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$p66^{Shc}$ gene has a pro-apoptotic role in human cell lines and it is activated by a p53-independent pathway ☆

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Abstract

$p66^{Shc}$ protein has been proposed to be an indispensable factor for *p53*-dependent, mitochondria-mediated apoptosis in mice. Here, we show that $p66^{Shc}$ plays a pro-apoptotic role also in cell lines of human origin such as SaOs-2 and HeLa, where *p53* is either absent or inactivated, thus, suggesting that $p66^{Shc}$ pro-apoptotic role is independent from the presence of a functional form of *p53*. The active form of $p66^{Shc}$ is phosphorylated in Serine 36. We confirm the importance of Serine 36 phosphorylation for $p66^{Shc}$ pro-apoptotic role, and our results suggest that the kinase involved in this process is activated independently from *p53*.

Keywords: $p66^{Shc}$; Oxidative stress; Apoptosis; Human cell lines; Serine phosphorylation

Shc proteins have been implicated in longevity in mammals [1] and in a variety of processes such as stress response, proliferation, and apoptosis [2]. The *shcA* locus encodes three isoforms *p46*, *p52*, and $p66^{Shc}$, sharing the same modular structure PTB-CH1-SH2 [3]. In particular, $p66^{Shc}$ contains a unique amino-terminal region (CH2) [4] which is phosphorylated at Serine 36 residue (Ser36) after UV rays and hydrogen peroxide (H_2O_2) exposure [5]. Activation of ERKs is necessary for Ser36 phosphorylation after H_2O_2 treatment and $p66^{Shc}$ seems to inhibit ERKs with a feed-back loop [6]. $p66^{Shc}$ is thought to be a crucial mediator of the apoptotic response to oxidative damage, since it has been observed that $p66^{Shc-/-}$ mice have increased resistance to oxidative stress and prolonged lifespan. Mouse embryo fibroblasts (MEFs) derived from $p66^{Shc-/-}$ mice display lower levels of intracellular reactive oxygen species (ROS) and increased resistance to oxidative stress [1]. The overexpression of $p66^{Shc}$ in Jurkat cells increases the percentage of apoptosis after H_2O_2 exposure [7]. On the whole, these data suggested a pro-apoptotic role for $p66^{Shc}$ in response to oxidative stress [7]. In fact, it has been reported that $p66^{Shc}$ is required for the elevation of the intracellular oxidants and for cytochrome *c* release [5]. Indeed, $p66^{Shc}$ appears to be localized at mitochondrial level, where it forms a complex with mtHsp70 and regulates the transmembrane potential [8]. More recently, a model for $p66^{Shc}$ pro-apoptotic action at mitochondrial level has been proposed [9]. As far as the role of p53 in $p66^{Shc}$ activation, experimental evidence showed that $p66^{Shc}$ protein levels are up-regulated in *p53* wt, but not

☆ *Abbreviations:* H_2O_2, hydrogen peroxide; MEFs, mouse embryonic fibroblasts; ROS, reactive oxygen species; PI, propidium iodide; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; RNAi, RNA inhibition technique; siRNA, small interfering double-stranded RNA.

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in *p53*−/− MEFs after H_2O_2 exposure [5]. Taking into account all the above-mentioned data, we wish to investigate if $p66^{Shc}$ requires *p53* to play its role in cells of human origin and to investigate the role of $p66^{Shc}$ Ser36 in oxidative stress-induced apoptosis. To this aim, we overexpressed or silenced $p66^{Shc}$ in human cell lines lacking a functional *p53* and thereafter we evaluated the susceptibility to apoptosis and mitochondrial membrane potential (MMP) after exposure to hydrogen peroxide. Finally we have evaluated the status of Ser36 residue of $p66^{Shc}$ protein and the results obtained allowed us to speculate that Ser36 function is independent from *p53* gene function.

Materials and methods

Cell cultures. Human cell lines SaOs-2 and HeLa were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) heat-inactivated for 30 min at 56 °C, 100 μg/ml streptomycin–100 IU/ml penicillin (PenStrep; BioWhittaker, Verviers, Belgium), and 2 mM L-Gln (BioWhittaker); cells were kept at 37 °C in a humidified atmosphere (5% CO_2).

Apoptosis was induced by treatment with 50 μM H_2O_2. In the case of transfected cells, H_2O_2 treatment was performed after 24 h from plasmid transfection. In the case of siRNA experiments, treatment was performed after 48 h from siRNA transfection. Before H_2O_2 exposure, culture medium was removed and replaced with PBS + H_2O_2 50 μM. After 30 min of incubation at 37 °C, PBS and H_2O_2 were discarded, fresh medium was supplied, and cells were allowed to recover for 4 or 24 h.

RNA extraction and reverse transcription. Total RNA was extracted from cells using TRIzol™ Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Reverse transcription reactions were performed with 2 μg of total RNA using the M-MLV Reverse Transcriptase (Invitrogen), following the manufacturer's instructions.

Protein extraction and Western blot analysis. Total proteins were extracted with Co-IP Buffer (10 mM Tris–HCl, pH 7.6, 140 m NaCl, 0.5% NP-40, and 5 mM EDTA) added with Protease Inhibitor Cocktail (Sigma). Pellets were resuspended in Co-IP Buffer and incubated on ice for 10 min; after centrifugation, the supernatant containing proteins is recovered. Thirty micrograms of proteins is used in Western blot analysis, using the following antibodies: $p66^{Shc}$ antibody (Upstate Biotechnology, Charlottesville, VA, USA), ShcA antibody (BD, S. Josè, CA, USA), β-actin antibody (Santa Cruz Biotechnology, S. Cruz, CA, USA), and Caspase-3 Antibody (Cell Signaling Technologies, MA, USA).

Plasmid transfection. HeLa and SaOs-2 cells were plated in six-well plates (9.6 cm^2/well) and the following day were transfected with 0.2 μg of plasmid DNA/dish using Effectene™ (Qiagen, Valencia, CA, USA) according to the manufacturer's standard protocol. Plasmids used were *pcDNA3p66Shc* (kindly provided by Dr. Yoshikuni Nagamine, Friedrich Miescher Institute, Basel, Switzerland), *pcDNA3.1* empty vector, and *pcDNA3p66S36A* (S36 A36, provided by Dr. Yoshikuni Nagamine).

siRNA transfection. To perform isoform-specific knockdown, the following 21-mer oligoribonucleotide pairs (Qiagen) were used: $p66^{Shc}$ *siRNA* 5′-gAA UgA gUC UCU gUC AUC gUC-3′ and 5′-CgA UgA CAg AgA CUCAUU CCG-3′, from nt 236–256 (in the CH2 domain); *non-silencing siRNA* 5′-UUC UCC gAA CgU gUC ACg U-3′ and 5′-ACg UgA CAC gUU Cgg AgA A-3′ as negative control.

HeLa and SaOs-2 cells were plated in six-well plates in DMEM 10% FBS, 2 mM L-Gln (without antibiotics) at 2×10^5 cells/well. The following day siRNAs were introduced into cells using the Oligofectamine™ Reagent (Invitrogen) according to the manufacturer's instructions, using 10 μl of 20 μM siRNA and 3 μl of transfection reagent/well.

Cytofluorimetric analysis. Flow cytometric analyses were performed using a FACScan flow cytometer (FACScalibur, BD). A minimum of 10,000 cells was acquired in list mode for each sample. Analysis was performed using CellQuest software (BD).

The percentage of apoptotic cells was detected by Annexin V-FITC (ANX-V) and PI double staining (rh-annexin V/FITC kit, Bender MedSystems™, Vienna, Austria). Cells were detached by trypsinization, centrifuged, and resuspended in 200 μl of appropriate binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and incubated for 10 min at room temperature in the dark with 5 μl of Annexin V-FITC-conjugated. Cells were washed in PBS and finally resuspended in 300 μl of binding buffer plus 10 μg/ml propidium iodide.

Mitochondrial membrane potential was assessed by JC-1 staining (Molecular Probes, Eugene, OR, USA). Briefly, cells were resuspended in 1 ml of complete medium and incubated for 10 min at room temperature in the dark with 5 μM JC-1, then washed and resuspended in 200 μl PBS, and analysed as previously described [9].

Results

$p66^{Shc}$ overexpression in p53−/− human cell lines is able to increase H_2O_2-mediated apoptosis

To investigate the apoptotic role of $p66^{Shc}$ in cells lacking *p53* function, we evaluated the exposure of phosphatidylserine residues (considered as an early apoptotic marker) in HeLa and SaOs-2 cells transfected with an empty plasmid (pcDNA3.1) or a plasmid encoding $p66^{Shc}$ (pcDNA3p66shc; provided by Dr. Yoshikuni Nagamine, Friedrich Miescher Institute, Basel, Switzerland [10]). The $p66^{Shc}$ sequence present in the pcDNA3 vector is of mouse origin and it contains a mutation in the potential translation initiator methionine codons of the $p46^{Shc}$ and $p52^{Shc}$ isoforms (converted into leucine codons), and thus, it is able to express only the $p66^{Shc}$ form. Mouse amino acidic $p66^{Shc}$ sequence displays a high degree of homology (94%) with human $p66^{Shc}$ protein, particularly in CH2 domain (GenBank®/Mouse 14211983 and Human NM_183001). Cells were transfected and $p66^{Shc}$ protein expression analysed by Western blot. After 24 h from transfection with pcDNA3p66shc plasmid, an increase of $p66^{Shc}$ expression was detected by Western blotting in both HeLa and SaOs-2 cells, as compared to cells transfected with the empty vector (data not shown). Cytofluorimetric analysis of cells co-stained with Annexin V-FITC and propidium iodide (PI) showed that a negligible percentage of apoptotic cells was detectable after transfection with the control empty vector (Figs. 1A and E). Similarly, after 24 h from transfection with pcDNA3p66shc, cells did not show any dramatic increase of apoptosis or necrosis (Figs. 1B and F). On the contrary, when treated with 50 μM H_2O_2 for 30 min and allowed to recover for further 24 h, cells transfected with the empty vector showed a consistent percentage of cell death (Figs. 1C and G). When treated with 50 μM H_2O_2 for 30 min and allowed to recover for further 24 h, cells transfected with pcDNA3p66shc showed a greater increase in the percentage of apoptotic and necrotic cells, as compared to cells transfected with the empty vector (Figs. 1D and H, compared with Figs. 1C and G). Fig. 1I summarizes the results of three separate experiments and reports the percentage of cell death (apoptosis + necrosis) in SaOs-2 and HeLa cells transfected with pcDNA3.1 empty vector or pcDNA3p66shc and treated