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A nuclear export signal and oxidative stress regulate ShcD subcellular localisation: A potential role for ShcD in the nucleus

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ABSTRACT

Tumour cells alter their gene expression profile to acquire a more invasive and resistant phenotype. Overexpression of the signalling adaptor protein ShcD in melanoma was found to be a prerequisite for melanoma migration and invasion. In common with other Shc proteins, ShcD has been shown to be involved in coupling receptor tyrosine kinases to the Ras–mitogen activated protein kinase signalling pathway, and to have a predominant cytoplasmic distribution. Here we report that ShcD can exist within the nucleus, and show that its CH2 domain has a critical role in nuclear export of ShcD. Analysis of GFP-tagged ShcD mutants containing deletions or amino acid substitutions within the CH2 domain revealed ^{83}LCTLIPRM^{90} as a functional nuclear export signal. We have further demonstrated that ShcD accumulates in the nucleus upon hydrogen peroxide treatment in FLAG–ShcD expressing HEK293 cells, as well as 518.A2 melanoma cells. Cross linking experiments showed that a proportion of ShcD is associated with DNA. Moreover we have shown that ShcD fused to the GAL4 DNA binding domain can drive transcription of a GAL4 site-driven luciferase reporter, suggesting a role for ShcD in regulating gene transcription. We suggest that ShcD nuclear translocation might provide melanoma cells with a mechanism that enables them to resist DNA damage due to oxidative stress.

1. Introduction

ShcD, also known as Rai like protein, RaLP, was recently identified as a fourth member of the Shc family of adaptor proteins [1,2]. Shc adaptors participate in signalling pathways mediated by a wide variety of receptors including receptor tyrosine kinases, cytokine receptors, antigen receptors, integrins and G-protein coupled receptors [3]. ShcA, the first Shc protein to be identified, is widely expressed, and exists as three distinct isoforms, p66Shc, p52Shc and p46 Shc, generated as a result of alternative splicing and initiation codon usage [4]. All isoforms contain a phosphotyrosine binding (PTB) domain, a central collagen homology (CH1) domain, and a C-terminal Src homology 2 (SH2) domain. The largest isoform, p66Shc, contains an additional collagen homology domain (CH2 domain) at its N-terminus [3]. The modular nature of the Shc adaptors confers upon them the ability to interact with different proteins through their distinct domains. The PTB and SH2 domains typically bind to tyrosine phosphorylated receptors. The CH1 domain is phosphorylated by tyrosine kinases and contains binding sites for SH2 domain containing proteins, the best characterised of these being Grb2 [5]. Grb2 recruitment to Shc can lead to activation of the Ras–mitogen activated protein (MAP) kinase pathway [6] and phosphatidylinositol 3-kinase [7], and promote cell proliferation and survival. The CH2 domain unique to p66Shc is able to bind to cytochrome c, resulting in the production of reactive oxygen species and apoptosis, in a process that is regulated by phosphorylation of p66Shc at serine residue 36 [8,9]. This accounts for the very different properties of p66Shc when compared to shorter isoforms, as it is the only isoform to promote apoptosis in response to cell stress, and is not involved in inducing cell proliferation [10]. While ShcA is widely expressed, except in the nervous system (11), ShcB and ShcC are mainly expressed in neuronal tissues [12].

ShcD (p69ShcD) has a similar domain structure to the other Shc family members, and also possesses an N-terminal CH2 domain, making it most similar to p66ShcA. Shorter forms are also generated when a plasmid encoding p69ShcD is expressed in HEK293T cells, termed p69#2ShcD, p59ShcD and p49ShcD, which arise as a result of alternative start codon usage [2]. It is not known whether these shorter forms exist naturally, as most expression studies have used an antibody directed against the CH2 domain which is truncated or missing in the shorter isoforms, but by analogy with ShcA, it would seem likely. Interestingly, ShcD also displays a selective tissue distribution pattern. In one report, ShcD was found to be expressed in the muscle tissue and to interact with muscle specific kinase (MuSK) to regulate clustering of acetylcholine receptors at the neuromuscular junction [2]. A study of mRNA expression levels of ShcD in mouse adult and embryonic tissue revealed high levels of expression in the muscle and neuronal tissues [11]. A role for ShcD in neuronal cell signalling is supported by evidence for its association with TrkB receptors [13].

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Interestingly large scale screening of tissue microarrays derived from normal and malignant tissues revealed that ShcD was only expressed at a significant level in malignant melanoma. Of particular interest was the observation that high levels were found at the transition between the radial and vertical growth phase, and very low levels were found in normal melanocytes and benign nevi [1]. When ShcD expression was silenced in melanoma cell lines using siRNA, the tumour cells showed reduced migratory behaviour, and a greatly reduced ability to colonise the lung when injected into mice, when compared to tumour cells expressing ShcD. ShcD was shown to be phosphorylated by IGF1 and EGF receptors and to induce activation of the Ras–MAP kinase pathway, but not Akt. Silencing of ShcD in a melanoma cell line resulted in no effect on Erk phosphorylation, yet suppressed migration suggesting that ShcD induced migration involves pathways other than the MAP kinase pathway [1]. Clearly a better understanding of the function of ShcD could identify new avenues to explore for the treatment of malignant melanoma.

Although most Shc family proteins have been shown to play a role in cytoplasmic signalling, coupling to membrane bound receptors, p46Shc is present in the mitochondria [14], and translocation of p66Shc to mitochondria is required for its pro-apoptotic function [15]. In addition ShcA isoforms have recently been reported to be present in the nuclear compartment, although their role in the nucleus has yet to be determined [16–18]. Cell fractionation studies and western blotting using an antibody directed against the ShcD CH2-domain have indicated that ShcD is predominantly cytoplasmic [1]. Using an antibody directed against the CH1 domain of ShcD we have identified a fraction of ShcD located within the nucleus. Here we report the identification of the nuclear export sequence which determines the predominantly cytoplasmic localisation of p69ShcD, and demonstrate that a p69ShcD is able to accumulate in the nucleus upon oxidative stress. Moreover, preliminary evidence suggests that nuclear p69ShcD could have transcriptional activity.

2. Materials and methods

2.1. Plasmids and transfections

GFP, FLAG and mCherry tagged ShcD constructs were generated by the Protex facility (Biochemistry Department, University of Leicester). All the tags were present at the amino terminus of the ShcD sequence. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). All truncated and ShcD mutants were confirmed via DNA sequencing. Plasmid DNA was transfected into ~70–90% confluent cells for 24 h using an ExGen 500 in vitro Transfection Reagent (Fermentas).

2.2. Cell lines and culture conditions

518.A2, DAUV cell lines are primary cell lines obtained from human metastatic melanomas. They were kindly provided by Dr. Mike Browning (Infection, Immunity and Inflammation Department, University of Leicester) and have been reported previously [19,20]. To establish FLAG–ShcD stably expressing cells, human embryonic kidney cells (HEK293) were transiently transfected with a FLAG–ShcD construct containing the neomycin selection gene. After 24 h, the transfected cells were selected by adding G418 (1 mg/ml). Colonies formed were harvested with cloning discs (Sigma). FLAG–ShcD expressing clones were identified by western blotting with an anti-FLAG antibody (Sigma). All cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% foetal bovine serum and 1% penicillin/streptomycin from Life Technologies. The cells were grown in a humidified incubator at 37 °C provided with 5% CO_2.

2.3. Nuclear cytoplasmic fractionation

Cells were lysed with hypotonic buffer comprising 10 mM HEPES (pH 7.8), 25 mM β-glycerolphosphate, 25 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM EDTA and 0.1% protease inhibitor cocktail (Sigma), and the nuclear membrane was disrupted by addition of 10% NP40 to a final concentration of 0.625% followed by separation of the nuclear fraction by centrifugation at 13,000 ×*g* for 30 s. The nuclear proteins were released by addition of high salt extraction buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 20 mM M NaF, 0.1 mM Na_3VO_4 and 0.1% protease inhibitor cocktail). Both the cytoplasmic fraction and the nuclear fraction were resolved on a 10% SDS-PAGE gel and western blots were then performed by using α-GAPDH (Millipore, MAB374), α-Histone-H1 (AE-4) (Santa Cruz, sc-8030) or rabbit polyclonal anti-ShcD antibody (TB2/4443). The anti-ShcD antibody was raised against a His-tagged fusion protein corresponding to the CH1 domain of ShcD (Cambridge Biosciences). Specificity was verified by knocking down endogenous ShcD using siRNA oligonucleotides supplied by Invitrogen, as described previously [1] and TurboFect siRNA Transfection Reagent (Fermentas).

2.4. Cell imaging

Melanoma cells were seeded for 24 h on acid-treated coverslips in 6 well plates, then transiently transfected with either GFP or mCherry tagged p69ShcD (full length), truncation or mutated versions thereof. The cells were then fixed with 3.7% formaldehyde for 20 min at room temperature followed by mounting and sealing the coverslips on glass slides. The cells were visualised by a Nikon fluorescence microscope using a 100 × 1.4 oil immersion objective or by confocal microscopy. For immunostaining endogenous ShcD, the cells were permeabilised after fixing with 0.1% Triton prior to adding polyclonal rabbit anti-ShcD antibody (1:50) or pre-immune serum from the same rabbit. Alexa Fluor 594-conjugated anti-rabbit antibody (Invitrogen) was used to detect bound anti-ShcD antibody.

2.5. ShcD–DNA association

The nuclear fraction was separated from the cytoplasmic fraction using the protocol described. The extraction of the DNA associated proteins was performed essentially as described previously [21]. Briefly, the nuclear fraction obtained from HEK293 cells that stably express FLAG–RaLP was treated with 1% formaldehyde. The nuclei were disrupted, by sonication, and the DNA–protein complexes were separated from the insoluble nuclear material by centrifugation for 5 min at 13,000 ×*g*. The supernatant was incubated overnight at 4 °C with isopropyl alcohol to precipitate the DNA and bound proteins. The DNA and its associated proteins were then electrophoresed on an SDS-PAGE gel. Western blotting was performed and the proteins were detected with specific antibody against GAPDH, Histone H1 or FLAG (Sigma, F1804).

2.6. Co-immunoprecipitation of Histone H1 and ShcD from chromatin extracts

The nuclear pellet was obtained from DAUV cells as described above, then washed twice with PBS followed by two washes with the chromatin extraction buffer with no nuclease (10 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.25 M sucrose) supplemented with 1 μM okadaic acid. The washed nuclear pellet was then digested by adding 200 μl of chromatin extraction buffer along with nuclease (0.008 units/μl) at 30 °C for 15 min. The soluble chromatin fraction was obtained after centrifugation at 20,000 ×*g* for 10 min at 4 °C. The soluble chromatin fraction was then incubated with immobilised anti-ShcD antibody overnight at 4 °C. Immunoprecipitates were analysed