Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 10 (1): 1-16, 2015
ISSN 1815-9923 / DOI: 10.3923/ajb.2015.1.16

Identifying the Putative Interacting Partners of the Heat Shock Protein, HtpG through Phage Display Library Screening

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ABSTRACT

Heat Shock family of Proteins (HSPs) is conserved from prokaryotes to eukarutes and used as an antitumores. The mechanism of action of many antitumor proteins is still obscure *in vivo*. HtpG protein is a prokaryotic member of HSPs. In the present study, the *htp*G gene from *E. coli* was amplified by polymerase chain reaction and cloned in frame into the pGEX-2T DNA vector. The recombinant plasmid used to overexpress the GST-HtpG fusion protein. The GST-HtpG fusion and GST proteins were purified by chromatographic technique and applied with 15 mer phage library to identify polypeptidies which bind with the HtpG protein. Identification of such polypeptieds helped us to identify partner proteins that bind with the HSPs *in vivo*. Thirteen of the 15 mer amino acids sequences were also identified to bind with HtpG protein. Proteins that have homology sequences with the 15 mer amino acids sequences were identified from protein data base. The binding domain of the HSPs with client or partner proteins such as thyroid hormone receptor, protein kinase, nitric oxide synthase, SMC protein family, DNA polymerase, DNA topoisomerase, 50S ribosomal protein L2, ATP synthase, P53 and a quite number of proteins were detemined. This data indicate that the HtpG and HSPs bind with many proteins *in vivo* which could facilitate their protein folding.

Key words: HSP, phage display, cloning, purifcation, GST, GST-HtpG

INTRODUCTION

Proteins play a vital role in various cellular processes such as cell division and prolifiration in both eukaryotes and prokaryotes. Cell division and proliferation are carried out in eukaryotes by the aid of a large number of protein families. Some of these proteins complete their function alone but others often achive these functions by binding with another protein partners to form a complex through protein-protein interaction that able to complete their entire role *in vivo* (Marcotte *et al*., 1999; Huang and Oliff, 2001).

Many powerful techniques are being used to investigate protein-protein interaction. The powerful technology of phage display has been extensively used and previous studies have shown that recombinant libraries displaying short random peptides can be valuable resources for identifying peptide sequences that have affinity to various targets. These libraries have yielded peptide sequences that are similar to the epitopes of antigens to define the binding specificity of monoclonal antibodies (Devlin *et al*., 1990; Lane and Stephen, 1993; Chowdhary *et al*., 2007). The phage display was previously used to identify short peptides which inhibit vascular endothelial tumor growth factor activity, interleukin-6 receptor (Su *et al*., 2005), targeting neuropilin as an

antitumor strategy in lung cancer (Chen *et al*., 2005a), tumor necrosis factor-alpha (Shibata *et al*., 2004) and to elucidate the mechanism of action of anti-HER2 monoclonal antibodies (Baselga and Albanell, 2001).

HSP90 proteins, named according to the 90 kDa normal molecular weight of their members, are highly conserved molecular chaperones that account for 0.5-2% of total cellular proteins in most cells under non-stress conditions (Csermely *et al*., 1998; Buchner, 2010). Molecular heat shock proteins, such as eukaryotic protein 90 (HSP90), give confidence the accurate folding of client proteins. HSP90 proteins compose one of the most preserved Heat Shock Protein (HSP) families (Scheibel and Buchner, 1997). These proteins participate in the regulation of the stress response (Ali *et al*., 1998; Chen *et al*., 2005b) and when associated with other co-chaperones function in correctly folding newly synthesized proteins, stabilizing and refolding denatured proteins after stress, preventing misfolding and aggregation of unfolded or partially folded proteins (Zuehlke and Johnson, 2010) and assist in protein transport across the Endoplasmic Reticulum (ER) and organellar membranes (Nadeau *et al*., 1993; Colombo *et al*., 2008). These proteins participate in the regulation of the stress response. However, when associated with other co-chaperones they play a major role in folding the newly synthesized proteins correctly, stabilizing and refolding denatured proteins after stress, preventing misfolding and aggregation of unfolded or partially folded proteins and assist in protein transport across the Endoplasmic Reticulum (ER) and organellar membranes HSP90 protein is found in Eubacteria.

Eubacteria have a single homologue of HSP90 known as high temperature protein G (HtpG) located on the chromosomal genome (Phillips *et al*., 1987). HtpG is encoded by the *htp*G gene which located between *dna*ZX and *adh* at 11.1 min on the *E. coli* chromosome (Bardwell and Craig, 1987). The *htp*G gene can be detected from *E. coli* with little effect on viability; *htp*G deletion mutants are feasible up to 1°C below the maximum temperature for feasibility of wild type cells (Bardwell and Craig, 1988). The *E. coli* HtpG has physicochemical and functional properties which are comparable to its eukaryotic homologues (Jakob *et al*., 1995). However, its *in vivo* functions, even though not clear, may be quite different from those of its eukaryotic counterparts. It is extremely preserved all the way through evolution, 41% of its residues are similar among those of *Drosophila* hsp83 and 42% are the same by means of the human hsp83 (Bardwell and Craig, 1987).

The present study, aims to look for structural functional relationship of the HtpG protein and its protein partners *in vivo*. Morover, to identify protein partners that combine with the HtpG protein to achieve its entire role *in vivo*.

MATERIALS AND METHODS

Chemicals: All the chemicals, restriction enzymes, DNA polymerase and DNA ligase used were AR or molecular biology grade and obtained from Sigma, BDH chemicals LTD, Bio-Rad and stored as directed by supplier.

Bacterial strains and plasmid DNA: AB1157 (F-*thi-1 thr-1 araC14 leuB6* Δ (*gpt proA2*) *62 lacY1 tsx-33 galK2 λ-Rac-hisG4 rfbD1 rpsl31 str*^{R} *kdgK51 xylA5 mlt-1 argE3*) (Bachmann, 1972), BL21 (F-*ompT hsdS* (r_B-m_B^-) *gal dcm*) (Phillips *et al*., 1984), DH5 (*supE44 hsdR17 recA1 endA1 gyrA96thi-1 relA1*) (Hanahan, 1983), W3110 (F-*IN* (*rrnD-rrnE*)*1*) (Bachmann, 1972) were kindly provided by Dr. Picksley, S.M. (Biomedical Science Department, Bradford University, UK). pGEX-2T (pGEX-2T GST gene fusion plasmid IPTG inducible Ap^R) Amersham Pharmacia Biotech, pMMY001 (pGEX-2T-*htp*G IPTG-inducible Ap^R).

Media and growth conditions

Luria-bertani medium: LB medium was made by dissolving 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl in 1 L deionised water and sterilized by autoclaving. The LB agar plates were prepared by adding 20 g agar to 1 L of LB medium. The LB media was supplemented with 100 μg mL^{-1} ampicillin (LBA).

Yeast-Tryptone (YT) medium: The 2x YT medium was made by dissolving 10 g yeast, 16 g bacto-tryptone extract and 5 g NaCl in 950 mL deionised water. The pH was adjusted to 7.0 with 5 N NaOH and the volume of the solution completed to 1 L with deionised water and sterilized by autoclaving.

DNA manipulation: Extraction, purification and manipulation of both chromosomal and plasmid DNA were carried out as described by Sambrook *et al.* (1989).

Polymerase Chain Reaction (PCR): Oligonucleotide DNA forward (5'TGAGGGGATC CTACATGAAAGGAC3') and reverse (5'GAAGGTCATCCGGGATTCCATCA3') primers were designed in frame to amplify the *htp*G gene from *E. coli* AB1157 chromosomal DNA. The PCR reaction was carried out according to Youssef and Al-Omair (2008). Briefly, The PCR reaction protocol includes a denaturation step at 92°C for 5 min; 22 cycles of 92°C for 45 sec, 55°C for 60 sec and 72°C for 2 min and a final extension step at 72°C for 5 min. After electrophoresis on a 1.5% agarose gel, the PCR products were excised, extracted and purified.

***Htp*G gene cloning into pGEX-2T DNA:** An appropriate DNA fragment containing the *htp*G gene treated with the *Eco*RI and *Bam*HI restriction enzymes. A plasmid DNA vector was purified and linearized with *Eco*RI and *Bam*HI restriction enzymes. The *htp*G gene was cloned into pGEX-2T DNA according to the method reported by Youssef and Al-Omair (2008).

Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970).

Overexpression and purification of GST-HtpG fusion protein: The overexpression and purification of GST-HtpG fusion protein was carried out according to Youssef and Al-Omair (2008).

Phage display of affinity ligands: Polystyrene petri dishes (33 mm) were coated with 500 μL of carbonate buffer (pH 9-9.8) containing 0.1-10 μg of purified protein and then the dishes were incubated overnight in a humid box at 4°C to allow protein to bind. The unbound protein was washed three times with 2 mL of PBS (Phosphate-Buffered Saline: 25 mM phosphate, 125 mM NaCl pH 7.4) containing 0.1% v/v Tween 20. The dishes then were blocked with 2 mL of PBST-M (PBS-buffer, 0.1% v/v tween 20, 5% w/v Marvel low fat milk powder) for 1 h at room temperature. The dishes were washed six times with 2 mL of PBS buffer containing 0.5% v/v Tween 20 and 100 μL of the 15 mer fd phage (4×10^{12} TU mL^{-1}) encoded a single random 45 b sequence and expressed 15 residue peptide as a fusion protein complex with coat protein PIII (Devlin *et al.*, 1990) in 500 μL TBST-M buffer were added. The dishes were incubated at room temperature for 3 h to allow the phage to bind. After washing ten times with 2 mL of PBS, 0.5% v/v Tween 20 and five times with 2 mL of PBS to remove unbound phage, the bound phage were eluted with 800 μL

elution buffer (0.1 M HCl-Glycine pH 2.2, 1 mg mL^{-1} BSA) for 20 min by gentle rocking. The eluates were neutralized with 48 μL of 2 M Tris-base (pH unadjusted).

For the amplification of selected phage, the neutralized eluants were used to infect 2 mL of mid-log phase *E. coli* W3110 cells. The cells were left at room temperature for 10 min and then at 37°C for a further 10 min. The whole suspension was transferred to 50 mL Falcon tubes with 10 mL 2xYT medium supplemented with 0.2 μg mL^{-1} tetracycline and incubated at 37°C with shaking at 220 rpm for 30 min. The concentration of tetracycline was increased upto 20 μg mL^{-1} and the incubation continued with shaking for 24 h at 37°C. The tube lids were punctured for extra aeration. The purification of the phage was carried out by centrifugation of the culture at 3800 rpm and 4°C for 45 min to remove the cells. The supernatant was transferred into 15 mL Falcon tubes containing 2 mL PEG-NaCl (Polyethylene glycol 6000: PEG 20% w/v, 2.5 M NaCl) per 10 mL supernatant. The tubes were mixed by inversion more than 30 times and left at 4°C overnight to precipitate the phage. The precipitated phage was centrifuged at 3800 rpm, 4°C for 45 min and the supernatant was removed. The phage pellet was resuspended in 1 mL PBS buffer and transferred into 1 mL eppendorf tube for centrifugation at 4°C and 21,000 RCF for 10 min to remove any residual cells. The supernatant was transferred to another eppendorf tube containing 200 μL PEG-NaCl per 1 mL supernatant, mixed well and placed at 4°C for 2 h for phage precipitation. The precipitated phage was centrifuged at 14,000 rpm for 30 min at 4°C, the supernatant was removed and the phage pellet was resuspended in 1 mL PBS buffer.

Phage ELISA: A 96-well PVC assay ELISA plate was coated with 3 μg/well purified protein in 50 μL 0.1 M carbonate buffer pH (9-9.8) and left overnight at 4°C. The unbound protein was removed by washing three times with 200 μL PBS buffer containing 0.1% v/v Tween 20. The plate was blocked with 200 μL PBST-M buffer for 1 h at room temperature. The plate then washed six times with 200 μL PBS buffer containing 0.5% v/v Tween 20. About 25 μL of phage suspension was diluted with PBST-M buffer and added to each well for 3 h at room temperature. The plate was washed six times with 200 μL PBS buffer containing 0.5% v/v Tween 20 and 50 μL of peroxidase conjugated anti-M13 antibody added for 1 h at room temperature. The plate was washed six times with PBS buffer followed by adding 100 μL substrate development TMB/H_2O_2 (3, 3', 5, 5' tetramethylbenzidine: TMB 0.1 mg mL^{-1}, 0.3% v/v H_2O_2 in 0.1 M sodium acetate pH 6.0) for 30 min. The reaction was stopped by adding 100 μL 1 M H_2SO_4 and the absorbance was measured at 450 nm using a plate reader.

Preparation of phage single stranded DNA: A single colony of bacteria infected with fd phage was picked up and grown for 5 h in 3 mL LB broth supplemented with 20 μg mL^{-1} tetracycline at 37°C, 200 rpm. About 1.5 mL of the infected culture was transferred to an eppendorf tube and centrifuged for 5 min at 10000 rpm. The supernatant was transferred to a fresh eppendorf tube containing 200 μL of 20% (w/v) Poly ethylene glycol (PEG 8000) in 2.5 M NaCl and the components were mixed gently by inverting several times. After 15 min the phage particles was precipitated by centrifugation, 4°C at 10000 rpm for 5 min. The pellet was suspended in 100 μL buffer pH 8.0 and mixed with 50 μL of phenol previously equilibrated with Tris-HCl (pH: 8.0) and left for 30 min at room temperature. After centrifugation the aqueous layer was transferred into eppendorf tube containing 300 μL of 25:1 mixture of absolute ethanol and 3 M sodium acetate (pH 5.2) and mixed well. After 15 min the single stranded DNA was precipitated by centrifugation for 10 min at 10000 rpm. The pellet was rinsed in 200 μL of 70% ethanol and recovered again. The single stranded DNA was dissolved in 50 μL TE buffer and kept at -20°C.

DNA sequencing: The ssDNA sequences was performed by Macrogen Inc. (Macrogen Inc. 1001, World Meridian Venture Center, No. 60-24, Gasan dong, Geumchun Seoul, 153-781, Republic of Korea) and determined by the dideoxynucleotide chain termination method using an automated procedure involving differential fluorescent label. Nucleotide sequences were analysed by using CHROMAS program and DNA was translated into amino acid by ExPASy Proteomics tools (http://kr.expasy.org/) web site.

RESULTS AND DISCUSSION

The aim of the present study is to try to identify a protein partner that might bind to the HtpG protein by using the technique of phage display (Parmley and Smith, 1988; Scott and Smith, 1990). The technique of phage display allows high affinity ligands that interact with the HtpG protein to be identified. Prior to starting such an approach an important step was to express HtpG protein in an easily purified form. One affinity tag commonly used to purify proteins for phage display is the GST (glutathione S transferase) tag (Smith and Johnson, 1988; Bottger *et al*., 1996). To achieve this *E. coli Htp*G gene was cloned into the pGEX-2T vector to generate a GST-HtpG fusion protein.

Genome sequence of *E. coli* MG1655 strain has been reported and the nucleotide sequence of the *htp*G gene was identified as a part of the complete genome sequence (Blattner *et al*., 1997). The *htp*G gene contain 1875 nucleotide base pair (bp) distributed in the following number and percentage 508G (27.09%), 483C (25.76%), 493A (26.29%) and 391T (20.85%).

The PCR oligonucleotide forward and reverse primers were used to amplify the entire *htp*G gene of bacterial strain AB1157 18 bp upstream of the *htp*G gene to 20 bp downstream of the *htp*G gene. The primers incorporated with *Bam*HI and *Eco*RI restriction sites to facilitate the subsequent cloning of the *htp*G gene. To minimize the mutagenic effect of the PCR procedure we utilized cloned *Pfu* DNA polymerase as a proof reading DNA polymerase and the number of PCR cycles was limited to 22 cycles to reduce the effect of polymerase induced errors in the amplification of *htp*G gene. The amplified PCR product was DNA fragment of ~1.9 kbp (1875 bp of the *htp*G gene plus flanking regions upstream and downstream).

Cloning the *htp*G gene into pGEX-2T DNA: The pGEX-2T is a suitable vector for the cloning process because its complete sequence is known. The pGEX-2T contains a useful and unique *Eco*RI and *Bam*HI restriction sites and express GST protein tagged of the target protein of interest HtpG. The pGEX-2T DNA was digested with *Eco*RI and *Bam*HI to linearize 4.3 kbp DNA of the vector. The 4.3 kbp *Eco*RI and *Bam*HI DNA fragment of pGEX-2T and the 1.9 kbp *Eco*RI and *Bam*HI DNA fragment of *htp*G PCR product were ligated using T4 DNA ligase as illustrated in Fig. 1. The ligation mixture was transformed into competent cells of the *E. coli* strain DH5 and plated onto LB agar plates supplemented with 100 μg mL^{-1} ampicillin. The putative clone of interest would be expected to be ampicillin resistant. The recombinant plasmid DNA was designated pMMY001 and isolated on large scale.

Over-expression of GST-HtpG protein: The pMMY001 plasmid was then introduced into the *E. coli* B protease deficient host strain, BL21 and used to express the GST-HtpG fusion protein. A time course of the overproduction of GST-HtpG fusion protein was studied by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration 1 mM at 0 h. The results of

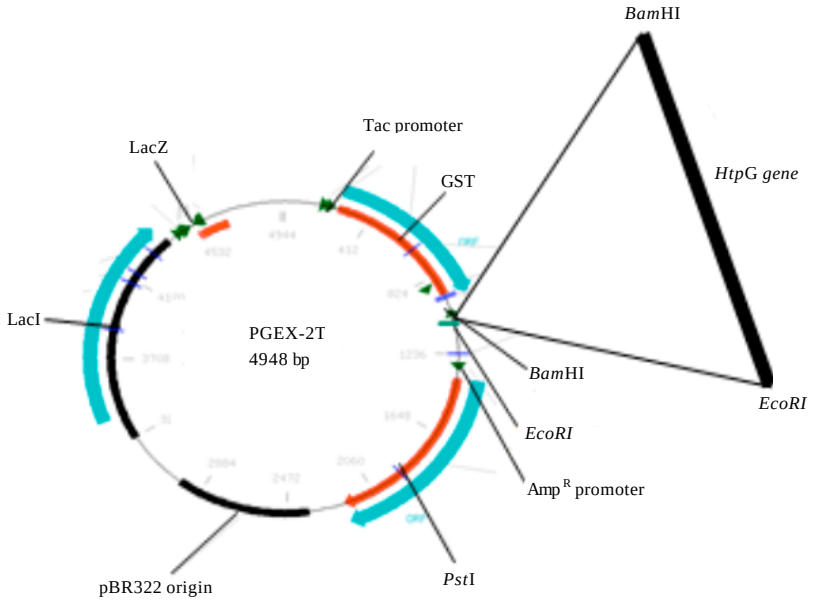

Fig. 1: Schematic diagram of the construct used for overexpression of recombinant lipase. The HtpG gene fused to GST sequence and was cloned downstream of the Tac promoter in pGEX-2T DNA expression vector which also contained the genes for lacI and lacZ repressors, pBR322 origin and ampicillin resistance

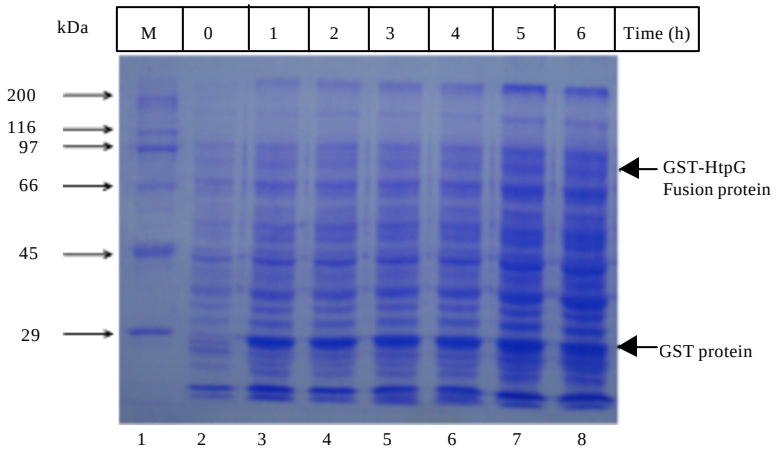

Fig. 2: Induction time course for overexpression of GST-HtpG fusion protein. Early to mid-log culture of *E. coli* BL21with pMMY001 and overexpressing GST-HtpG fusion protein was induced at time 0 hours with IPTG at a final concentration of 1 mM and samples were taken and analysed by 10% SDS-PAGE gel at times indicated (Lane 2-8), protein marker (Lane 1) Sigma SD6H2 (M.Wt 30,000-200,000 kDa)

overproduction of the GST-HtpG fusion protein (~96 kDa) are shown in Fig. 2. The overproduction of GST-HtpG is evident after 1 h of IPTG induction (Fig. 2 Lane 3) and GST-HtpG fusion protein is optimally expressed after 6 h of IPTG induction (Fig. 2 Lane 8).

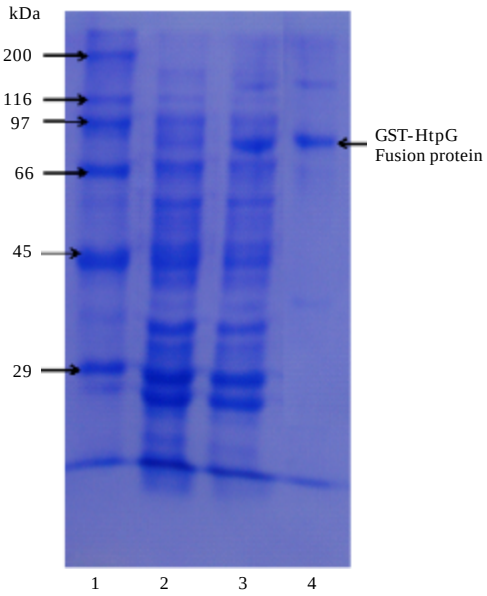

Fig. 3: Purification profile of GST-HtpG fusion protein. Lane 1: protein marker Sigma SDS6H2-1VL (M.Wt 30,000-200,000 kDa). Lane 2: Crude extract of *E. coli* strain overexpressing GST-HtpG protein. Lane 3: GST-HtpG protein eluted from DEAE-sepharose column by using 100 mL gradiant of 50-500 mM of NaCl in buffer A. Lane 4: GST-HtpG protein eluted from glutathione sepharose 4B affinity column by using 25 mL 10 mM reduced glutathione in buffer C

Purification of GST-HtpG fusion and GST proteins: The GST-HtpG fusion protein purified using glutathione-sepharose, after initially removing nucleic acid by applying the cell extract to a DEAE-sepharose column. The results of the GST-HtpG fusion protein purification scheme are shown in Fig. 3. The yield of the full length GST-HtpG fusion protein was modest as compared with the strong band in the cells lysed in loading buffer. It was noticeable that the purification led to the purification of 96 kDa protein corresponding to the GST-HtpG fusion protein (Fig. 3 Lane 4). The purification of GST-HtpG fusion protein was confirmed by Western blot using an anti-GST antibody (data not shown).

To carry out the phage display technique with GST-HtpG fusion protein we had to purify the GST protein to be used as a control. Therefore, the bacterial strain BL21 carrying pGEX-2T vector was utilized to over-express the GST protein by induction with IPTG to final concentration 1.0 mM. The overexpression of the GST protein (26 kDa) is shown in Fig. 4. GST protein purification is evident from the single band in Fig. 4, Lane 4.

Phage display with GST-HtpG fusion protein: The purified GST protein was utilized as a control for GST-HtpG fusion protein study with the phage display technique. The 15 mer phage library (5×10^{11} TU mL^{-1}, kindly provided by Dr. S.M. Picksley Bradford University, UK) was used for this purpose. To examine the specificity of the 15 mer first round selected phage with GST-HtpG fusion protein, the 15 mer phage pool selected from these round (1st round) was plated onto an ELISA plate wells previously coated with GST, BSA, ovalbumin, Marvel as a non specific control proteins and GST-HtpG protein.

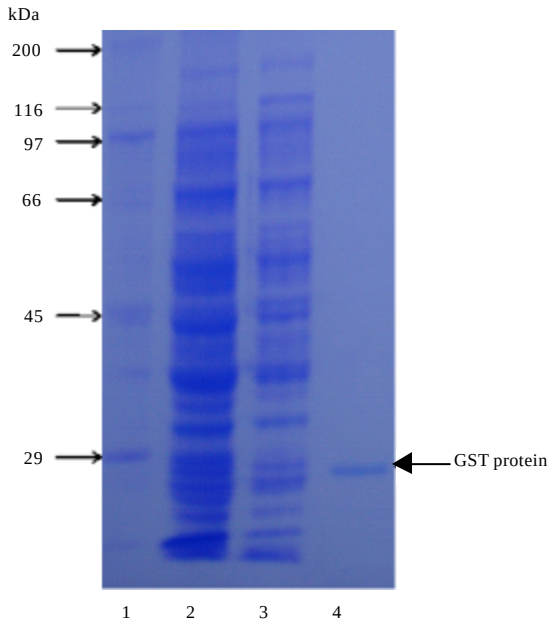

Fig. 4: Purification profile of GSTprotein. Lanes 1: Protein marker Sigma SDS6H2-1VL (M.Wt 30,000-200,000 kDa). Lane 2: Crude extract of *E. coli* strain overexpressing GST protein. Lane 3: GST protein eluted from DEAE-sepharose column by using 100 mL gradiant of 50-500 mM of NaCl in buffer A. Lane 4: GST protein eluted from glutathione sepharose 4B affinity column by using 25 mL 10 mM reduced glutathione in buffer C

The ELISA signal of the 15 mer phage (Fig. 5a) plated onto a well coated with GST-HtpG fusion protein in the ELISA plate is approximately 2 fold greater than the ELISA signal detected from plating the phage onto wells coated with GST protein and 4 fold greater than that observed with BSA, ovalbumin and Marvel proteins (controls). From the results of the first round the 15 mer (Fig. 5a) it was possible that some phage specifically bound to the HtpG protein in the GST-HtpG fusion protein. So, the 15 mer phage pool produced from the first round were taken to a further round of selection by plating 50 µL of the 15 mer phage pool onto small dishes previously coated with 5 µg of the purified GST-HtpG fusion protein and the phage were eluted, neutralised and amplified (second round). The specificity of the 15 mer second round selected phage was examined as described above for the first round.

As shown in Fig. 5b the ELISA signal produced from the second round 15 mer phage plated onto the GST-HtpG protein was about 4 fold greater than the ELISA signals obtained 15 mer phage onto GST and 6 fold greater than the ELISA signals produced with BSA, ovalbumin, Marvel (controls).

It can be predict from these results that the 15 mer phage selected by the GST-HtpG protein should have an affinity ligand displayed on the surface of the phage that binds tightly to the HtpG protein. It was decided to take the 15 mer phage to a further round (third round) of selection with the GST-HtpG protein. The third round of selection was performed by plating 15 µL of 15 mer phage pools from the previous round of selection onto small dishes previously coated with 0.5 µg of the purified GST-HtpG fusion protein. The phage from single colonies were amplified and

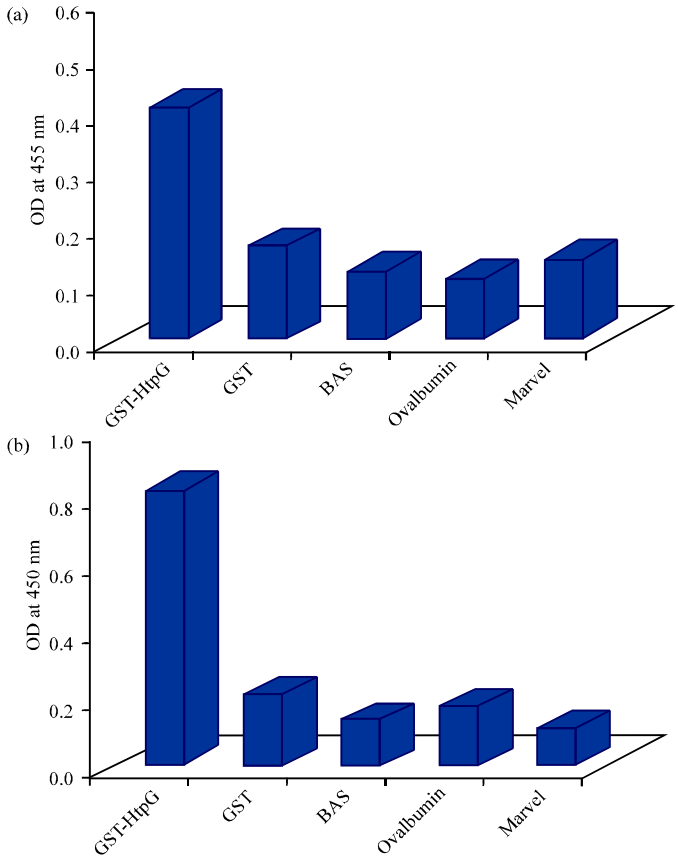

Fig. 5(a-b): Binding affinity of the (a) 1st and (b) 2nd round 15 mer phage library selected with GST-HtpG fusion protein to bind with GST-HtpG, GST, BSA, Ovalbumin and Marvel proteins

the specificity of the 15 mer phage amplified from each clone was examined by plating 25 µL/well onto an ELISA plate wells previously coated with GST, BSA (controls) and GST-HtpG proteins. The bound phage were detected at 450 nm by peroxidase conjugated anti-M13 antibody and TMB substrate. The ELISA signal produced from plating the third round 15 mer phage onto GST-HtpG fusion protein was about 6-8 fold greater than the ELISA signals obtained from plating the 15 mer phage onto GST or BSA controls (Fig. 6). The individual 15 mer phage selected after third round each showed strong ELISA signals with GST-HtpG protein rather than the GST or BSA. It was concluded that the 15 mer phage library after the third round of selection had a high affinity ligand(s) on their surface that might interact with the HtpG protein.

To identify the sequence of the high affinity ligands displayed on the surface of the 15 mer phage library (insert), 25 phage clones of the 15 mer phage library were chosen that gave 8 or 7 fold ELISA signals (strong signals) greater than the controls, for sequencing their ssDNA. The following oligonucleotides primer 5'CCCTCATAGTTAGCGTAA3' was designed on the basis of the identified sequence of bacteriophage fd (Beck *et al*., 1978) to identify the sequence of the insert in the 5' end of the gene III (Parmley and Smith, 1988). The DNA sequence of the region, that encoded for the amino terminal in PIII (contain insert) was carried out for the 25 phage clones of the 15 mer library to identify the nucleotide sequence of the insert that fused to the gene of coat protein PIII.

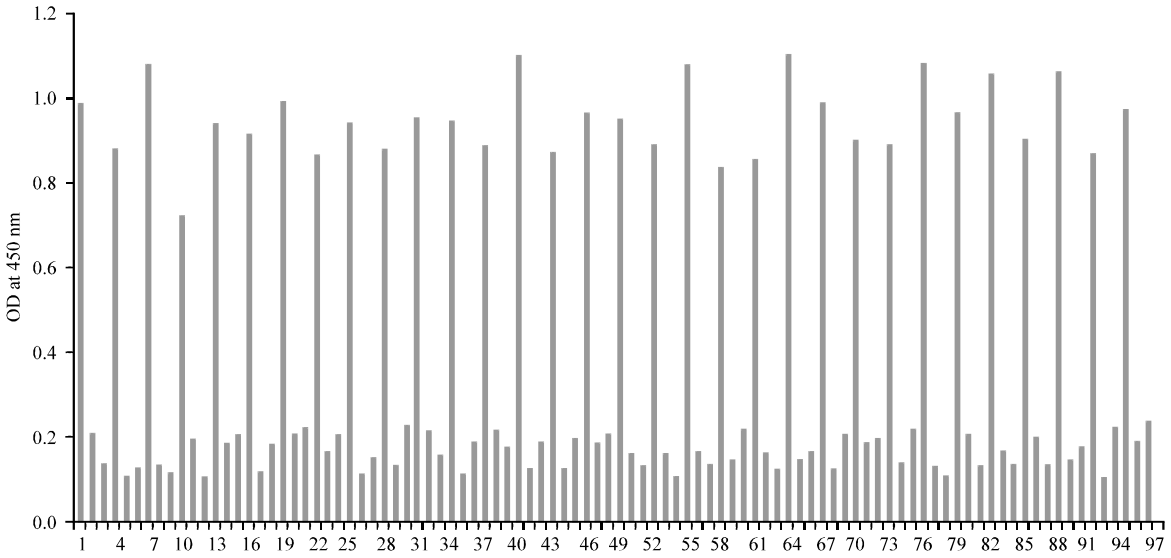

Fig. 6: Binding affinity of the amplified phage single colonies of the 3rd round 15 mer phage library selected with GST-HtpG fusion protein to bind with GST-HtpG (1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85, 88, 91, 94, 96), GST (2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, 89, 92, 95) and BSA (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 93, 94) proteins

Table 1: Nucleotide sequence of ssDNA of inserts in gene III of the 15 mer phage library clones and their corresponding encoded amino acid sequences that bind tightly with HtpG protein

Nucleotide sequence of ssDNA inserts in gene III of the 15 mer phage library clones	Encoded amino acids sequences of inserts in gene III of the 15 mer phage library clones	No. of clones
5'GAAACCTATGTGATGCGCAGCTGGGATAACGATCATGCGTGCCGC3'	ETYVMRSWDNDHACR	1
5'CGCGGCTATGATACCAGCGTGCCGGATAGCACCTGGCGCATTAGC3'	RGYDTSVPDSTWRIS	2
5'AAAGATTGGGATGCGCGCCTGCATGCGGAACCGGCGAGCGCGGAT3'	KDWDARLHAEPASAD	1
5'TGCCATCCGATTCTGTTTCGCCGCATGAGCGATCGCGATAAAGCG3'	CHPILFRRMSDRDKA	2
5'GTGAGCATGTGGAGCAAACCGGATAAAGGCTTTAAAGAACGCAAA3'	VSMWSKPDKGFKERK	3
5'CGCACCGTGAAAAGCGATCTGTATGCGCGCCCGCAGCTGATGCAT3'	RTVKSDLYARPQLMH	1
5'TTTGCGGATGCGCCGGAAACCAGCGCGAGCTATATGCAGGAAGGC3'	FADAPETSASYMQEG	3
5'GAAACCTGGCGCTTTCAGAGCAACGCGAAACAGAGCCTGCATCTG3'	ETWRFQSNAKQSLHL	2
5'AAAGATGAAGCGGAACATAGCCAGACCTTTTGGCTGCAGTTTGGC3'	KDEAEHSQTFWLQFG	3
5'CAGGAACGCGATACCCTGGAAGATAGCAGCCTGTTTATTCGCAAC3'	QERDTLEDSSLFIRN	1
5'AGCATTTGGGATCTGCCGCTGCAGTATCGCGGCTTTGGCACCAGC3'	SIWDLPLQYRGFGTS	3
5'AAAGATGGCCAGGCGGGCAAAACCCCGGGCGGCAGCCGCAGCGAT3'	KDGQAGKTPGGSRSD	2
5'CGCGAATGCAACTGCGCGCTGGAACTGAGCGATGCGCAGGTGGGC3'	RECNCALELSDAQVG	1

The DNA sequences of the insert and their corresponding amino acids in all the positive clones were represented in Table 1 for the 15 mer insert. The DNA sequences were translated into the corresponding amino acids by ExPASy Proteomics tools (http://kr.expasy.org/) web site and the results for the DNA sequence of the insert region with its corresponding amino acids are represented in Table 1. The amino acids sequences in Table 1 reveals that these 15 mer amino acids sequences interacts strongly with the HtpG protein and might exist within the sequence of the

HtpG protein partners. Are there proteins that have a sequence homology to these the 15 mer amino acid sequences? To answer that question, these sequences were used to search for protein homology using Swissport protein database (http://web.expasy.org/blast/) web site. The thirteen 15 mer amino acids sequences represented in Table 1 were originated to bind definitely to the HtpG protein.

Proteins identified in databases that have homology sequence with the sequence of the 15 mer amino acids sequence CHPILFRRMSDRDKA are nitrate reductase, sulfite reductase flavoprotein, bifunctional reductase, oxidoreductase, nitric oxide synthase. HSP90 proteins play an important role in the maturation of signal transduction proteins, like nitric oxide synthase and calcineurin (Wegele *et al*., 2004). Throughout these substrates, they control diverse cellular processes. The arrangement of these functions appears to consequence in a role as a capacitor of phenotypic variation: Decreasing cytosolic HSP90 function in *Drosophila melanogaster* and *Arabidopsis thaliana* results in the appearance of phenotypes that depend on the genetic background but which would normally be cryptic (Rutherford and Lindquist, 1998).

Proteins predicted permease, condensin complex subunit 1, oxidoreductase, phenylalanyl-tRNA synthetase have a homology sequence with the identified 15 mer amino acids KDWDARLHAEPASAD insert displayed on the surface of the phage that show strong binding to the HtpG protein. It was reported that (Zhao *et al*., 2005) Hsp90 protein family appears to associate with client proteins at their latest stages of folding. The Hsp90 proteins has been shown to form complexes with the inactive forms of a wide array of cellular signaling proteins and chaperones the conformational changes essential for their signaling dependent activities, including ligand binding and association with partner proteins. Hence, Hsp90 proteins play a unique role in cellular physiology by preferentially stabilizing near resident state structures and aiding the dynamic assembly and disassembly of signaling complexes. Since many Hsp90 client proteins play critical roles in cellular proliferation and survival, pharmacological suppression of Hsp90 activity and hence the many Hsp90 dependent signaling pathways, blocks growth of cancerous cells (Whitesell and Lindquist, 2005).

The amino acids sequence GYDSSVPDSTWRI of glucocorticoid receptor protein (from 542 to 554) was identified to have a homology sequence with 15 mer amino acids sequence RGYDTSVPDSTWRIS. In support to our results (Pratt and Toft, 2003) reported that the glucocorticoid receptor forms a complex with Hsp90 protein family to have high affinity steroid binding activity. Hsp90 interaction with glucocorticoid receptors is not limited to the cytoplasm but continues during translocation into the nucleus where the chaperone assists in the receptor's DNA binding, transcription control and nuclear recycling (Grad and Picard, 2007). The GYDSSVPDSTWRI sequence is the domain of glucocorticoid receptor that bind with the Hsp90 family of proteins and this require more effort to elucidate the identified GYDSSVPDSTWRI binding site in the of glucocorticoid receptor.

Many proteins have been identified to have a homology sequence with the 15 mer amino acids sequence ETYVMRSWDNDHACR. These proteins are O-methyltransferase, pyruvate, phosphate dikinase, transcriptional regulator, aminotransferase, RecJ, transcriptional regulator family TetR and chromosomal replication initiator protein dnaA. Hsp90 proteins bind to exposed hydrophobic surfaces of their client proteins, thereby preventing nonspecific intermolecular interactions that could lead to aggregation.

Proteins that have a homology sequence with the 15 mer amoino acids sequenc SIWDLPLQYRGFGTS are aminoethyltransaminse, thiamine phosphate synthase, sulfonylurea

receptor, deaminopimelate decarboxylase, β-fructofuranosidase, coat protein VP2, aminomethyl transferase and Heat shock protein HtrC. Moreover, the amino acida sequenc SIWDLPLQYRGFGTS was recognized to have sequence homology with the following proteins DNA polymerase V, ATP binding cassette, transcriptional activator and DNA polymerase II. The Hsp90 proteins may have a role in DNA repair and recombination process by binding to proteins involved in DNA repair recombination mechanism. Given the significant differences in the interactions with partner proteins, it is probable that Hsp90 has an essential mechanism different from either heat schock protein families as Hsp70 or GroEL. Although nucleotide binding and hydrolysis are necessary for the function of all three molecular chaperones, the relatively trivial structural transformations effected by Hsp90 on native like client proteins may need a different mechanism for coupling ATP to chaperone conformational state.

The amino acids VSMWSKPDKGFKERK sequence was originated to have a homology sequence with ribosomal protein L2, 50S ribosomal protein L2 and ATP/GTP-binding protein. Recently, it was reported that L2, a protein component of 50S ribosomal subunit, was found as an associated protein with His-tagged HtpG expressed in *E. coli* cells. HtpG specifically recognized ribosomal protein L2 and its ATPase activity that was activated more than ten folds (Motojima-Miyazaki *et al*., 2010). This is similar to the case of the Hsp90-client protein interaction (McLaughlin *et al*., 2002). Molecular chaperones are necessary with the purpose of maintaining the correctly folded state of other proteins in the cell. This is generally accomplished by recognition of hydrophobic surfaces on the client protein and then frequent rounds of ATP hydrolysis dependent conformational alterations by the chaperone, assisting substrate folding (Young *et al*., 2001, 2004). Moreover, the 15 mer amino acids sequence VSMWSKPDKGFKERK was found to have a homology sequence with other proteins as sulfatase, coenzyme F420 hydrogenase/dehydrogenase beta subunit, pyruvate kinase, serine/threonine-protein phosphatase, acyltransferase, increased rDNA silencing protein 4, malate synthase and glycosyl hydrolase family. The HtpG protein may bind to these proteins for protein folding.

The 15 mer amino acids sequence RTVKSDLYARPQLMH of the insert that displayed on the surface of the phage and bind with the HtpG protein has been recogonized to have a homology sequence with the following proteins, uroporphyrinogen decarboxylase, NADH dehydrogenase, transposase IS4 family protein, ABC superfamily ATP binding cassette transporter, putative uncharacterized protein and Type II restriction endonuclease. It was proposed that (Saito *et al*., 2008) the HtpG protein is involved in fine modification of tetrapyrrole biosynthesis through regulation of the uroporphyrinogen decarboxylase activity.

Proteins identified in data base and have a sequence homology with the 15 mer amino acids FADAPETSASYMQEG insert displayed on the surface of the phage and show strong binding to the HtpG protein. These proteins are phosphoesterase phosphatase related protein precursor, transcriptional regulator, transcriptional activator MltR, 3-oxoacyl-(Acyl-carrier-protein) reductase, transforming growth factor beta regulator 4, TBRG4, alcohol dehydrogenase subunit I, ATP-dependent DNA helicase RecQ, ATP binding cassette sub-family D member 1, UTP-GlnB uridylyltransferase and mannose-1-phosphate guanylyltransferase/mannose-6-phosphate.

The 15 mer amino acids sequence etwrfqsnakqslhl of the insert that show strong binding to the HtpG protein and exhibit homology sequence with proteins identified in data base as reverse transcriptase, phosphoribulokinase/uridine kinase family enzyme, RNA-directed DNA polymerase (Reverse transcriptase) and 3-polyprenyl-4hydroxybenzoate decarboxylase.

The sequence homology of the 15 mer amino acids RECNCALELSDAQVG has been identified to have sequence homology with other proteins identified in data base as represented in. These proteins include Asp/Glu racemase, homoserine dehydrogenase, hydroxymethylglutaryl-CoA synthase, protein-(Glutamine-N5) methyltransferase, prolyl-tRNA synthetase, chaperone protein DnaK (HSP70) (*Shuttleworthia satelles*), Long-chain fatty acid transport protein 4, Acetoin catabolism regulatory protein, glutathione S-transferase domain, L-carnitine dehydratase/bile acid-inducible protein F precursor and DNA repair helicase.

Proteins identified in databases and have homology with the sequence of the 15 mer amino acids KDGQAGKTPGGSRSD insert are cellular tumor antigen p53, excisionase/Xis, DNA-binding, predicted protein, alpha/beta hydrolase, serine-aspartate repeat-containing protein F and cytosolic phospholipase A2 zeta. In the early 1990s, several groups observed that Hsp90 was overexpressed at 2-10 fold higher levels in a wide variety of cancer cells and in virally transformed cells (Ferrarini *et al*., 1992; Yufu *et al*., 1992), suggesting a crucial role of Hsp90 for growth and/or survival of tumor cells. Hsp90 expression is also connected with many types of tumors counting breast cancer, pancreatic carcinoma, human leukemia, systemic lupus erythematosus as well as multidrug resistance (Csermely *et al*., 1998). Hsp90 inhibition provides in recent times urbanized, important pharmacological proposal for anticancer therapy (Sreedhar *et al*., 2004). Inhibition of Hsp90 with petite molecules such as geldanamycin and its chemically similar compounds has been shown to be antitumorigenic and several of these compounds are presently in clinical trials (Solit *et al*., 2008). Hsp90 is involved in a diverse array of cellular processes such as signal transduction, cell cycle control and transcriptional regulation (Schatz and Dobberstein, 1996; Pratt and Toft, 1997; Buchner, 1999; Mayer and Bukau, 2005). It is essential for the functions of a growing number of client proteins, of over 100 are currently reported. These include steroid hormone receptors and the other transcription, protein kinases, nitric oxide synthase, tumor suppressors' p53 and retinoblastoma and telomerase (Pratt and Toft, 2003).

CONCLUSION

The 15 phage library was utilized effectively with the HtpG protein in the form of GST-HtpG fusion proteins. The HtpG partner proteins binding domains as DNA polymerase, thyroid hormone receptor interactor 12, ATPase central domain protein, DNA gyrase subunit A, DNA topoisomerase, RuvB helicase, nitrate reductase, nitric-oxide synthase, glucocorticoid receptor and cellular tumor antigen p53 were identified. All of these data need more work to recognize the exact mechanism of HSP90 proteins with all the partner proteins. Whereas the molecular mechanisms of HSP60 and HSP70 are well characterized, the conformational changes and determinants of substrate recognition associated with Hsp90 function remain unclear. For HSP60 and HSP70 partner protein binding stabilizes amorphous states; only upon release can folding occur. Thus, in the bound state, the partner protein adapts its conformation to the binding surfaces for these chaperones. Given the considerable differences in the interactions with partner proteins, it is possible that HSP90 has an essential mechanism. That is differently commencing either GroEL or HSP70. Although, nucleotide binding and hydrolysis are necessary for the function of all three molecular chaperones, the relatively slight structural transformations effected by HSP90 on native like client proteins may require a different mechanism for coupling ATP to chaperone conformational state. On the other hand, Hsp90 substrates have already achieved an incompletely folded or almost fully folded conformation before they interact with HSP90, suggesting that Hsp90 must adapt its conformation

to match each substrate or that different conformations recognize different substrates. This issue is particularly pronounced for the bacterial Hsp90, HtpG, for which there are no known cochaperones to improve the efficiency of client protein recruitment. Hsp90 communication with glucocorticoid receptors is not inadequate to the cytoplasm but continues during translocation into the nucleus where the chaperone assists in the receptor's DNA binding, transcription control, DNA release and nuclear recycling.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support by General Directorate of Research Grants Programs, King AbdulAziz City for Science and Technology (KACST), Saudi Arabia (Project Number 08-Bio13-6). We would like to thank Dr. Picksley, S.M. (Bradford University, UK) for his generous gifts of some bacterial strains and phage library used in this study.

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