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The nuclear exporter, Crm1, is regulated by NFY and Sp1 in cancer cells and repressed by p53 in response to DNA damage

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ABSTRACT

The nuclear exporter protein, Crm1, plays a key role in normal cell functioning, mediating the nucleo-cytoplasmic transport of cargo proteins. Elevated Crm1 expression has recently been identified in various tumours; however, the mechanisms driving its expression have not been investigated to date. In this study we identified the Crm1 promoter and factors associated with its elevated expression and with its repression under conditions of DNA damage. The −1405 to +99 Crm1 promoter region was found to be significantly more active in cancer and transformed cells compared to normal, and the −175 to +99 region identified as responsible for the differential activity. Mutation of two CCAAT boxes and a GC box within this region significantly diminished Crm1 promoter activity and ChIP analysis revealed binding of NFY and Sp1 to these sites, with increased binding in transformed and cancer cells. In addition, p53 was found to repress Crm1 promoter activity, after induction with doxorubicin, with p53 siRNA blocking the effect. Crm1 promoter constructs with mutated CCAAT boxes were significantly less responsive to p53 repression, and *in vivo* binding of NFY to the CCAAT boxes was diminished upon p53 binding, suggesting that p53 mediates repression of the Crm1 promoter via interfering with NFY. This was confirmed using NFY knock-down cells, in which Crm1 promoter activity was significantly less responsive to p53. *In vitro* EMSAs revealed that NFY and p53 bind the CCAAT boxes as a single complex under conditions of DNA damage. In summary, this study is a first to analyse Crm1 promoter regulation and reveals NFY and Sp1 as contributors to Crm1 overexpression in cancer. In addition, this study reveals that Crm1 transcription is inhibited by DNA damage and that the mechanism of inhibition involves p53 interfering with NFY function.

1. Introduction

Crm1 (the Chromosome region maintenance 1 protein or Exportin 1) is a member of the Karyopherin β protein family and the major nuclear export receptor in the cell [1]. It mediates the nuclear export of cargo proteins and certain RNAs from the nucleus into the cytoplasm, across the nuclear pore complex (NPC), and thus facilitates protein and RNA subcellular localisation. Crm1 recognises cargo proteins that carry a nuclear export signal (NES) [2], typically containing three to four critically spaced leucines ($LX_{(1-3)}LX_{(2-3)}LXJ$, L: leucine, X: spacer, J: leucine, valine or isoleucine) [3,4]. Such cargo proteins include various transcription factors [5], cell cycle proteins [6,7], and signalling proteins [8], which require timely translocation across the nuclear envelope. Many of the integral processes in the cell thus rely on Crm1 expression and function [9].

Recent studies have reported that the expression of Crm1 is altered in cancer. Crm1 protein levels are elevated in cervical cancer [10], ovarian cancer [11], osteosarcoma [12], glioma [13] and pancreatic cancer [14], with high levels of Crm1 being found to associate with poor patient survival [11–14]. Moreover, Crm1 expression has been found to be elevated in transformed fibroblasts compared to normal fibroblasts, suggesting that the increased expression of Crm1 is a general feature of the transformed phenotype [10]. The high expression of Crm1 in cancer and transformed cells is functionally relevant, as the inhibition of its expression results in cell death via apoptosis, while inhibition of its expression in normal cells does not [10]. This implicates Crm1 as a potential anti-cancer drug target, and currently studies are underway aiming to develop effective inhibitors of Crm1 [15].

The increased expression of Crm1 protein in cancer derives from increased transcription of Crm1 mRNA [10], suggesting that a transcriptional regulatory mechanism exists for differential Crm1 expression in normal and cancer cells, possibly at the level of promoter control. However, little is known about the factors that regulate the Crm1 promoter. The aim of this study was therefore to investigate Crm1 promoter activity and to identify cis- and trans-elements necessary for high Crm1 expression in cancer cells. We report that NFY/CBP, Sp1 and p53 transcription factors bind the Crm1 promoter and play an important role in Crm1 promoter regulation in cancer and transformed cells.

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2. Materials and methods

2.1. Cell culture

WI38 normal lung fibroblasts, SVWI38 transformed WI38 fibroblasts, and CaSki cervical cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% Fetal Bovine Serum (FBS) (Gibco, Paisley, Scotland). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Protein harvest and Western blot analysis

Cells in culture were grown to 80% confluency and lysed on ice in RIPA buffer (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1% deoxycholate, 0.1 % SDS, 1 % Triton X-100, 1× Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)). Western blot analyses were performed using the rabbit anti-Crm1 (H-300) (sc-5595, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-β-tubulin (H-235) (sc-9104, Santa Cruz Biotechnology), rabbit anti-NFYA (H-209) (sc-10779, Santa Cruz Biotechnology), rabbit anti-Sp1 (H-225) (sc-14027, Santa Cruz Biotechnology), mouse anti-p65 (F-6) (sc-8008, Santa Cruz Biotechnology), and mouse anti-p53 (M7001, DakoCytomation, Glostrup, Denmark) antibodies.

2.3. Construction of the Crm1 (−1405 to +99) promoter construct

Primers were designed for amplification of the region from −1405 to +99 (with the transcription start site designated +1) of the Crm1 gene (GenBank Accession Number: NC_000002). Primer sequences were: Crm1 F 5′-AGGCTAGCGCGTTTTTCTTTATTGGAG-GG-3′ (NheI site is underlined) and Crm1 R 5′-AG**AAGCTT**CTGTTGCTCTTGCT-GATGCT-3′ (HindIII site is highlighted in bold). PCR was performed using normal blood DNA as template and the high fidelity Expand Plus DNA Polymerase (Roche). Promoter PCR products were subcloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and excised with NheI and HindIII restriction enzymes for subcloning into the luciferase reporter plasmid pGL3-Basic (Promega). Cloned promoter constructs were verified by sequencing using the UCT Human Genetics Sequencing Unit.

2.4. Generation of Crm1 promoter deletion constructs

Promoter deletion constructs were generated using either restriction sites common to both the cloned promoter fragment and the pGL3-Basic multiple cloning site (SacI for the −394 to +99 construct; NheI for the −82 to +99 construct), or gene-specific forward primers (5′-AGGCTAGCTCTCCGTCACCGAAGAGG-3′ for the −500 to +99 construct; 5′-AGGCTAGCGTGAGGCGGGATTGACTG-3′ for the −175 to +99 construct; NheI restriction site is underlined) and the Crm1 R primer.

2.5. Luciferase assays

100 ng of each promoter construct was transfected into 30000 cells/well of a 24-well plate, using 0.3 μl Transfectin (Bio-Rad, Hercules, CA, USA). To normalise for transfection efficiency the cells were co-transfected with 10 ng of the pRL-TK plasmid (Promega) that encodes Renilla luciferase. Total cell lysates were prepared from cells 24 h post-transfection using Passive Lysis Buffer (Promega) and firefly luciferase activity was assayed using the Dual Luciferase Kit (Promega). Luminescence was monitored using the Glomax 96 microplate luminometer (Promega).

2.6. Site-directed mutagenesis of potential transcription factor binding sites

Mutations in potential transcription factor binding sites in the Crm1 (−175 to +99) promoter construct were prepared by site-directed mutagenesis, using mutagenic primers. Primer sequences were:

CCAATbox1mut: 5′-GACTGGCGCTCGGGCtAgcGAGAGATAGGCT-CATG-3′ and
5′-CATGAGCCTATCTCTCgcTaGCCCGAGCGCCAGTC-3′,
YYmut: 5′-CAATGAGAGATAGGCTagcGGCGCGGCTGTAGCTG-3′ and
5′-CAGCTACAGCCGCGCCgctAGCCTATCTCTCATTG-3′,
CCAATbox2mut: 5′-CTGTAGCTGGAACTGAC**gAATtc**GAGGGCGGG-CTGGGGG-3′ and 5′-CCCCCAGCCCGCCCTC**gaATTc**GTCAGTTCCAGCTA-CAG-3′,
Sp1site1mut: 5′-GGCGGGCTGGGGGCtagcGCCCAGGTTCCAAAC-3′ and
5′-GTTTGGAACCTGGGCgctaGCCCCCAGCCCGCC-3′,
Sp1site2mut: 5′-GC-TGGAACTGACCAATgctAGcGCGGGCTGGGGGC-GGAG-3′ and
5′-CT CCGCCCCCAGCCCGCgCTagcATTGGTCAGTTCCAGC-3′,
GCboxmut: 5′-GTTTGAGGCACTAGctcGAGGGGGAGAAGCGG-3′ and
5′-CCGCTTCTCCCCCTCgagCTAGTGCCTC-AAAC-3′

(mutated bases are indicated in lowercase; NheI restriction sites are underlined; EcoRI restrictions sites are in bold; XhoI restriction sites are double underlined). Following PCR amplification, 10 U DpnI (Promega) was added and digestion carried out at 37 °C for 90 min, before transformation into JM109 highly competent cells (Promega). Restriction digestion analysis was used to identify clones carrying the respective mutations.

2.7. siRNA transfection

For the effect of siRNA on Crm1 promoter activity, 40 000 CaSki cells/well of a 24-well plate were transfected with 30 nM control siRNA-A (sc-37007, Santa Cruz Biotechnology), NFYA siRNA (sc-29947), Sp1 siRNA (sc-29487) or p53 siRNA (sc-29435), and 24 h later transfected with 50 ng Crm1 (−175 to +99) promoter construct and 5 ng pRL-TK plasmid. Luciferase activity was measured 24 h later and expressed relative to Renilla luciferase in the same extract.

2.8. Chromatin immunoprecipitation (ChIP) assay

Cells were grown to approximately 90 % confluency and protein–DNA complexes cross-linked with 1% formaldehyde for 10 min, followed by the addition of 0.125 M Glycine, pH 2.5. Cells were harvested, lysed in lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris–Cl, pH 8.1, 1× Complete Protease Inhibitor (Roche)), and sonicated to lengths of between 400 and 1000 bp. Cell lysates were diluted in dilution buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–Cl, pH 8.1, 1× Complete Protease Inhibitor) and then precleared with protein-A agarose beads (Merck, NJ, USA) for 2 h. Beads had been previously blocked in 100 μg/ml salmon sperm DNA and 5% BSA. Chromatin was incubated with 2 μg antibody (α-NFYA (H-209 X, sc-10779 X, Santa Cruz Biotechnology); α-Sp1 (H-225, sc-14027, Santa Cruz Biotechnology); or no antibody negative control) at 4 °C overnight. Protein-A agarose beads were added for a further 2 h at 4 °C, and immunocomplexes bound by the beads recovered by centrifugation and washed twice sequentially in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–Cl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–Cl, pH 8.1, 500 mM NaCl), Buffer III (0.25 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris–Cl, pH 8.1) and TE, pH 7.4. Bound material was eluted using elution