Intra- and intermolecular domain interactions among novel two-component system proteins coded by Rv0600c, Rv0601c and Rv0602c of *Mycobacterium tuberculosis*

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Received 1 April 2008

Revised 29 October 2008

Accepted 12 November 2008

Two-component signal transduction pathways comprising a histidine kinase and its cognate response regulator play a dominant role in the adaptation of *Mycobacterium tuberculosis* to its host, and its virulence, pathogenicity and latency. Autophosphorylation occurs at a conserved histidine of the histidine kinase and subsequently the phosphoryl group is transferred to the conserved aspartate of its cognate response regulator. Among the twelve two-component systems of *M. tuberculosis*, Rv0600c (HK1), Rv0601c (HK2) and Rv0602c (TcrA) are annotated as a unique three-protein two-component system. HK1 contains an ATP-binding domain, and HK2, a novel Hpt mono-domain protein, contains the conserved phosphorylable histidine residue. HK1 and HK2 complement each other's functions. Interactions among different domains of the HK1, HK2 and TcrA proteins were studied using a yeast two-hybrid system. Self-interaction was observed for HK2 but not for HK1 or TcrA. HK2 was found to interact reasonably well with both HK1 and TcrA, but HK1 interacted weakly with TcrA. The conserved aspartate-containing receiver domain of TcrA interacted well with HK2 but not with HK1. These results suggest the existence of a novel signalling mechanism amongst HK1–HK2–TcrA, and a model for this mechanism is proposed.

INTRODUCTION

Tuberculosis is an increasing and major world-wide problem, especially as AIDS facilitates its spread. The emergence of multi-drug resistant *Mycobacterium tuberculosis* strains has driven the need to develop new drugs and drug targets. Understanding the mechanisms of signal transduction that allow *M. tuberculosis* to resist host immunity or chemotherapy may help to identify new drug targets. Eubacteria, archaea and a few eukaryotes possess so-called two-component signal transducing systems, consisting of a histidine kinase and a response regulator. In almost all cases histidine kinases and their cognate response regulators are encoded by juxtaposed genes

(Grebe & Stock, 1999). The histidine kinase is the signal sensor and the response regulator is the effect-producing component, usually a transcriptional regulator. Environmental alterations are sensed by the sensor part of the histidine kinase, and result in the ATP-mediated trans-autophosphorylation of a conserved histidine residue. The high-energy phosphoryl group is then transferred to a conserved aspartate on the cognate response regulator receiver domain, in turn resulting in a conformational change enabling the response regulator effector domain to bind DNA and act as transcriptional regulator of specific genes, thus producing an appropriate response to the environmental signal (Stock *et al.*, 2000).

Histidine kinases and response regulators are modular in nature, with different permutations and combinations of basic domains. Most histidine kinases are membrane bound, with an extracytoplasmic signal-sensing domain at the N terminus and a cytoplasmic kinase core domain at the C terminus. The cytoplasmic kinase core can be further dissected into the HAMP domain, the histidine-containing dimerization domain and an ATP-binding catalytic domain (Khorchid & Ikura, 2006). The HAMP domains are linkers connecting the transmembrane regions of some histidine kinases to their kinase core (Aravind & Ponting,

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; GAL4 AD, GAL4 BD, activation domain and DNA-binding domain of GAL4 transcription activator; HAMP, domain present in histidine kinases, adenylyl cyclases, methyl-accepting proteins and phosphatases; HK, histidine kinase; HK1, histidine kinase encoded by Rv0600c; HK1n, HK1 with 40 residues deleted from N terminus; HK2, histidine kinase encoded by Rv0601c; HPT, histidine-containing phosphotransfer (domain); RR, response regulator; RR1, receiver domain of response regulator TcrA; RR2, effector domain of TcrA; TcrA, response regulator coded by Rv0602c.

Supplementary tables of strains and plasmids are available with the online version of this paper.

1999). The kinase core bears the phospho-accepting His residue and the conserved sequence fingerprints called homology boxes, namely H-, N-, D- and G boxes, based upon highly conserved histidine, asparagine, aspartic acid and glycine residues, respectively. The conserved sequence of the H-box is Fhxxh(S/T/A)H(D/E)h(R/K)TPLxxh, that of the N box is (D/N)xxxhxxhxxNLhxNAh(F/H/Y)(S/T), that of the D box is DxGxGhxxxxxxhFxxF and that of the G-box is GGxGLGLxhhxxhxxxxG, where x signifies any residue and h is any hydrophobic residue. The H-box contains the conserved histidine, which is the site of phosphorylation and is located on a helix. N-, D- and G-boxes form the parts of the nucleotide-binding domain (Grebe & Stock, 1999). The two-component system response regulators are mostly cytoplasmic transcription factors that modulate expression of the target gene to produce the desired response (Mizuno, 1997). Response regulators usually contain two domains: the conserved N-terminal receiver domain and the variable C-terminal effector domain. The receiver domain interacts with the phosphorylated histidine kinase and catalyses the transfer of the phosphoryl group to its own conserved aspartate residue. The effector domain is usually a DNA-binding domain. The phosphorylation of the response regulator results in the conformational changes that expose the DNA-binding site of the effector domain and thus result in the output response (Kern *et al.*, 1999; Zhang *et al.*, 2003).

Two-component systems play an important role in adaptation of *M. tuberculosis* to its host, and its virulence, pathogenicity and latency (Zahrt & Deretic, 2001; Parish *et al.*, 2003; Perez *et al.*, 2001; Malhotra *et al.*, 2004). The analysis of the complete genome of *M. tuberculosis* H37Rv (Cole *et al.*, 1998; Morth *et al.*, 2005) revealed the presence of 12 complete pairs of sensor histidine kinases and response regulators. Specific reports on the functional characterization of two-component systems in mycobacteria are limited only to a few of these systems (Perez *et al.*, 2001; Himpens *et al.*, 2000; Haydel *et al.*, 2002; Dasgupta *et al.*, 2000; Via *et al.*, 1996; Ewann *et al.*, 2002).

A unique three-protein two-component system has been identified in the *M. tuberculosis* H37Rv genome. The genes encoding two-component proteins are identical among the H37Rv, CDC1551 and other 210 strains of *M. tuberculosis* examined, except for the two putative histidine kinases Rv0600c (207 aa) and Rv0601c (156 aa) of H37Rv, which are merged in CDC1551 into a single protein with a distinct N-terminal sequence (Tyagi & Sharma, 2004). Rv0600c/HK1 and Rv0601c/HK2 are annotated to phosphorylate a single response regulator, Rv0602c/TcrA. The Rv0601c gene has been found to be downregulated under hypoxic conditions (Sherman *et al.*, 2001). The two histidine kinases, HK1 and HK2, are incomplete in themselves but complementary to each other. HK1 possesses an ATPase domain comprising conserved N-, D-/F- and G-boxes but no H-box, and HK2 bears a HAMP domain and only the H-box, with the conserved phosphorylatable His-131 at its C terminus, all other boxes being

absent (Shrivastava *et al.*, 2006). HK2 shows structural similarity with the histidine-containing phosphotransfer (HPt) domains. HPt domains exist as part of hybrid histidine kinases and never as a single protein in bacteria, except HK2 in *M. tuberculosis* H37Rv (Shrivastava *et al.*, 2006). TcrA is a typical bi-domain response regulator with an N-terminal response receiver domain with a conserved Asp-73 and a C-terminal effector domain. Radioactive phosphotransfer experiments show that phosphorylation of HK2 occurs in the presence of HK1 and the phosphate group is subsequently transferred to TcrA (Shrivastava *et al.*, 2007). Here, we report the interactions among the HK1, HK2 and TcrA proteins and their domains, studied by a yeast two-hybrid system (Fields & Song, 1989), and show that self-interaction occurs in HK2 but not in HK1 and TcrA. We also show that HK2 interacts with both HK1 and the receiver domain of TcrA, to exert its signal transduction effect in this unique three-protein two-component system.

METHODS

Strains and media. Strains and plasmids used in this study are listed in Supplementary Table S1, available with the online version of this paper. *Escherichia coli* DH5 α was used for maintenance and propagation of the two-hybrid plasmids pGBKT7, pGADT7 (Clontech) and their derivatives (constructed in this study). *E. coli* DH5 α was grown in Luria–Bertani (LB) medium (Himedia) (pH 7.0) at 37 °C, transformed with pGADT7 and pGBKT7 and selected on LB medium supplemented with ampicillin (100 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹), respectively. Yeast strains, *Saccharomyces cerevisiae* Y187 and AH109, were used for interaction analysis. *S. cerevisiae* Y187 bears the *lacZ* reporter gene under the control of the *GAL1* UAS and *GAL1* TATA box. *S. cerevisiae* AH109 bears four reporter genes, *ADE2*, *HIS3*, *MEL1* and *lacZ*, under the control of three completely heterologous *GAL4*-responsive *GAL2*, *GAL1* and *MEL1* UASs and TATA boxes, respectively. The yeast strains were grown at 30 °C in yeast peptone dextrose (YPD) medium (pH 6.5) (Clontech) or synthetically defined (SD) dropout selection medium (pH 5.8) (Clontech). According to the stringency of the selection required, SD double dropout medium (–Leu/–Trp), SD triple dropout medium (–His/–Leu/–Trp), SD triple dropout medium supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT) or SD double dropout medium supplemented with X- α -Gal (Sigma; 80 μ g ml⁻¹) was used. SD/–Leu/–Trp was used to select double transformants or diploids bearing both pGBKT7 and pGADT7 vectors. SD triple dropout medium and supplements were used to detect interaction by expression of the reporter genes.

Construction of the two-hybrid plasmids.

(i) **pGAD-HK1 and pGBK-HK1.** The Rv0600c gene was amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv, using the primer pair ECOOC-F/BAM0CR (see Supplementary Table S2 for primer sequences). The amplicons and the *E. coli*/yeast shuttle vectors (pGADT7 or pGBKT7) were double digested with *EcoRI* and *BamHI*, analysed by agarose gel electrophoresis, purified with the Qiaquick gel extraction kit (Qiagen) and ligated using T4 DNA ligase, resulting in formation of pGAD-HK1 and pGBK-HK1 (see Table S1 for details of plasmids).

(ii) **pGAD-HK2, pGBK-HK2, pGAD-RR and pGBK-RR.** The Rv0601c gene was amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv, using the primer pair NDE1C-F/ ECO1C-R. The

Rv0602c gene was amplified using primer pair NDE2C-F/BAM2C-R. The Rv0601c amplicon was digested with *NdeI* and *EcoRI* and cloned into similarly digested pGADT7 and pGBKT7, yielding pGAD-HK2 and pGBK-HK2 respectively. The Rv0602c amplicon was digested with *NdeI* and *BamHI* and cloned into pGADT7 and pGBKT7 digested with the same restriction enzymes, generating pGAD-RR and pGBK-RR, respectively.

(iii) **pGAD-ΔHK2_{40–156}, pGBK-ΔHK2_{40–156}, pGBK-ΔHK1_{50–207} and pGBK-ΔHK1_{50–177}.** The N-terminally truncated Rv0601c (ΔHK2_{40–156} or HK2n) was amplified by PCR from the genomic DNA of *M. tuberculosis* using primers NDE6011-F and ECO1C-R and cloned in *NdeI*- and *EcoRI*-digested pGADT7 and pGBKT7 to generate recombinant plasmids pGAD-ΔHK2_{40–156} and pGBK-ΔHK2_{40–156}, respectively. The N-terminally truncated Rv0600c (ΔHK1_{50–207} or HK1n) and the ATPase domain of HK1 (ΔHK1_{50–177} or HK1D) were amplified by PCR from the recombinant plasmids having full-length Rv0600c using the primer pairs ECO6001-F/BAM0C-R and ECO6001-F/BAM6001-R, respectively, double digested with *EcoRI* and *BamHI* and cloned into pGBKT7 digested with the same restriction enzymes, to generate recombinant plasmids pGBK-ΔHK1_{50–207} and pGBK-ΔHK1_{50–177}, respectively.

(iv) **pGAD-RR1_{21–144} and pGAD-RR2_{163–250}.** The receiver domain with conserved Asp (RR1_{21–144}) and the effector domain (RR2_{163–250}) of TcrA were amplified from recombinant plasmid bearing full-length Rv0602c using primer pairs NDE6021-F/BAM6021-R and NDE6022-F/BAM6022-R, respectively, digested with *NdeI* and *BamHI* and cloned into the corresponding restriction sites of pGADT7 to generate pGAD-RR1_{21–144} and pGAD-RR2_{163–250}, respectively.

The recombinant plasmids were transformed into *E. coli* DH5α. Positive transformants with the pGADT7 backbone were selected on LB plates containing ampicillin (100 µg ml⁻¹) and those with the pGBKT7 backbone were selected on LB plates containing kanamycin (50 µg ml⁻¹). Plasmids were isolated from selected bacterial colonies using the QIAprep plasmid isolation kit (Qiagen). Plasmids pGBKT7-53 (Clontech), encoding the GAL4 DNA-binding domain (DNA-BD) fused with murine p53, and pGADT7-T (Clontech), encoding the GAL4 activation domain (AD) fused with SV40 T-antigen, were used as positive controls in the yeast two-hybrid assay (Li & Fields, 1993). pGBKT7-Lam, encoding GAL4 DNA-BD fused with human lamin C, and pGADT7-T were used as a negative control during the interaction study.

All the recombinant plasmids were sequenced in an ABI automated DNA sequencer using the ABI BigDye terminator kit to check insertion in the proper reading frame. Plasmids with pGADT7 backbone (preys) were sequenced using the T7 and 3' AD sequencing primers, and plasmids with pGBKT7 backbone (baits) were sequenced using the T7 and 3' BD sequencing primers (provided in the kit).

Yeast transformation and mating. Plasmid pGBKT7 and its recombinant derivatives were transformed into *S. cerevisiae* Y187, using the lithium acetate method according to the protocol provided by the manufacturer (Clontech). Similarly, pGADT7 and its recombinant derivatives were transformed into *S. cerevisiae* AH109. *S. cerevisiae* Y187 transformants were selected on SD/-Trp plates and AH109 transformants on SD/-Leu plates. Transcriptional activation of the reporter genes by single transformants (self-activation) was analysed by the formation of blue colonies on SD/-Leu or SD/-Trp plates (as applicable) containing X-α-Gal. Mating of the respective bait- and prey-containing *S. cerevisiae* generated diploids with both bait and prey vectors, which were selected on SD/-Leu/-Trp plates. Mating was done by placing a colony of 2–3 mm from a fresh culture plate of each bait- and prey-containing *S. cerevisiae* in a 1.5 ml microcentrifuge tube containing 0.5 ml YPD medium (pH 6.5). The

tubes were vortexed to resuspend cells and incubated overnight at 30 °C with shaking at 200 r.p.m.

Confirmation of protein expression. Expression of the desired protein in yeast was verified by Western blotting of the protein extracts using anti-GAL4BD and anti-GAL4AD monoclonal antibodies (Clontech). The diploid *S. cerevisiae* were grown in 5 ml SD double-dropout selection medium, pelleted by centrifugation and resuspended in 5 × SDS-PAGE loading dye. The samples were boiled for 10 min, analysed by SDS-15% PAGE and electrotransferred on to membrane (Hybond C, GE Healthcare). The membranes were probed with anti-GAL4 AD (1:2500 dilution) and anti-GAL4 BD (1:5000 dilution) monoclonal antibodies (Clontech). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bangalore Genei) was used as secondary antibody (1:2000 dilution). Detection was carried out by the formation of an insoluble coloured precipitate at the reaction site as a result of the HRP activity on the substrate 3-3'-diaminobenzidine tetrahydrochloride in the presence of hydrogen peroxide (Bangalore Genei).

Screening for positive interaction. The diploids generated by mating were tested for interaction by analysing expression of reporter genes, *HIS3* and *MEL1*. The expression of the *HIS3* reporter was analysed by growth on SD/-His/-Leu/-Trp plates. The interaction observed on SD/-His/-Leu/-Trp plates was also confirmed by using 5 mM 3-AT as competitor in SD/-His/-Leu/-Trp medium. The expression of *MEL1* reporter was observed by development of blue colonies in the presence of X-α-Gal on plates of SD/-Leu/-Trp medium.

Quantification of the strength of protein-protein interaction. Liquid cultures of the diploid yeast strains were assayed for β-galactosidase activity to verify and quantify the relative strength of the protein-protein interactions. Two millilitres of overnight cultures in SD selection media were subcultured into 8 ml YPD medium and grown at 30 °C until the cells reached the mid-exponential phase (OD₆₀₀ 0.5–0.8). The cells were harvested from 4.5 ml culture and resuspended in 300 µl Z buffer (0.1 M Na₂HPO₄, 35 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0). One hundred microlitres of cell suspension was lysed by repeated freeze/thaw cycles and then 160 µl *o*-nitrophenyl β-D-galactopyranoside (ONPG, 4 mg ml⁻¹ in Z buffer) was added to the lysate and incubated at 30 °C until the colour developed. The reaction was stopped by addition of 0.4 ml 1 M sodium carbonate. After centrifugation, the A₄₂₀ of the supernatant was measured. As a negative control yeast with non-interacting partners were used. The β-galactosidase units were calculated according to Miller (1972). The reaction was repeated with three individual colonies for each diploid, and three sets of readings were taken per individual colony.

RESULTS

Construction of hybrid plasmids and test for self-activation

The genes of the targeted histidine kinases and response regulators along with their truncated regions and domains were amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv. As a prerequisite for the yeast two-hybrid study, the targeted full-length proteins, their domains and truncated forms were cloned into the *E. coli*/yeast shuttle vector plasmids pGBKT7 and pGADT7 (Fig. 1; Supplementary Table S1). Sequencing of all the recombinant plasmids confirmed the cloning of the inserts in the proper reading frame. In order to analyse the ability

Fig. 1. Constructs used in the two-hybrid assay to produce different fusion proteins. The numbers above the boxes indicate the amino acid residues of different proteins fused with either the GAL4 AD or GAL4 DNA BD domain, as indicated on the left.

of the fusion proteins to activate reporter gene expression (self-activation) the hybrid bait vectors with the pGBKT7 backbone were transformed into *S. cerevisiae* Y187 and the hybrid prey vectors with the pGADT7 backbone into *S. cerevisiae* AH109, and the transcriptional activation of the reporter genes was analysed by growth on histidine-deficient selective dropout plates and by the formation of blue colonies on plates containing X- α -Gal. None of the fusions tested in this study showed background transcriptional activity (self-activation), and therefore all were deemed suitable for the yeast two-hybrid studies. Yeast mating was done and positive diploids were selected based on reporter gene expression.

Protein expression in *S. cerevisiae*

Western blotting experiments using anti-GAL4 BD and anti-GAL4 AD monoclonal antibodies were done to confirm the expression of fusion proteins in diploid yeast strains. The fusion proteins detected by Western blotting are indicated by arrows and marked at the top of each blot in Fig. 2. Each pair is named as 'domains fused with GAL4 BD/domains fused with GAL4 AD'. The size of GAL4 DNA BD alone was approximately 16 kDa and that of GAL4 AD was 13 kDa. As seen in Fig. 2, fusion of GAL4 BD with HK1, HK1n, HK1D, HK2, HK2n and TcrA produced proteins of approximately 39, 33.3, 30, 33, 29 and 44 kDa, respectively. Similarly, fusion of GAL4 AD with HK1, HK2n, HK2, RR, RR1 and RR2 produced proteins of

Fig. 2. Western blot analysis of GAL4 fusion proteins expressed in diploid *S. cerevisiae* obtained by mating of different bait and prey containing Y187 and AH109 respectively. The membrane was probed with both anti-GAL4 BD and anti-GAL4 AD antibodies. Arrows indicate the detection of the proteins labelled at the top of each blot. The positions of protein molecular mass markers are shown on the right.

approximately 36, 26, 30, 41, 27 and 23 kDa, respectively. The blot showed weak expression for HK2n and HK1D.

Self-interaction

The interaction as analysed by growth on SD/–His/–Leu/–Trp plates supplemented with 5 mM 3-AT showed that there was no leaky expression of *HIS3*. The yeast two-hybrid analyses of GAL4 BD–HK2 and GAL4 AD–HK2 showed that HK2 can self-interact to form homodimers (Table 1). The interaction was of moderate strength as detected by the β -galactosidase activity assay for the *lacZ* reporter gene expression (Fig. 4). The interaction did not result in expression of *MEL1* reporter and no blue colonies could be observed on SD/–Leu/–Trp plates overlaid with X- α -Gal (Fig. 3b, last column). HK2 has an N-terminal transmembrane region. To analyse the region essential for dimer formation, a transmembrane region deletion construct of HK2 (Δ HK2_{40–156}/HK2n) was created and the interaction between GAL4 BD- Δ HK2_{40–156} and GAL4 AD- Δ HK2_{40–156} was studied. These results showed that the C-terminal region of HK2 (not the N-terminal region) is associated with dimer formation (Fig. 3a). No growth of *S. cerevisiae* on SD triple-dropout medium was observed for self-interaction between RR and HK1 (Fig. 3a).

Interacting partners and strength of interaction

It has been shown that HK1 phosphorylates HK2 and then HK2 transfers the phosphoryl group to TcrA (RR) (Shrivastava *et al.*, 2007). For this phosphotransfer physical contact must occur between HK1 and HK2 and between HK2 and TcrA. To detect these interactions and to study the domains involved, a yeast two-hybrid assay was done. Pairing of HK1, HK1n and HK1D with HK2 showed growth of yeast colonies on SD/–His/–Leu/–Trp plates (row 1, Table 1). Quantification of the interaction strength using *MEL1* expression (Fig. 3b) and ONPG assay for *lacZ*

Table 1. Interactions between fusion proteins by growth of diploids on SD/–His/–Leu/–Trp medium supplemented with 3-AT (5 mM) after 4 days

Columns represent proteins fused with the GAL4 BD and rows represent proteins fused with the GAL4 AD expressed in the diploids. HK1n denotes Δ HK1_{50–207}, HK1D denotes Δ HK1_{50–177}, RR1 represents RR1_{21–144} and RR2 indicates RR2_{163–250}. Growth of yeast colonies (as indicated by average colony diameter) was scored as: + + +, very good; + +, good; +, fair; –, none.

GAL4 BD/GAL4 AD	HK1	HK1n	HK1D	HK2
HK2	+++	+++	++	++
RR	++	–	–	+++
RR1	++	+	++	+++
RR2	+++	+++	+++	–

reporter expression (Fig. 4) showed relatively strong interaction between HK1 and HK2 and between HK1n and HK2 but a relatively weak interaction between HK1D and HK2. The interaction between HK1 and RR was detected by growth on histidine-deficient medium and development of blue colonies on X- α -Gal-supplemented SD/–Leu/–Trp plates. The *HIS3* reporter was not expressed in the case of diploids having HK1n and HK1D with RR but a weak interaction was detected by light blue colonies on X- α -Gal-supplemented SD/–Leu/–Trp medium (row 2, Table 1; Fig. 3b). The interaction analysis between the receiver domain RR-1 and effector domain RR-2 of TcrA with HK1, HK1n and HK1D showed very little growth and a relatively weaker interaction with RR-1 as compared to RR-2 (rows 3 and 4, Table 1; Fig. 4). It was observed that the domains of TcrA interact with HK1 more strongly than the full-length TcrA. This may be because the interaction site of HK1 on each domain of TcrA was blocked by the other domain in complete TcrA and was exposed when individual domains were studied. A strong interaction was evident between HK2 and RR and between HK2 and RR1 from the expression of the *HIS3* and *MEL1* selection marker (column 4, Table 1; Fig. 3b). Quantification of the interaction strength by β -galactosidase activity showed that the interaction between HK2 and TcrA is strongest among the interactions between other proteins of the targeted two-component system (Fig. 4). The interaction between HK2 and the effector domain of TcrA (RR2) was not detectable on histidine-deficient selection plates (Table 1, last row, last column) or on X- α -Gal-supplemented SD/–Leu/–Trp plates (Fig. 3b, last row, last column), but a weak interaction was detected by activation of the *lacZ* gene (Fig. 4). This showed that the HK2–RR2 interaction was weak and activated only a single reporter under the *MEL1* promoter but could not activate *HIS3* under the *GAL1* promoter.

Yeast transformants with plasmids pGBKT7-53 and pGADT7-T (both from Clontech), which encode the murine p53 protein and SV40 large T antigen, respectively, and known to physically associate strongly (Li & Fields, 1993), showed growth on SD/–His/–Leu/–Trp medium and also developed blue colonies on SD/–Leu/–Trp X- α -Gal-supplemented plates. This was used as a positive control to assess the assay conditions. Similarly a negative control showing no interaction between human lamin C coded by PGBKT7-Lam and SV40 large T antigen (did not produce any blue colonies on selected plates) was used during the assay for verification of the interactions (Fig. 3c).

DISCUSSION

Based on our results, a model of the interaction assembly is proposed for the novel three-protein two-component system from *M. tuberculosis* comprising HK1, HK2 and TcrA/RR (Fig. 5). HK2 is a membrane-bound signal-sensing protein with its N-terminal region being the extracellular signal sensor. HK2 dimerization occurs at its

Fig. 3. (a) Self-dimerization of the GAL4BD and GAL4AD fusions of Δ HK2₄₀₋₁₅₆ (HK2n), HK1 and TcrA (RR) analysed by growth on SD/-His/-Leu/-Trp medium supplemented with 5 mM 3-AT. (b) Expression of *MEL1* reporter analysed by development of blue colonies on SD/-Leu/-Trp supplemented with X- α -Gal. Light blue colonies indicate weak interaction among HK1n-RR, HK1n-RR1, HK1D-HK2, HK1D-RR and HK1D-RR1. No blue colonies were observed for the HK2-HK2 and HK2-RR2 pairs. Dark blue colonies indicate strong interaction among the other pairs. (c) Analysis of positive control and negative control by growth on SD/-His/-Leu/-Trp medium supplemented with X- α -Gal.

Fig. 4. Expression of *lacZ* reporter gene (β -galactosidase assay) for diploids containing different interacting partners. Each bar represents the mean $\pm$ SD of β -galactosidase activity from triplicate assays of three independent transformants. HK1n denotes Δ HK1₅₀₋₂₀₇, HK1D denotes Δ HK1₅₀₋₁₇₇, HK2n denotes Δ HK2₄₀₋₁₅₆, RR1 denotes RR1₂₁₋₁₄₄ and RR2 indicates RR2₁₆₃₋₂₅₀.

C terminus. HK2 forms dimers and each monomer interacts with HK1 and the receiver domain of TcrA.

HK2 bears a helical HPT domain-like structure (Shrivastava *et al.*, 2006). The interaction of HK2 and TcrA is likely to be similar to that between phosphotransferase domains and acceptor-like Spo0B and Spo0F. Structural studies have shown that Spo0F of *B. subtilis* interacts with α 1 and α 2 of the two Spo0B protomers, as well as two α - β loops in the C-terminal domain of the phosphotransferase (Varughese *et al.*, 2006). The interaction surface of Spo0B-Spo0F was shown to be hydrophobic, involving unconserved residues (Zapf *et al.*, 2000). The study between non-cognate pair of HPT domain response regulator as evident by crystal structure of the HPT domain of ArcB and CheY (Kato *et al.*, 1999) shows the interaction interface of the kidney-shaped HPT domain and the response regulator receiver domain and describes the four regions of contact. This ArcB-CheY interaction study thus provides support for the interaction observed between HK2 and TcrA in the present yeast two-hybrid screen.

The interaction strength as determined by the expression of β -galactosidase is weaker for the HK1-HK2-TcrA system than for other homologous proteins studied, such as interaction between NtrB and NtrC of *Klebsiella pneumoniae* (Martínez-Argudo *et al.*, 2001), and between KdpD and the Lpr family of *M. tuberculosis* (Steyn *et al.*, 2003),

Fig. 5. Proposed model of the protein-protein interaction assembly in the novel three-protein two-component system from *M. tuberculosis* based on interaction studies. The model indicates that the histidine kinase HK2 forms a dimer and interacts with ATP-binding HK1, resulting in phosphorylation of the conserved His residue on HK2. The interaction of HK2 with the receiver domain of the response regulator TcrA results in transfer of the phosphoryl group to the conserved Asp residue on TcrA.

where average β -galactosidase expression detected was around 40–50 units.

The interaction assembly of HK2 and HK1 is similar to the dimerization domain and catalytic domain arrangement of the autophosphorylation assembly in typical histidine kinases (Fig. 5). Structural analysis of the two-component system proteins suggested that the histidine kinases form homodimers, with their bi-helical histidine-containing domains forming the four-helix bundle at the interaction interface (Tomomori *et al.*, 1999). The catalytic ATP-binding domain of one monomer interacts with the dimerization domain of the other monomer and thus participates in trans-autophosphorylation of the conserved histidine (Hoch & Silhavy, 1995; Bilwes *et al.*, 1999; Tanaka *et al.*, 1998). Bacterial long phosphorelay pathways bear an additional histidine-containing helical domain, the HPT domain, as part of the hybrid kinases. HPT domains are helical domains with structural similarity to the four-helix bundle formed by the dimerization domains of the typical histidine kinases. This suggested that the helical bundles facilitate histidine phosphorylation. An *in silico* and circular dichroism study (Shrivastava *et al.*, 2006) indicated that HK2 is a complete helical protein having structural similarity with the HPT domains, and it was thus termed a HPT-mono-domain protein. HK1 is similar to the catalytic domain of the homologous histidine kinases. HK2 bears the conserved phosphorylatable His at its C terminus. The interaction assembly thus mimics the assembly of autophosphorylation in homodimeric histidine kinases, but is different in having an HPT-like domain replacing the four-helix assembly at the dimeric interface and in the catalytic domain being on a separate protein.

ACKNOWLEDGEMENTS

This work was financially supported by the Department of Biotechnology (Government of India). We thank the University Grants Commission (UGC), India, for providing a senior research fellowship to R.S. We acknowledge Professor Harald Wiker for providing the genomic DNA of *M. tuberculosis* and critical discussion regarding the manuscript.

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Edited by: T. Msadek