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SHORT COMMUNICATION

The activity of *hsp90α* promoter is regulated by NF-κB transcription factors

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Heat-shock proteins (HSP) 90 exert a relevant role in the survival and response to therapy of many neoplastic cell types. Here, we show that the promoter of *hsp90α* gene, that encodes the inducible form of HSP90, is regulated by nuclear factor-κB (NF-κB) activity. Indeed, we found that NF-κB factors bound to one of the two putative consensus sequences present in the *hsp90α*-flanking region; mutation of such motif hampered the phorbol-myristate-13-acetate-stimulated expression of a luciferase reporter gene under the control of the *hsp90α* promoter. Furthermore, the downmodulation of NF-κB (p65) levels by a specific small interfering (si) RNA resulted in reducing the levels of endogenous HSP90α protein. These findings disclose a previously unrecognized mechanism that contributes to connect NF-κB factors and HSPs in cell defence machinery.
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The heat-shock protein (HSP) 90 belongs to the class of molecular chaperones that participate in the normal folding, intracellular localization and proteolytic turnover of several proteins, including key regulators of cell growth and survival (Pearl and Prodromou, 2003; Richter *et al*., 2007). Two major cytoplasmic forms, α (inducible) and β (constitutive), of the protein are recognized; a third isoform, HSP90N, appears to derive from a very recent gene rearrangement of *hsp90α*, while two HSP90 analogues, Grp94 and HSP75/TRAP1, are located in the mitochondrial matrix (Young *et al*., 2004). Owing to its inducible expression, HSP90α exerts a relevant role in cell homeostatic responses to stressful conditions. In neoplastic cells, this chaperone is implicated in cell protection against proapoptotic stimuli, originating from de-regulated proliferation or chemotherapeutic agents (Whitesell and Lindquist, 2005).

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Among proteins that participate in antiapoptotic pathways, there are nuclear factor-κB (NF-κB) transcription factors (Maiuri *et al*., 2004; Karin and Greten, 2005). Two NF-κB putative consensus sequences (seq. 1 and 2) are present in the *hsp90α* (and not in the *hsp90β*) flanking region (Figure 1A). Nuclear extracts from tumor necrosis factor (TNF)-α-stimulated HeLa cells bound selectively to seq. 1 in electrophoretic mobility shift assays (EMSA) (Figure 1B). To confirm the interaction of NF-κB factors with seq. 1, we stimulated HeLa cells with 10 ng/ml TNF-α for 2 or 4 h and used an anti-p65 antibody for chromatin immunoprecipitation (ChIP) (Tanaka and Nasmyth, 1998); rabbit IgGs were used as a negative control. p65 binding to Hsp90 ακB seq. 1 was evident in immunoprecipitates from 2 to 4 h stimulated cells (Figure 1C) Promoter sequences from nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha (IκBα) and β-actin were used as positive and negative controls, respectively.

To investigate the activation of the *hsp90α* promoter by NF-κB factors, we performed site-directed mutagenesis of either seq. 1 or 2 in a construct (HspLuc1430) carrying a luciferase reporter gene driven by the *hsp90α* promoter and analysed luciferase levels in HEK-293-transfected cells. As shown in Figure 2, cells transfected with the wild type (HspLuc1430) or mutated (HspLuc1430 no. 1; HspLuc1430 no. 2) constructs displayed similar basal levels of luciferase activity. When the cells were incubated with the NF-κB activating agent phorbol-myristate-13-acetate (PMA), we detected increased levels of luciferase activity in cells transfected with either HspLuc1430 or HspLuc1430 no. 2, but not in cells transfected with HspLuc1430 no. 1 (Figure 2). These experiments confirmed that seq. 1 regulated the activity of the *hsp90α* promoter.

We then verified whether NF-κB activity influenced HSP90α protein levels. First, we analysed the time- and dose-dependent effect of TNF-α on HSP90α amounts: HSP90α increase was particularly evident after 4 h of cell incubation with the cytokine (Figure 3a) and using a TNF-α concentration of 10 ng/ml (Figure 3b). We then transfected the cells with a small interfering (siRNA) specific for p65 or, for comparison, with a heat shock protein (HSF)1-specific siRNA, since several HSP-1-responsive elements (HSE) are present in the *hsp90α* promoter (NCBI GenBank U25822gi|793941|gb|U25822.1|HSU25822, TESS) and regulate its acti-

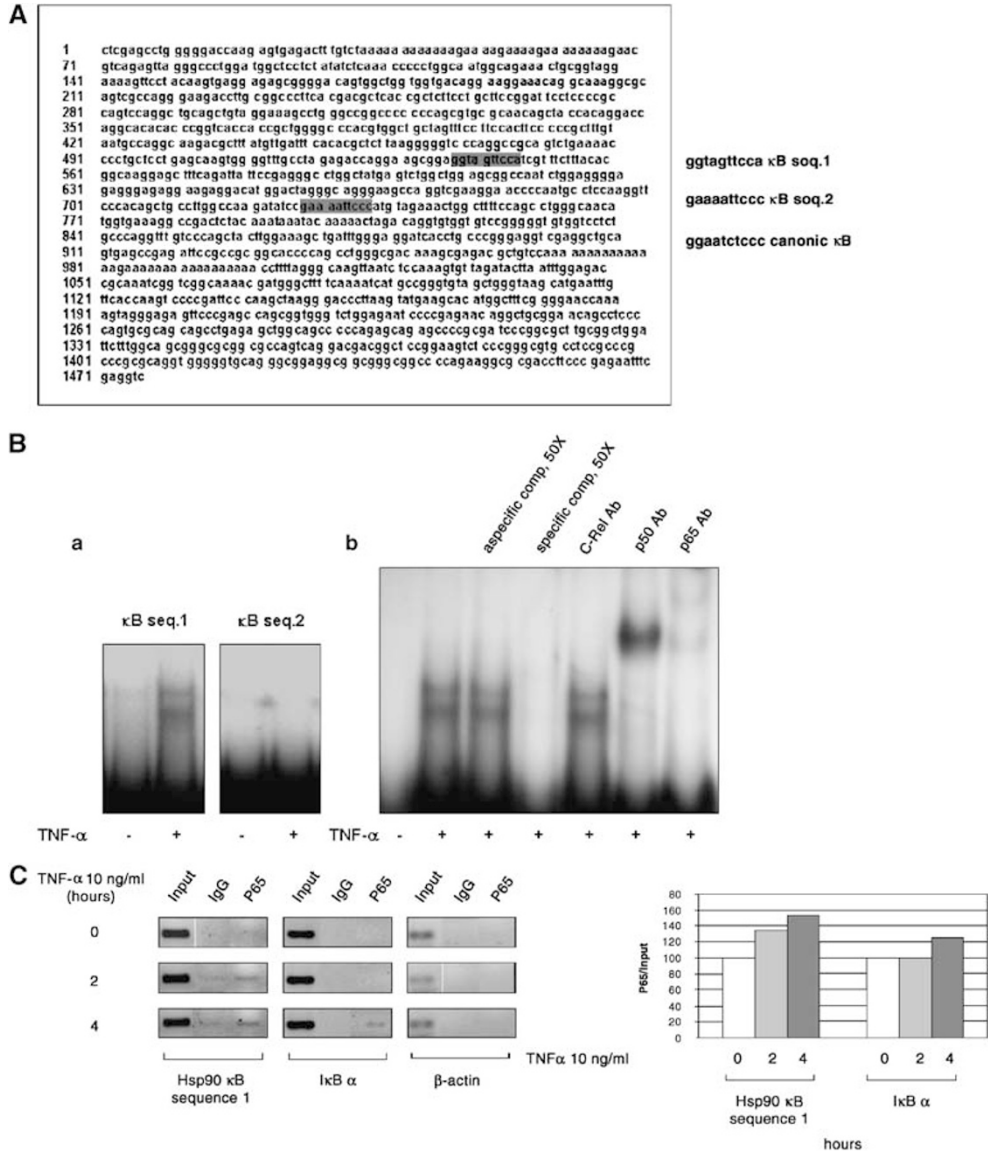

Figure 1 Binding of NF-κB factors to a consensus sequence in the *hsp90α* gene promoter. (**A**) Sequence of the human *hsp90α* gene promoter (NCBI GenBank U25822gi|793941|gb|U25822.1|HSU25822, TESS): two putative NF-κB-binding motifs are outlined. (**B**) HeLa cells (American Tissue Culture Collection, Rockville, MD, USA) were plated in 60 mm Petri dishes at 80% of confluency and incubated with 10 ng/ml TNF-α (Roche, Indianapolis, IN, USA) for 1 h. (a) Nuclear extracts were obtained and analysed as described (Romano *et al*., 1999) for their binding to ^{32}P-labelled-κB oligonucleotides, corresponding to sequences 1 (5′-AGCGGAGG TAGTTCCATCGTTT-3′ and 5′-TCGCCTCCATCAAGGTAGCAAA-3′) or 2 (5′-ATATCCGAAAATTCCCATGTAG-3′ and 5′-TATAGGCTTTTAAGGGTACATC-3′), in EMSA. (b) Nuclear extracts from TNF-α-stimulated cells were incubated with 50-fold concentrated specific or non-specific (Oct-1 consensus) cold competitors, or with antibodies recognizing NF-κB subunits (anti-c-Rel, -p50 or -p65 polyclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein-oligo complexes were analysed by EMSA. Supershifted complexes are indicated by the arrows. (**C**) HeLa cells were stimulated with 10 ng/ml TNF-α for 60 and 120 min. ChIP assay was performed according to the manufacturer's (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) instruction. Briefly, HeLa cells were collected at the indicated times and nuclear extracts were obtained using NE-PER Kit (Pierce, Rockford, IL, USA). Chromatin from HeLa cells was formaldehyde crosslinked, shared by sonication (four times, 15 s each, one-third power) and immunoprecipitated with p65 antibody (anti-p65 polyclonal antibody C-20, Santa Cruz Biotechnology) followed by incubation with protein A-Sepharose saturated with salmon sperm DNA. IgGs from a rabbit serum (DAKO A/S, Glostrup, Denmark) were used as negative control. After reversal of the crosslinking, the purified DNA fragments were subjected to PCR to amplify a segment spanning the κB seq. 1 on the Hsp90α promoter. Input DNA from fragmented chromatin before immunoprecipitation was used to monitor equal starting conditions. Primers used in PCR were: *hsp90α* κB seq. 1, FWD: 5′-CCAGGCAAGACGCTTTATGT-3′ and REV: 5′-CTCCAGCCAGACTCATAGCC-3′; IκBα promoter (−316 to −15), FWD 5′-GACGACCCCAATTCAAATCG-3′ and REV 5′-TCAGGCTCGGGGAATTTCC-3′; β-actin promoter (−980 to –915), FWD 5′-TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGC CATAAAAGGCAA-3′. IκBα promoter primers were designed to amplify the κB responsive elements as positive control. β-Actin promoter primers were used as negative control. The products were analysed on 1.5% (w/v) agarose gels and stained with ethidium bromide. The graph depicts the values obtained by PCR bands using densitometry. Bands obtained by immunoprecipitation with anti-p65 antibody were normalized against DNA input; the baseline (T_0) derived from untreated cells was set at 100. ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor.

vity (Morimoto *et al*., 1992). Transfected cells were incubated with 10 ng/ml TNF-α for 4 h and total lysates were analysed by western blotting. Downmodulation of p65 levels resulted in reducing HSP90α levels by $>50\%$.

A number of evidence indicated that HSP90α is required for inhibitor of kappa light polypeptide gene

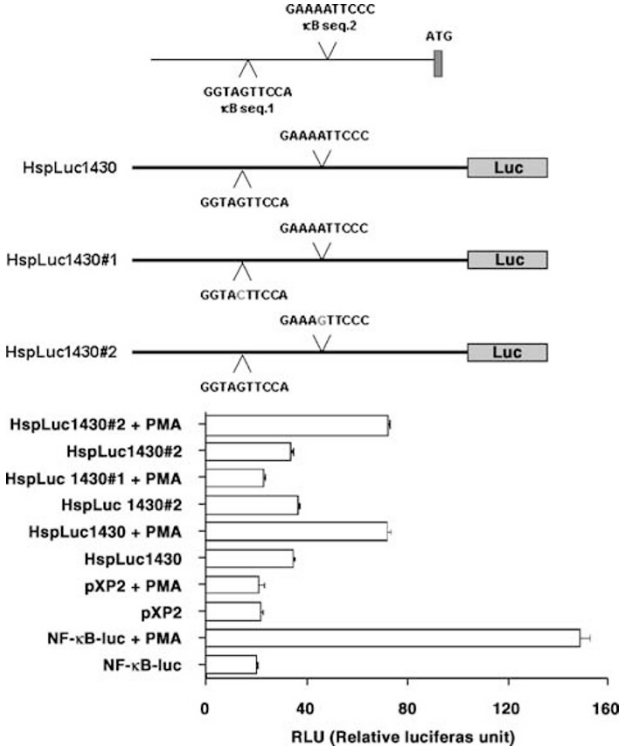

Figure 2 Analysis of the expression of a luciferase reporter gene under the control of wild type or mutated *hsp90α* gene promoter. The 5′-flanking region of the human *hsp90α* gene promoter (HSPLuc1430) and promoterless vector (pXP2) for luciferase assays were kindly provided by Professor KJ Wu, IBMB, National Yang-Ming University, Taiwan. NF-κB-luciferase reporter construct (NF-κB-luc) containing five κB elements was a gift from Dr Andrew Bowie, Department of Biochemistry and National Pharmaceutical Biotechnology Centre, Trinity College, Dublin 2, Ireland. Site-directed mutagenesis of the *hsp90α* promoter inserted in HspLuc1430 was performed by PCR using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's directions. The mutagenic primers used for generating HspLuc1430 no. 1 and HspLuc1430 no. 2 mutants were respectively: sense 5′-GGA AGCGGAGGTACTTCCATCGTTTCT-3′, antisense 5′-AGAA ACGATGGAAGTACCTCCGCTTCC-3′; and sense 5′-CCAAG ATATCCGAAAGTTCCCATGTAGAAAC-3′, antisense 5′-GT TTCTACATGGGAACTTTCGGATATCTTGG-3′. PCR was performed using PfuTurbo DNA polymerase, HspLuc1430 as template and the proper mutagenic primers. PCR products were digested with DpnI endonuclease and vectors DNA containing the desired mutations were then transformed into XL1-Blue supercompetent cells. The presence of the correct mutated sequence and the absence of PCR-derived alterations in the promoter sequence were confirmed by completely sequencing both strands of each construct. The mutations chosen generated sequences without homology to κB-binding sites, as assessed by a search made in TESS (Transcription Element Search System, UPENN, Philadelphia, PA, USA, http://www.cbil.upenn.edu/cgibin/tess/tess database). HEK-293 cells (ATCC) (5×10^6) were transiently transfected with luciferase reporter gene under the control of the *hsp90α* gene promoter region, either wild type (HspLuc1430) or mutated in the NF-κB consensus seq. 1 (HspLuc1430 no. 1) or 2 (HspLuc1430 no. 2), using FuGene 6 transfection reagents (Roche). Cells were stimulated with the NF-κB inducer PMA (Sigma Chemical Co., St Louis, MO, USA) (10 ng/ml) and after 48 h luciferase activity was assayed using the Dual-GloTM Luciferase Assay System (Madison, WI, USA), according to the manufacturer's instruction, and a Perkin Elmer luminometer (Boston, MA, USA). NF-κB, nuclear factor-κB.

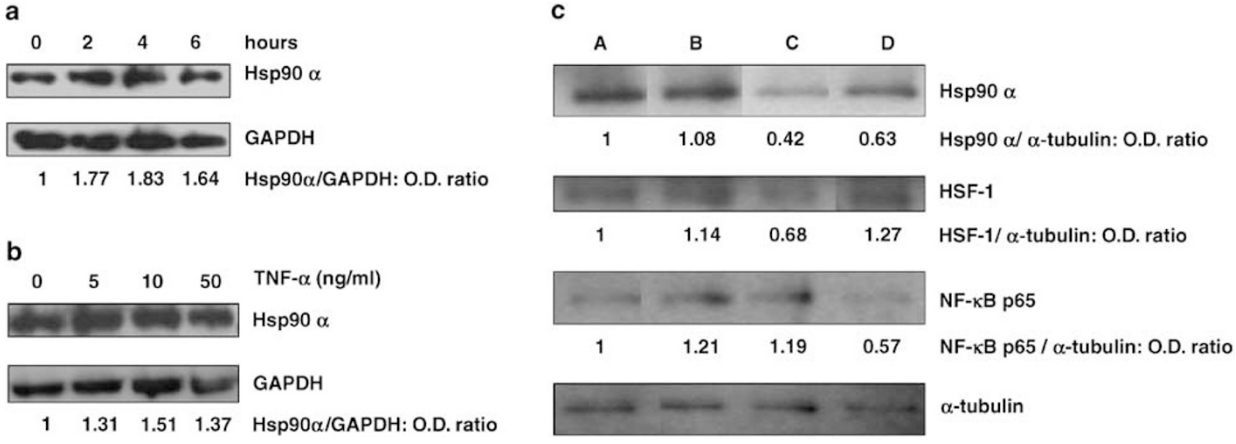

Figure 3 Effect of a p65-specific siRNA on HSP90α protein levels. (**a**) HeLa cells were incubated with 10 ng/ml TNF-α for 2, 4 or 6 h; then whole cell lysates were obtained and analysed with an anti-HSP90α antibody (rat anti-Hsp90α monoclonal antibody, Stressgen, Victoria, Canada) in western blot. (**b**) HeLa cells were incubated with 10 ng/ml TNF-α for 2, 4 or 6 h; then lysates were obtained and analysed with anti-HSP90α antibody in western blot. (**c**) Cells were transfected with siRNAs targeting the p65 subunit of NF-κB mRNA (5′-GCCCUAUCCCUUUACGUCA-3′) or HSF-1 mRNA (5′-AAAUCAAGAGGAAAGUCCTT-3′), respectively, or with a negative control siRNA (5′-CAGUCGCGUUUGCGACUGG-3′) (Dharmacon, La Fayette, CO, USA), at the final concentration of 20 nM, using TRANSIT-TKO (Mirus, Madison, WI, USA). After 48 h, the cells were treated with TNF-α 10 ng/ml for 4 h. Whole cell lysates were obtained and analysed with anti-HSP90α, -p65 (C-20, Santa Cruz Biotechnology) or HSF-1 (E-4, Santa Cruz) in western blot. A = no siRNA; B = control siRNA; C = HSF-1-targeting siRNA; D = p65-targeting siRNA. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor.

enhancer in B cells kinase (IKK) biosynthesis (Broemer *et al*., 2004) and for constitutive and inducible IKK and NF-κB activation (Chen *et al*., 2002; Field *et al*., 2003; Broemer *et al*., 2004; Pittet *et al*., 2005; Mitsiades *et al*., 2006). Our results demonstrate that, in turn, NF-κB activity can influence *hsp90α* expression. The reciprocal interactions of HSP90α and NF-κB activities are likely to constitute a regulatory loop, able to influence cell survival and response to stressful agents.

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