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The protein kinase inhibitor SB203580 uncouples PMA-induced differentiation of HL-60 cells from phosphorylation of Hsp27

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Abstract HL-60 cells are an attractive model for studies of human myeloid cell differentiation. Among the well-examined parameters correlated to differentiation of HL-60 cells are the expression and phosphorylation of the small heat shock protein Hsp27. Here we demonstrate that PMA treatment of HL-60 cells stimulates different MAP kinase cascades, leading to significant activation of ERK2 and p38 reactivating kinase (p38RK). Using the protein kinase inhibitor SB 203580, we specifically inhibited p38RK and, thereby, activation of its target MAP kinase-activated protein kinase 2 (MAPKAP kinase 2), which is the major enzyme responsible for small Hsp phosphorylation. As a result, PMA-induced Hsp27 phosphorylation is inhibited in SB 203580–treated HL-60 cells indicating that p38RK and MAPKAP kinase 2 are components of the PMA-induced signal transduction pathway leading to Hsp27 phosphorylation. We further demonstrate that, although PMA-induced phosphorylation is inhibited, SB 203580-treated HL-60 cells are still able to differentiate to the macrophage-like phenotype as judged by decrease in cell proliferation, induction of expression of the cell surface antigen CD11b and changes in cell morphology. These results indicate that, although correlated, Hsp27 phosphorylation is not required for HL-60 cell differentiation. However, the results do not exclude that increased Hsp27 expression is involved in HL-60 cell differentiation.

INTRODUCTION

After its establishment from a patient with acute myeloid leukaemia in 1977, the HL-60 cell line developed into a useful model for terminal differentiation from promyelocytes to monocyte/macrophage-like as well as granulocyte-like cells in vitro (Collins 1987; Birnie 1988). Interestingly, the direction of in vitro differentiation depends on the stimulus applied to the cells: the phorbol ester myristate (PMA) induces differentiation to the macrophage-like phenotype (Huberman and Callaham 1979; Rovera et al 1979), whereas dimethyl sulfoxide (DMSO) and retinoic acid direct the HL-60 cell line to the granulocyte-like phenotype (Vorbrodt et al 1979; Breitman et al 1980).

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The molecular signaling mechanisms involved in the regulation of HL-60 cell differentiation are not entirely understood. However, it became clear very early that protein phosphorylation is correlated with this process, because a rapid increase in serine phosphorylation of two different intracellular proteins could be detected a few minutes after treatment of HL-60 cells with PMA (Feuerstein and Cooper 1983; Feuerstein et al 1984). Protein kinase C (PKC) seems to be involved in PMA-induced differentiation to the macrophage-like phenotype, since sustained activation of PKC enhances this differentiation process (Aihara et al 1991) and PMA-resistant variants of HL-60 are deficient in the PKC-β isoform (Tonetti et al 1992, 1994). A contribution of MAP kinase to this process is also probable, since PMA stimulates the activity of this enzyme in HL-60 cells but not in the PMA-resistant variant HL-525 (Kharbanda et al 1994).

The finding that heat shock on its own as well as thermotolerance-inducing agents were also able to induce differentiation of HL-60 cells (Richards et al 1988) gave

the first indication that heat shock proteins (Hsps) could be involved in the molecular mechanism underlying this differentiation process. Subsequent analysis of the various intracellular changes in HL-60 cells during the differentiation process revealed significant changes in the mRNA-level of *hsp27, hsp60, hsp70* and *hsp90α, β* (Shakoori et al 1992; Mivechi et al 1994). This is in accordance with the finding that in PMA-stimulated U937 cells which represent the transition from monocytes to macrophages heat shock protein transcription and synthesis are also significantly stimulated (Twomey et al 1993).

Recently it has become evident that one of the two initially ascribed PMA-induced proteins, which shows a molecular mass of 27 kDa and was phosphorylated at serine residues, is identical to the human small heat shock protein Hsp27 (Spector et al 1993). Accordingly, it was proposed that this protein, the expression and phosphorylation of which is intermediary in the myelomonocytic pathway, could be a unique marker of macrophage differentiation in HL-60 cells (Spector et al 1993, 1994). This assumption was further supported by the known correlation between expression and phosphorylation of small Hsps and growth and differentiation in several cell lines and organisms (Arrigo and Landry 1994). In the above studies (Spector et al 1993, 1994) for both PMA- and retinoic acid-induced differentiation of HL-60 cells, increased expression and phosphorylation of the human small heat shock protein Hsp27 has been observed. However, another recent study (Minowada and Welch 1995) confirmed the PMA-induced stimulation of Hsp27 expression and phosphorylation but, probably since they performed their analysis at a later time after treatment, found no changes in Hsp27 expression and phosphorylation during retinoic acid- or dimethyl sulfoxide (DMSO)-induced differentiation. Taken together, the data obtained at least indicate that the small heat shock protein (sHsp) could be specifically involved in the transition to the macrophage-like phenotype.

It is known that sHsp-phosphorylation is a downstream event of a newly described, stress-dependent signal transduction pathway homologous to the yeast HOG1–pathway (Brewster et al 1993) which apparently parallels the classical MAP kinase cascade (Han et al 1994; Rouse et al 1994). The known components upstream to Hsp27 in this phosphorylation pathway are the sHsp-phosphorylating enzyme MAP kinase-activated protein kinase 2 (Stokoe et al 1992), its activator, the p38 reactivating kinase (Freshney et al 1994; Rouse et al 1994) and the activators of p38RK, protein kinases designated MKK3 and MKK6 (Raingeaud et al 1996).

In this paper, we blocked this signal transduction pathway using a highly specific inhibitor for p38RK, the pyridinyl-imidazole compound SB 203580 (Lee et al 1994; Cuenda et al 1995), and analyzed whether inhibition of p38RK is sufficient to also block PMA-induced phosphorylation of Hsp27. In a second step, provided PMA-induced phosphorylation of Hsp27 was influenced by SB 203580, we were able to examine the role of sHsp-phosphorylation by determining the effect of the inhibitor on PMA-induced differentiation of HL-60 cells.

MATERIALS AND METHODS

Cell culture

HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. To stimulate HL-60 cells to differentiate into a macrophage-like phenotype, phorbol myristate acid (PMA, SIGMA, St Louis, USA) was added to a final concentration of 160 nM to the cell culture medium. Where indicated, SB 203580 (4-(4-Fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole (SmithKline Beecham Pharmaceuticals, King of Prussia, USA) was used in a final concentration of 10 µM and was added to the cell culture 2 h before PMA-stimulation. SB 203580 was present in the medium during the entire differentiation time.

MAP kinase assay

Approximately 1×10^6 cells were serum starved for 18 h, treated where indicated with SB 203580 for 2 h and subsequently stimulated by PMA. After 10 min the cells were harvested, lysed in 100 µl of lysis buffer LB (20 mM Tris acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM Na-pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.27 M sucrose, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride) for 20 min on ice and centrifuged for 5 min at 15 000 × *g*. Fifty microliters of the supernatant was diluted in 500 µl of immunoprecipitation (IP) buffer (50 mM Tris/HCL, pH 7.4, 150 mM NaCl, 5 mM EDTA, 25 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100) and incubated with a rabbit antiserum raised against the Xenopus p38RK-homolog Mpk2 (Rouse et al 1994) or anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4°C. Immunocomplexes were precipitated by incubation with 25 µl protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 1 h. The IP-pellet was washed four times with 500 µl of IP buffer and redissolved in 20 µl assay mixture (50 mM β-glycerophosphate, 0.1 mM EDTA, 0.1 mM ATP, 4 mM magnesium acetate, 1.5 µCi of [γ^{33}P] ATP) containing 10 µg of the appropriate substrate, MBP (SIGMA) or recombinant His-MAPKAP kinase 2 expressed in *Escherichia coli* BL21(DE3) using the plasmid pRSET-B

(Invitrogen, San Diego, USA). The kinase reaction was incubated for 15 min at 30°C. The reaction was terminated by addition of 8 µl 4 × SDS loading buffer. ^{33}P labeling of the substrate was detected and quantified after SDS-PAGE using the Bio Imaging Analyser BAS 2000 (Fuji, Tokyo, Japan).

Assay of MAPKAP kinase 2 activity

The cell treatment and immunoprecipitation (IP) were carried out as described above with the following modifications: cells were subjected to heat shock (44°C, 15 min) or PMA treatment (160 nM, 20 min) and IP was performed with 4 µl of a rabbit antiserum raised against recombinant GST-MAPKAP kinase 2 Δ3B (Plath et al 1994). The immunoprecipitate was redissolved in 20 µl of kinase assay mixture as described above, containing 10 µg recombinant Hsp27 (Jakob et al 1993) as substrate, and incubated for 15 min at 30°C.

FACS analysis of cell surface marker

HL-60 cells were cultivated in the absence and presence of SB 203580 for 72 h after PMA stimulation. The cells were then washed with phosphate buffered saline (PBS, pH 7.4) and incubated at 0°C for 30 min with a primary monoclonal antibody against CD11b (SIGMA) in a dilution of 1:20 in PBS, pH 7.4 containing 3% bovine serum albumin (BSA) and 0.02% sodium azide. Incubation with the FITC conjugated secondary goat anti-mouse IgG (SIGMA) was subsequently performed for 30 min. After washing 3 times with PBS, cells were redissolved in PBS containing 2% formaldehyde and analyzed on a fluorescence-activated cell sorter (FACS, EPICS XL Coulter, Coultertech, Florida, USA).

2D-gel electrophoresis and immunoblotting

Cells (10^6 per sample) were harvested at different times after stimulation by heat shock or PMA and used for analysis. Two-dimensional electrophoresis was performed as described (Knauf et al 1994). Immunoblotting was carried out with a rabbit antiserum against Hsp25/27 (Engel et al 1991). The immunoblots were developed with a secondary goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, USA) and NBT/BCIP as colorigenic substrate.

Colorimetric assay

The MTT bioassay (Mosmann 1983) was used according to the description of the manufacturer for the non-radioactive quantification of cell proliferation and metabolic activity after treatment with PMA and/or SB 203580. PMA-induced cells were grown in 96-well microtiter plates (Nunc, Roskilde, Denmark) in a final volume of 100 µl culture medium in the presence or absence of SB 203580 (10 µM) for 4, 24, 48 and 72 h (37°C, 5% CO_2). Ten microliters of MTT reagent (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) was added to each well to a final concentration of 0.5 mg/ml and incubated for 4 h. After this time the formazan crystals were completely solubilized by addition of 100 µl isopropanol/HCl/SDS solution. The absorbance at 570 nm was measured using a microtiter plate reader M 5000 (DYNATECH, Billingshurst, UK).

RESULTS

The stress activated HOG1-homolog kinase cascade is stimulated by PMA-treatment

To investigate the role of small Hsp phosphorylation in PMA-induced differentiation of HL-60 cells, we first analyzed whether the HOG1-homologous kinase cascade, which is responsible for activation of MAPKAP kinase 2 and subsequent sHsp phosphorylation, is activated in HL-60 cells as a result of PMA-treatment. For that reason we immunoprecipitated the activator of MAPKAP kinase 2, the p38RK, from lysates of HL-60 cells before and after stimulation with PMA. The kinase activity in the immunoprecipitate was measured using recombinant His-tagged MAPKAP kinase 2 as substrate. As shown in Figure 1, a stimulation of the activity of p38RK, as measured by phosphate incorporation into MAPKAP kinase 2, to more than 150% could be observed. In parallel, we could also detect a PMA-induced increase in the activity of the related component of the MAP-kinase cascade, the enzyme ERK2 (Fig.1). In the immunoprecipitate of ERK2 a greater than 2-fold increase in kinase activity, using the substrate myelin basic protein (MBP), could be measured. Hence, it seems that PMA stimulates different MAP kinase cascades in HL-60 cells in parallel.

PMA-induced activation of MAPKAP kinase 2 is blocked by SB 203580

To further understand the specific role of the kinase cascade that leads to Hsp27 phosphorylation, we used a specific inhibitor of the HOG1-homologous cascade. This inhibitor, SB 203580, is a pyridinyl imidazole which shows very high specificity for the p38RK and which, at a final concentration of 10 µM, does not significantly inhibit any other protein kinase tested so far, including ERK2, jun N-terminal kinases (JNKs) and MAPKAP kinase 2 (Cuenda et al 1995). PMA stimulation of HL-60 cells was carried out in the absence and presence of this inhibitor. The binding of SB 203580 to p38RK is

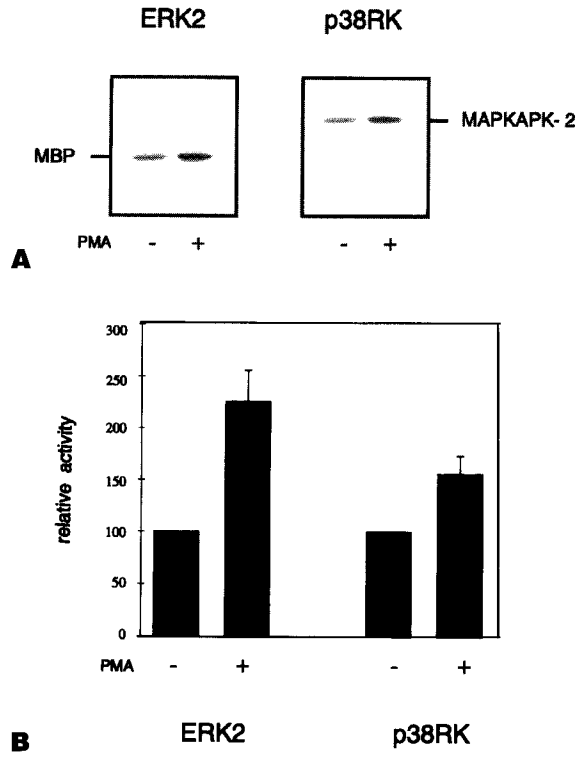

Fig. 1 Phorbol ester activates different MAP kinases in HL-60 cells. Cells were treated with PMA (160 nm) for 10 min. Subsequently, the kinase activity was analyzed by an immunocomplex protein kinase assay using [γ^{33}P]-ATP and MBP (for ERK2) or His-MAPKAP kinase 2 (for p38RK) as substrates. The phosphorylated substrates were detected after SDS-PAGE by phospho imaging (A). The phosphorylation was quantified in three independent experiments by phospho imaging analysis and is presented as relative kinase activity compared to untreated control cells (B).

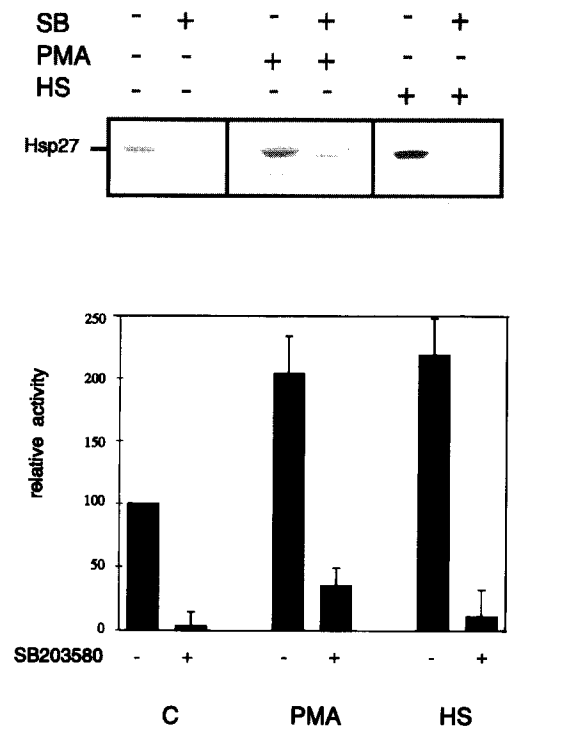

Fig. 2 SB 203580 is an potent inhibitor for PMA- and heat shock-induced MAPKAP kinase 2 activity. HL-60 cells were pretreated with SB 203580 for 2 h and subsequently subjected to PMA- (160 nM) or heat shock-treatment (44°C) for 20min. MAPKAP kinase 2 activity was analyzed by an immunocomplex protein kinase assay using [γ^{33}P]-ATP and Hsp27 as a substrate. The phosphorylated Hsp27 was detected after SDS-PAGE by phospho imaging (A). The phosphorylation was quantified in three independent experiments by phospho imaging analysis and is presented as relative kinase activity compared to untreated control cells (B).

reversible. Hence, it is not possible to measure the inhibiting effect of this compound by a direct p38RK immunoprecipitation kinase assay, since p38RK activity is recovered during the washing and dilution steps of the assay. Another indirect possibility to detect the in vivo inhibition of p38RK by SB 203580 is to measure the activity of the p38RK target MAPKAP kinase 2, which is phosphorylated and activated by p38RK. We immunoprecipitated MAPKAP kinase 2 from the cells stimulated with heat shock or PMA in the presence and absence of the inhibitor SB 203580 and determined the MAPKAP kinase 2 activity in the immunoprecipitate using recombinant Hsp27 as substrate. Figure 2 demonstrates that PMA-treatment and, as a control, heat shock both stimulate MAPKAP kinase 2 activity in HL-60 cells. The stimulation of MAPKAP kinase 2 activity as a result of PMA-treatment is about 2-fold. Figure 2 further shows the efficient inhibition of heat shock- and PMA-induced activation of MAPKAP kinase 2 by SB 203580. In both cases, the MAPKAP kinase 2 activity in stimulated cells in the presence of SB 203580 is clearly below the basal activity in non-stimulated HL-60 cells. In addition, even in non-stimulated cells a decrease of the basal activity of MAPKAP kinase 2 could be observed as a result of the addition of SB 203580. These data indicate that SB 203580 effectively inhibits p38RK and that this kinase is the main activator of the MAPKAP kinase 2 in HL-60 cells.

SB 203580 suppresses Hsp27 phosphorylation in HL-60 cells

In a next step we examined the effect of SB 203580 on the protein Hsp27 which is phosphorylated in vivo by MAPKAP kinase 2 (Stokoe et al 1992; Cuenda et al 1995). Cell lysates were prepared from PMA- and heat-stimulated

HL-60 cells and the phosphorylation of Hsp27 was analyzed by combined two-dimensional polyacrylamide electrophoresis and Western blot to detect the different phosphorylated isoforms of Hsp27 (Fig. 3). In control cells Hsp27 is present in the non-phosphorylated (0 in Fig. 3) and, to a lower extent, also in the mono-phosphorylated isoforms (1 in Fig. 3). Heat shock, as a classical inducer for Hsp27 phosphorylation, clearly shifts Hsp27 towards the mono- and bis-phosphorylated (2 in Fig. 3) isoforms. Even small amounts of the triple-phosphorylated isoform could be detected. As already described (Spector et al 1993; Minowada and Welch 1995), PMA treatment also shifts Hsp27 towards the higher phosphorylated isoforms. This shift can be observed already after 30 min and is still detectable 24 h after PMA-treatment (Fig. 3). A 2-h pretreatment of the cells with 10 μM of the inhibitor SB 203580 (+ SB 203580 in Fig. 3) leads to a clear reduction of Hsp27 phosphorylation both in heat shock- or PMA-treated cells leaving the pattern of isoforms almost unchanged compared to the control. Furthermore, long-time treatment of the cells with SB 203580 suppresses Hsp27 phosphorylation even below the basal level (24 h, 48 h in Fig. 3). Hence, under the experimental conditions chosen SB 203580 is an effective and specific inhibitor for Hsp27 phosphorylation in HL-60 cells during the entire time necessary for the PMA-induced differentiation process.

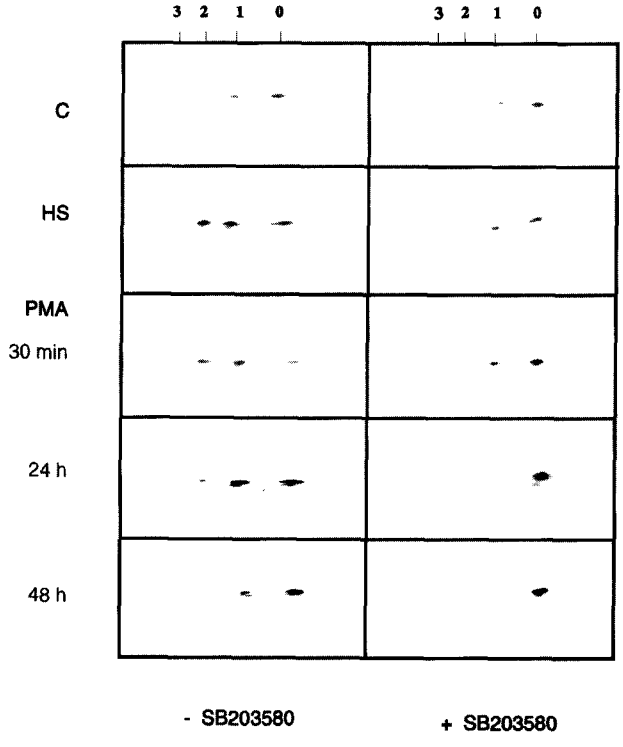

Fig. 3 PMA- and heat shock-induced phosphorylation of Hsp27 was inhibited by SB 203580. After 2 h of cultivation of HL-60 cells in the presence and absence of 10 μM SB 203580, the cells were stimulated with heat shock (44°C) for 20 min or with PMA (160 nM) for 30 min. In the case of PMA stimulation cells were washed after 4 h, further incubated in culture medium with or without 10 μM SB 203580 and Hsp27 phosphorylation was analyzed also after 24 h and 48 h. 10^6 cells were applied to two-dimensional gel electrophoresis and Western blot analysis using an Hsp25/27 antiserum. The numbers indicate the relative position of the non-phosphorylated (0), mono-phosphorylated (1), bis-phosphorylated (2) and triple-phosphorylated isoform (3) of Hsp27.

SB 203580 does not inhibit PMA-induced proliferation arrest of HL-60 cells

To prove whether Hsp27 phosphorylation is involved in the differentiation process of HL-60 cells as proposed (Spector et al 1993), we analyzed the influence of the p38RK inhibitor on the PMA-induced differentiation of HL-60 cells. For that reason we first looked at the PMA-induced proliferation arrest by determining the number of living cells after different times of stimulation of the cells with PMA in the presence and absence of SB 203580. As seen in Figure 4, the presence of SB 203580 in the medium does not significantly influence cell proliferation. PMA treatment leads to an arrest of cell proliferation representing the differentiation of the HL-60 cells. Interestingly, PMA can also induce this arrest in cell proliferation in the presence of SB 203580, where no increased MAPKAP kinase 2 activity (cf. Fig. 2) and Hsp27 phosphorylation (cf. Fig. 3) could be detected. This

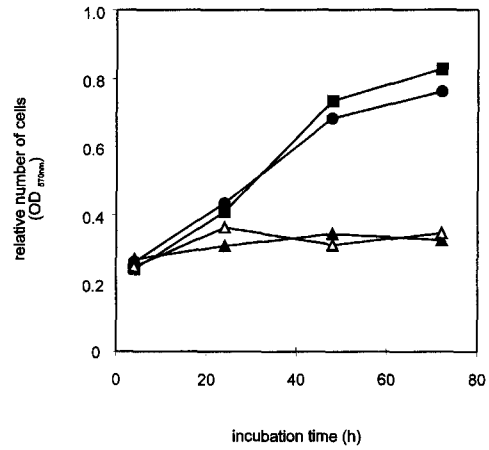

Fig. 4 PMA-induced growth arrest of HL-60 cells is not affected by SB 203580. HL-60 cells were preincubated with SB 203580 (10 μM) for 2 h and subsequently subjected to PMA treatment (160 nM). Four hours later the cells were washed and further incubated in culture medium with or without 10 μM SB 203580. After 4 h, 24 h, 48 h or 72 h the number of living cells was determined using a MTT-assay. Untreated control, ●; SB 203580-treated cells, ■; PMA-treated cells, ▲; SB 203580- and PMA-treated cells, Δ. Results are expressed as mean of three independent experiments (standard deviation < 0.052).

finding was rather unexpected and suggests that Hsp27 phosphorylation is correlated to differentiation but not a necessary intermediate for this process.

SB 203580 does not inhibit the PMA-induced expression of the cell surface marker CD11b and the development of the macrophage-like phenotype

To further prove the unexpected finding that Hsp27 phosphorylation is not necessary for HL-60 cell differentiation we examined the PMA-induced changes in expression of a differentiation specific cell surface marker as well as HL-60 cell morphology after PMA treatment in the presence and absence of SB 203580. As a surface marker we chose the β2–integrin CD11b, which is not expressed in promyelotic cells but is expressed during the monocyte stage of the macrophage-specific differentiation (Hickstein et al 1989). Using a specific antibody against CD11b and a fluorescence-labeled secondary antibody, cells expressing the surface antigen CD11b were detected by an increase in fluorescence using FACS-analysis. The shift of the fluorescence maximum to higher values in Figure 5A demonstrates that PMA-treated cells (PMA, –SB 203580) show an increased expression of CD11b compared to the control (C, –SB 203580). Cells treated with SB 203580 show a similar PMA-induced increase in CD11b surface marker expression (PMA, +SB 203580). This supports the notion that SB 203580 and, hence, Hsp27 phosphorylation does not influence the process of PMA-induced HL-60 cell differentiation.

Finally, we also examined the process of HL-60 cell differentiation to the macrophage-like phenotype by phase contrast microscopy (Fig. 5B). It is clearly seen that in the presence of the inhibitor SB 203580, PMA treatment led to an arrest in proliferation and a cellular morphology which is characteristic for the macrophage-like phenotype. In addition to the lower cell density of the PMA-treated cultures, the cells show the formation of granular or vacuole structures, the enlargement of the nuclei and an increased adherent growth. This finding further confirms that Hsp27 phosphorylation is not necessary for PMA-induced differentiation of HL-60 cells.

DISCUSSION

In this paper we investigated the signaling mechanism which underlies PMA-induced Hsp27 phosphorylation in HL-60 cells. We present evidence that PMA stimulates not only the classical MAP kinase cascade resulting in activation of ERKs but also a stress-induced cascade homologous to the yeast HOG1-pathway that leads to activation of the MAP kinase homologue p38RK. A similar result was recently obtained for PMA-stimulation of HeLa cells (Raingeaud et al 1995). Using SB 203580, a specific inhibitor for the p38RK, we achieved almost complete suppression of the heat shock- or PMA-induced activation of the p38RK downstream target MAPKAP kinase 2 indicating that SB 203580 effectively blocks p38RK in these cells. SB 203580 also inhibits the phosphorylation of the MAPKAP kinase 2 substrate Hsp27. Taking into account the high specificity of the inhibitor SB 203580 (Cuenda et al 1995), these data indicate that the PMA-induced phosphorylation of Hsp27 is probably the result of stimulation of the HOG1-homologous kinase cascade leading to subsequent phosphorylation/activation of p38RK, MAPKAP kinase 2 and Hsp27.

However, until now it has not been clear which upstream events lead to the PMA-induced activation of p38RK in HL-60 cells. A possible explanation could be PKC-dependent stimulation of the HOG1-homologous MAP kinase cascade upstream to p38RK, as shown for other MAP kinase cascades (Kolch et al 1993; Kharbanda et al 1994; Marquardt et al 1994). In analogy to these findings, a PKC-dependent stimulation of the p38RK activators MKK3 or MKK6 seems possible. On the other hand, it cannot be excluded that this activation is the result of cross-talk between the activated JNK/SAPK pathway (Kyriakis et al 1994) and the HOG1-homologous kinase cascade as described for MKK4/JNKK (Derijard et al 1995; Lin et al 1995) as an activator of both JNK and p38RK. A PKC-dependent stimulation of the JNK/SAPK pathway by PMA seems likely, since a jun-phosphorylating activity has been characterized that is stimulated by PMA in HL-60 cells but not in the PMA-resistant cell line HL-525 (Kharbanda et al 1994). A third possibility, which cannot be excluded, is the PKC-independent activation of the HOG1-homologous cascade by PMA via the presumed receptor(s)/sensor(s) directly linked to this cascade. However, the finding that down-regulation of PKC by prolonged PMA-treatment suppresses the induction of Hsp27 phosphorylation by PMA but not by heat shock (Welch 1985) supports the idea of the involvement of PKC in the case of PMA. To decide between these different possibilities it would be interesting to analyze PMA-induced activation of p38RK in the PMA-resistant cell line HL-525 to define the role of PKC-β in this process.

Very recently a second enzyme similar to MAPKAP kinase 2, designated MAPKAP kinase 3 or 3pK, has been described (McLaughlin et al 1996; Sithanandam et al 1996). This enzyme is also expressed in HL-60 cells (Sithanandam et al 1996), is activated by p38RK and can phosphorylate Hsp27 (McLaughlin et al 1996). However, since PMA-induced phosphorylation of Hsp27 is almost completely blocked by SB 203580, it seems likely that MAPKAP kinase 2 is the dominant kinase responsible for Hsp27 phosphorylation or that the way of activation of

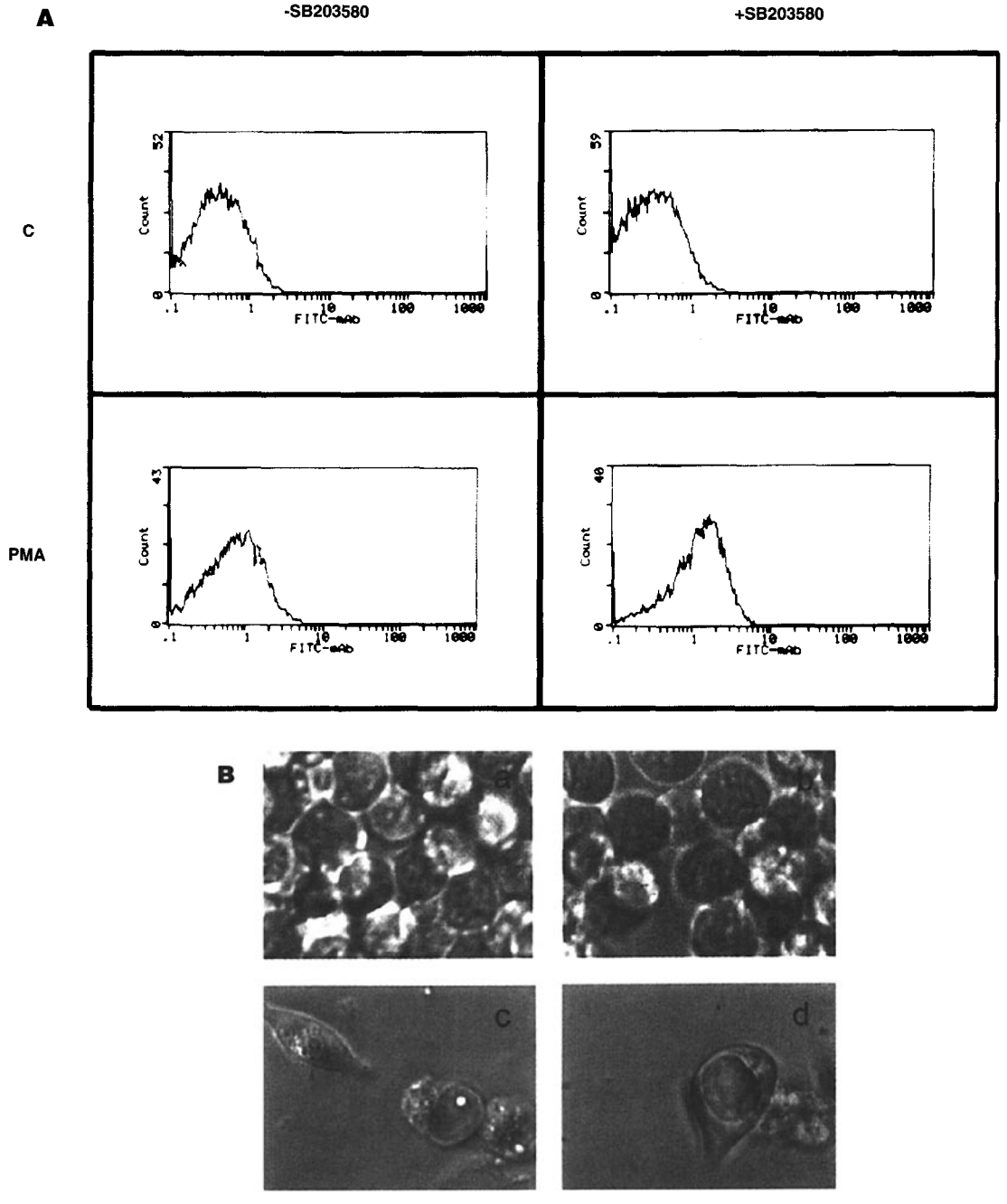

Fig. 5 SB 203580 does not inhibit PMA-induced differentiation of HL-60 cells. Cells were treated as described in Figure 4 followed by (A) FACS analysis of the differentiation surface antigen CD11b and (B) phase contrast microscopic analysis 72 h after induction by PMA. In (A) the number of cells is plotted against the logarithm of fluorescence intensity as a relative measure of surface marker expression. In (B) the sub-panels a, b, c and d correspond to untreated cells (C), cells treated with SB (C + SB 203580), cells stimulated with PMA (PMA) and cells stimulated with PMA after pre-incubation with SB 203580 (PMA + SB 203580), respectively.

MAPKAP kinase 2 and MAPKAP kinase 3/3pK is very similar in HL-60 cells. It is interesting to note that after a 2 h pre-incubation the inhibitor SB 203580 seems not to alter the basal phosphorylation of Hsp27. This could mean that the basal phosphorylation is due to another Hsp27-kinase in HL-60 cells, which is an element of an SB 203580-resistant pathway, or, more likely, that the basal Hsp27 phosphorylation is stable during the time of SB 203580 pretreatment. The latter explanation is supported by the finding that long-term treatment of

HL-60 cells by SB 203580 leads to almost exclusive accumulation of the non-phosphorylated isoform of Hsp27 (Fig. 3, 24 h and 48 h).

Although SB 203580 exhibits high specificity for p38RK, it is not necessarily specific for MAPKAP kinase 2 or Hsp27 since it could also lead to inhibition of phosphorylation of other targets of p38RK and/or MAPKAP kinase 2. Furthermore, direct side-effects of SB 203580 on other cellular targets could not be excluded. However, one would have to make the unlikely assumption that other targets could mimic the specific influence of Hsp27 phosphorylation on PMA-induced differentiation to explain the above results alternatively.

In summary, we demonstrate that HL-60 cells in which the phosphorylation of Hsp27 is effectively suppressed using SB 203580 could still be induced by PMA treatment to differentiate as judged from proliferation arrest, expression of the cell surface marker CD11b and morphological criteria. Hence, differentiation of HL-60 cells to the macrophage-like phenotype is independent of activation of the HOG1-homologous kinase cascade and Hsp27 phosphorylation. These data do not argue against the observation that PMA-induced differentiation leads to increased expression and rapid phosphorylation of Hsp27 and, therefore, that Hsp27 expression and phosphorylation is a unique marker of growth arrest during PMA-induced macrophage-like differentiation of HL-60 cells (Spector et al 1993). Indeed, in our experiments an increased expression of Hsp27 as a result of PMA-stimulation could also be detected. Therefore, an influence of the degree of expression of Hsp27 on the above differentiation process could not be excluded. However, the correlation between Hsp27 phosphorylation and differentiation of HL-60 cells seems not to hold for DMSO- or retinoic acid-induced differentiation (Minowada and Welch 1995) and, as shown in this paper, seems not to have any functional relevance for PMA-induced differentiation. Hence, the physiological role of Hsp27 phosphorylation in these cells is still an unresolved question.

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