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p130Cas acts as survival factor during PMA-induced apoptosis in HL-60 promyelocytic leukemia cells

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Abstract

Phorbol 12-myristate 13-acetate (PMA) stimulates the differentiation of promyelocytic leukemia HL-60 cells by inducing adhesion followed by cell aggregation and, importantly, apoptosis. p130Cas (Crk-associated substrate) is an adapter molecule that controls cell growth, attachment and apoptotic programs. Notably, elevated p130Cas activity is associated with leukemias and lymphomas. Since p130Cas regulates cell adhesion, we tested the hypothesis that it participates in the differentiation of hematopoietic cells. Here we show that PMA mediates the late induction of p130Cas expression in HL-60 cells, which coincided with cell aggregation and the onset of apoptosis. Ectopic p130Cas expression led to increased cell adhesion and earlier cell aggregation potentially contributing to the observed increased cell viability in these transductants. p130Cas expression concurred with the induction of its own regulator the transcription factor EGR1, its coregulator NAB2, and apoptosis. NF- κ B inhibition in PMA-treated HL-60 cells promoted the loss of cell aggregation and cell death. We further showed a reduction of p130Cas, EGR1, and NAB2 levels in response to NF- κ B inhibition during PMA treatment. Hence, p130Cas acts as survival factor by limiting PMA-mediated cell cluster disruption and resulting cell death in HL-60 cells.

Keywords

p130Cas family; HL-60; leukemia; differentiation; EGR1; apoptosis

1. Introduction

Diverse compounds can induce the differentiation of normal and tumoral promyelocytic cells leading to the maturation of these cells (Tsiftoglou et al., 2003). Differentiation therapy approaches showed high efficacy in clinical trials (Castaigne et al., 1990; Leszczyniecka et al., 2001) and were shown to increase the sensitivity to chemotherapeutics by enhanced apoptosis (Depraetere et al., 1995; Nakaya et al., 1990). However, recurrence of the disease mediated by persistent leukemia stem cells and/or acquired resistance are still major obstacles to therapy (Buss and Ho, 2011). Human promyelocytic leukemia HL-60 cells are a widely used model system to study various aspects of leukemia cell differentiation and apoptosis in malignancies with promyelocytic characteristics (Hansson et al., 2005; Bertagnolo et al., 2011). HL-60 cells differentiate upon treatment with phorbol 12-

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myristate 13-acetate (PMA¹) into monocytes/macrophages accompanied by increases in cell adhesion and aggregation (Zheng et al., 2002; Kwon et al., 2006). During this differentiation process apoptosis is induced in a substantial fraction of these cells (Hansson et al., 2005).

The Cas family members p130Cas/*BCAR1* (Crk-associated substrate/breast cancer antiestrogen resistance 1) and HEF1/*NEDD9* (human enhancer of filamentation/neural precursor cell expressed, developmentally downregulated 9) play crucial roles in cell growth, attachment and migration, and apoptotic programs (reviewed in Tikhmyanova et al., 2010). In response to stimuli such as growth factors, mimicked in part by PMA, or integrin engagement, proteins of this family function as scaffolds and integrate large multi-protein complexes (Tikhmyanova et al., 2010; Bouton et al., 2001; Cabodi et al., 2010). Changes in function and expression of p130Cas and HEF1 are associated with leukemias and lymphomas (Tikhmyanova et al., 2010). To decipher molecular mechanisms leading to PMA-mediated differentiation, adhesion/aggregation, and apoptosis of leukemia cells, the roles of these Cas family members were investigated. Here we report that p130Cas acts as a survival factor during PMA-induced apoptosis in HL-60 cells by promoting/maintaining cell aggregation leading to enhanced cell viability.

2. Materials and Methods

Cell Lines and culture/treatment conditions

Human promyelocytic leukemia HL-60 cells were obtained from Matthew D. Layne (Boston University School of Medicine). The cells were grown in phenol-red free RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained at 37°C and 5% CO₂ and tested for mycoplasma contamination.

Differentiation of HL-60 cells was induced after 4 h of plating by the addition of PMA (Sigma, 16 nM) for indicated times. For Bay 11-7082 (Sigma) studies the cells were pretreated for 30 min prior to PMA treatment with Bay 11-7082 at the indicated concentrations or the corresponding volume of ethanol/vehicle. Experiments were performed at least three times.

Generation of expression constructs

I.M.A.G.E. clone ID #6428300 containing human full length cDNA of p130Cas was purchased from Open Biosystems. The cDNA was amplified by PCR using *Pfu* polymerase and the primers: forward 5'-GCGGCCGCGCCCGCCGACACCATGA-3', reverse 5'-GTTAACAACCTGCACTGGCCCTGTCAGG-3' and ligated into pCR Blunt II Topo vector using the Zero Blunt end TOPO PCR cloning kit (Invitrogen). Subsequently, the PCR product was subcloned into the retroviral pC4_{bsr}R(TO) expression vector digested with *EcoR* I and *Not* I and validated by sequencing. QIAGEN Plasmid Maxi Kit (Qiagen) was used for DNA preparation.

Retroviral transduction

Retroviral transduction was carried out as described (Soni et al., 2009; Kumbrink and Kirsch, 2012). Briefly, inducible expression of p130Cas or empty vector (ev) control in HL-60 cells was achieved by transduction with equal volumes of supernatant containing amphotropic C4_{bsr}R(TO) virus particles that carry the cDNAs for p130Cas or ev and the regulator CX_{neo}TR2 virus. Cells were selected twice with 8 µg/ml Blasticidin (Invitrogen) for 2 days and subsequently with 1 mg/ml Geneticin (Invitrogen) for 5 days.

Cell adhesion assay

HL-60 transductants (50,000 in 100 μ l) were seeded in triplicate into 96-well plates and treated with PMA and Bay 11-7082 (1.5 μ M) or Ethanol (vehicle) as described above for 15 h or left untreated. Non-adherent cells after PMA/Bay 11-7082 treatment were removed by inverting the plate. Wells were washed twice with medium and subsequently 90 μ l medium plus 10 μ l of 10x AlamarBlue (Invitrogen) were added. After 2 h, fluorescence was measured with a Synergy HT Microplate Reader (BioTek). Average fluorescence after background subtraction of untreated cells was set to 100%. The experiment was performed three times with similar results.

Cell viability assay

HL-60 transductants (15,000 in 100 μ l) were seeded in triplicate into 96-well plates and treated as described above for up to 3 days or left untreated. At each time point cell viability was determined by the addition of 10 μ l of 10x AlamarBlue (Invitrogen) and subsequent reading of fluorescence after 3.5 h as described above. Average fluorescence after background subtraction of untreated cells at day 0 was set to 1. The experiment was performed twice with similar results.

Protein analysis

Whole cells extracts (WCEs) were prepared and analyzed by Western blot analysis (WB) as described (Kumbrink and Kirsch, 2012). Antibodies (abs) used were: monoclonal abs against β -Actin (Sigma), NAB2 (Kirsch et al., 1996), p130Cas (clone 21, BD, Transduction Laboratories), PARP1 (clone Y17, recognizes 116 kDa full-length, Millipore), and PARP1 (clone C-2-10, detects 116 kDa full-length and 85 kDa cleaved fragment, Calbiochem); polyclonal EGR1 (588, Santa Cruz Biotechnologies). Experiment were performed at least three times.

Reverse transcription (RT)-PCR

RNA preparation, RT-PCR, and PCRs specific for *BCAR1* (total) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA were performed at least three times as described previously (Kumbrink and Kirsch, 2012). Products were separated by electrophoresis through 1.2% agarose gels.

Phase Contrast Microscopy

Cells were imaged by using an Olympus IMT-2 inverted phase contrast microscope, and images recorded with a Nikon Coolpix 4300 CCD camera attached to the microscope.

Densitometric analysis

Densitometric analysis of gel bands was performed using Quantity One software (Bio-Rad). Equal adjustments of contrast and brightness of microscope images, gel pictures, and scanned films were applied to all parts of the image using Adobe Photoshop CS2 version 9.0. Horizontal black lines indicate that results from different WB are shown.

3. Results and Discussion

3.1 Involvement of p130Cas during PMA-mediated differentiation, adhesion, and apoptosis induction in HL-60 cells

The Cas family members p130Cas and HEF1 integrate signaling pathways involved in adhesion and apoptosis and are associated with hematological malignancies (Tikhmyanova et al., 2010). Thus, we investigated whether p130Cas and HEF1 are important during PMA-

mediated differentiation of HL-60 leukemia cells into monocytes/macrophages, which is associated with cell aggregation, and apoptosis induction. HL-60 cells were treated with PMA for up to 60 h and cell morphology was observed by microscopy (Fig. 1A). Initial induction of cell adhesion and a migratory phenotype (in >10% of the cells), as indicated by mesenchymal morphology with polarization/leading edges, started at 14 h (Fig. 1A, 18 h shown). Continuous treatment led to further cell adhesion (24 h) and profound cell clustering at 48 h. HEF1 and p130Cas protein expression and *BCAR1* mRNA levels were monitored during PMA treatment by WB, using abs that are cross-reactive with p130Cas and the p105 and p115 isoforms of HEF1 (Law et al., 1996), and RT-PCR, respectively (Fig. 1B). In untreated cells only the p105 HEF1 isoform was found. PMA treatment resulted in the induction and persistence of the p115 isoform of HEF1 for up to 60 h. This isoform represents a phosphorylated form of p105 HEF1 which is associated with mediating cell adhesion (Tikhmyanova et al., 2010). Consistent with the fact that p130Cas levels are usually undetectable or low in more immature hematopoietic cells (Tikhmyanova et al., 2010) no p130Cas/*BCAR1* expression was observed in untreated HL-60 cells. With ongoing differentiation *BCAR1* mRNA induction was detected at 24 h continuously increasing until reaching maximum levels at 48 h, which were stable for up to 60 h tested. Concordantly, p130Cas protein induction was first observed at 36 h reaching its plateau at ~48 h (Fig. 2A, lower panel). Of note, dimethyl sulfoxide (DMSO) treatment of HL-60 cells for up to five days, inducing the differentiation into metamyelocytes and banded neutrophils (Collins et al., 1978), resulted in the up-regulation of p105 but not p115 HEF1 and neither induced cell adhesion/aggregation nor p130Cas expression (data not shown). This suggests, that these Cas family members are distinctly regulated during diverse differentiation programs and may direct cell aggregation in HL-60 leukemia cells. To test this, HL-60 cells were stably transduced with an expression plasmid for p130Cas and empty vector (ev) as control. Adhesion and cell aggregation were investigated by microscopy and adhesion assay following PMA stimulation. PMA treatment led to a strong induction of ectopic p130Cas in the p130Cas transductants (Fig. 1C). This induction of p130Cas correlated with significantly enhanced cell clustering and adhesion at 15 h of PMA stimulation in the HL-60 p130Cas transductants (47%) compared to ev cells (16%) (Fig. 1D).

PMA induces significant apoptotic cell death in HL-60 cells by 72 h after treatment (Zheng et al., 2002). Depending on the cellular context and stimulus p130Cas exhibits both pro- and anti-apoptotic activities (Tikhmyanova et al., 2010). Thus, we investigated whether a correlation between PMA-induced p130Cas expression and apoptosis exists by measuring Poly [ADP-ribose] polymerase 1 (PARP1) expression. Interestingly, decreased expression of full length PARP1 (115 kDa), indicative of PARP1 cleavage and apoptosis induction, coincided with p130Cas up-regulation at 36 h of treatment (Fig. 2A and 1B). To test the role of p130Cas in PMA-mediated cell death, the viability of HL-60 p130Cas and ev transductants was determined for 3 days using the AlamarBlue assay (Fig. 2B). Upon PMA treatment the cell viability was greatly decreased in ev cells (by 28% and 26% at day two and three, respectively), while the reduction in p130Cas cells was significantly lower (9% and 11%). These results suggest that the late induction of endogenous p130Cas mediates increased adhesion and cell aggregation to limit PMA-mediated apoptosis.

In summary, the induction of the p115 HEF1 isoform may be important for the early activation of adhesion/differentiation in HL-60 cells, whereas p130Cas may be involved in the later steps of differentiation/aggregation. Moreover, p130Cas may be important for modulating PMA-mediated apoptotic pathways in HL-60 cells and thus its potential role in it was further investigated.

3.2 Influence of nuclear factor (NF)- κ B inhibition on p130Cas expression and PMA-mediated cell aggregation and apoptosis in HL-60 cells

Inhibition of NF- κ B activity by the compound Bay 11-7082 (Bay) enhances PMA-mediated apoptosis in HL-60 cells showing the potential to improve the therapeutic efficacy of PMA in certain leukemia patients (Hansson et al., 2005). Nevertheless, the molecular mechanisms by which Bay increases PMA effectiveness are poorly understood. The expression kinetics of p130Cas in response to PMA correlates with apoptosis induction (Fig. 1B and 2A). Therefore, Bay was utilized as apoptosis enhancer and its effect on PMA-induced p130Cas and HEF1 expression and apoptosis was investigated. The transcription factor early growth response 1 (EGR1/NGFI-A) and its coregulator NGFI-A binding protein (NAB) 2 were recently identified as regulators of p130Cas expression in breast cancer cells (Kumbrink and Kirsch, 2012) and in HL-60 cells the late induction pattern of *BCAR1*/p130Cas overlaps with EGR1 and NAB2 (Fig. 1B and 2A). Thus, the protein levels of EGR1 and NAB2 were also determined following Bay application. Treatment of HL-60 cells with PMA (16 nM) or Bay (1 and 1.5 μ M) significantly downregulated p105 HEF1 levels when analyzed at 24 and 48 h by WB (Fig. 2C). Moreover, the PMA-mediated induction of the p115 HEF1 isoform was significantly reduced after Bay treatment (1.5 μ M) for 48 h. In addition, the up-regulation of p130Cas, EGR1, and NAB2 at 48 h was attenuated in a dose-dependent manner (Fig. 2C, right panels). The reduction of p115 HEF1 and p130Cas inducibility was accompanied by reduced PARP1 levels due to increased cleavage as indicated by the enhanced detection of the 85-kDa fragment of PARP1, compared with single PMA or Bay application. PARP1 cleavage was detectable already at 24 h of combined PMA and Bay (1 and 1.5 μ M) treatment. Nonetheless this early apoptosis induction by PMA and Bay (1 μ M) did not substantially inhibit EGR1, NAB2, and p130Cas induction (Fig. 2C). This further suggests, that the EGR1/NAB2/p130Cas system may be activated by survival pathways in response to apoptosis. Morphological changes suggestive of apoptosis-mediated cell death were also confirmed by microscopy (Fig. 2D). Notably, Bay treatment greatly inhibited PMA-induced cell aggregation. In addition, the early adhesion (15 h of PMA stimulation) of the HL-60 transductants was significantly reduced (ev, 17% to 8%; p130Cas 48% to 30%) after the application of Bay (1.5 μ M) (Fig. 2E). Nevertheless, the percentage of adherent p130Cas transductants (30%) was significantly higher than that of only PMA-treated ev cells (17%). Loss of adhesion and cell aggregation of HL-60 cells in response to PMA is commonly used as an indicator of the effectiveness of potential therapeutics such as antibodies targeting ICAM-1 (Kwon et al., 2006). This further supports our observations that p130Cas acts as “survival factor” in PMA-mediated cell death by limiting the reduction of cell adhesion and aggregation. These results suggest that Bay promotes PMA-mediated apoptosis in HL-60 cells in part by downmodulation of the EGR1/NAB2/p130Cas system and HEF1 activation. Furthermore, this shows for the first time that NF- κ B may be involved in the regulation of the expression of these two proteins of the Cas-family. Because p115 HEF1 represents a phosphorylated form of p105 HEF1 (Tikhmyanova et al., 2010), the influence of NF- κ B on HEF1 is probably indirect by transcriptional activation of direct target genes. The immediate early gene EGR1 and the delayed early gene NAB2 were shown to mediate the late p130Cas/*BCAR1* induction which in turn upregulates EGR1 and NAB2 (Kumbrink and Kirsch, 2012). Because co-regulation of various target genes by EGR1 and NF- κ B has been described (Damm et al., 2010; Ma et al., 2009), it is plausible that both transcription factors may act in concert to regulate p130Cas expression in differentiating HL-60 cells. EGR1 and NAB2 are key factors in promoting the macrophage program in myeloid progenitor cells (Laslo et al., 2006) and in T cell activation (Collins et al., 2006). The EGR1/NAB2/p130Cas regulatory circuit may serve to maintain EGR1 and NAB2 levels to activate genes that determine differentiation programs in hematopoietic cells.

To summarize, our results suggest that during PMA-mediated differentiation of HL-60 cells NF- κ B and EGR1 up-regulate NAB2 expression (Kumbrink et al., 2005; Kumbrink et al., 2010) and subsequently the induction of p130Cas (Fig. 2A, lower panel) which in turn further promotes cell aggregation to limit cell death. Current differentiation therapy involving all-*trans* retinoic acid (ATRA), differentiating HL-60 cells into neutrophils similar to DMSO or DMF, have improved the outcomes for a subset of leukemia patients (Kamimura et al., 2011). Because ATRA-mediated differentiation of HL-60 cells does not induce cell adhesion and cluster formation (Breitman et al., 1980) our identified mechanism for PMA-mediated differentiation might not be applicable for the neutrophilic pathway.

Nonetheless, future studies deciphering the detailed mechanisms of the NF- κ B/EGR1/NAB2/p130Cas system in leukemia cells may lead to results that can be translated into novel strategies for leukemia treatment. For example, currently developed approaches to target p130Cas signaling in different types of malignancies (Cabodi et al., 2010; Kumbrink and Kirsch, 2011) may block the protective effects of p130Cas during PMA and/or Bay application.

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The abbreviations used were

BCAR1	breast cancer antiestrogen resistance 1
p130Cas	Crk-associated substrate
EGR	early growth response
ev	empty vector
NAB2	NGFI-A binding protein 2
NF-κ B	nuclear factor- β B
PMA	phorbol 12-myristate 13-acetate
RT-PCR	reverse transcription PCR
WB	Western blotting
WCE	whole cells extracts

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Figure 1.

p130Cas induction is important for PMA-mediated differentiation and adhesion in HL-60 cells. (A–C) HL-60 cells (5×10^5) were seeded in 6-well plates and treated with PMA for indicated times or left untreated as described in Material and Methods. (A) Cell morphology and growth pattern was analyzed by phase contrast microscopy. Scale bars: 100 μ m. (B) Upper panel, WCEs (30 μ g) were analyzed by WB for p130Cas, HEF1, and actin (loading control) protein expression. Lower panel, total *BCAR1* and *GAPDH* (control) mRNA levels were determined by RT-PCR. –DNA, negative control for PCR. Relative mean expression levels from three experiments of p130Cas protein and *BCAR1* mRNA to actin or *GAPDH*, respectively, determined by densitometric analyses are indicated. Relative expression (Value $\pm$ standard deviation) for p130Cas are (WB): 36 h, 0.22 ± 0.18 ; 48 h, 0.73 ± 0.13 ; 60 h, 0.88 ± 0.1 ; for *BCAR1* (mRNA): 24 h, 0.12 ± 0.08 ; 36 h, 0.31 ± 0.14 ; 48 h, 0.66 ± 0.24 ; 60 h, 0.86 ± 0.31 . (C and D) HL-60 cells were transduced with p130Cas cDNA or ev. Transductants were treated with PMA for indicated times. (C) WCEs (20 μ g) were analyzed by WB for ectopic p130Cas and actin expression. (D) Left panels, phase contrast images of HL-60 ev and HL-60 p130Cas cells at 15 h PMA treatment. Scale bars: 100 μ m. Right panel, quantification of adhesion was performed as described previously. *P* values were calculated using Student's *t* test. ***, *P* < .001.

Figure 2. p130Cas acts as survival factor during PMA-induced apoptosis in HL-60 cells. (A, C, and D) HL-60 cells (5×10^5) were seeded in 6-well plates and treated with PMA and/or the NF- κ B inhibitor Bay 11-7082 (1 and 1.5 μ M) or vehicle (ethanol) for indicated times or left untreated as described previously. (A) Upper panel, WCEs (30 μ g) were analyzed by WB for PARP1 (clone Y17), EGR1, NAB2, and actin expression. Lower panel, relative mean protein levels from three experiments of PARP1, EGR1, NAB2, and p130Cas (determined in Fig. 1A) normalized to actin and standard deviation determined by densitometric analyses are shown. (B and E) Viability and adhesion measurements of HL-60 transductants expressing p130Cas or ev was performed as described previously (C) Left panel, WCEs (30 μ g) were analyzed by WB for p130Cas, HEF1, PARP1 (clone C-2-10), EGR1, NAB2, and actin expression. Right panel, densitometric analysis from three independent experiments. (D) Cell morphology and growth pattern were analyzed by phase contrast microscopy. Scale bars: 100 μ m. (E) Cells were treated with PMA and 1.5 μ M Bay or vehicle as indicated. (B, C and E) *P* values were calculated using Student's *t* test. *, *P* < .05; **, *P* < .01; ***, *P* < .001.