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Expression in T-cells of the proapoptotic protein p66SHC is controlled by promoter demethylation

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Abstract

p66Shc plays a key role in oxidative stress-induced apoptosis. p66Shc gene expression is tissue-specific and controlled by promoter methylation. In T-cells p66Shc expression is induced by a variety of apoptotic stimuli. We have addressed the mechanisms regulating p66Shc expression in T-cells. We show that the increase in p66Shc protein following stimulation with a Ca^{2+} ionophore results from enhanced gene expression, which is primarily dependent on DNA replication-independent promoter demethylation. Our data underline the role of CpG methylation in the control of p66Shc gene expression and provide evidence that Ca^{2+} signaling may lead to epigenetic modifications in nondividing cells.

Keywords: CpG methylation; Chromatin; Gene expression; Apoptosis; Lymphocyte; Shc proteins

p66Shc is the longest of the three protein isoforms encoded by *shcA*. The N-terminal CH2 domain endows p66Shc with unique functional properties compared to p52Shc/p46Shc. Indeed, p66Shc antagonizes mitogenic signaling by p52Shc [1–3], which couples growth factor receptors to Ras [4]. Furthermore, p66Shc is a key component of a p53-dependent apoptotic pathway triggered by oxidative stress [5]. The apoptogenic activity of p66Shc results from its capacity to promote ROS production [6–9].

Expression of the three ShcA isoforms is regulated both transcriptionally and translationally. p52Shc/p46Shc are coexpressed from the same transcript by alternative usage of two in-frame translation initiation codons. An alternative promoter drives the generation of p66Shc mRNA. The two promoters are differentially regulated, resulting in constitutive and ubiquitous expression of p52Shc/p46Shc and tissue-specific expression of p66Shc, which is absent in neuronal and hematopoietic cells [1]. A close inverse correlation has been established between p66Shc expression and the methylation state of eight CpG dinucleotides in the gene promoter, suggesting an epigenetic mechanism of regulation of p66Shc expression. In support of this notion, treatment with demethylating agents results in p66Shc expression in cells where it is normally undetectable [10].

T-lymphocytes express low to undetectable amounts of p66Shc. Analysis of the methylation state of the p66Shc gene promoter in quiescent human PBL shows a significant fraction of methylated CpG [10], suggesting that promoter methylation contributes to p66Shc gene silencing in these cells. We have recently shown that both human and murine T-cells inducibly express p66Shc in response to a variety of apoptogenic stimuli [3]. Expression of p66Shc results

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in T-cell priming to apoptosis [3,11]. Here we have investigated the mechanisms controlling the induction of p66Shc expression in human T-cells.

Materials and methods

Cells, antibodies, plasmids and reagents. PBMC were isolated from whole blood by density gradient centrifugation and depleted of macrophages by adherence. For the genome bisulfite sequencing experiments T-lymphocytes were further purified using the RosetteSep® cocktail for T-cell enrichment (StemCell Technologies, Vancouver, Canada). The Jurkat T-lymphoma line was also used. The p66Shc promoter construct (spanning positions −434/+101) was previously described [10]. The luciferase reporters pGL3basic and pGL3prom were from Promega Italia srl (Milan, Italy), the pCR2.1-TOPO cloning vector from Invitrogen Ltd (Paisley, UK).

p66Shc was immunoprecipitated using an anti-CH2 polyclonal antiserum [1]. Anti-Erk antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), secondary, unlabeled, and peroxidase-labeled antibodies were from Cappel (Durham, NC) and Amersham Pharmacia Biotech. IgG from OKT3 hybridoma supernatants were affinity-purified on Mabtrap (Amersham Pharmacia Italia srl). Anti-human CD28 mAb was from Becton-Dickinson Biosciences (Heidelberg, Germany).

RNA purification and RT-PCR. RNA was extracted using the RNA WIZ™ isolation reagent (Ambion Inc., Austin, TX). Semiquantitative RT-PCR was carried out using ImProm-II™ reverse transcriptase and Taq DNA polymerase (Promega). Housekeeping controls included the genes encoding GAPDH, actin, RNA polymerase II, and 18S RNA. RT-PCR products were separated by agarose gel electrophoresis and, when required, recovered using the Nucleo Spin extraction kit from Macherey-Nagel Srl (Hoerdt, France). The identity of the RT-PCR products was confirmed by automatic sequencing. Gel images were acquired by laser densitometry (Kodak Digital Science™ Electrophoresis Documentation and Analysis System 120), and the band intensities quantitated using ImageQuant Version 5.0 (Molecular Dynamics, Sunnyvale, CA). The levels of p66Shc mRNA were normalized to the levels of 18S RNA.

Transfections, luciferase assays, flow cytometry. Transient transfections were carried out as described [3]. A CMV-β-galactosidase reporter was cotransfected as a transfection control.

For cell cycle analysis, PBL were treated with 500 ng/ml A23187 or activated by CD3/CD28 coengagement as described [12]. Cells were washed, fixed in ice-cold 70% ethanol, treated for 30 min at RT with 100 μg/ml RNase and stained with 20 μg/ml propidium iodide. Samples were processed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Data were acquired using CellQuest software and analyzed and plotted using Flowjo (Tree Star, Inc).

Metabolic labeling, immunoprecipitations, and immunoblots. For metabolic labeling PBL ($1–2 \times 10^7$/sample) were treated with carrier or A23187 for 24 h, then resuspended in methionine-free medium, added with 3–4 μCi [^{35}S]methionine/10^6 cells and incubated for 3 h at 37 °C. After two washes cells were resuspended in complete medium and chased for different lengths of time. Cells were lysed in 1% (v/v) Triton X-100 in 20 mM Tris–HCl, pH 8, 150 mM NaCl (in the presence of a protease inhibitor cocktail) and p66Shc was immunoprecipitated using an anti-CH2 polyclonal antiserum and protein-A–Sepharose. Immunoprecipitated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and exposed to a Phosphorimager (Molecular Dynamics). Immunoblots were carried out using a chemiluminescence detection system (Pierce, Rockford, IL).

Genome bisulfate sequencing. Genomic DNA was extracted from carrier- or A23187-treated T-lymphocytes as described [12] and subjected to sodium bisulfite treatment and subsequent desulfonation essentially as described [13]. DNA was recovered by ethanol precipitation and subjected to PCR using the GoTaq DNA polymerase (Promega) and primers designed to amplify the p66Shc promoter region. The PCR products were cloned in the pCR2.1-TOPO vector. Plasmid DNA was recovered from individual colonies and subjected to automated sequencing. At least 10 clones from independent PCR products were sequenced for each donor.

Results

Apoptogenic stimulation of human PBL causes an increase in the levels of p66Shc mRNA

p66Shc is inducibly expressed in T-cells following apoptogenic stimulation [3]. To understand whether the increase in p66Shc protein results from enhanced gene transcription, a time course analysis of the steady-state p66Shc mRNA levels was carried out by semiquantitative RT-PCR on human PBL treated with the Ca^{2+} ionophore, A23187, which acts as a potent inducer of apoptosis in lymphocytes. As shown in Fig. 1, p66Shc mRNA was detectable in untreated PBL. A23187 treatment resulted in an average 3-fold increase in p66Shc mRNA, which was first detectable 4 h after A23187 addition and peaked at 24 h.

Induction of p66Shc expression correlates with DNA replication-independent promoter demethylation

The p66Shc gene promoter is hypermethylated in resting human PBL [10]. To address the possibility that a change in promoter methylation underlies the A23187-induced increase in p66Shc mRNA, we analyzed the methylation

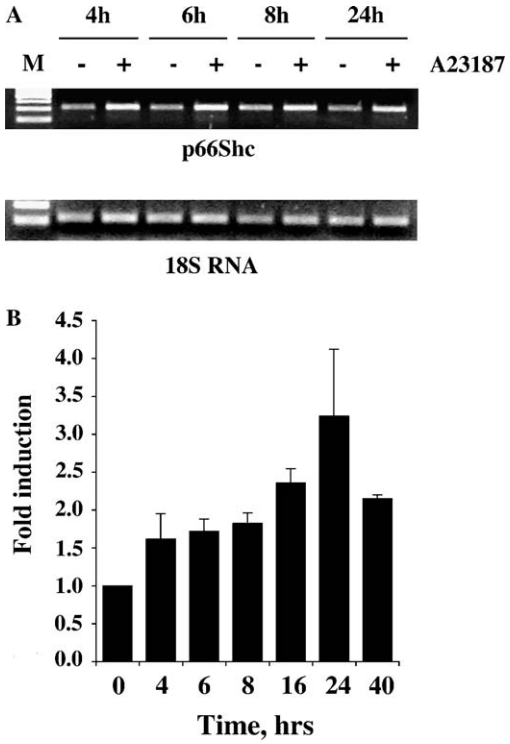

Fig. 1. A23187 treatment results in an increase in the steady-state p66Shc mRNA levels in human PBL. Semiquantitative RT-PCR analysis of p66Shc mRNA in human PBL treated with carrier or A23187 (500 ng/ml) for the indicated times. 18S RNA was used as internal control. (A) Data from a representative time course experiment. (B) RT-PCR products from multiple time course experiments were resolved by agarose gel electrophoresis, quantitated by laser densitometry, and normalized to the levels of 18S RNA. Data are expressed as fold induction of A23187-treated samples to the respective carrier-treated samples ($n \geqslant 3$).

state of the eight CpG dinucleotides in the region encompassing position −139 to +66 of the p66Shc gene promoter on genomic DNA purified from PBL isolated from three donors and treated for 24 h with A23187. The overall level of CpG methylation of the p66Shc gene promoter was ~60% in untreated PBL (Fig. 2A). Although some variation in the methylation pattern was observed, cytosines at positions −127, −53, −30, +23, and +30 (CpG2, 4, 5, 7, 8) were consistently hypermethylated, as were, to a lesser extent, cytosines at positions −138 (CpG1) and −78 (CpG3) (Fig. 2B,C). A23187 treatment resulted an overall ~30% reduction in CpG methylation, which affected primarily CpG1–6 (Fig. 2). The most striking effect of A23187 treatment was observed on CpG3 which, albeit not heavily methylated in quiescent cells, became fully unmethylated in A23187-treated cells (24.25% ± 8.4% methylation in control cells, 0% methylation in A23187-treated cells; $p<0.05$; data from three donors). Conversely, methylation of CpG7 and CpG8 was largely unaffected (Fig. 2). Hence the A23187-dependent increase in p66Shc

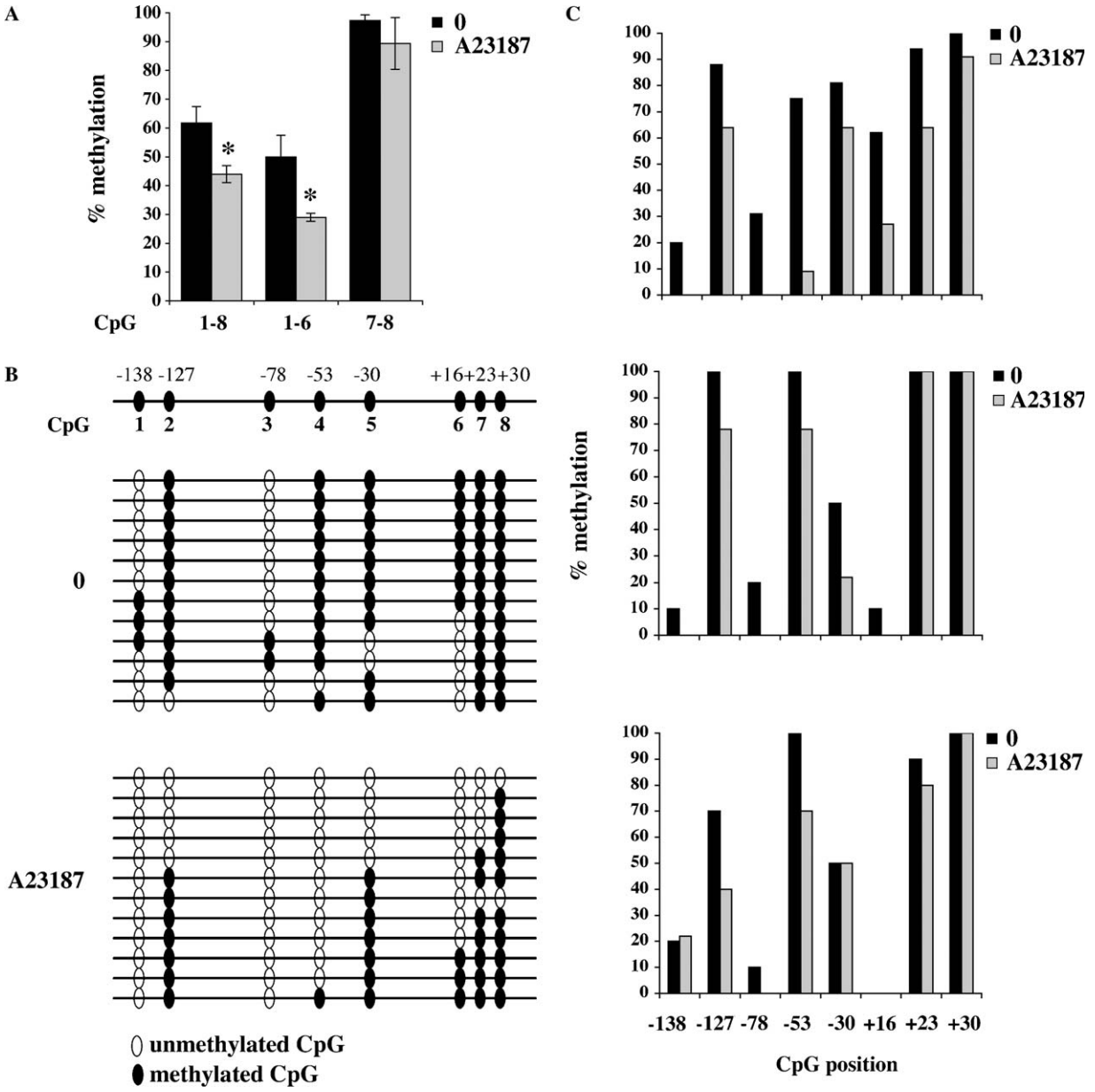

Fig. 2. A23187 induces p66Shc gene promoter demethylation in PBL. (A) Relative methylation of the p66Shc gene promoter in T-lymphocytes from three healthy donors. PBL were either untreated (0) or treated for 24 h with A23187 (500 ng/ml). The data are presented as % methylated CpG. Values are means ± SD of the levels of methylation of all eight CpGs (CpG1–8) in the p66Shc gene promoter, or of the subsets corresponding to the first six CpG (CpG1–6) and the last two CpG (CpG7-8) . Statistically significant differences of A23187-treated samples to controls, as assayed by Student's *t* test, are indicated by asterisks ($p < 0.01$). (B) CpG methylation pattern in 12 representative clones of a PCR from bisulfite-treated genomic DNA from T-lymphocytes of one of the three donors. A scheme of the p66Shc gene promoter highlighting the methylatable CpGs is shown. (C) Relative methylation of each CpG in the p66Shc gene promoter in T-lymphocytes from three individual donors. The A23187-dependent p66Shc gene promoter demethylation was confirmed on two additional donors.

mRNA is associated with a decrease in promoter methylation, with a preferential effect on specific CpGs.

DNA hypomethylation is believed to occur primarily as a passive process associated with DNA replication. Methylation can however be reversed in non-dividing, terminally differentiated cells through active promoter demethylation [14,15]. Although elevation of $[Ca^{2+}]_c$ is *per se* not sufficient to promote cell cycle entry, to rule out that the A23187-dependent reduction in p66Shc gene promoter methylation was associated with DNA synthesis, PBL treated for 24 h with A23187 were subjected to cell cycle analysis. As a positive control, PBL were costimulated with anti-CD3 and anti-CD28 mAbs. The S-phase subpopulation was 0% in A23187-treated PBL, while at the same time point a significant fraction of cells was found in S- or G2/M-phase following CD3/CD28 costimulation. As expected, a fraction of the A23187-treated PBL were apoptotic, as assessed by their hypodiploid DNA complement (Fig. 3). The data demonstrate that the reduction in p66Shc gene promoter methylation triggered by A23187 is not associated with DNA replication.

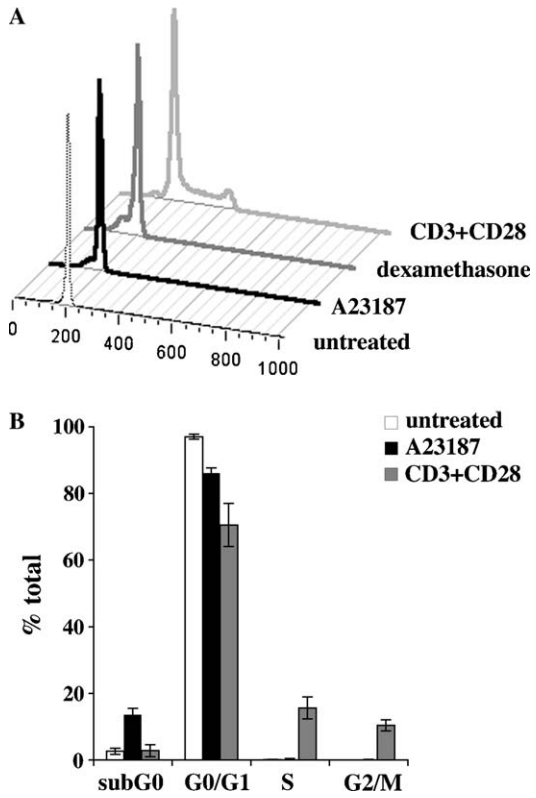

Fig. 3. A23187 does not affect DNA replication in human PBL. (A) Cell cycle analysis by flow cytometry of human PBL treated with either A23187 (500 ng/ml), or anti-CD3+anti-CD28 mAb (mitogenic stimulation), or 0.5 μM dexamethasone (apoptotic stimulation) and subsequently permeabilized and stained with propidium iodide. Data were analyzed and plotted using Flowjo (Tree Star, Inc). Histograms from a representative experiment are shown. (B) Quantitation of the relative number of cells at the different stages of the cell cycle, as determined by flow cytometric analysis of PBL treated as in A ($n = 3$).

A23187 does not enhance p66Shc gene transcription through direct activation of the transcriptional machinery

To understand whether, in addition to inducing a decrease in promoter methylation, an increase in $[Ca^{2+}]_c$ might also enhance its activity, the effect of A23187 on p66Shc gene transcription was studied using a p66Shc promoter-driven luciferase reporter. As previously reported and shown in Fig. 4A, this construct is constitutively active when transfected in Jurkat T-cells, while its activity is suppressed following *in vitro* methylation with the CpG methylase SstI [10]. The unmethylated construct allows therefore to study the transcriptional apparatus responsible for p66Shc gene expression. Jurkat cells were transiently transfected with the p66Shc promoter reporter and treated

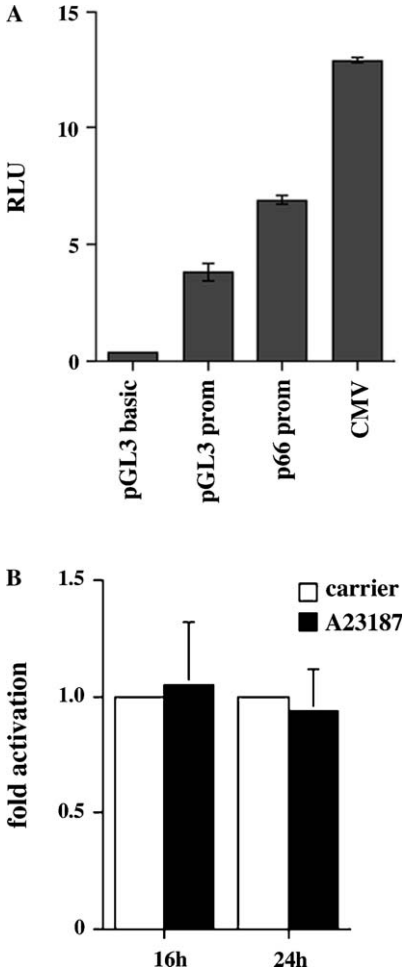

Fig. 4. A23187 does not affect the transcriptional activity of the p66Shc gene promoter. (A) Quantitation of luciferase activity in Jurkat cells transiently transfected with luciferase reporter constructs under the control of the SV40 minimal promoter (pGL3basic), the SV40 enhancer (pGL3prom), the p66Shc gene promoter (p66prom) or the CMV enhancer (CMV) ($n = 2$). RLU, relative luciferase units. (B) Quantitation of luciferase activity in Jurkat cells transiently transfected with the p66Shc promoter luciferase reporter and treated for the indicated times with carrier or A23187 (500 ng/ml). The data are expressed as fold induction of A23187-treated samples to the respective carrier-treated samples (taken as 100%) ($n > 3$).

with carrier or A23187. No increase in luciferase activity above the basal levels was detected in A23187-treated cells, even at low plasmid concentrations (Fig. 4B and data not shown). The results indicate that the A23187-dependent increase in p66Shc mRNA in PBL is unlikely to result from the activation of the transcriptional machinery that controls p66Shc gene expression.

mRNA but not protein stabilization contributes to the enhancement of p66Shc expression by A23187

mRNA stabilization represents a major post-transcriptional mechanism of control of gene expression [16]. To address the possibility that enhanced mRNA stability may contribute to the A23187-dependent increase in the steady-state p66Shc mRNA levels, PBL preincubated for 16 h with A23187 were treated with actinomycin-D, which blocks *de novo* gene transcription. The kinetics of p66Shc mRNA decay after actinomycin-D addition was analyzed by semiquantitative RT-PCR. The same analysis was carried out on four housekeeping genes, *i.e.*, actin, GAPDH, RNA polymerase II, and 18S RNA. As opposed to the other three transcripts, 18S RNA showed no significant variation after actinomycin-D treatment, and was therefore used as internal control. p66Shc-specific transcripts were reduced by ~70% in PBL pretreated with carrier and further treated for 24 h with actinomycin-D, and undetectable by 72 h (Fig. 5A). In the same conditions actin and RNA polymerase II mRNA decayed with a similar kinetics, while GAPDH mRNA showed a slower decay (Fig. 5B). A23187 treatment resulted in a moderate delay in p66Shc mRNA decay, with ~55% reduction 24 h after actinomycin-D addition and a ~90% reduction at 72 h. A stabilizing effect of A23187 treatment was also observed for RNA polymerase II mRNA and, to a larger extent, on GAPDH and actin mRNA (Fig. 5B). The data indicate that, besides activating p66Shc gene expression by promoting CpG demethylation, A23187 further sustains this process by enhancing mRNA stability.

Oxidants have been reported to enhance p66Shc protein stability in fibroblasts [6]. To address the potential role of protein stabilization in the induction of p66Shc expression in T-cells, PBL were pretreated for 24 h with carrier or A23187 to induce maximal levels of p66Shc mRNA and subsequently subjected to a 3 h-pulse with [^{35}S]methionine. PBL were then chased for different lengths of time with unlabeled methionine. p66Shc was immunoprecipitated and resolved by SDS–PAGE, and the levels of incorporated [^{35}S]methionine were quantitated. Measurable amounts of p66Shc were found in carrier-treated PBL, indicating that p66Shc is expressed in quiescent PBL at levels close to the lower detection limits of

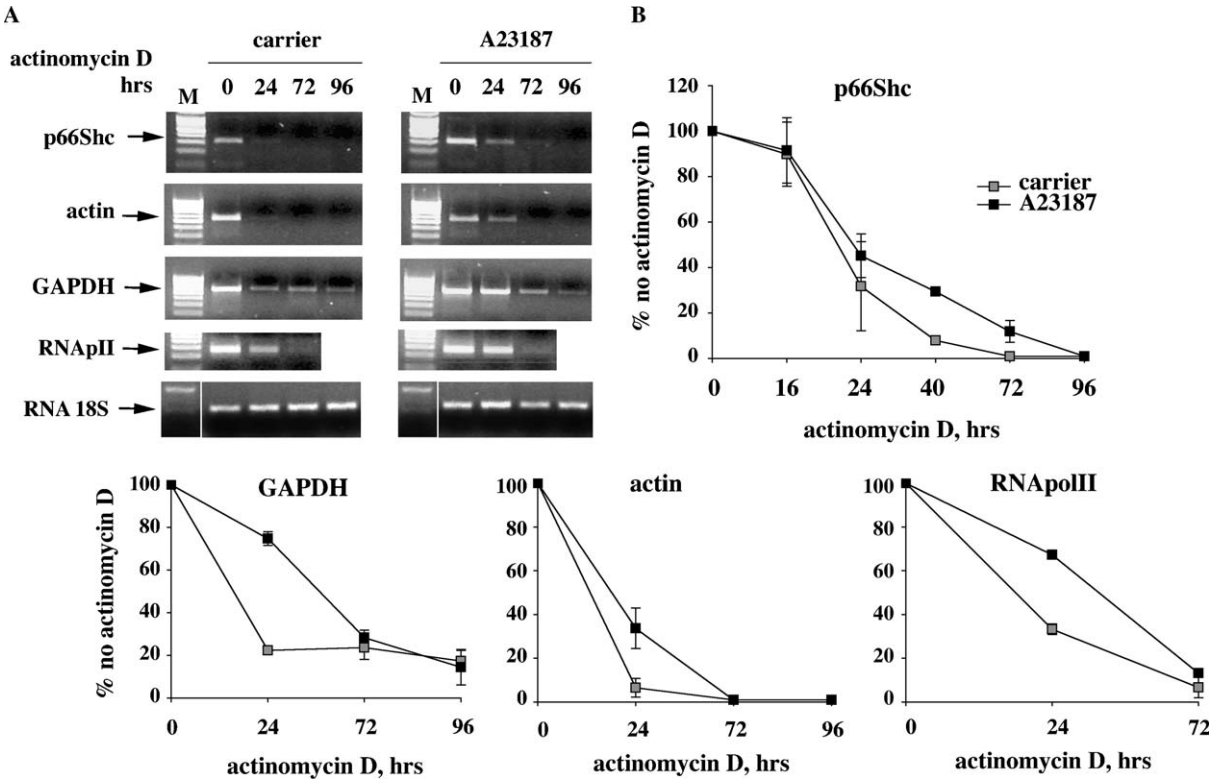

Fig. 5. A23187 treatment enhances p66Shc mRNA stability in PBL. (A) Semiquantitative RT-PCR analysis of p66Shc, actin, GAPDH, RNA polymerase II mRNA, as well as 18S RNA, in freshly purified human PBL treated with carrier or A23187 (500 ng/ml) for 16 h and subsequently added with actinomycin-D (10 μg/ml). Data from a representative experiment are shown. (B) RT-PCR products from two independent experiments were resolved by agarose gel electrophoresis, quantitated by laser densitometry and normalized using 18S RNA. For each treatment (carrier or A23187) the data are expressed as the ratio of actinomycin-D-treated *vs* untreated samples (no actinomycin-D taken as 100%).

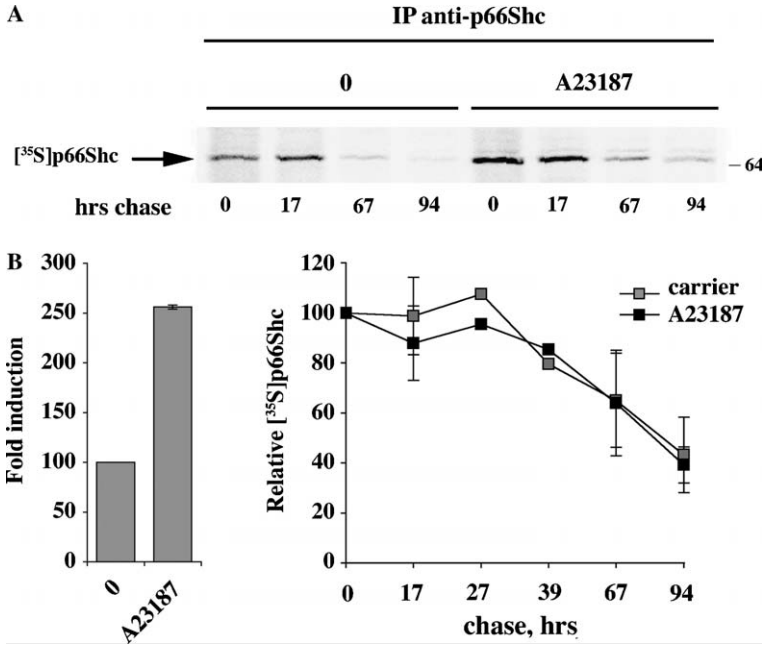

Fig. 6. A23187 treatment does not affect p66Shc protein stability in PBL. (A) Autoradiography of p66Shc-specific immunoprecipitates from human PBL metabolically labeled with [^{35}S]methionine. Cells were treated for 24 h with carrier or A23187 (500 ng/ml), metabolically labeled for 3 h in methionine-free medium added with [^{35}S]methionine, and chased for the indicated times. A representative experiment is shown ($n \geqslant 3$). (B) *Left*, Quantitation of [^{35}S]methionine incorporation in p66Shc-specific immunoprecipitates at the end of the metabolic labeling. The Phosphorimager data were quantitated using Imagequant and normalized to the levels of Erk in each lysate, as determined by immunoblot analysis and laser densitometry. The data are expressed as fold induction of A23187-treated samples to the respective carrier-treated samples (taken as 100%) ($n = 2$). *Right*, Quantitation of [^{35}S]methionine incorporation in p66Shc-specific immunoprecipitates metabolically labeled as above and chased for the indicated times. For each treatment (carrier or A23187) the data are expressed as relative [^{35}S]-labeled p66Shc at each time point of chase *vs* the "0" time point (end of metabolic labeling, no chase; taken as 100%). The results of multiple experiments have been pooled to cover the time points indicated ($n > 3$).

immunoblot. A23187 treatment resulted in a ~3-fold increase in [^{35}S]-labeled p66Shc (Fig. 6A, B). A ~20% decrease in labeling was first observed after 40 h chase, that reached ~60% at the latest time point analyzed. The kinetics of [^{35}S]-labeled p66Shc decay following A23187 treatment was comparable to that observed in carrier-treated cells (Fig. 6B). Hence, protein stabilization does not appear to contribute to the Ca^{2+}-dependent enhancement of p66Shc expression in PBL.

Discussion

CpG methylation functions in concert with histone modification and chromatin remodeling at multiple steps of lymphocyte development, activation, and differentiation [17,18]. The data presented here show that the inducible expression of p66Shc in T-cells is regulated by promoter demethylation. Compared to other genes, such as *IL-2*, where methylation of five critical CpGs is complete in naive T-cells and reduced drastically in activated T-cells [19], the methylation pattern of the p66Shc gene promoter is less clear-cut. A partial methylation level can indeed be observed in quiescent T-cells, which involves to a variable extent all CpGs. Furthermore, with the exception of CpG3, the A23187-dependent CpG demethylation is not complete. It is however noteworthy that methylation of the eight CpGs in the p66Shc gene promoter is differentially affected by A23187 with a significant level of demethylation of CpG1–6, suggesting that methylation of these CpGs plays an important role in controlling promoter accessibility. Of these, CpG3 lies within a putative CAAT box, while both CpG2 and CpG4 are part of putative Sp1 binding sites [10]. On the other hand, taken together with the observation that, albeit inducible, the expression of p66Shc in PBL is modest, the extensive hypermethylation of cytosines +23 and +30 even after A23187 treatment suggests that methylation of these CpGs is crucial in the negative control of p66Shc gene expression.

The most common mechanism of CpG demethylation is passive. This process is initiated by DNA-binding proteins which block access to maintenance DNA methyltransferases on newly synthesized DNA [15]. Emerging evidence underscores however the existence of an active, DNA replication-independent mechanism of CpG demethylation, involving a specific 5-methylcytosine demethylase [14]. Whether this enzyme is the methyl-CpG binding domain protein, MBD2, which demethylates DNA both *in vitro* and *in vivo* [20], is still controvertial. The specific instances of active CpG demethylation are as yet very sparse. In the immune system active demethylation has been demonstrated to occur at the IL-2 gene promoter at very early times during naive T-cell activation [19]. Furthermore, one specific CpG in the Th2 control locus undergoes active demethylation

within hours of activation in Th2 polarizing conditions [21]. Our data strongly support a mechanism of active demethylation for the p66Shc gene promoter.

Ca^{2+} has been implicated in mRNA stabilization in many cell types, including immune cells [16,22]. Our data show a stabilizing effect of Ca^{2+} also on p66Shc mRNA. The half-life of the p66Shc gene transcript in both untreated and A23187-treated PBL is close to 24 h. Since the increase in steady-state mRNA levels can be detected as early as 4 h following A23187 treatment, RNA stabilization is unlikely to play a significant role in the induction of p66Shc gene expression but may rather be instrumental in sustaining this process. Collectively, the data highlight promoter demethylation as the initiating event in p66Shc gene expression.

Acknowledgments

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