Involvement of ERK1/2, p38 and PI3K in megakaryocytic differentiation of K562 cells

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ABSTRACT

Megakaryocytic differentiation of myelogenous leukemia cell lines induced by a number of chemical compounds mimics, in part, the physiological process that takes place in the bone marrow in response to a variety of stimuli. We have investigated the involvement of MAPKs (ERK1/2 and p38) and PI3K signaling pathways in the differentiated phenotypes of K562 cells promoted by phorbol 12-myristate 13-acetate (PMA), staurosporine (STA), and the p38 MAPK inhibitor SB202190. In our experimental conditions, only STA-treated cells showed the phenotype of mature megakaryocytes including GPIbα expression, DNA endoreduplication, and formation of platelet-like structures. We provide evidence supporting that basal activity, but not sustained activation, of ERK1/2 is required for expression of megakaryocyte surface markers. Moreover, ERK1/2 signaling is not involved in cell endomitosis. The PI3K pathway exerts dual regulatory effects on K562 cell differentiation: it is intimately connected with ERK1/2 cascade to stimulate expression of surface markers and it is also necessary, but not sufficient, for polyploidization. Finally, apoptosis and megakaryocytic differentiation exhibit different sensitivity to p38 down-regulation: it is required for expression of early specific markers but is not involved in cell apoptosis. The present work with K562 cells provides new insights into the molecular mechanisms regulating megakaryocyte differentiation. The results indicate that a precise orchestration of signals, including ERK1/2 and p38 MAPKs as well as PI3K pathway, is necessary for acquisition of features of mature megakaryocytes.

Key words: Megakaryocytic differentiation, K562, ERK1/2, p38, PI3K

Introduction

Megakaryopoiesis is a thrombopoietin (TPO)-regulated multistep process consisting of cellular proliferation and expression of specific differentiation markers in the early stages of megakaryocyte (MK) development, followed by polyploidization and acquisition of additional lineage-specific late markers (1,2). The αIIbβ3 integrin subunits represent early markers of MK differentiation, but components of the GPIb-IX-V complex appear later, at the same time than endomitotic cell cycles (3-5). To date, signaling pathways controlling initiation and progression of MK differentiation remain unclear.

Although the extracellular signal-regulated protein kinase (ERK1/2) pathway was initially related to mitogenic processes, it is also implicated in several models of differentiation (6). TPO stimulates ERK1/2 and p38 mitogen-activated protein kinase (p38 MAPK) pathways in primary cells and megakaryoblastic cell lines expressing the TPO receptor Mpl (7-9). Recently, ERK1/2 has been shown to play a critical role in TPO-induced differentiation of primary megakaryocytes derived from bone marrow and fetal liver (10). In addition, ERK1/2 activation has been shown to be either sufficient for (11,12) or independent of (13) megakaryocytic differentiation of leukemia cell lines by a variety of stimuli.

An important role for ERK in TPO-induced endomitosis was first reported in primary MKs and in a Mpl-expressing cell line (7). Then, the phosphoinositide 3-kinase (PI3K) pathway was suggested to play a role in G1-S transition during megakaryocyte proliferation (14). Moreover, PI3K activation was reported to be involved in ERK-dependent endomitosis induced by TPO (15). However, more recently, inhibition of TPO-induced ERK or PI3K activity has been shown to display opposite effects on ploidy of differentiating MKs (16).

Among human myelogenous cell lines, K562 has been extensively used as a model for the study of megakaryocytic differentiation. K562 cells behave as pluripotent precursors with marked phenotypic plasticity. Under basal conditions they express early markers for erythroid and MK lineages and can experience additional differentiation depending on the stimulus (17,18). It has been reported that PMA-induced differentiation along the MK lineage requires sustained activation of ERK1/2 (19) and, more recently, down-regulation of p38 has been suggested to be necessary for full differentiation of K562 cells by PMA (20).

In the present study, to gain insights into the molecular regulation of late MK differentiation, we analyzed the involvement of ERK1/2, p38, and PI3K activation in the distinct phenotypic features induced by PMA, staurosporine (STA) and the p38 inhibitor SB202192 in K562 cells.

Materials and methods

Materials

Phorbol 12-myristate-13-acetate (PMA) and the p38 specific inhibitor SB202190 were obtained from Sigma-Aldrich (Alcobendas, Spain). The MEK1 inhibitor PD98059 was from Cell Signaling, and staurosporine (STA) from Roche (Penzberg, Germany). Indomethacin, the PI3K inhibitor LY294002, and the MEK1/2 inhibitor U0126 were from Calbiochem-Novabiochem (Darmstadt, Germany).

Monoclonal antibodies (mAbs) specific for αIIb (2BC1) and β3 (H1AG11) were raised in our laboratory using as antigen αIIbβ3 heterodimer isolated from human platelets (21). mAb AK2 (anti-GPIbα) was obtained from Serotec (Oxford, UK), and the polyclonal antibodies (pAbs) against total or phosphorylated ERK1/2 and p38 MAPK were from Cell Signaling (Danvers, MA, USA).

Cell cultures and differentiation

The human leukemia K562 and MEG-01 cells were grown at 37ºC under 5% CO_2 in DMEM and RPMI media (Gibco BRL, Paisley, UK), respectively, supplemented with 10% FCS, 100 U/mL penicillin and 10 μg/mL streptomycine.

For differentiation experiments, $1.5x10^5$ cells were resuspended in 0.5 mL of medium supplemented with 1% serum and seeded in 12-well plates. After 16 h, when indicated, inhibitors PD98059 (20 μM), LY294002 (10 μM), indomethacin (10-50 μm), or U0126 (2.5 μM) were added and, after 2 h, cells were stimulated with PMA (2 nM), STA (20 nM), or SB202190 (10 μM) alone or combined as indicated and incubation continued for 3 days.

Flow cytometry

Expression of surface markers was determined using the mAbs 2BC1 (anti-αIIb), H1AG11 (anti-β3), and AK2 (anti-GPIbα). Cells ($2.5x10^5$/100 μL) were incubated with the specific mAb for 30 min, washed, and treated with a 1:500 dilution of Alexa Fluor 488-anti-mouse IgG (Molecular Probes, Eugene, Oregon, USA) for 20 min. Samples were analyzed in a Coulter flow cytometer model EPICS XL.

Cell viability was evaluated by flow cytometry after staining intact cells with 5 μg/mL propidium iodide (PI).

Apoptosis was determined measuring binding of annexin-V-FITC (Vibrant apoptosis assay kit, Molecular Probes) by flow cytometry.

For ploidy analysis, cells were washed and resuspended in PBS-0.5% Triton X-100. After 5 min, cells were pelleted by centrifugation, resuspended in PBS-0.5% Triton X-100 containing 25 μg/mL RNase and 25 μg/mL PI, incubated for 20 min and, then, analyzed by flow cytometry.

Immunoblot assays

Activation of ERK1/2 and p38 MAPKs was analyzed by western blotting. Cells were estimulated with the indicated inducer(s) in the absence or presence of PD98059 and/or LY294002 inhibitors and, after 2, 6, and 24 h, 100 μL aliquots were pelleted and solubilized in 2x Laemmli buffer containing 10% β-mercaptoethanol. Lysates were boiled, resolved on 10%-SDS-PAGE, and blotted with pAbs againts total or phosphorylated ERK1/2 and p38.

Cell transfections

Wild type (WT) p38α and dominant-negative (DN) p38α mutant, and DN and constitutively active (CA) MKK6 mutants cloned into expression plasmids were provided by Roger J. Davis (Uviversity of Massachusetts, Worcester, MA, USA).

K562 cells ($1.5x10^6$) were transiently transfected with DN mutant forms of p38α and MKK6 or with WT p38α and CA mutant form of MKK6, for down- and up-regulation of p38 activity, respectively, using the Amaxa nucleofection system (Koeln, Germany). 24-h after transfections, cells were resuspended in medium supplemented with 1% FCS and seeded in 12-well plates before inducing differentiation with PMA, STA or SB 202190.

Statistical analysis

All experiments were performed, at least, three times. The results are expressed as the means ± SE. Statistical significance was estimated by analysis of variance (ANOVA) followed by the Fischer's LSD test for multiple comparisons. Significance was defined as $p<0.05$.

Results

Differentiation phenotypes induced by PMA, STA, and SB202190 in K562 cells

Incubation of K562 cells with 2 nM PMA, 20 nM STA, or 10 μM SB202190 induced distinct differentiation phenotypes, summarized in Fig. 1. We previously determined the optimal concentrations promoting differentiation changes and causing minimal cell death. As previously reported, treatment with PMA and STA (22) resulted in cell growth arrest (data not shown). PMA enhanced surface expression of β3, and induced a morphology characterized by a net increase in cell size, the presence of vacuoles, and eccentrically located nuclei. Although this phenotype has been usually referred to as megakaryocytic (12,20,23), we agree with other authors in conferring monocyte/macrophage-like morphology to the majority of K562 cells treated with PMA (22). Cells treated with STA exhibited large size, emission of extensions resembling proplatelet structures, release of platelet-like particles, and increase of ploidy and expression of β3 and αIIb, as well as the late GPIbα megakaryocytic marker. Finally, cells incubated with SB202190 displayed increased surface expression of the three megakaryocytic markers without morphological changes. Thus, phenotypes induced by PMA, STA and SB202190 shared features like increase of β3 expression, but only cells treated with STA showed phenotypic characteristics that resemble complete megakaryocyte differentiation. As shown in Fig. 2, simultaneous treatment with STA and SB202190 had an additive effect on β3 expression; however, GPIbα expression (Fig. 2) and ploidy (not shown) were reduced. Moreover, cell treatment with PMA and either STA or SB202190 did not enhance β3.

In an attempt to gain some insight into the molecular regulation of megakaryocyte differentiation, we next considered of interest to relate the phenotype features of these *in vitro* models with the activation state of the main signaling pathways involved in the differentiation program.

Inhibition of ERK signaling cascade blocks K562 cell differentiation

It is currently admitted that ERK signaling pathway plays a role in megakaryocyte differentiation, and it has been suggested that sustained kinetics of ERK activation may be essential for this process (6,12,19). In our experimental conditions, treatment of K562 cells with PMA or STA caused an increase in ERK phosphorylation lasting more than 24 h, whereas SB202190 did not significantly modify the basal activation state of the kinase. Cells incubated with the MEK1 inhibitor PD98059 showed a reduction of ERK phosphorylation in response to PMA or STA stimulation (Fig. 3A). In addition, PD98059 markedly reduced the expression levels of megakaryocytic markers stimulated not only by PMA and STA, but also by SB202190 (Fig. 3B). Similar effects were found by using the MEK1/2 inhibitor U-0126 (Fig. 3C). Incubation with indomethacin did not modify β3 expression in K562 cells (Fig. 3C), ruling out the possibility that the effect of PD98059 was mediated by inhibition of cyclooxygenase activity (24). The results support a major role of ERK activity in differentiation of K562 cells. However, the effect of SB202190 suggests that sustained activation of ERK is not an essential requirement for expression of surface markers.

A dual regulatory role of the PI3K pathway in K562 cell differentiation

K562 cells were incubated with the differentiation inducers in the absence or presence of the specific pharmacological PI3K inhibitor LY294002. Fig. 3B shows that LY294002 reduced the expression of β3, αIIb, and GPIbα, although to a lesser extent than the ERK inhibitor PD98059. The simultaneous addition of both inhibitors resulted in a higher decrease of the surface marker levels. In view of the reported PI3K-dependent activation of ERK in some cell systems (25-27), we next explored whether the inhibitory effect of

LY294002 was mediated by down-regulation of the ERK cascade. Results in Fig. 4A demonstrate that ERK phosphorylation induced by both PMA and STA is partially blocked in cells incubated in the presence of LY294002, indicating that the PI3K pathway may regulate differentiation of K562 cells, at least in part, through ERK activation.

As the PI3K pathway has been suggested to play a role in G1-S transition during megakaryocyte proliferation (14), we analyzed its participation in STA-induced endomitosis of K562. As shown in Fig. 4B, cells incubated with LY294002 showed a marked reduction of the polyploidization induced by STA. Nevertheless, since PI3K is also activated during differentiation induced by PMA and SB202190, the results suggest that signaling through PI3K is necessary but not sufficient for endomitosis. Cell DNA content was not modified by the concentration of PD98059 found to drastically block the expression of surface markers (Fig. 4B).

Megakaryocytic differentiation and apoptosis of K562 cells are processes with different sensitivity to p38 MAPK inhibition

Exposure of K562 cells to SB202190, a highly specific inhibitor of p38 MAPK (28), induced the expression of both early and late megakaryocyte markers (Fig. 1A). To investigate the role of this signaling pathway in K562 cell differentiation, p38 phosphorylation was manipulated by expressing a combination of mutant forms of MKK6 and p38 kinases. Downregulation of p38 activity by transient transfection of dominant-negative (DN) mutants did not induce β3 expression in untreated cells, but significantly improved expression in PMA- and STA-stimulated cells (Fig. 5A). Conversely, cells overexpressing p38α and a constitutively active (CA) mutant of MKK6 showed reduced β3 expression in response to PMA and STA. SB202190-induced β3 expression was not affected by transfection of either DN or CA mutants, indicating that in these experimental conditions p38 activity is completely blocked by SB202190. In agreement with the lack of additional effects of STA and SB202192 on GPIbα expression (Fig. 2), downregulation of p38 activity by transfecting DN mutants did not increase expression of this late marker (not shown). The results suggest that downregulation of p38 phosphorylation improves expression of megakaryocytic markers, but is not sufficient for the cell to enter into the differentiation program.

PMA and STA stimulate p38 phosphorylation in K562 cells (Fig. 5B). While the effect of STA lasts for few hours, p38 phosphorylation by PMA is maintained for more than 24 hours. SB202190 acts blocking p38 activity and, therefore, p38 autophosphorylation, but it does not affect p38 phosphorylation by other kinases (28); thus, the inhibitory effect of SB202190 on PMA-induced p38 phosphorylation (Fig. 5C) might indicate that PMA activates an alternative pathway leading to p38 autophosphorylation, as described in other cell models (29). Consistent with this, STA- but not PMA-induced p38 phosphorylation was blocked in cells expressing DN MKK6 (Fig. 5B).

p38 activation has been reported to modulate both cell survival and apoptosis (30). To gain some insight into the role of p38 in these processes, we determined β3 expression and annexin-V binding in cells transfected with DN or CA forms of p38 and MKK6 kinases (Fig. 6). In our experimental conditions, K562 differentiation is accompanied by a variable degree of cell death, but propidium iodide (PI)-permeable cells do not express more β3 than viable cells, indicating that cell death is not necessarily associated to terminal differentiation. The percentage of apoptotic cells in PMA- and STA-differentiated cultures was markedly higher than in untreated or SB202190-treated cells. p38 activation by transfection of CA mutants increased the number of apoptotic cells in untreated as well as SB202190-treated cultures, but β3 expression remained unchanged. In contrast, although the state of p38 activation has not a significant effect on cell apoptosis induced by STA or PMA, it seems to

control the final cell fate: necrotic death when p38 is constitutively active and increase of surface markers when its activity is down-regulated (Fig. 6).

Effect of PMA, STA and SB202190 in MEG-01 cell differentiation

In a series of experiments, we compared the expression of β3 and GPIbα in K562 cells with that in human megakaryoblastic leukemia cell line MEG-01, which has also been widely used as a model of megakaryocyte differentiation (31). Consistent with their more differentiated state, MEG-01 cells exhibited higher basal expression of β3 than K562 cells (Fig. 7A), and STA and PMA produced only a 2- to 3-fold increase in β3 expression, in contrast to the 10- to 30-fold increase obtained in K562 cells. In MEG-01 cells, the highest β3 expression levels were obtained with SB202190, which promoted a similar 4- to 5-fold increase in both cell types. Similarly to β3, basal GPIbα expression was higher than in K562 cells, but relative increases induced by STA and PMA were minor. PD98059 blocked the stimulatory effect of SB202190 but inhibition of STA- or PMA-induced ERK activation (not shown) was not accompanied by a significant reduction of MK markers (Fig. 7A).

In consonance with these results, experiments shown in Fig. 7B indicate that down-regulation of ERK phosphorylation later than 24 h after stimulation of K562 cells with STA or PMA did not blocked expression of megakaryocyte markers. In contrast, the effect of SB202190 was inhibited by PD98059 at any time during the course of differentiation.

Discussion

In order to gain insights into the molecular mechanisms controlling terminal megakaryocyte (MK) differentiation, we correlated the phenotypic properties of K562 cells induced by several differentiating agents with the activation of signaling pathways involved in MK differentiation. In our experimental conditions, PMA, STA and SB202190 induced distinct phenotypes sharing increased expression of the β3 MK marker. The highest β3 expression was obtained with PMA, but αIIb and the late MK marker GPIbα were undetectable in PMA-differentiated cells. Expression of αIIb in K562 cells treated with PMA is a matter of controversial. Although some authors have reported its detection (1,19,23), our results are in line with the originally described uncoupled expression of β3 and αIIb in these cells (32,33). Thus, although the phenotype induced by PMA in K562 cells has traditionally been referred to as megakaryocytic, we agree with Lerga A et al. (22) that most of the PMA-differentiated cells resemble the monocyte-macrophage lineage with some features of immature MKs. The ability of STA to induce some MK markers in leukemia cells has been previously reported (22,34,35). In our study, STA-treated K562 cells showed the phenotype of mature MKs including GPIbα expression, DNA endoreduplication, and formation of platelet-like structures. Finally, we report for the first time that a high percent of K562 cells treated with the p38 inhibitor SB202192 display surface expression of MK markers, but not changes in cell morphology and ploidy.

PMA and STA are, respectively, activator and inhibitor of PKC activity. According to a recent report, differentiation and ERK1/2 phosphorylation induced by PMA in K562 cells are mediated by new PKC isoforms (20). However, the MK phenotype induced by STA is not due to PKC down-regulation, since neither the expression pattern nor the phosphorylation status of ERK1/2 were modified by other PKC inhibitors such as H-7 and the STA-related BIM-1 and RO-31-8220 (results not shown). Although induction of MK markers by PMA and STA is, apparently, dependent on sustained ERK1/2 phosphorylation, experiments with SB202190 suggest that sustained activation of ERK1/2 is not obligatory for surface expression of MK markers. These results aggree with a previous report showing that differentiation of myeloid leukemia cells is independent of ERK1/2 activation (13). However, in the present study, the blocking effect of PD98059 in SB202190-treated cells suggests that a minimal or basal ERK1/2 activity is required. In the more differentiated MEG-01 cell line, induction of MK markers by PMA and STA was proportionally lower and did not show the stringent ERK-dependency observed in K562 cells. Consistent with this, SB202190, which does not activate ERK, induced the highest β3 expression in these cells. Moreover, inhibition of ERK failed to block expression of MK markers in K562 cells when added 24 h after stimulation with STA or PMA. Altogether, these findings suggest that ERK activity is essential to initiate MK differentiation but, once cells enter the differentiation program, signaling through this pathway is not critical.

The decrease of MK markers induced by the PI3K inhibitor LY294002 is consistent with a previous report showing activation of PI3K in TPO-stimulated cells expressing the c-mpl receptor (36). We show that the effect of LY294002 in STA- and PMA-treated cells is, at least in part, mediated by down-regulation of ERK activation, suggesting that PI3-kinase and ERK signaling cascades are intimately connected in modulating K562 cell differentiation. Relation between these pathways has been reported in other experimental models (25-27). In addition, we show that increased cell size and ploidy induced by STA are also partially dependent on PI3K, but not ERK1/2, activity. Since the PI3K pathway is also activated in the differentiation processes triggered by PMA and SB202190, we may conclude that activation of this signaling pathway is necessary but not sufficient for endomitosis. As an alternative explanation, as reported for TPO stimulation (37), it is

possible that signals generated by PI3K activation to induce endomitosis could be only sensed by STA-differentiated cells.

Recently, it has been proposed that inhibition of p38 MAPK plays an important role on MK differentiation (20). In the present study, results from experiments with SB202190 suggested that inhibition of p38 activity might be enough to initiate MK differentiation; however, nucleofection assays showed that inhibition of this kinase is not sufficient to induce MK markers. Thus, the effect of SB202190 on K562 cell differentiation cannot be only a consequence of p38 inhibition. In contrast to SB202190, STA and PMA phosphorylated p38 MAPK. The distinct p38 kinetics induced by PMA and STA may be decisive in the progress and final phenotype of the differentiation program triggered by the sustained ERK1/2 phosphorylation. Sustained up-regulation of p38 phosphorylation in transfection experiments significantly blocked β3 and GPIbα expression induced by STA or PMA. Although blocking STA- or PMA-induced p38α activation by transfection of dominant-negative (DN) forms of MKK6 and p38 was accompanied by enhanced expression of β3, expression of GPIbα in STA-treated cells did not change. So, down-regulation of p38 activity may be important to stimulate expression of early, but not late, MK markers. Inhibiting p38 with SB202190 also increased β3 in STA- but not in PMA-differentiated cells, which may be due to the considerable increase of necrotic cell death in cultures simultaneously treated with PMA and the p38 inhibitor (Fig. 2, lower panel), as previously reported (20). Similarly to STA, TPO induces transient activation of p38 in leukemia cells (9). In addition, increasing evidence supports a critical contribution of p38 in the negative regulation of cell cycle progression (38,39). Considering all these findings, p38 signaling may have an important role in regulating megakaryocytopoiesis. Our results are consistent with a model in which transient p38 activation may induce cell cycle arrest, triggering a differentiation program in which subsequent p38 down-regulation is required for acquisition of MK features.

It is unclear whether apoptotic cell death during leukemia cell differentiation occurs spontaneously or is a differentiation-dependent process coupled to cell maturation (40). Caspase-dependent (41) or -independent (42) apoptosis has been also considered as the final physiological fate of mature MK. In our study, STA and PMA treatments resulted in a number of necrotic cells and an increased fraction of apoptotic cells. However, we showed that β3 and GPIbα expression was similar in differentiated cells regardless of their viability degree; therefore, apoptosis and cell death do not appear to be a direct consequence of terminal cell differentiation. p38 signaling can promote apoptosis (43,44) as well as cell survival (45) and differentiation (46), and it has been suggested that its regulatory role depends on cell type and conditions. In the present report, neither transfection of DN p38α and MKK6 mutants nor inhibition of p38 MAPK activity with SB202190 reduced apoptosis in response to STA or PMA stimulation. Thus, though p38 activation in differentiating K562 cells may negatively contribute to proliferation (39), it does not appear to be involved in cell apoptosis, suggesting that differentiation and apoptosis are simultaneously induced through parallel but distinct pathways. In agreement with these results, apoptosis and erythroid differentiation have been reported to exhibit different sensitivity to p38 inhibition (47).

In summary, we provide evidence supporting that MK differentiation requires a precise orchestration of signals including ERK1/2 and p38 MAPKs as well as PI3K pathways. Basal activity, but not sustained activation, of ERK1/2 is required for expression of MK surface markers. In contrast, ERK1/2 signaling is not involved in cell endomitosis. The PI3K pathway exerts dual regulatory effects on K562 cell differentiation: it feeds into the ERK1/2 cascade to stimulate expression of MK markers and, also, it is necessary for ploidy. Finally, down-regulation of p38 is required for expression of early MK markers but is not involved in cell apoptosis.

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The authors state that they have no conflict of interest.

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Figure legends

Figure 1. Differentiating effects of PMA, staurosporine (STA) and SB202190 (SB) on K562 cells. K562 cells were treated with 2 nM PMA, 20 nM STA, or 10 μM SB202190 for 3 days as described in "Materials and methods". (A) Flow cytometry analysis of β3, αIIb and GPIbα surface expression. Numbers indicate the values of fluorescence calculated as a product of the percent of gated positive cells and the value of the mean channel of fluorescence intensity. (B) Morphology of K562 cells untreated or treated for 3 days with the indicated inducer. Objective: 10x. (C) Flow cytometric analysis of DNA content. Numbers represent the percent of cells with DNA content $\geq$ 2N. (D) Platelet-like particle formation by K562 cells. Bars represent the percent of β3- or GPIbα-positive particles referred to the total number of events. All numerical results are means $\pm$ SD of, at least, three independent experiments. Data from the same representative experiment are shown in panels A, B and C.

Figure 2. Surface marker expression and cell death induced by combinations of PMA, staurosporine (STA) and SB202190 (SB) on K562 cells. Cells were treated with the indicated combinations of PMA (2 nM), STA (20 nM) and SB202190 (10 μM) for three days as described in "Materials and methods". Expression of β3 and GPIbα was evaluated by flow cytometry. The values of fluorescence were calculated as products of the percent of gated positive cells and the value of the mean channel of fluorescence intensity. In the lower panel, death cells were detected by measuring PI-cell permeabilization by flow cytometry, and bars represent the percentage of gated positive cells. All values are means $\pm$ SD of three independent experiments. * $p<0.05$ significantly different from cells treated with STA alone.

Figure 3. Effect of ERK1/2 and PI3K inhibitors on K562 cell differentiation induced by PMA, staurosporine (STA) and SB202190 (SB). In experiments represented in panels A and B, cells were treated with the MEK1 inhibitor PD98059 (PD) (20 μM) and/or the PI3K inhibitor LY294002 (LY) (10 μM) for 2 h before addition of differentiating agents. (A) Western analysis of phospho-ERK1/2 was carried out 2-, 6- and 24-h after stimulation with PMA (2 nM), STA (20 nM) and SB202190 (10 μM). Control of protein loading was performed by western analysis of total ERK1/2. The figure shows a representative blot of at least three independent experiments. (B) Flow cytometry analysis of β3, αIIb and GPIbα surface expression. The values of fluorescence are calculated as products of the percent of positive cells and the value of the mean channel of fluorescence intensity. Results are means $\pm$ SD of three experiments. * $p<0.05$, § $p>0.01$, significantly different from their respective control cells incubated without inhibitor. In cells incubated with STA+LY/PD, * $p<0.05$, † $p<0.02$, § $p<0.01$, significantly different from cells incubated with STA+LY. (C) Cells were incubated with PD98059 (PD) (20 μM), the cyclooxigenase inhibitor indomethacin (Indo) (10 μM), or the MEK1/2 inhibitor U0126 (2.5 μM) for 16 h before addition of differentiating agents. Flow cytometry analysis of β3 was performed after 3 day-incubation. Results are means $\pm$ SD of three experiments.

Figure 4. Effect of PI3K inhibitor on ERK1/2 phosphorylation and cell ploidy. Cells were treated with the MEK1 inhibitor PD98059 (PD) (20 μM) or the PI3K inhibitor LY294002 (LY) (10 μM) for 2 h before addition of the differentiating agents. (A) Western analysis of phospho-ERK1/2 was carried out 2-, 6- and 24-h after stimulation with PMA (2 nM) or STA (20 nM). Control of protein loading was performed by western analysis of total ERK1/2. The figure shows a representative blot of at least three independent experiments.

(B) DNA content was determined by flow cytometry as described in "Materials and methods". The figure shows a representative experiment and numbers represent the percent of cells with DNA content $\geq$ 2N. Results are means $\pm$ SD of, at least, three independent experiments.

Figure 5. p38 activation modulates expression of β3 induced by PMA and staurosporine (STA). K562 cells were transfected with void vector (Mock), with dominant negative variants of both p38α and MKK6 (DN), or with wild type p38α plus constitutively active MKK6 mutants (CA) as described in "Materials and methods" and, 24 h after transfection, PMA (2 nM), STA (20 nM), or SB202190 (SB) (10 μM) were added. (A) β3 surface expression was measured by flow cytometry after 3-day incubation. The values are calculated as products of the percent of positive cells and the value of the mean channel of fluorescence intensity, and expressed as fold increase over expression in control cells (untreated cells transfected with void plasmids). Results are means $\pm$ SD of three experiments. * $p<0.05$ significantly different from their respective mock transfected cells. (B) p38 phosphorylation induced by PMA is not blocked by down-regulation of MKK6-dependent activation of p38. Western analysis of phospho-p38 in non-transfected cells (Mock) and cells transfected with DN variants of p38α and MKK6 (DN) was carried out 2-, 6- and 24-h after stimulation with the differentiating inducers. Control of protein loading was performed by western analysis of total p38. (C) p38 phosphorylation induced by PMA is partially blocked by the p38 inhibitor SB202190. SB202190 (10 μM) was added 2 h before addition of STA and PMA to non-transfected cells. Blots are representative of at least three experiments.

Figure 6. p38 differently regulates apoptosis and differentiation of K562 cells. K562 cells were transfected as previously described and, 24 h after transfection, differentiation was induced for 3 days with PMA (2 nM), STA (20 nM) or SB202190 (SB) (10 μM). β3 surface expression, annexin-V-FITC binding and propidium iodide (PI) staining were measured by flow cytometry. β3 expression values are calculated as products of the percent of positive cells and the value of the mean channel of fluorescence intensity, and referred to total cell population or to cells non-permeable to PI (viable cell population). Results are means $\pm$ SD of three experiments. * $p<0.05$, ** $p<0.02$.

Figure 7. Activation of ERK1/2 by PMA and STA is important in early, but not late, differentiation stages. (A) K562 and MEG-01 cells were first treated with PD98059 (PD) (20 μM) and, then, incubated with PMA (2 nM), STA (20 nM) or SB202190 (SB) (10 μM) for 3 days. Results are means $\pm$ SD of four experiments.* $p<0.05$ significantly different from cells incubated without PD. (B) Time-dependency of the effect of ERK1/2 inhibition on β3 expression. PD98059 (PD) (20μM) was added one day before (-1), at the same time (0), or 1, 2 or 3 days after addition of differentiation inducers, and expression of β3 and GPIbα was determined by flow cytometry. Unfilled bars correspond to cells incubated without PD. Results are means $\pm$ SD of three experiments.

Figure 1

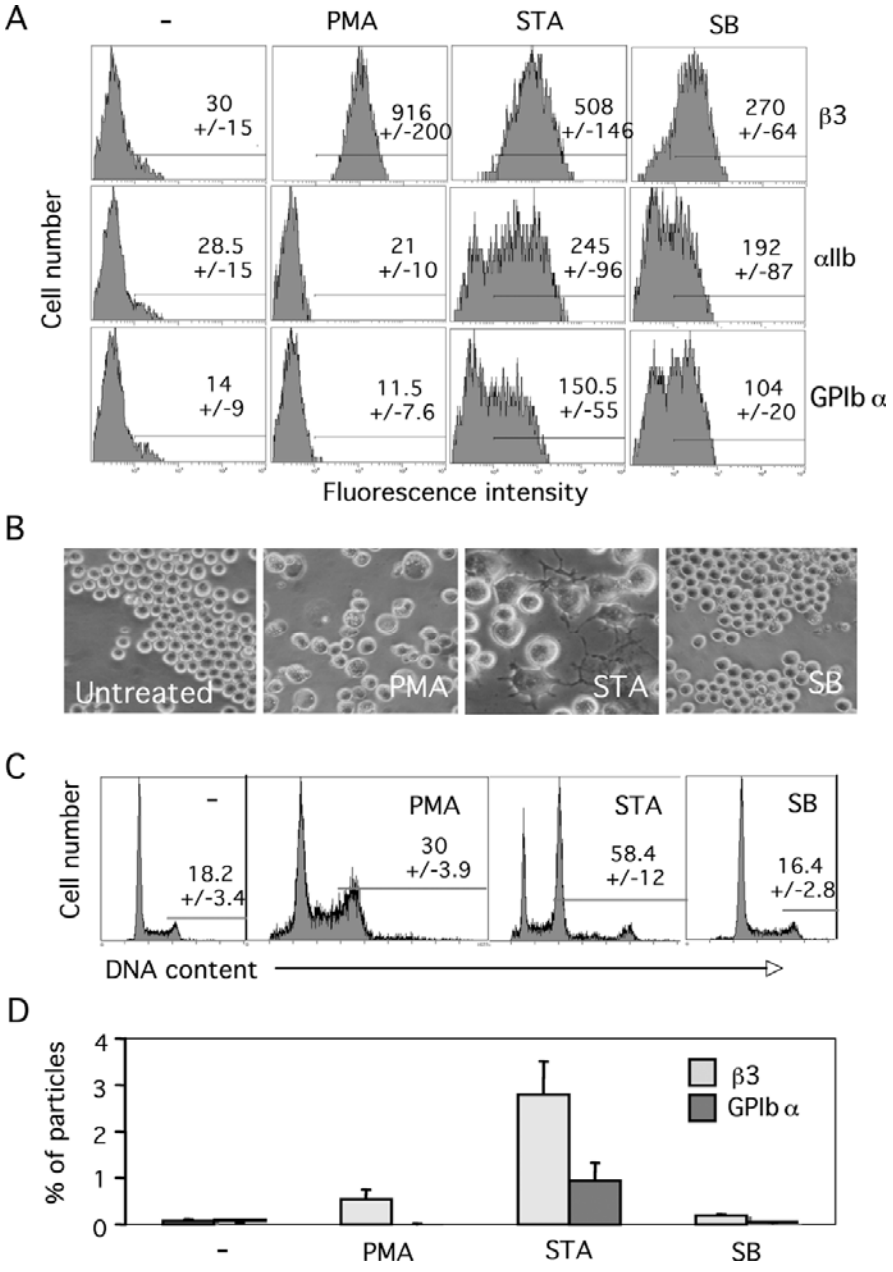

A
-
PMA
STA
SB
30 +/-15
916 +/-200
508 +/-146
270 +/-64
β3
28.5 +/-15
21 +/-10
245 +/-96
192 +/-87
αIIb
14 +/-9
11.5 +/-7.6
150.5 +/-55
104 +/-20
GPIb α
Cell number
Fluorescence intensity
B
Untreated
PMA
STA
SB
C
-
PMA
STA
SB
18.2 +/-3.4
30 +/-3.9
58.4 +/-12
16.4 +/-2.8
Cell number
DNA content
D
% of particles
0
1
2
3
4
β3
GPIb α
-
PMA
STA
SB

Figure 2

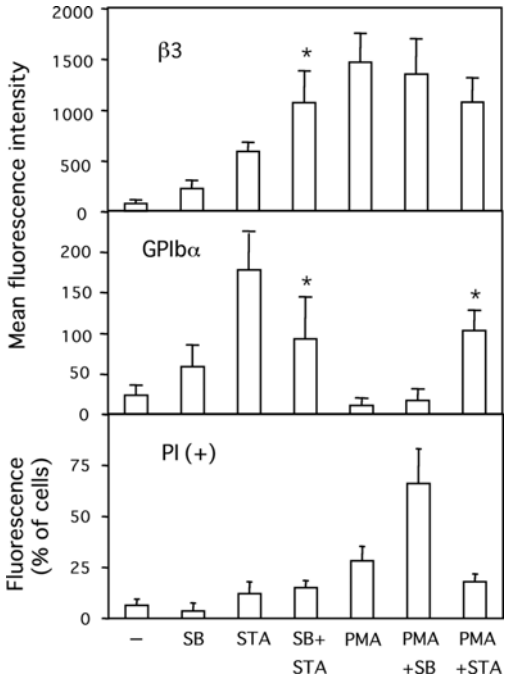


Figure 3

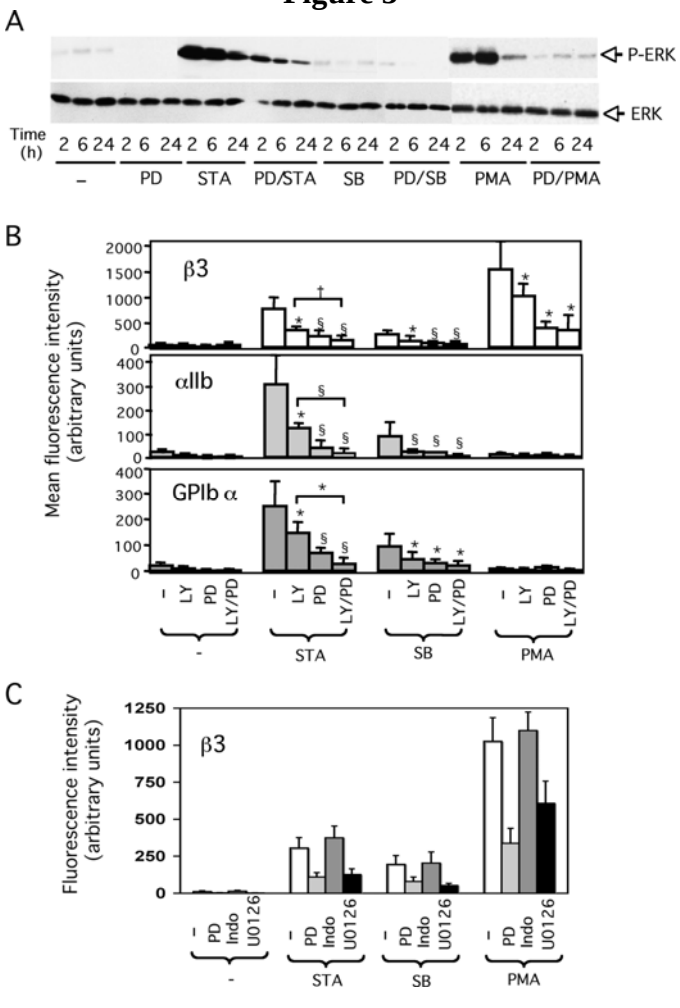


Figure 4

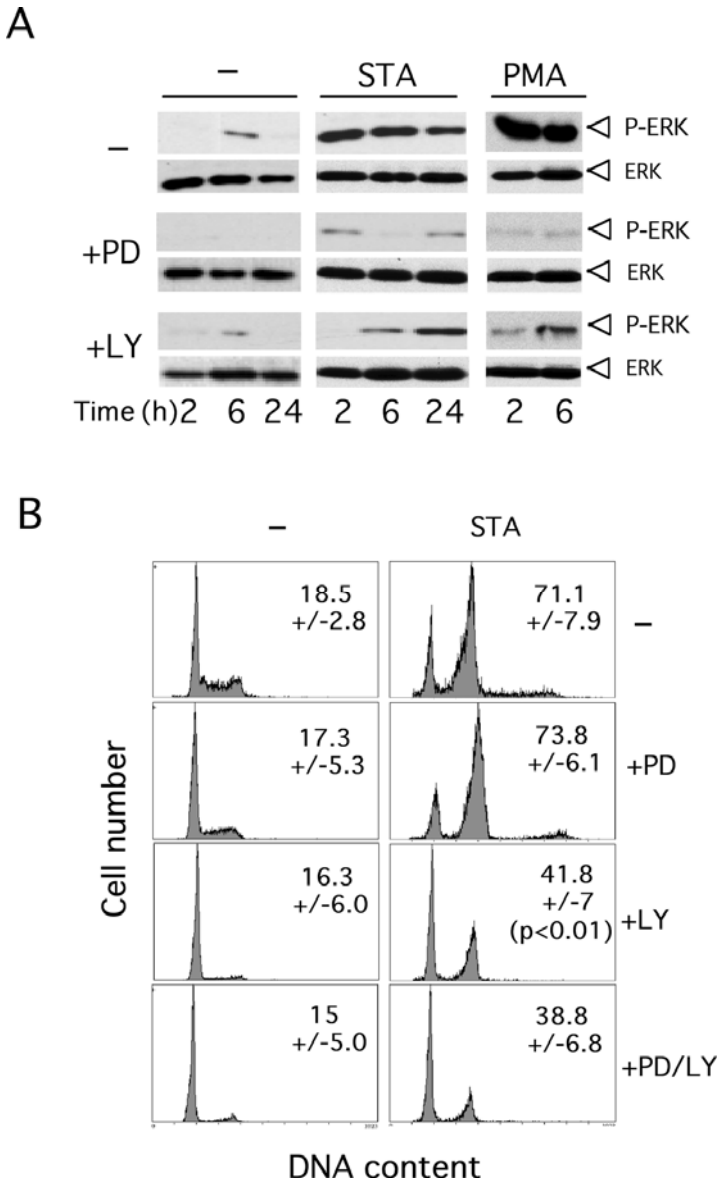
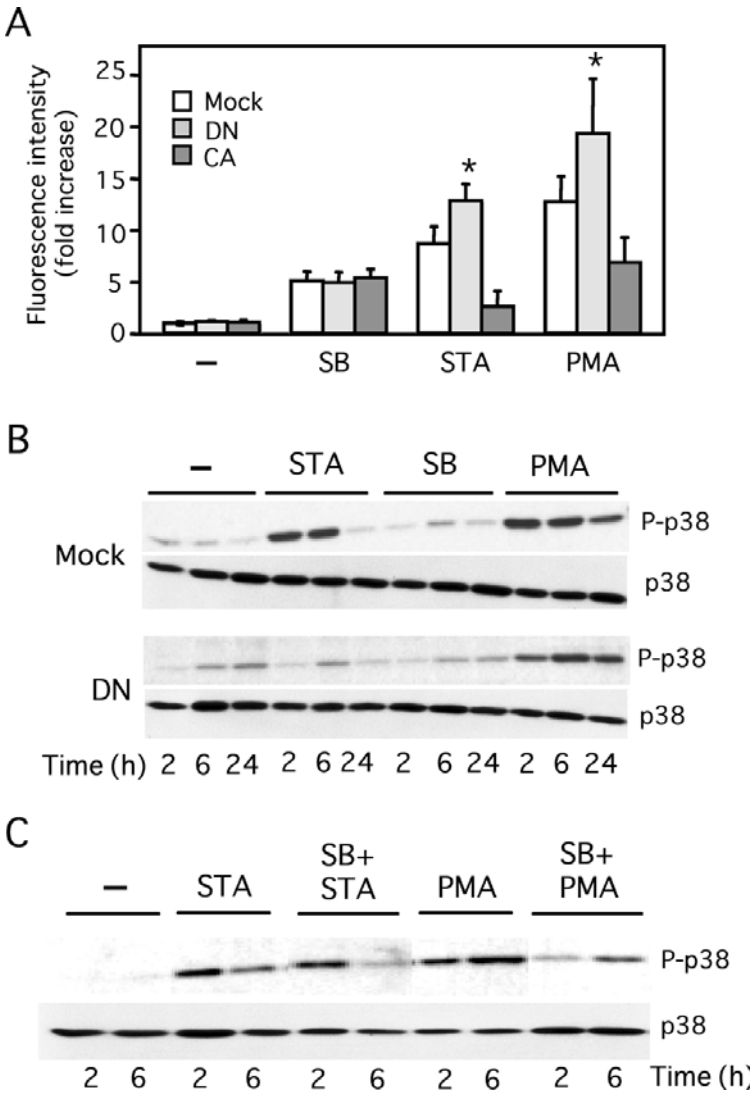

A
–
STA
PMA
–
+PD
+LY
P-ERK
ERK
Time (h)2 6 24 2 6 24 2 6
B
–
STA
18.5 +/-2.8
71.1 +/-7.9
17.3 +/-5.3
73.8 +/-6.1
16.3 +/-6.0
41.8 +/-7 (p<0.01)
15 +/-5.0
38.8 +/-6.8
+PD
+LY
+PD/LY
Cell number
DNA content

Figure 5


A
Fluorescence intensity (fold increase)
25
20
15
10
5
0
Mock
DN
CA
*
*
–
SB
STA
PMA
B
–
STA
SB
PMA
Mock
P-p38
p38
DN
P-p38
p38
Time (h) 2 6 24 2 6 24 2 6 24 2 6 24
C
–
STA
SB+ STA
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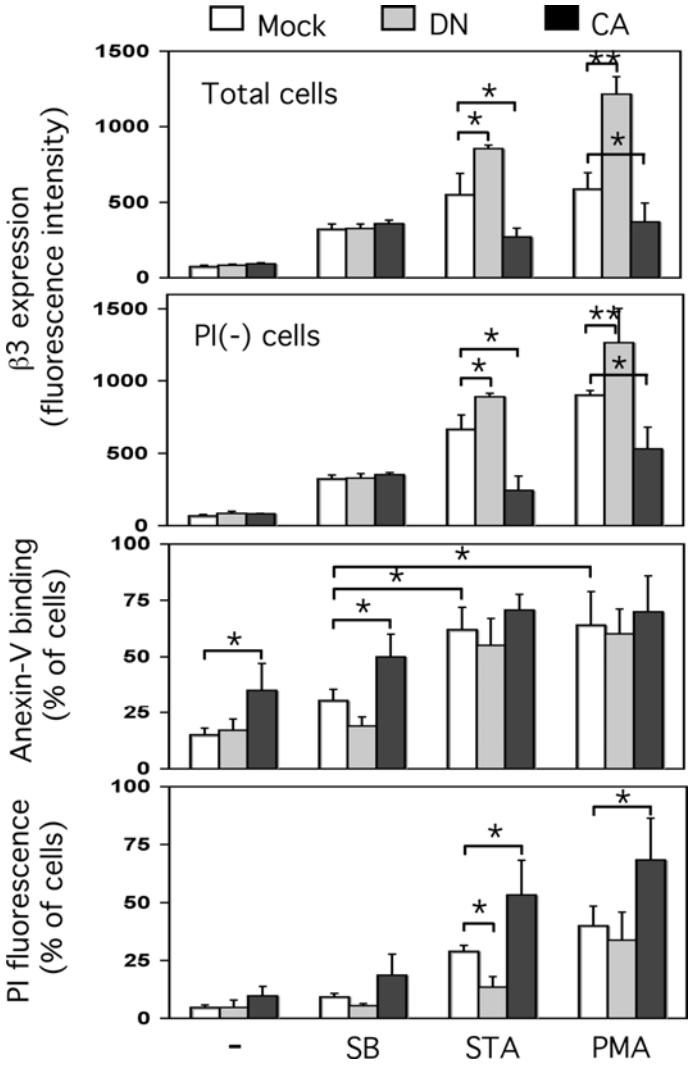
P-p38
p38
2 6 2 6 2 6 2 6 2 6 Time (h)

Figure 6


Mock
DN
CA
Total cells
PI(-) cells
β3 expression (fluorescence intensity)
Anexin-V binding (% of cells)
PI fluorescence (% of cells)
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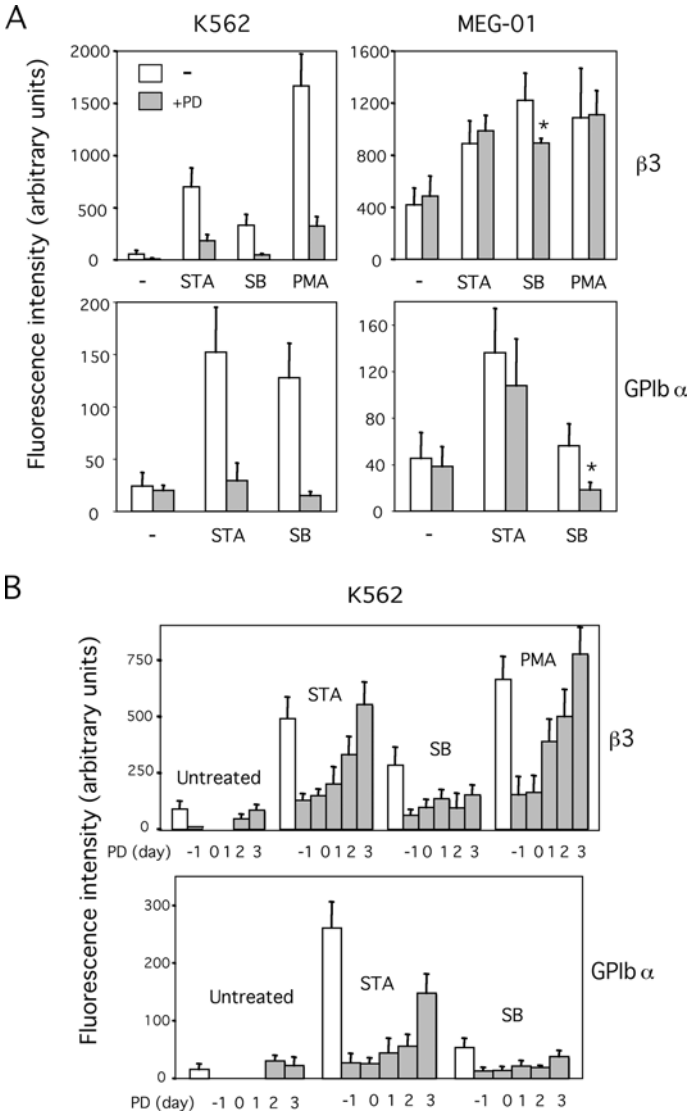
1000
500
0
100
75
50
25
0
-
SB
STA
PMA

Figure 7


A
K562
MEG-01
Fluorescence intensity (arbitrary units)
-
+PD
β3
GPIb α
STA
SB
PMA
B
K562
Untreated
PD (day) -1 0 1 2 3