslips were dried with ethanol, mounted in glycerol-DABCO, and analyzed with a fluorescence microscope (Nikon Eclipse TE2000-E).

To analyze F-actin/Vav1 co-localization, fixed cells were washed with PBS, permeabilized with 1% Triton X-100 in PBS for 5 min at room temperature, incubated with 1% BSA for 30 min, and reacted with the specific anti-Vav1 antibody for 3 h in PBS at room temperature. Samples were then incubated with a mixture containing both the FITC-conjugated secondary antibody and the TRITC-conjugated phalloidin in PBS for 30 min at room temperature in the dark. After three washes with PBS, cells on coverslips were dried with ethanol, mounted in glycerol-DABCO, and analyzed with a confocal microscope (Zeiss 510). Images were processed by using the LSM Image Browser (Zeiss).

Confocal microscopy sections at the apical, mid-height, and basal surfaces of PMA-treated adherent cells were obtained as described by Khatau et al. (2009).

F-actin quantification by flow cytometry

Cells (5×10^5) were harvested, and the pellets were washed with PBS. For permeabilization, cells were incubated for 5 min at room temperature in the dark with 100 μl of Perm Solution (Immunotech) and then stabilized with 50 μl of Stab Solution (Immunotech). For F-actin staining, cells were incubated for 30 min at room temperature in the dark with 82.5 nM Alexa-488-labeled phalloidin (Invitrogen, Paisley, UK). After incubation, cells were washed and fixed with 0.5% formaldehyde and analyzed on a FACSCan (Becton Dickinson). Gating was performed to exclude, by forward and side scatter criteria, cell debris, and cell clumps. Mean fluorescence intensity values collected from 10,000 cells were expressed as the percentage of control values.

Statistical analysis

The results were expressed as means±standard deviations of three independent experiments. Statistical analysis was performed by using the two-tailed Student's *t*-test for unpaired data. *P* values <0.05 were considered statistically significant.

Results

Vav1 is up-regulated during differentiation to monocytes/macrophages of APL-derived cells

Maturation of AML-derived cells along the monocytic/macrophagic lineage was obtained by treating HL-60 and NB4 cells with 100 nM PMA. A preliminary time-course analysis of the maturation-related adhesion and expression of CD11b indicated that, as expected, 48 h of PMA administration induced the highest differentiation level (data not shown). The treatment time of 48 h was then used in all the subsequent experiments.

The analysis of cell morphology showed that, after 48 h treatment with PMA, both HL-60 (Fig. 1a, b) and NB4 (Fig. 1c, d) cell populations displayed increased heterogeneity; this was particularly evident in adherent cells, which showed a flattened/enlarged shape or a variety of profiles with long thin processes extending from the margins of many cells.

The immunochemical analysis of total lysates of untreated and PMA-treated cells showed a drug-induced increase of Vav1 expression, which was much higher in adherent cells and paralleled the acquisition of a differentiated phenotype (Fig. 1e). Immunocytochemical analysis with the anti-Vav1 antibody confirmed that PMA administration induced the increase of Vav1 and showed its accumulation close to the nucleus, particularly in NB4 cells (Fig. 1f-m).

Since almost all the known roles of Vav1 depend on its tyrosine phosphorylation, the involvement of tyrosine phosphorylated Vav1 in the maturation of APL-derived cells to monocytes/macrophages was investigated. As reported in Fig. 2a, the immunochemical analysis performed on immunoprecipitates with the anti-Vav1 antibody from adherent cells, which constituted the differentiated population, showed an increase of tyrosine phosphorylated

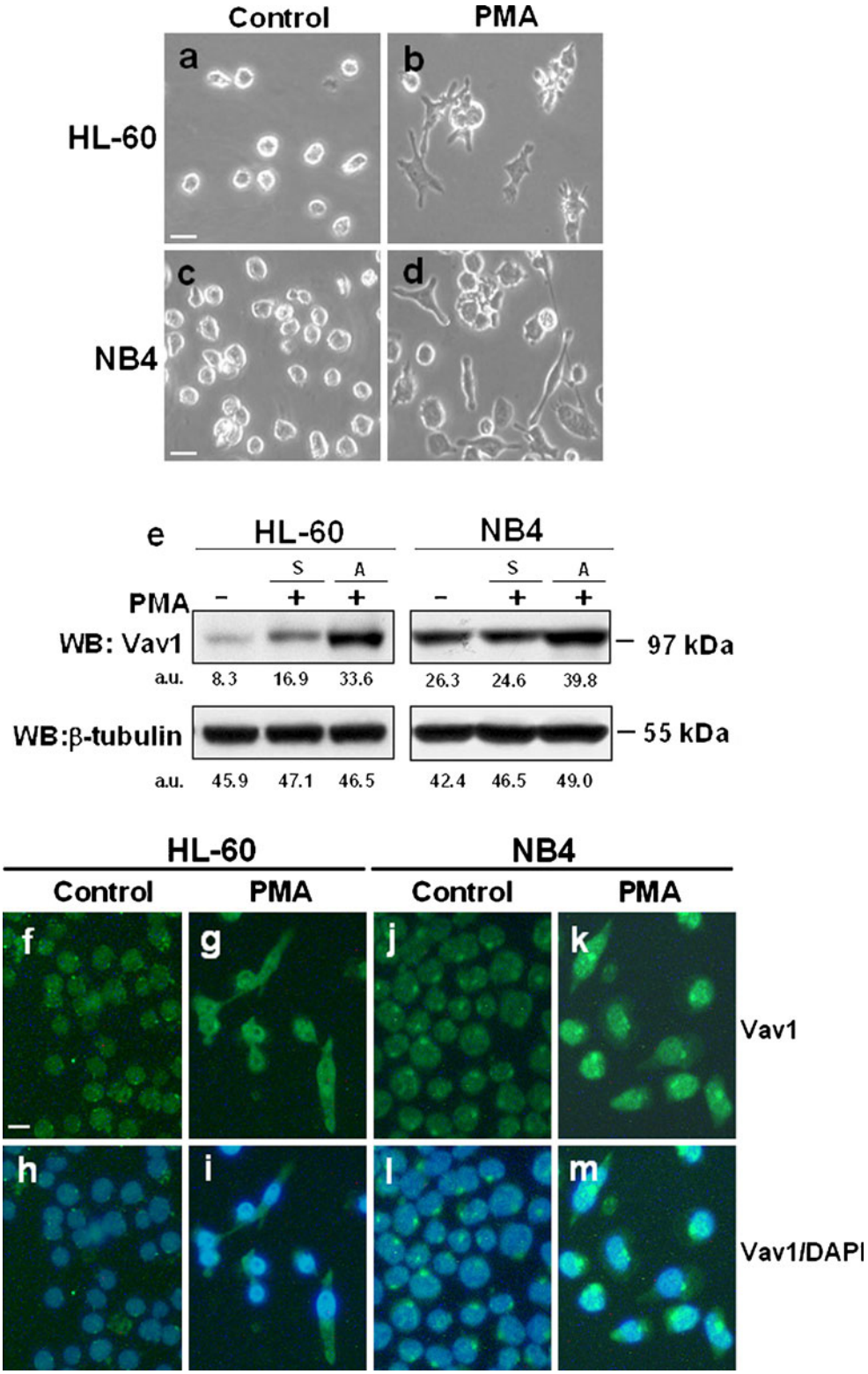

Fig. 1 Vav1 in phorbol-12-myristate-13-acetate (*PMA*)-treated cells. **a-d** Phase-contrast images of HL-60 (**a**, **b**) and NB4 (**c**, **d**) cells cultured under control conditions or in the presence of 100 nM PMA for 48 h. *Bars* 20 μm. **e** Lysates of control (−) and PMA-treated (+) cells evaluated for Vav1 expression by means of Western blot analysis (*WB*). Lysates were also analyzed for β-tubulin content, as an internal control of equal protein loading (*S* cells in suspension, *A* adherent cells). Densitometric analysis was expressed as arbitrary units (*a.u.*). **f-m** Representative fluorescence microscopy images of control and differentiating (48 h PMA) cells subjected to immunocytochemical analysis with the anti-Vav1 antibody. After staining of the nucleus with 4,6-diamino-2-phenylindole (*DAPI*), merged anti-Vav1/DAPI images were obtained. *Bar* 15 μm. All the data are representative of 3 separate experiments

Vav1 as a consequence of PMA administration in both HL-60 and NB4 cells. The same investigation performed with an antibody able to recognize specifically Vav1 phosphorylated in the Y174 residue showed a PMA-induced increase of phosphorylated Vav1 only in NB4 cells (Fig. 2a).

The potential role of Syk in regulating the tyrosine phosphorylation level of Vav1 was investigated by performing PMA treatment in the presence of PIC, a specific inhibitor of Syk. As reported in Fig. 2a, neither the overall tyrosine phosphorylation of Vav1, nor that on its Y174 residue were

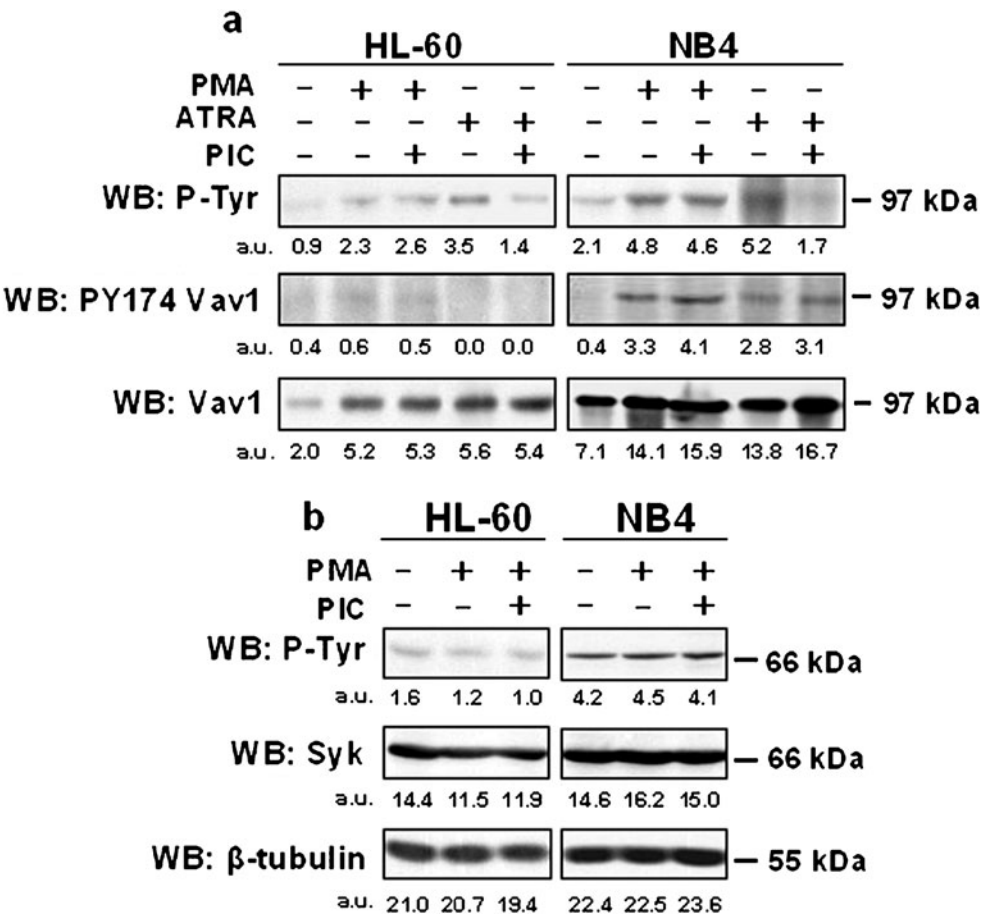

Fig. 2 Tyrosine phosphorylation of Vav1 in PMA-treated cells. **a** Immunochemical analysis with the indicated antibodies of anti-Vav1 immunoprecipitates from HL-60 and NB4 cells treated with PMA or all *trans*-retinoic acid (*ATRA*) in the presence (+) or absence (−) of piceatannol (*PIC*). **b** Lysates from HL-60 and NB4 cells treated with PMA in the presence (+) or absence (−) of PIC were subjected to immunochemical analysis with the indicated antibodies to evaluate expression and tyrosine phosphorylation of Syk. β-Tubulin content constituted an internal control of equal protein loading. Densitometrical analysis was expressed as arbitrary units (*a.u.*). The data are representative of three separate experiments

significantly affected by PIC. On the other hand, a role of Syk inhibition on the total tyrosine phosphorylation levels of Vav1 was observed in HL-60 and NB4 cells induced to neutrophil differentiation with ATRA (Fig. 2a), confirming our previously published data (Bertagnolo et al. 2008). The Syk-dependent phosphorylation of Vav1 in PMA-treated cells was definitely ruled out by the data reported in Fig. 2b, indicating that PMA treatment failed to up-regulate both expression and tyrosine phosphorylation of Syk.

Vav1 is crucial for phenotypical differentiation to monocytes/macrophages of APL-derived cells

To assess the role of Vav1 in the monocytic/macrophagic maturation of tumoral myeloid precursors, the PMA-induced increase of Vav1 amount was counteracted with specific siRNAs. As shown in Fig. 3a, the down-modulation strategy, particularly efficient in NB4 cells, reduced the expression of Vav1 in the whole cell populations for the entire duration of the differentiation treatment (48 h).

The effects on the differentiation process of the down-modulation of Vav1 expression during PMA administration were evaluated by monitoring maturation-related features. As reported in Fig. 3b, when the PMA-induced increase of Vav1 expression was counteracted, a substantial decrease of the percentage of cells that became adherent was observed, particularly in the NB4 cell line. Concerning cells which, despite their reduced amount of Vav1, retained their adhesion capabilities, a significant decrease of CD11b expression was observed (Fig. 3c), together with a decreased level of the maturation-related morphological changes (Fig. 3d-g). In particular, the down-modulation of Vav1 during the differentiating treatment reduced, in both HL-60 and NB4 cells, the number of enlarged and elongated cells in favor of cells with a morphology similar to that of untreated (compacted) cells.

The results of the morphometric analysis of HL-60 and NB4 adherent cells under the above-reported experimental conditions are presented in Table 1. As deduced by the analysis of phase contrast images after PMA administration, NB4 cells showed an adhesion area higher than that of HL-60 cells and, unlike these cells, rarely displayed an elongated fibroblast-like morphology, having predominantly a flattened shape. When the expression of Vav1 was down-modulated during the differentiation treatment, the total adhesion area decreased significantly in both cell lines, as did the number of enlarged and elongated cells, in parallel with an increased number of cells showing a round compacted shape (Table 1).

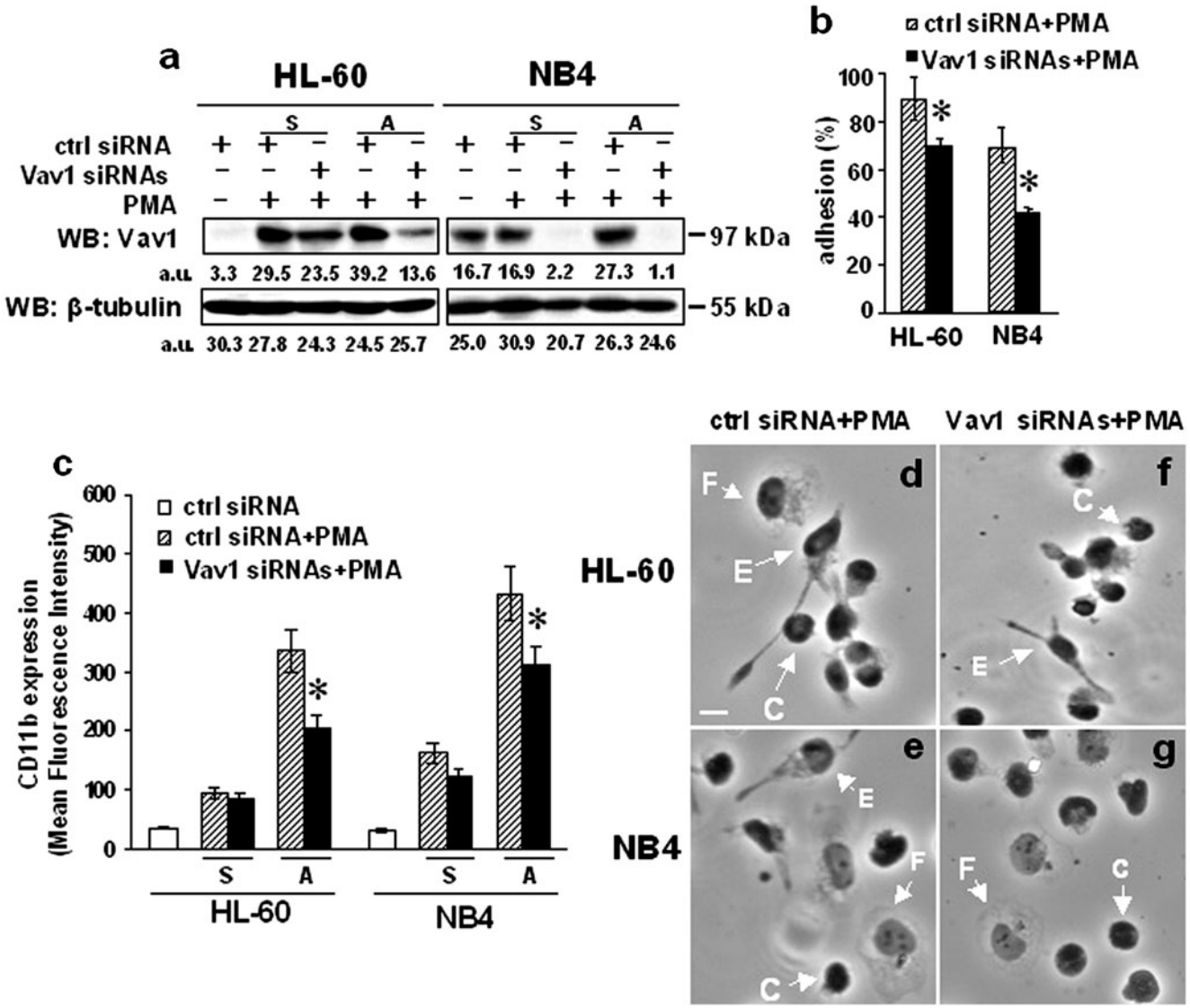

Fig. 3 Silencing of Vav1 during PMA treatment. **a** Exponentially growing HL-60 and NB4 cells were subjected to RNA interference assays with specific siRNAs for Vav1 (*Vav1 siRNAs*), then treated with PMA, and subjected to immunochemical analysis to evaluate Vav1 expression. The analysis of β-tubulin content constituted an internal control of equal protein loading. Densitometric analysis was expressed as arbitrary units (*a.u.*) (*ctrl siRNA* non-silencing control siRNA, *S* cells in suspension, *A* adherent cells). Cells under the same experimental conditions were analyzed for adhesion capability (**b**) and CD11b expression (**c**). Data are the means of three separate experiments±SD; *statistically significant values at $P<0.05$. **d-g** Representative phase-contrast images (of three separate experiments) of adherent HL-60 (**d**, **f**) and NB4 (**e**, **g**) cells transfected with a scrambled non-silencing RNA (*ctrl siRNA*, **d**, **e**) or with siRNAs specific for Vav1 (*Vav1 siRNAs* **f**, **g**) and then treated with PMA (*arrows* "compacted" [*C*], "flattened" [*F*], and "elongated" [*E*] cells, identified as described in Materials and methods). *Bar* 10 μm

Vav1 regulates morphology of PMA-treated cells by modulating actin-based cytoskeleton

Since adhesion and spreading of monocytes-macrophages require orderly changes in cytoskeleton structures, further experiments investigated whether Vav1, in addition to controlling the expression of adhesion-related surface antigens, regulated the cytoskeleton reorganization at the basis of the morphological changes induced by PMA in APL-derived cells. Since, as above reported, PMA induced similar phenotypical modifications in HL-60 and NB4 cells, and because we were able to down-modulate Vav1 more efficiently and with larger effects on cell morphology in NB4 cells, the subsequent experiments were performed exclusively on this latter cell line.

At first, we focused on α-tubulin, also stemming from our previous data demonstrating that, during ATRA-induced granulocytic differentiation of APL-derived cells, Vav1 affected its expression (Bertagnolo et al. 2008) and from the reported evidence that the microtubule cytoskel-

Table 1 Effect of Vav1 down-modulation on the shape changes of adherent HL-60 and NB4 cells treated with PMA for 48 h (*ctrl siRNA* non-silencing control siRNA, *Vav1 siRNAs* siRNAs specific for Vav1 mRNA). Data are mean values±SD of four separate experiments

Conditions		Adhesion area (μ^2/cell)	Percentage of compacted cells	Percentage of flattened cells	Percentage of elongated cells
HL-60	ctrl siRNA	362.3±44.5*	31.9±1.2	31.2±7.4	37.0±8.6
	Vav1 siRNAs	273.0±16.2*	50.5±9.9	22.1±0.6	27.4±10.4
NB4	ctrl siRNA	481.0±56.3*	39.4±3.7*	54.0±1.5	6.6±3.2*
	Vav1 siRNAs	374.1±14.8*	50.8±6.9*	47.5±7.2	1.7±0.5*

*$P<0.05$ (P values were obtained by comparing diffusion coefficients from Vav1 siRNAs experiments with ctrl siRNA data by using a two-sample t-test)

eton was impaired in macrophages from Vav1-deficient mice (Wells et al. 2005). Immunochemical analysis failed to demonstrate any effect on the amount of α-tubulin after PMA administration and/or after Vav1 down-modulation (Fig. 4a). This lack of response of α-tubulin to Vav1 was also confirmed by immunocytochemical analysis, showing that the PMA-induced organized network of filaments, starting from a gathering area nearby the nucleus, was not affected by the down-modulation of Vav1 in cells that retained the ability to attach to the plastic, even if their morphology appeared modified and more similar to that of untreated cells (Fig. 4c-e).

Since adhesion and migration of monocytes require actin remodeling, an event involved in the Vav1-dependent regulation of cytoskeleton (Bustelo 2001), the expression and polymerization of actin were compared after PMA treatment of wild-type and Vav1-deficient NB4 cells. As reported in Fig. 4a, immunochemical analysis of adherent/mature cells demonstrated that PMA induced, in addition to Vav1, the expression of actin that, on the other hand, was counteracted by the down-modulation of Vav1 during the differentiation treatment.

To establish whether Vav1 may have a role in regulating actin polymerization, the effects of reduced expression of Vav1 on the formation of F-actin was investigated. As shown in Fig. 4f, i, in untreated cells, F-actin represents a thin layer that surrounds the nucleus; this layer is difficult to appreciate because of the small volume of cytoplasm present in these hematopoietic tumoral precursors. PMA treatment induced a significant increase of F-actin that seemed to give rise to an organized network, starting from an assembly area located near the nucleus (Fig. 4g, j). The F-actin scaffold well defined the nuclear periphery and the cytoplasmic cell border and also showed a significant accumulation inside the thin and long cell processes (Fig. 4g, j). In cells in which PMA treatment was performed in the presence of a reduced amount of Vav1, a minor decrease of F-actin content was observed, particularly at the nuclear and cellular periphery (Fig. 4h, k). The increase in the amount of F-actin in PMA-treated NB4 cells and its slight decrease as a consequence of the down-modulation of Vav1 expression was quantified by cytofluorimetrical analysis, as reported in Fig. 4b.

Vav1 and actin might synergize in determining nuclear morphology of adherent cells

Since, as above demonstrated, the expression of both Vav1 and actin is induced by PMA in NB4 cells and, on the other hand, the actin amount is dependent on the presence of Vav1 during the differentiation treatment, we tried to establish whether the two proteins might collaborate in this cell model. With this aim, the Vav1/F-actin association was investigated by means of confocal microscopy of PMA-treated adherent NB4 cells.

As deduced from Fig. 5a, b, the co-localization of Vav1 and filamentous actin substantially increased after PMA treatment. In particular, even if red (F-actin) or green (Vav1) areas were still present in the majority of adherent cells, large regions in which the two proteins co-localized (yellow areas) were highlighted (Fig. 5a, b), possibly located at the actin assembly areas as previously described in Fig. 4g, j.

A more detailed analysis of Vav1/F-actin co-localization (Fig. 5c-h) showed that, in PMA-treated adherent cells with an elongated (Fig. 5c) or flattened (Fig. 5f) shape, F-actin well defined the nuclear periphery, but in elongated cells only, it seemed to be localized at the cytoplasmic level, particularly in the longest cell processes. Vav1, like filamentous actin, well defined the nuclear periphery but, at variance with F-actin, showed a homogeneous distribution in the cytoplasm of both elongated (Fig. 5d) and flattened (Fig. 5g) cells. The simultaneous confocal analysis of the Vav1 and F-actin signals (Fig. 5e, h) clearly indicated that Vav1/F-actin were co-localized at the assembly area located near the nucleus. Only a partial Vav1/F-actin co-localization was present at the nuclear periphery and in the thin processes of the elongated cells (Fig. 5e). On the other hand, the two molecules were largely dissociated inside the nucleus and in the cytoplasm of both elongated and flattened cells (Fig. 5e, h).

Recent data suggest that the actin cytoskeleton is involved in regulating the nuclear shape of adherent cells

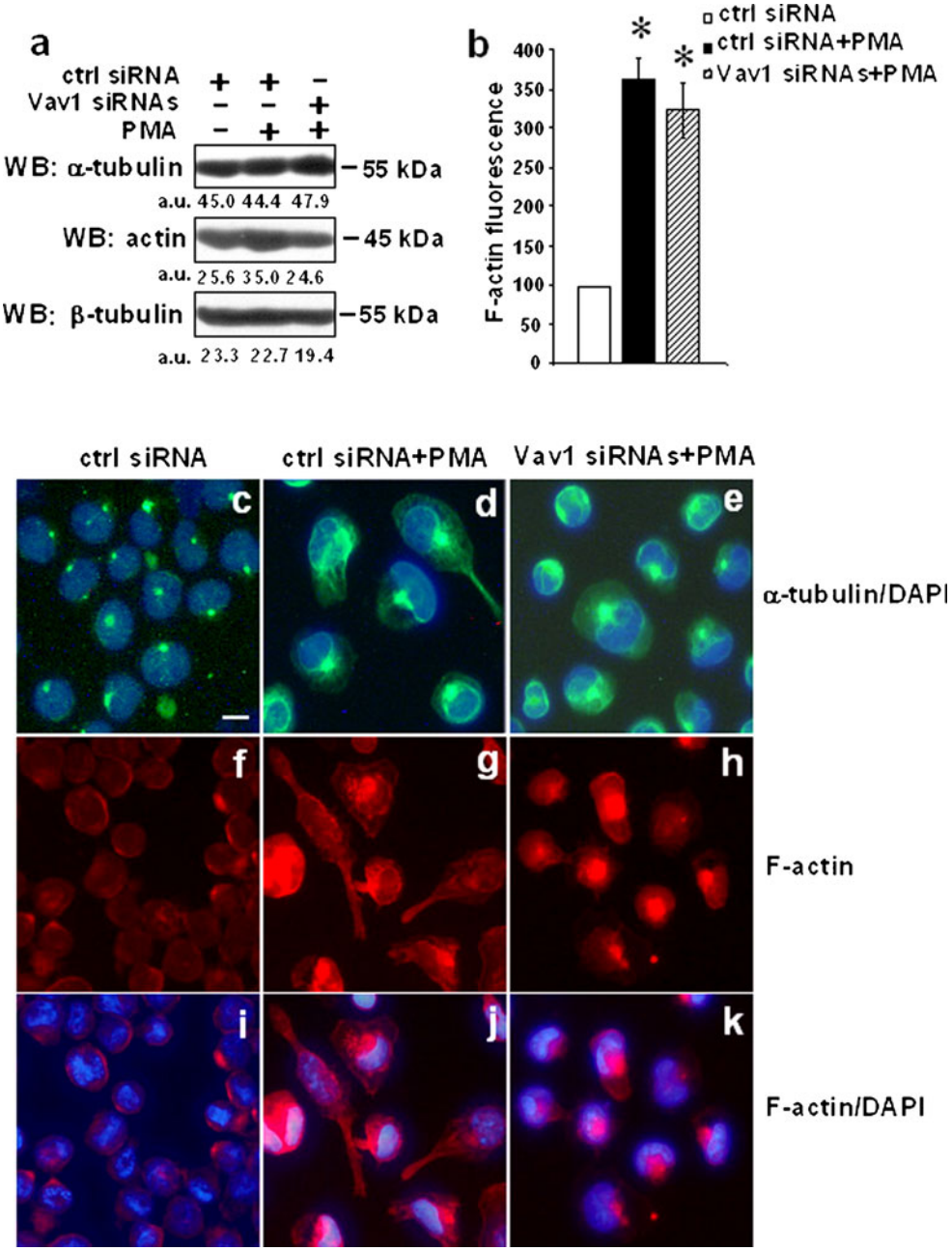

Fig. 4 Cytoskeleton in Vav1-silenced NB4 cells. **a** NB4 cells transfected with a scrambled non-silencing RNA (*ctrl siRNA*) or with siRNAs specific for Vav1 (*Vav1 siRNAs*) were treated with PMA and subjected to immunochemical analysis with the indicated antibodies. The β-tubulin content was used as an internal control of equal protein loading. Densitometric analysis was expressed as arbitrary units (*a.u.*). Data are representative of three separate experiments. **c-k** Cells under the same experimental conditions were subjected to immunocytochemical analysis to evaluate α-tubulin (**c-e**). F-actin staining was performed by using tetramethylrhodamine isothiocyanate (TRITC)-coupled phalloidin (**f-h**). After nuclear staining with DAPI, merged F-actin/DAPI (*red/blue*) images were obtained (**i-k**). All images are representative of three separate experiments. *Bar* 10 μm. **b** F-actin was quantitatively evaluated by cytofluorimetrical analysis after staining with Alexa-488-labelled phalloidin. Data are the means of three separate experiments± SD; *statistically significant values at $P<0.05$

through an actin filament structure, the perinuclear actin cap, which is located above and around the interphase nucleus (Khatau et al. 2009). Stemming from this notion and from the above reported results indicating that Vav1 and F-actin are co-localized around the nucleus of PMA-treated cells, we performed a further analysis to determine whether, in our cell model, Vav1 might synergize with actin to regulate nuclear morphology. With this intent, confocal sections obtained at the apical surface, mid-height, and basal surface of treated NB4 cells were analyzed for Vav1 and F-actin content and localization. As shown in Fig. 5, sections at the basal level revealed a homogeneous distribution of both Vav1 (Fig. 5n) and F-actin (Fig. 5k) and a Vav1/F-actin co-localization almost exclusively restricted to the assembly areas (Fig. 5q). Sections at the mid-height cell level showed an increased amount of F-actin, mainly located around and close to the nucleus (Fig. 5j). In the same sections, Vav1 defined the nuclear periphery, without any evident modification of its total amount in comparison with the basal sections (Fig. 5m). Concerning the apical sections, a further increase of F-actin content was observed, whose distribution almost completely covered the cell area (Fig. 5i). In these sections, we were not able to discern any significant changes of the amount of Vav1 (Fig. 5l), which, on the other hand, almost completely co-localized with F-actin (Fig. 5o).

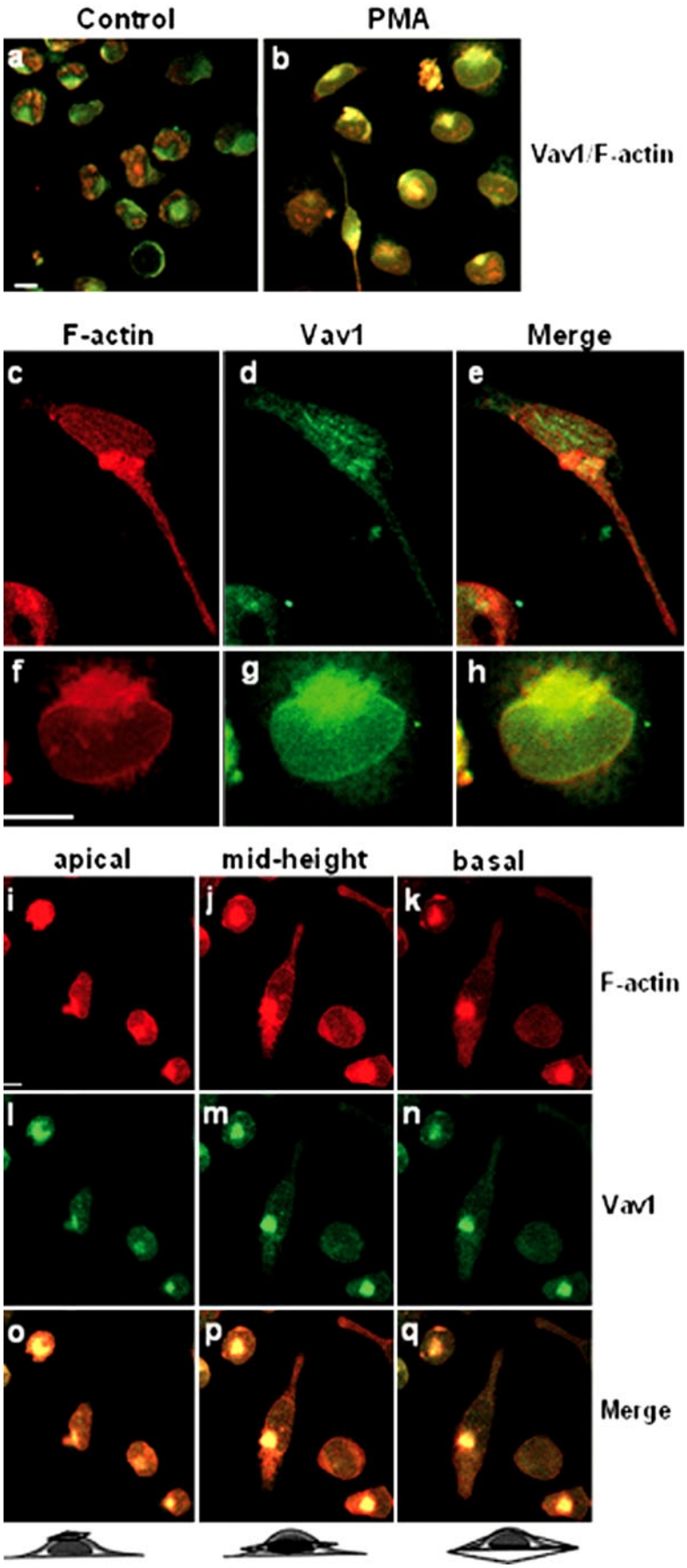

Fig. 5 Co-localization of Vav1 with F-actin. Untreated (**a**) and adherent PMA-treated (**b**) NB4 cells were subjected to immunocytochemical analysis for the evaluation of Vav1/F-actin co-localization. Immunofluorescence staining was performed with anti-Vav1 antibody followed by fluorescein-isothiocyanate-coupled anti-rabbit IgG (*green*). F-actin was detected by using TRITC-coupled phalloidin (*red*). Cells were then analyzed by confocal microscopy, and merged Vav1/F-actin images were obtained. **c-h** Representative confocal images of F-actin and Vav1 distribution and co-localization (*Merge*) in "elongated" (**c-e**) and "flattened" (**f-h**) PMA-treated adherent NB4 cells, according to the criteria described in Materials and methods. **i-q** PMA-treated NB4 cells were subjected to immunocytochemical analysis with both anti-Vav1 antibody and phalloidin. Confocal microscopy sections of the F-actin- and Vav1-stained cells were collected at the apical (**i**, **l**, **o**), mid-height (**j**, **m**, **p**), and basal (**k**, **n**, **q**) levels. Merged images (*Merge*) are shown (**o-q**) evaluating the co-localization between filamentous actin (*red*) and Vav1 (*green*). All images are representative of three separate experiments. *Bars* 10 μm

Discussion

Vav1 participates in the development and function of many types of immune cells, in which its complex structure is predictive of many functions that are only in part related to its GEF activity (Fujikawa et al. 2003; Hall et al. 2006; Katzav 2009; Kim et al. 2003; Miletic et al. 2009). In addition to playing a physiological role in normal hematopoietic cells, Vav1 is a key molecule in the ATRA-induced differentiation of APL-derived tumoral promyelocytes, being involved in multiple events ending in the acquisition of a neutrophil-like phenotype (Bertagnolo et al. 2004, 2005, 2008; Brugnoli et al. 2010).

This work has been aimed at establishing whether, as for other ATRA-induced molecules, the function of Vav1 is agonist-restricted or whether a broader role is played by this protein in the maturation of tumoral promyelocytes. Since the human promyelocytic leukemia cell lines HL-60 and NB4 can be differentiated toward neutrophils by ATRA and to monocytes/macrophages by PMA (Murao et al. 1983; Song and Norman 1998), they are useful models for investigating the involvement of Vav1 in the maturation of myeloid precursors along the monocytic/macrophagic lineage.

The results obtained by means of immunochemical and immunocytochemical analysis demonstrate that, in both HL-60 and NB4 cells, the PMA-induced acquisition of a monocyte-like phenotype is accompanied by the increased expression of Vav1. This is consistent with the notion that mature myeloid cells from peripheral blood, including monocytes, express Vav1, and that proper amounts of the protein are necessary for their inflammation-related functions (Bhavsar et al. 2009; Hall et al. 2006). As observed in the treatment of HL-60 and NB4 with ATRA, a relevant increase of tyrosine-phosphorylated Vav1 is induced by PMA. On the other hand, contrary to what we have demonstrated in ATRA-treated cells (Bertagnolo et al. 2001, 2008), we have failed to reveal any role for Syk in this event, consistent with the notion that, at least in HL-60 cells, Syk might exert a unique role in directing cells toward granulocyte differentiation (Qin and Yamamura 1997). These results are also in agreement with previous data indicating that, in macrophage-like differentiated HL-60 cells, the activity of Syk is focused on the roles played by mature cells, including their complement-mediated phagocytosis, in which the kinase regulates both actin dynamics and the Vav-RhoA activation pathway (Shi et al. 2006).

A crucial role for Vav1 in determining the acquisition of maturation-related features has been demonstrated by counteracting the expression of Vav1 induced by PMA by means of specific siRNAs. In particular, the expression of CD11b, which is induced by PMA and constitutes a marker of myeloid differentiation, is significantly reduced, similar to results that we have previously demonstrated during the treatment of both HL-60 and NB4 cells with ATRA (Bertagnolo et al. 2005, 2008). This suggests that, in differentiating APL-derived cells, Vav1 plays a role in regulating the expression of the CD11b surface antigen, regardless of the agonist employed and the maturation lineage. Since we have recently found the recruitment of Vav1 to protein/DNA complexes on the *CD11b* promoter in NB4 cells (Brugnoli et al. 2010), we speculate that Vav1 plays a specific role in driving the expression of this surface marker.

Moreover, cell adhesion is affected by the down-modulation of Vav1 during PMA treatment of HL-60 and NB4 cells, in terms of both the number of adherent cells and of the adhesion area of cells that remain attached to the flask bottom. These results are in agreement with the data obtained with macrophages from $Vav1^{-/-}$ mice, showing a smaller adhesive area or a decreased adhesion efficiency (Wells et al. 2005).

The main known functional role of Vav1 is to regulate cytoskeleton reorganization (Bustelo 2001), a phenomenon at the basis of both adhesion and migration of monocytes/macrophages (Park et al. 2008). Vav1 regulates cell architecture not only by means of its GEF activity, but also by modulating the expression of cytoskeleton proteins. In particular, in both HL-60 and NB4 cells, we have recently demonstrated the involvement of Vav1 in the ATRA-induced expression of the microtubule component α-tubulin (Bertagnolo et al. 2008). Contrary to what we have observed during granulocytic differentiation, no effects of Vav1 down-modulation have been found in either the expression or the architectural organization of α-tubulin during the PMA-induced monocytic/macrophagic maturation of NB4 cells. This indicates that, during the maturation process of APL-derived cells, Vav1 exerts an agonist/lineage specific role in regulating α-tubulin. Our results also suggest that, concerning microtubule organization, the role of Vav1 is restricted to the motility of mature cells, according to the data indicating that changes in microtubule dynamics contribute to the reduced migration speed of $Vav1^{-/-}$ macrophages in response to CSF-1 (Wells et al. 2005).

Dynamic regulation of the filamentous actin cytoskeleton, which encompasses a variety of different structures essential for many aspects of cell physiology, is critical to numerous physical cellular processes, such as adhesion, migration and phagocytosis, each of which requires precise regulation of cell shape (Cougoule et al. 2006; Stricker et al. 2010).

A role of Vav1 in regulating actin organization has been demonstrated in both lymphoid and myeloid cells. In

particular, actin cytoskeleton rearrangements are reported to be defective in *Vav1*$^{-/-}$ lymphocytes (Holsinger et al. 1998), and Vav1-deficient neutrophils show reduced in vitro and in vivo activities mediated by filamentous actin, such as motility and mobilization into peripheral blood (Gakidis et al. 2004). Recent data demonstrate that Vav proteins, including Vav1, are required for actin cytoskeleton reorganization during the migration of macrophages, by coupling RhoA and Rac1 activity to adhesion receptors (Bhavsar et al. 2009).

Moreover, in the ATRA-induced maturation of cells derived from APL, Vav1 seems to regulate actin organization. In particular, a specific role for Vav1 in this cell model concerns its ability to affect the cellular and nuclear morphology of differentiating cells by recruiting phosphoinositide 3-kinase to actin cytoskeleton, an event ending in the modulation of the actin-associated phosphoinositides and, ultimately, actin polymerization (Bertagnolo et al. 2004).

Here, we demonstrate an unprecedented involvement of Vav1 in regulating the increase of actin expression induced by PMA in both HL-60 and NB4 cells. This constitutes a further indication that Vav1, in addition to being involved in the formation of filaments, takes part in cytoskeleton reorganization as a modulator of protein expression.

A wide variety of contractile F-actin networks with different architectures and organization of F-actin polarity has been found near cell adhesion surfaces, and the modifications of cell shape in the various cell processes seem to be regulated by the existence of the F-actin cortex, a thin membrane-bound F-actin network (Stricker et al. 2010). Furthermore, recent work in which adhesive micropatterned surfaces have been used to control the overall shape of fibroblasts, has demonstrated that the shape of the nucleus is tightly regulated through a perinuclear actin cap that is located above and around the interphase nucleus (Khatau et al. 2009).

By means of confocal analysis of PMA-treated adherent NB4 cells, we have demonstrated the existence of an agonist-induced F-actin network that well defines the cytoplasmic cell border, accumulates inside the thin and long cell processes, and surrounds the nuclear compartment, starting from an assembly area located near the nucleus. Vav1/F-actin co-localization has been observed at the F-actin assembly area, at the nuclear periphery, and in the thin process of elongated cells. Since the existence of cytoplasmic processes in PMA-treated adherent cells is indicative of migratory activity (Stricker et al. 2010), Vav1/F-actin co-localization in cytoplasm protrusions is suggestive of a synergy of the two molecules in modulating cell motility. This is in agreement with the role described for Vav proteins in the maintenance of normal morphology and migratory behavior in macrophages (Bhavsar et al. 2009).

Confocal analysis of sections obtained at the apical surface, at the mid-height level, and at the basal surface of PMA-treated NB4 cells has demonstrated Vav1/F-actin co-localization almost exclusively restricted to the nuclear periphery and to the F-actin assembly areas located close to the nucleus. In addition, as deduced by the analysis of apical sections, the almost complete co-localization of Vav1 with F-actin in the region above the nucleus suggests that the two proteins cooperate in regulating the shape of the nucleus through an actin filament structure similar to the perinuclear actin cap described by Khatau et al. (2009).

F-actin structures are involved in various steps of cell migration, including those essential for adhesion (Stricker et al. 2010). Confocal analysis of basal sections of PMA-treated cells has failed to demonstrate a significant Vav1/F-actin co-localization, suggesting that, in our cell model, the role of Vav1 in modulating cell adhesion is related to its ability to regulate the expression of integrins, such as CD11b, rather than directly affecting the actin-based cytoskeleton.

The reported results constitute the first evidence that Vav1 plays a crucial role in the maturation of leukemic promyelocytes to monocytes/macrophages. Since Vav1 is also critical for the maturation of tumoral myeloid precursors along the granulocytic lineage, our data highlight the key role for this protein during the completion of the differentiation program of tumoral myeloid cells along the various hematopoietic lineages and suggest that Vav1 is a common target for developing future therapies of the diverse subtypes of myeloid leukemias. This may be of interest, since the ATRA- and PMA-mediated differentiation of human myeloid leukemia cell lines has recently been reported to result in changes of their sensitivity to chemotherapeutic drugs, suggesting new therapeutic strategies aimed at the rational combination of differentiating agents and conventional anticancer drugs (Jasek et al. 2008; Kogan 2009).

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