The SH3-Like Domain Switches Its Interaction Partners to Modulate the Repression Activity of Mycobacterial Iron-Dependent Transcription Regulator (IdeR) in Response to Metal Ion Fluctuations*

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Running head: Partner switching of the SH3-like domain in IdeR

Iron-dependent regulator (IdeR), a metal ion-activated pleiotropic transcription factor, plays a critical role in maintaining the intracellular iron homeostasis in Mycobacteria, which is important for the normal growth of the cells. This study was initially performed in an attempt to elucidate all potential interactions between the various domains of IdeR that occur in living mycobacterial cells. This led to a hitherto unidentified self-association for the SH3-like domain of IdeR. Further studies demonstrate that the SH3-like domain interacts with different partners in the dimeric forms of IdeR depending on the levels of metal ions in the environment: it undergoes inter-subunit self-association in the metal-free DNA-non-binding form, but interacts with the N-terminal domain in the metal-bound DNA-binding form in an intra-subunit manner to finely modulate the transcription repression activity of IdeR. Our more detailed mapping studies reveal that the SH3-like domain uses an overlapping surface to participate in these two interactions, which therefore occur in a mutually exclusive fashion. This novel mechanism would allow an effective and cooperative interconversion between the two functional forms of IdeR. Our data also demonstrate that a disturbance of the interactions involving the SH3-like domain impairs the transcription repression activity of IdeR and delays the growth of mycobacterial cells.

Ferrous ion (Fe2+) is an essential cofactor for a large number of proteins, functioning in many vital physiological processes (1). Nevertheless, excessive free Fe2+ is extremely harmful to cells under aerobic conditions by catalyzing the formation of highly toxic hydroxyl free radicals (2). As a result, strict cellular homeostasis of free Fe2+ must be effectively maintained for all living organisms.

In mycobacteria, the Iron-dependent Regulator (IdeR) has been identified as a pleiotropic transcription regulator that regulates the expression of a number of iron-acquisition genes, thus playing a critical role in maintaining the intracellular iron homeostasis (3-6). IdeR has also been found to regulate the expression of genes functioning in the storage of iron and resistance to oxidative stress (4, 5, 7). Deletion of the ideR gene in the pathogenic M. tuberculosis was found to be lethal for the strain (7). Similar deletion of the ideR gene in M. smegmatis, a non-virulent and fast growing mycobacterium, engendered the cells defective in repressing the expression of the iron-acquisition genes even in the presence of excessive iron (4).

Homologues of IdeR have also been found in a variety of other gram-positive bacteria (8-12), with the best-characterized being DtxR from Corynebacterium diphtheriae (12), which is highly similar to IdeR in structure and function (3). The binding of Fe2+ to IdeR/DtxR transforms it from a metal-free DNA-non-binding form to a DNA-binding form that is able to specifically bind to a conserved “iron box” operator DNA sequence (5, 13). Besides Fe2+, other transition metal ions such as Co2+, Ni2+, Zn2+, Cd2+ and Mn2+ are also able to activate IdeR/DtxR to its DNA-binding form under in vitro conditions (13, 14).

Data of crystal structure determination revealed that the DNA-binding form of IdeR/DtxR exists as homo-dimers with each monomer consisting a N-
terminal domain and a C-terminal domain connected via a highly flexible linker (15-18). The highly conserved N-terminal domain with two metal binding sites (15-18, 19) has been considered responsible for the dimerization and DNA-binding processes, and can be further divided into two corresponding sub-domains. The C-terminal domain was also designated as the SH3-like domain, in view of its similar folding pattern but hardly any sequence similarity to that of the commonly existing SH3 domains of eukaryotic proteins (17, 18, 20).

In contrast to that of the DNA-binding form, the structural information of the DNA-non-binding form of IdeR/DtxR is still very limited. Data of NMR studies revealed that the N-terminal domain of DtxR undergoes a significant conformation change during the transformation from its DNA-binding to DNA-non-binding form, changing from a well-organized structure to a partially disordered “molten globule” state (21). It has been generally believed that the IdeR/DtxR dimers dissociate into monomers during this structural transformation. Nevertheless, dimers were also occasionally observed for the DNA-non-binding form of IdeR/DtxR (13, 21-24), which was interpreted as such that the dimerization sub-domain in the molten globule state somehow is still able to weakly mediate the dimerization process (13, 24). Whether regions other than the dimerization sub-domain also contribute to this dimerization process is a question that has not been addressed.

The SH3-like domain has been known to play a role in forming the DNA-binding form of IdeR/DtxR, mainly based on the determination of its crystal structure (17, 18). Although hardly visible in the initially reported crystal structures of the DNA-binding forms of IdeR/DtxR (15, 25), the SH3-like domain was later found to interact with the N-terminal domain, contributing to metal ion binding in the better-refined structures (16-18). In such structures, the orientation of the SH3-like domain was found to be slightly different in each of the IdeR/DtxR monomer, suggesting a dynamic nature of the interaction between the SH3-like and N-terminal domains, with the meaning of which hardly known.

In an attempt to examine whether the domain-domain interactions in IdeR/DtxR revealed by these in vitro studies (17, 18, 20) indeed occur in vivo, we unexpectedly revealed the hitherto unidentified self-association of the SH3-like domain of IdeR. Further in vitro studies demonstrate that this novel interaction largely occurs in the DNA-non-binding form of IdeR in the absence of metal binding, using a surface that overlaps with the one used by the SH3-like domain to interact with the N-terminal domain. The physiological significance of these interactions involving the SH3-like domain was also demonstrated in both M. smegmatis and M. tuberculosis.

**EXPERIMENTAL PROCEDURES**

**Strains, plasmids and culture conditions** - All strains and plasmids used in this study were listed in table S1. *Escherichia coli* DH5α, Top10 and BL21(DE3) strains were cultured routinely in Luria-Bertani (LB) broth at 37°C. *M. smegmatis* ATCC607 was grown in Middlebrook 7H9 liquid media or on Middlebrook 7H11 agar plates (supplemented with 0.5% glycerol, 0.5% Tween 80 and the indicated antibiotics). M63 minimal media (supplemented with 0.5% glycerol, 1 mM MgSO₄ and the indicated antibiotics) were used in survival assays for protein-protein interaction detection. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 liquid media or on Middlebrook 7H10 agar plates (supplemented with 10% OADC, 0.5% glycerol and 0.5% Tween 80 and the indicated antibiotics). For cfu counting, *M. tuberculosis* H37Rv cells were cultured on Löwenstein-Jensen (L-J) media. Concentrations for chemicals and/or antibiotics added in the culture media were as follows: ampicillin (Amp) at 100 µg/ml, kanamycin (Kan) at 50 µg/ml (for *E. coli*), or 25 µg/ml (for *M. smegmatis*), hygromycin (Hyg) at 200-250 µg/ml (for *E. coli*) or 50 µg/ml (for *Mycobacteria*), trimethoprim (TMP) at 10 µg/ml, anhydrotetracycline (Ate) at 50 ng/ml. The *M. smegmatis* strains were routinely cultured at 37°C except for protein-protein interaction detection, where the culture temperature was shifted to 30°C. *M. tuberculosis* H37Rv strains were routinely cultured at 37°C.

**Construction of the plasmids used for detecting protein-protein interactions in Mycobacteria** – In our developed mycobacterial mDHFR-PCA system, the two fusion proteins were expressed from two compatible *E. coli*-Mycobacteria shuttle vectors, designated as pLC_Teff1/2 and pLC3 respectively (See Fig. 1A). pLC_Teff1/2 is an extra-chromosomal plasmid with 4-8 copies in each mycobacteria cell; however, pLC3 is an integrative plasmid with only a single copy in each mycobacterial cell.

The pLC_Teff1/2 vector was constructed as follows.
The DNA fragment containing \( P_{\text{tetO}} \) sequence was prepared as described (26), and then was digested with HindIII and EcoRV before being sub-cloned into the pAL5,000 originated E. coli-mycobacteria shuttle vector pSUM40 (kindly provided by Prof. Ainsa, Ref. 27). Subsequently inserted into this plasmid was an EcoRV and Kpnl double digested DNA fragment encoding yeast GCN4 protein, a (Gly-Gly-Gly-Gly-Ser)\(_2\) flexible polylinker and the mDHFR F[1,2] fusion protein, as amplified from the plasmid pBluscript-mDHFR-F[1,2] (kindly provided by Prof. Michnick, 28). The region encoding GCN4 in this new plasmid was removed by digesting with EcoRV and ClaI and then replaced by an oligonucleotide fragment encoding the FLAG tag and a multiple cloning site (MCS, see Fig. 1A for its detailed sequence). The constructed plasmid was named as pDF\(_{myc}\)1/2. Finally, a DNA fragment encoding the tetR gene of the E. coli Tn10 transponson (amplified from the genome of E. coli XL1-blue and the intrinsic HindIII restriction site of the tetR gene was synonymously mutated via overlapping PCR) was fused to the downstream of a DNA fragment containing the Hsp60 promoter of M. bovis BCG (amplified from the genome of M. bovis BCG) via overlapping PCR, before being digested with HindIII and inserted into pDF\(_{myc}\)1/2. The plasmid with transcriptions controlled by \( P_{\text{hsp60}} \) and \( P_{\text{tetO}} \) promoters in opposite directions was selected and designated as pLC3 \(_{tetR}\)1/2.

The pLC3 vector was constructed as follows. The first step of the construction was with virtually the same procedure as that for generating pDF\(_{myc}\)1/2 except that the DNA fragment encoding yeast GCN4 protein, a (Gly-Gly-Gly-Gly-Ser)\(_2\) flexible polylinker and the mDHFR-F[3] fusion protein was amplified from the plasmid pBluscript-mDHFR-F[3] (kindly provided by Prof. Michnick, 28). The new constructed plasmid was thus designated as pDF\(_{myc}\)3. The DNA fragment encoding test protein (designated as X here) was then inserted into the multiple cloning site (all were inserted between NotI and BamHI in this study) of pDF\(_{myc}\)3. To avoid potential incompatibility between the pLC\(_{tetR}\)1/2 and pLC\(_{myc}\)3, the whole expression cassettes of the DHFR-F[3] fusion proteins was unloaded by cleaving with HindIII and Kpnl and then inserted into the pMV306\(_{Hyg}\) vector (kindly provided by Prof. Clifton Barry, NIH) to generate pLC3-X. The physical maps of pLC\(_{tetR}\)1/2 and pLC3 can be obtained upon request.

**Detection of protein-protein interactions in M. smegmatis** – pLC\(_{tetR}\)1/2 and pLC3 carrying the DNA-fragment encoding the indicated test proteins (each about 300 ng) were co-transformed into M. smegmatis competent cells by electroporation. Co-transformed bacteria were selected on Middlebrook11 agar media (containing 0.5% glycerol, 50 \( \mu \)g/ml Amp, 25 \( \mu \)g/ml Kan and 50 \( \mu \)g/ml Hyg) at 37°C. The well-separated colonies were then transferred into liquid Middlebrook 7H9 media (supplemented with 0.5% glycerol, 0.5% Tween 80 and the same amount of above three antibiotics) and grown at 37°C to late log phase. The cultured cells were washed twice with M63 medium, diluted to OD\(_{600}\) 0.001, before spotted onto M63 agar plates (supplemented with 0.5% glycerol, the three antibiotics, 10\( \mu \)g/ml TMP and 50\( \mu \)g/ml AAc) and cultured at 30°C for approximately 5-7 days. The test was performed at 30°C instead of 37°C to avoid any possible impairment of high temperature on the folding and subsequent interaction of the fusion proteins, as demonstrated by Pelletier et. al when applying mDHFR PCA in E. coli (28).

**Site-specific mutagenesis, expression and purification of recombinant proteins** – DNA encoding full-length IdeR was amplified from the genome of M. smegmatis ATCC607 directly. The DNA fragments encoding all IdeR variants were digested with NcoI and NotI before being sub-cloned onto pET21bm (a modified pET21b plasmid in which the initial Ndel restriction site was mutated to a NcoI restriction site) and transformed into E. coli BL21(DE3) cells. His-tags were fused to the C-terminals of all expressed proteins for the purposes of affinity purification and the Western blot detection. The sequences of encoding genes were all verified by DNA sequencing. The detailed sequences of all primers used in this study can be found in Table S2.

For protein expression, all E. coli transformants were cultured in LB/Amp media at 37°C to OD\(_{600}\) about 0.5, induced with 0.25 mM isopropyl \( \beta \)-D-thiogalactopyranoside (IPTG) for 4 hours before being harvested by centrifugation. All recombinant proteins were purified according to the following procedure. The cells were re-suspended in a buffer containing 50 mM phosphate, 150 mM NaCl and 15 mM imidazole, at pH 7.4 (5 mM \( \beta \)-mercaptoethanol was also added for the purification of the cysteine-substituted proteins) before being lysed by ultrasonic. The cell lysates were then clarified by centrifugation, loaded to a Ni-NTA column before
the bound IdeR proteins were eluted with 100-500 mM imidazole. Collected protein samples were then dialyzed against a dialysis buffer containing 50 mM phosphate, 150 mM NaCl, pH 7.4 to remove the imidazole (5 mM DTT was also added in the dialysis buffer for cysteine-substituted IdeR mutant proteins) and stored at -80°C before use. Protein concentration was determined with the BCA-based method using bovine serum albumin (BSA) as standard.

Size-exclusive chromatography - Analytical size-exclusive chromatography was performed on a ÄCTA Purifier system using a pre-packed Superdex 75 10/300 GL column (Amersham Biosciences Biotech). Protein sample (100 µl) was loaded and then eluted with buffer containing 50 mM sodium phosphate, 150 mM NaCl, pH 7.4 at a flow rate of 0.3 ml/min. The elution profile was recorded as the value of the light absorption at 220 nm (due to the absence of Trp, Tyr and Phe residues in the SH3-like domain of IdeR).

Glutaraldehyde cross-linking - Cross-linking of purified SH3-like domain of IdeR was carried out with glutaraldehyde by the following procedures. The purified proteins (1 mg/ml) were first treated with indicated concentration of glutaraldehyde at room temperature for 10 minutes, and then quenched on ice with 100 mM Tris-HCl (pH 8.0) for another 10 minutes. The cross-linked products were then analyzed by 12% SDS-PAGE.

Non-denaturing pore gradient polyacrylamide gel electrophoresis (PAGE) - Non-denaturing pore gradient polyacrylamide gel with a gradient from 6% to 45% was prepared mainly according to the methods described by Ausubel et al (29). Samples were electrophoresed at 100 V for indicated time at a temperature of 4°C.

Molecular docking and structure analysis - Homo-multimeric docking of the SH3-like domains of IdeR was carried out utilizing ClusPro program (http://nrc.bu.edu/cluster) (30-32), with one monomeric SH3-like domain of IdeR from M. tuberculosis (residues 151-230, PDB code number: 1U8R, Ref. 17) as the starting three-dimensional coordinates. The selection of residues for cysteine scanning mutagenesis described in Fig. 3 was based on following two criteria: (i) it should be located at or near the interaction surface of docked oligomers; (ii) Cα-Cα distances between two same residue on assembled oligomers should be less than 10 Å. The analysis of crystal structures and the calculation of the distances between indicated residues were all performed with PYMOL program.

Detection of disulfide bonds – E. coli BL21(DE3) cells were transformed with plasmid pET21m which carries the DNA fragment encoding the SH3-like domain with the indicated position being substituted with cysteine residue. The transformed cells were induced with 0.5 mM IPTG for 4 hours (37°C), collected and re-suspended in phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 1.4 mM K₂HPO₄, and 1 mM PMSF. The reducing condition was kept by the addition of 5X Laemmli loading buffer (supplemented with 25 mM N-ethylmaleimide and 25 mM EDTA). The reducing condition was maintained by the addition of 50 mM DTT in replaced of NEM and EDTA into the 5X loading buffer. The samples separated with 12% SDS-PAGE (50 µg each lane) were transferred to PVDF membrane and detected with monoclonal antibody against His-tag. The bands were visualized immunochromically by using AP conjugated IgG.

Cysteine specific cross-linking - The cysteine-specific cross-linking was performed according to methods previously described (33, 34) with minor modifications. Briefly, protein samples were treated with o- or p-PDM (at 500 µM, dissolved in DMSO) and then incubated at room temperature for 10 min (DMSO alone was added for negative control). The reaction was stopped by quenching with 10 mM DTT. The active or inactive forms of the IdeR proteins (of ~2 µM) were maintained respectively by adding 200 µM CoCl₂ or 3 mM DP for 10 minutes at room temperature before cross-linking treatment.

Spin labeling and EPR measurements – Spin-labeling of the IdeR(C102D/H173C) protein and the following EPR measurement were performed as previously described (35) with minor modification. Briefly, purified IdeR(C102D/H173C) protein (at 5 mg/ml) was first dialyzed against 50 mM phosphate buffer (containing 150 mM NaCl, pH 7.4) at 4°C for 3 hours to remove the trace amount of DTT before being labeled with 1 mM maleimide-PROXYL overnight at 4°C. The labeled samples were concentrated to a final concentration of 250 µM by ultra-filtration after all free probes were removed by

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thoroughly dialysis (against with 20 mM Tris-
HCl, containing 50 mM NaCl, pH 7.4). The EPR
spectra were collected over 80 G scan width (at
room temperature) or over 120 G scan width (at
150K) using the modulation amplitude of 2.5 G
and a microwave power of 8 mW.

EMSA – Binding of IdeR to operator DNA
was performed as described by Spiering, et al
(23). The 33 bp mbtA operator DNA was
prepared by directly annealing two synthesized
complemented oligonucleotides (one was labeled
with FAM at its 5’ end). The binding reaction
mixture was incubated at 25°C for 10 min before
being subjected to a 12% PAGE in 40 mM Tris-
acetate (pH7.5) / 2.5% glycerol and electrophoresed in the same buffer without
glycerol. 200 µM CoCl₂ was added in the
reaction mixture, PAGE gel and running buffer to
maintain IdeR proteins in their active forms. Gels
were visualized by scanning with Typhoon
scanner utilizing exciting wavelength at 488 nm.
Oxidized forms of IdeR mutants, in which certain
types of interactions were immobilized via
disulfide bonds (Fig. 5), were prepared by
dialyzing the samples under the ambient
oxidizing condition for 48 hours (at 4°C).
Reduction of disulfide bonds was achieved by
addition of 5mM DTT into oxidized samples and
then incubated on ice for 30 min.

In vitro transcription/translation assay –
The reporter plasmid, pPT-Luc, used in in vitro
transcription/translation reactions, was
constructed as follows. The region between the
BglII and XbaI sites on plasmid pET28a was
replaced by a DNA sequence containing the
hybrid promoter tacP/toxO (36), to generate
plasmid pPT. A DNA fragment encoding
luciferase was then obtained from plasmid pGL3-
Basic by digesting with NcoI and BamHI before
being inserted into pPT to obtain pPT-Luc. The
detailed physical map of pPT-Luc can be
obtained upon request. In vitro transcription and
translation reaction was performed as described
by Love, et al (36- 38) by using Promega S30
Extract system (Promega Co.). Briefly, pPT-luc
was added to 20 µl of premix and 5 µl of
complete amino acid mixture. Indicated amount
of IdeR proteins and/or DP were then added, and
the reaction volumes were brought up to 50 µl
with DEPC-treated water. Reactions were
incubated at room temperature for 10 min, and
then 15 µl of S30 extracts was added. Reaction
mixtures were incubated at 37°C for 1 hour
before halted by incubating on ice for 10 min. The
activity of luciferase was analyzed with Turner
luminometer (Terner 20/20®). All reactions were
performed in triplicate.

Tricine SDS - polyacrylamide gel
electrophoresis- Tricine SDS- PAGE (12%) was
performed as previously described (39).

RNA extraction and real time PCR – To analyze
the expression level of fxbA in M. smegmatis cells,
the bacteria transformed with indicated plasmids
were cultured in Middlebrook 7H9 medium
(supplemented 25 µg/ml kanamycin, 0.5% glycerol,
0.5% Tween 80 and 50 ng/ml Atc) at 37°C until the
late log phase. The collected cells were re-suspended
into TRNzol and broken via ultrasonication. RNA
extraction was performed according to
manufacturer’s instructions (Tiangen Co.). A mixture of the reverse primers of fxbA and mysA (with their
sequence to be found in Table S2) was added in each
extracted RNA sample to synthesize the cDNAs of
the two genes at the same time. mysA, as a housekeeping gene in mycobacteria (5), was used as
the internal standard to normalize the expression
level of fxbA in each RNA sample. The real time
PCR reactions were performed on DNA-Engine
Opticon-continuous Fluorescence detection system
(MJ research) by using a SYBR ExScript RT-PCR kit
(Takara Co.). Reaction mixtures were subjected to
PCR with the following program: 95°C 5s, 60°C 20s
for 45 cycles.

M. tuberculosis infection and pathogenicity
testing – M. tuberculosis H37Rv was transformed with
pLC3-IdeR(Δ1-140) or pLC3-GCN4 by
electroporation and then selected onto Middlebrook
7H10 agar plates (supplemented with 0.5% glycerol,
10% OADC and 50 µg/ml Hyg) at 37°C for 4-6
weeks. Well-separated colonies were picked into
Middlebrook 7H9 liquid media (supplemented with
0.5% glycerol, 0.5% Tween 80, 10% OADC and 50
µg/ml Hyg) and grown at 37°C until the late log
phase. Two groups of BALB/c mice (6-8 weeks old)
were then infected via tail vein injection with 1X10⁷
of the cultured bacteria. Mice (n=3) from each group
were sacrificed at the end of 1, 2 and 4 weeks after
the initial infection. Lungs and spleens were weighed
and homogenized. Bacterial loads in organs were
assessed by serial dilution of homogenates on L-J
media before being cultured at 37 °C for 4-6 weeks.

RESULTS

The SH3-like domain of IdeR self-associates in
living mycobacterial cells - In an attempt to
systematically examine all the possible pairwise interactions occurring between the domains of IdeR, including those observed by crystal structure determination, we developed a system that is able to detect protein-protein interactions in living mycobacterial cells. This system was designed as follows. On the one hand, the murine dihydrofolate reductase (mDHFR) is rationally split into two fragments (designated as F[1,2] and F[3]), which are unable to interact with each other by themselves to restore the enzymatic activity unless both are fused to two interacting proteins. On the other hand, the restored enzymatic activity of mDHFR would be essential for the growth of the \textit{M. smegmatis} cells under a selection condition, where the endogenous DHFR is specifically suppressed by trimethoprim (TMP), an inhibitor known to be highly effective towards prokaryotic DHFR but far less effective towards the eukaryotic ones such as mDHFR (28).

The expression of both fusion proteins from the pair of plasmids, designated as pLC\textsubscript{TetR1/2} and pLC3 (schematically presented in Fig. 1A), is directed by the \textit{P}_{\text{TetO}} hybrid promoter (26), towards which the TetR protein acts as a repressor. The TetR protein, being constitutively expressed from the pLC\textsubscript{Tet1/2} plasmid, will be inactivated by binding with its specific inactivator anhydrotetracycline (Ate, ref. 26) and thus allowing the expression of the fusion proteins to be initiated. The feasibility of this designed system was demonstrated by fusing yeast GCN4, which is known to self-associate (28), with the two mDHFR fragments: the \textit{M. smegmatis} cells co-transformed with the pair of plasmids expressing GCN4-F[1,2] and GCN4-F[3] grew on a selection medium containing both the inhibitor of prokaryotic DHFR (TMP) and the inactivator of TetR repressor (Ate), but not on that containing the former but not the latter (Fig. 1B). It should be noted that our protein-protein interaction system described above is similar to what was recently reported by Singh \textit{et al.} (40) in overall strategy, but different in details. For instance, the expression of the fusion proteins will be initiated only by induction in our system, but being constitutive in theirs.

Results of analysis of pairwise interactions of various forms of IdeR (including the full-length and the indicated truncation forms) in living \textit{M. smegmatis} cells are displayed in Fig. 2. Consistent with previous observations of \textit{in vitro} studies (15, 17, 18, 41), the full-length IdeR homo-dimerized in living \textit{M. smegmatis}, as indicated by the growth of cells co-expressing IdeR-F[1,2] and IdeR-F[3] on the selection medium (spot \textit{bb} in Fig. 2B); the N-terminal domain alone, containing the dimerization sub-domain characterized earlier, also underwent homo-dimerization in the cells (spot \textit{dd} in Fig. 2B).

Totally unhindered from previous studies are the following observations. On the one hand, a truncation form of IdeR lacking the dimerization sub-domain so far defined not only self-associated (spot \textit{cc} in Fig. 2B) but also interacted with the full-length IdeR in the living cells (spots \textit{bc} and \textit{cb} in Fig. 2B). On the other hand, a truncation form of IdeR solely containing the SH3-like domain also self-associated in mycobacterial cells (spot \textit{ee} in Fig. 2B). These observations taken together strongly suggest that the SH3-like domain represents one additional region, other than the previously characterized dimerization sub-domain, to be also able to mediate dimerization for IdeR in mycobacterial cells.

Interaction between the SH3-like domain and the N-terminal domain, despite its observation in the resolved crystal structures of full-length IdeR (17, 18), was not detected using our \textit{in vivo} assay, where the two domains were separately expressed and fused to the mDHFR fragments F[1,2] and F[3], as indicated by the failure of growth of mycobacterial cells co-expressing these proteins (spots \textit{de} and \textit{ed} in Fig. 2B). This result apparently helps to resolve an unanswered question of crystal structure studies, where it was not known whether the interacting SH3-like domain and the N-terminal domain come from the same or different subunits in the dimeric forms of IdeR, because of the invisibility of the highly flexible linker region between these two domains (17, 18). The failure of detecting interactions between these two domains when expressed separately implicates that coming from the same subunit and being connected together in one polypeptide chain are essential for the interaction of these two domains to occur (Data presented in Fig. 6 further demonstrate that the cleavage of the linker region almost completely abolished the interaction between the SH3-like and N-terminal domains).

The self-association of the SH3-like domain and its interaction with the N-terminal domain of IdeR are mediated by an overlapping surface – The capacity for the separately expressed SH3-like domain of IdeR to undergo self-association under \textit{in vitro} conditions was then examined via both size-exclusion chromatography (Fig. 3A) and chemical cross-linking (Fig. 3B) analyses, revealing the
presence of both monomeric and dimeric forms. This somehow suggests a dynamic and/or weak nature of the self-association for the SH3-like domain. This conclusion is apparently supported by results of non-denaturing pore gradient PAGE analysis (29), presented in Fig. 3C, where a dimeric form of the separately expressed SH3-like domain was visible only after a shorter time of electrophoresis but disappeared after a longer time of electrophoresis, as indicated by the gradual decrease in intensity of the upper bands (asterisk-labeled) from lanes 1 to 3. Such results reflect a typical undergoing of dynamic dissociation of oligomeric proteins as explained by us before (42-44).

To map the surface on the SH3-like domain mediating its self-association, cysteine scanning mutagenesis (45) was performed, for which the residue located at the interaction surface will be identified by its allowing the formation of disulfide bonds after being replaced by a cysteine residue, as reflected by the detection of cross-linked dimers under oxidized conditions (46). For this purpose, each of 21 positions on the SH3-like domain was substituted by a cysteine residue. The substituting positions were chosen as such that they are largely located at the surface of the SH3-like domain according to the determined structure of full-length IdeR (17, 18), with some having a predicted location at the self-association surface based on the models generated from homo-oligomeric docking (for more details see the Experimental Procedures).

Results presented in Fig. 3D demonstrate the formation of covalently linked dimers for the SH3-like domain under oxidizing (top panel) but not reducing (bottom panel) conditions when the residue at position 173, 192, 201, 202, 203, 218, 219 or 220 was replaced by a cysteine residue. The mapping of these positions (red colored in Fig. 3E) on the tertiary structure of the SH3-like domain reveals a remarkable clustering of them. Markedly, this surface nicely overlaps with that the SH3-like domain uses to interact with the N-terminal domain in the DNA-binding form of the full-length IdeR (17, 18; also see the right side in Fig. 3E). This apparently explains why the self-association of the SH3-like domain was not observed in the reported crystal structure of IdeR, in which the SH3-like domain interacts with the N-terminal domain (17, 18).

**The SH3-like domain self-associates in the metal-free DNA-non-binding form of full-length dimeric IdeR** — It was then examined whether the SH3-like domain in the full-length IdeR protein indeed undergoes self-association using the surface characterized above. Given that the SH3-like domain interacts with the N-terminal domain in the DNA-binding form of IdeR (17, 18), it is conceivable that the self-association of the SH3-like domain, if does happen in the full-length IdeR, might occur in its metal-free DNA-non-binding form, in which the N-terminal domain is believed to exist as a partially disordered molten globule state (21). As a result, the interaction between the SH3-like domain and the N-terminal domain would be unlikely to occur, and thus free the surface for the SH3-like domain to self-associate.

To test such a hypothesis, the cysteine-specific cross-linking method (33, 34) was applied to analyze the IdeR(C102D/H173C) mutant, where replacement of the single intrinsic Cys102 by aspartate would only allow the specific cross-linking to occur via the cysteine introduced at position 173, which was above revealed to be located at the self-association surface for the separately expressed SH3-like domain (see Fig. 3D and 3E). The cysteine to aspartate substitution at position 102 has been constructed in a few previous reports, with the mutant IdeR/DtxR protein retaining much of its activity in metal-dependent DNA binding and transcription repression (14, 21, 25, 47-48). Our own data of in vitro transcription/translation analysis (presented below in Fig. 7C) also demonstrate that IdeR(C102D) indeed retains its metal dependent repression activity against specific operator DNA sequences. Similarly, the IdeR(C102D/H173C) protein was also shown to exhibit a metal ion dependent binding activity towards the specific operator DNA fragment (see Fig. 5A below).

Results presented in Fig. 4A demonstrate that the self-association of the SH3-like domain in the full-length IdeR indeed occurs but only in its metal free DNA-non-binding form. This is indicated by the detection of a cross-linked dimeric form of IdeR(C102D/H173C) predominantly when all free metals were chelated by the addition of 2,2'-dipyridyl (DP) but hardly any in the presence of Co²⁺ (compare lanes 8 and 7 in Fig. 4A) or when IdeR(C102D) was subject to the same analysis (lanes 2 to 4 in Fig. 4A). It should be noted that a second cross-linked product (star-labeled in Fig. 4A), present in far less abundance and migrated at a slower rate than the dimeric form described above, is occasionally observed for both IdeR(C102D) and IdeR(C102D/H173C) (see lanes 3 and 7, Fig. 4A).
These second cross-linked products are likely the dimeric forms that are cross-linked via the amine groups at the N-terminal or internal lysine residues, in view that the functional group maleimide of cross-linking agent p-phenylenediamineimide (p-PDM) used here may also generate cross-linking between amino groups, although in a far less effective manner (49).

The self-association of the SH3-like domain in the DNA-non-binding form of full-length IdeR was further confirmed by detecting the occurrence of spin-spin interaction between a pair of spin probes specifically attached to the cysteine residue of each of the subunits of IdeR(C102D/H173C). Such spin-spin interaction can only be detectable as the two probes are very close to each other (within a distance of 8-25 Å, ref. 35, 50). For this purpose, Cys73 in IdeR(C102D/H173C) was labeled with spin probe PROXYL and the labeled protein was then subject to EPR measurement. The EPR spectrum of the frozen PROXYL-labeled IdeR(C102D/H173C) protein in the presence of metal chelator (DP), presented in Fig. 4B, reveals a strong spin-spin interaction effect, as indicated by having a dI to d ratio (dI/d) of 0.58. (a value of dI/d larger than 0.4 is commonly taken to indicate a spin-spin interaction between two probes, ref. 35, 50).

It was subsequently examined whether the lack of association between the SH3-like domains of the two subunits in the metal bound DNA-binding form (as indicated by results presented in lane 7, Fig. 4A) is really accompanied by the occurrence of interaction between the SH3-like and N-terminal domains of the same subunit. For this purpose, two cysteine residues were introduced into IdeR(C102D), substituting Lys185 in the SH3-like domain and Glu95 in the N-terminal domain respectively, to generate the IdeR(C102D/E95C/K185C) mutant protein. These two positions are expected to be proximal to each other in the DNA-binding form of IdeR, as guided by the data of crystal structure determination (17, 18).

The cysteine specific cross-linking produced a form of the IdeR(C102D/E95C/K185C) protein that migrated at a faster rate than the uncross-linked monomeric form and that is most predominant with the addition of Co2+ (compare results presented in lanes 7 and 8, Fig. 4C). The position of the cross-linked product unequivocally indicates intra-subunit nature of the cross-linking. This also demonstrates that the interacting SH3-like and N-terminal domains come from the same chain, as suggested by our results of in vivo protein-protein interaction assays, where the interaction between the separately expressed SH3-like and N-terminal domains was not detected (see Spots dE and eD in Fig. 2B). The detection of minute amount of intra-subunit cross-linked product in the presence of the chelator (lane 8 in Fig. 4C) most likely reflects the difficulty of a complete removal of the protein-bound metals by the chelators. The cross-linking agent α-PDM was chosen here because the length of its cross-linking arm (7.7 Å, Ref. 33) fits ideally the estimated spatial distance between positions 95 and 185 (7.4 Å) in the DNA-binding form of IdeR by referring to the determined crystal structure (17, 18).

**Immobilization of the self-association of the SH3-like domain weakens but that of the interaction between the SH3-like and N-terminal domains enhances the DNA-binding capacity of IdeR.** To understand its biological role, the self-association of the SH3-like domain in IdeR was immobilized by disulfide bond formation before examining its capacity to bind to specific mycobacterial mibA operator (O_mibA, ref. 5, 18) DNA fragment by electrophoretic mobility shift assays (EMSAs), again using the IdeR(C102D/H173C) protein.

The EMSA results, presented in Fig. 5A, clearly demonstrate that the DNA-binding capacity of IdeR(C102D/H173C) was significantly weakened after the self-association of its SH3-like domain was immobilized even in the presence of Co2+ (compare lanes 2 and 3, 4 and 5, 6 and 7, 8 and 9 respectively in Fig. 5A). In contrast, a similar immobilization of the intra-subunit interaction between the SH3-like and N-terminal domains significantly strengthened the capacity of IdeR(C102D/E95C/K185C) to bind to the same operator DNA sequence (compare lanes 2 and 3, 4 and 5, 6 and 7, 8 and 9 respectively in Fig. 5C). Formation of disulfide bonds in these interaction-immobilized protein samples was confirmed by SDS-PAGE analyses (Fig. 5B and 5D).

**Cleaving or lengthening the linker between the SH3-like domain and the N-terminal domain impairs their interaction as well as the metal non-responsive transcription repression activity of IdeR.** The failure of detecting interaction between the separately expressed SH3-like and N-terminal domains of IdeR under in vivo conditions (Fig. 2B) suggests a relatively weak interaction between them.
and thus needs a linker of proper length to connect them together for the intra-subunit interaction to occur in full-length IdeR. Two strategies were taken to test this hypothesis. One is to make a cleavage at the linker region and the other is to lengthen the linker region. The effects of such disturbances on the IdeR proteins were then examined by measuring the DNA-binding activity via EMSA or the transcription repression activity by using the \textit{in vitro} transcription/translation assay (51).

In order to make the cleavage, a six-residue thrombin-cleavage site was first inserted into the linker region of IdeR(C102D), generating the IdeR(C102D, thromA) protein (Fig. 6A). The results of EMSA, which is always performed in the presence of Co\textsuperscript{2+}, presented in Fig. 6C demonstrate that the cleaved products indeed exhibited a significantly decreased DNA-binding activity (compare lanes 2-5 with lanes 7-10) with the DNA probes being shifted by the cleaved products to a position lower than that by the intact proteins (also compare lanes 2-5 with lanes 7-10) but the same position as that by IdeR(C102D)ASH3 (compare lanes 7-10 with lanes 12-15). These results strongly suggest that the SH3-like domain no longer interacts with the N-terminal domain in the cleaved products (even in the presence of Co\textsuperscript{2+}) and only the N-terminal domain complexes with the operator DNA. Effective thrombin cleavage was confirmed by SDS-PAGE analysis (lanes 3-6 in Fig. 6B).

The transcription repression activity of these cleaved products of IdeR was then examined using the \textit{in vitro} transcription/translation system (51), being much closer to the real situation inside a living cell and quantitative in nature. In this system, the higher the level of transcription repression activity for IdeR, the lower the level of activity for the luciferase reporter whose expression is directed by a modified promoter of pTac integrated with two tandem IdeR-specific operator \(O_{\text{op}}\) sequences (36–38, 52).

Consistent with the results of EMSA, the cleaved products of IdeR(C102D, thromA) exhibited a transcription repression activity much lower than that of the intact protein (compare columns 1 and 3, Fig. 6D) in such \textit{in vitro} transcription/translation system, itself containing a certain level of metal ions (36, 38). Additionally, such repression activity of the cleaved products of IdeR was hardly brought to any further decrease when an excess amount of metal chelators (DP) were added (compare columns 3 and 4 in Fig. 6D), indicating an almost complete abolishment of repression activity for IdeR when the intra-subunit interaction between the SH3-like and N-terminal domains was eliminated. It should be noticed that although the N-terminal domain alone was apparently able to bind to the specific operator DNA sequences (as shown by data presented in Fig. 6C), it is unable to repress the transcription as assayed with the \textit{in vitro} transcription/translation system. These observations together suggest that interacting with the SH3-like domain is far more important than previously speculated for the N-terminal domain of IdeR to bind to the specific operator DNA sequences and in turn to exhibit the transcription repression activity in cells. In other words, the capacity of binding to the specific operator DNA sequences \textit{in vitro} (as usually detected by EMSA) for the N-terminal domain of IdeR should not be simply taken as to indicate its capacity to repress transcription \textit{in vivo}.

It is conceivable that optimal occurrence of the relatively weak intra-subunit interaction between the SH3-like and N-terminal domains is highly dependent on the connecting linker of proper length. It follows that extending the length of the linker between the SH3-like and N-terminal domains would weaken the interaction, thus decreasing the DNA-binding and transcription repression activities of IdeR. Our data indeed demonstrate that the transcription repression activity was decreased proportionally, as shown by data presented in Fig. 7C, when the length of the linker is increased by 2 or 4 folds (illustrated in Fig. 7A and confirmed by data shown in Fig. 7B).

\textbf{Heterologously over-expressing the SH3-like domain of IdeR up-regulates the expression of IdeR-controlling gene in \textit{M. smegmatis} cells – The physiological importance of the interactions involving the SH3-like domain of IdeR was then examined. For this purpose, the SH3-like domain of IdeR (as a fusion protein with mDHFR-F[1,2]) was heterologously over-expressed in \textit{M. smegmatis}. Our data of \textit{in vivo} protein-protein interaction studies (see Fig. 2B) have demonstrated that the separately expressed SH3-like domain predominantly interacts with the SH3-like domain instead of the N-terminal domain of IdeR in living mycobacterial cell. It follows that the heterologously over-expressed SH3-like domain would act in a dominant-negative manner to interact with the SH3-like domain of the endogenous IdeR protein in the cells. As a result, the interaction between the SH3-like domain and the N-terminal domain in the endogenous IdeR protein...}
would be impaired (schematically illustrated in Fig. 8) and in turn the formation of DNA-binding form of IdeR would be impaired, in view that the SH3-like domain uses an overlapping surface to mutually exclusively interact with its two alternative partners (Fig. 3).

The repression activity of the endogenous IdeR in *M. smegmatis* cells over-expressing the SH3-like domain was then detected by measuring the transcription level of the *fxbA* gene via quantitative PCR. This gene encodes an enzyme needed for iron acquisition (53) and is known to be negatively regulated by IdeR (54).

The significant increase (column C, Fig. 8) in the transcription level of the *fxbA* gene in these *M. smegmatis* cells, in comparison with the control cells over-expressing an unrelated protein GCN4 or the wild type IdeR (columns A and B, Fig. 8), demonstrates an impairment of the transcription repression activity of the endogenous IdeR. Serving as a positive control is the data showing the de-repression of the *fxbA* gene transcription in *M. smegmatis* cells over-expressing IdeRΔ(1-74), a truncation form of IdeR with the DNA-binding sub-domain deleted (column D, Fig. 8) and thus its interaction with the endogenous IdeR would form a hetero-dimer (as demonstrated by results of *in vivo* assays presented in Fig. S1) which lost the DNA binding capacity for exhibiting the transcription repression activity in view that one of the two subunits in the hetero-dimer lacks a DNA-binding sub-domain.

A heterologous over-expression of the SH3-like domain of IdeR delays the growth of *M. tuberculosis* both *in vitro* and *in vivo* – We subsequently examined the role of the SH3-like domain on the physiology and pathogenicity of *M. tuberculosis*, also via the dominant-negative strategy described above.

An over-expression of the SH3-like domain in *M. tuberculosis* (designated as *Mtb-SH3*) resulted in a significant delay of cell growth in comparison with that over-expressing GCN4 (designated as *Mtb-Ctrl*) when cultured on solid Middlebrook 7H10 medium (Fig. 9A). This observation somehow suggests that the interactions involving the SH3-like domain of IdeR are important for the normal physiology of *M. tuberculosis*.

The pathogenicity of the *M. tuberculosis* cells over-expressing the SH3-like domain was then examined using a mouse infection model. The survival curves, presented in Fig. 9B, showed that the median-time-to-death (i.e., the time when 50% of the mice had died) of the BALB/c mice infected with *Mtb-SH3* is significantly longer than that of the mice infected with the control strain *Mtb-Ctrl* (being 39 and 25.5 days respectively, T-test, P<0.05). Correspondingly, the mean weight of the mice infected with *Mtb-SH3*, measured at the 28th day after the initial infection, was significantly higher than that of the mice infected by *Mtb-Ctrl* (see the inset in Fig 9B). These observations suggest that the interactions involving the SH3-like domain are important for *M. tuberculosis* to exhibit its pathogenicity in mice.

Further examination of the bacterial loads in the infected mice demonstrated a ten-fold reduction of *M. tuberculosis* cells in the spleens (Fig. 9C) but no significant reduction in the lungs (Fig. 9D) for the mice infected with *Mtb-SH3* in comparison with those infected with *Mtb-Ctrl*, as counted at day 28 after infection. This might be explained as such that the function of IdeR is more important for *M. tuberculosis* to survive in spleen than in lung as a result of the differences in niches between these two organs (e.g., in levels of reactive oxygen species and free ferrous iron).

**DISCUSSION**

This study was conducted in an effort to elucidate how the IdeR protein, a pleiotropic transcription regulator that plays a critical role in maintaining the intracellular iron homeostasis in mycobacteria (3-6), interconverts between its DNA-binding and DNA-non-binding forms in response to the fluctuations of iron levels in the cells. For this purpose, both *in vitro* and *in vivo* studies were performed. Our data for the first time revealed that the SH3-like domain is able to undergo self-association to help to maintain the DNA-non-binding form of IdeR also in a dimeric instead of monomeric form under conditions where metal ions are lacking. Our results also demonstrate that the inter-subunit self-association of the SH3-like domain in IdeR protein will give way to the intra-subunit interaction between the SH3-like and N-terminal domains, which plays an important role for IdeR to bind to the specific operator sequences and thus repress the transcription of the dozens of regulated genes. Furthermore, these two types of interactions involving the SH3-like domain occur via an overlapping surface on the SH3-like domain and thus in a mutually exclusive manner.

In light of our findings on the new roles played by the SH3-like domain, a new working model was
proposed (schematically shown in Fig. 10) to explain how IdeR effectively interconverts between its two forms in response to the fluctuations of iron levels in mycobacterial cells. The most appealing feature of this model is that the inter-subunit self-association of the SH3-like domains would prevent its intra-subunit interaction with the N-terminal domain, and vice versa. It follows that the interconversion between the DNA-binding and DNA-non-binding forms of IdeR would occur in a cooperative nature, allowing an effective interconversion and sensitive response towards the physiological fluctuations of the iron levels to strictly maintain the critical cellular iron homeostasis.

Our data reported here (see Fig. 2, 3, 4, 6, and 7), in combination with observations of structure determination studies where the two SH3-like domains were found to be either invisible (15, 25) or each interacting with a N-terminal domain in a different orientation in the dimeric DNA-binding forms of IdeR/DtxR (16-18), strongly suggest that the two alternative interactions involving the SH3-like domain are all relatively weak and occur in a dynamic nature. Nevertheless, the interaction between two SH3-like domains seems stronger than that between the SH3-like and N-terminal domains, with the former but not the latter that was detected by our in vivo protein-protein interaction studies (see Fig. 2B). Being connected together by a linker region of proper length and high flexibility is needed to increase the strength of the interaction between the SH3-like and N-terminal domains to a level that is comparable to that of interaction between two SH3-like domains, thus to ensure an effective switch of the SH3-like domain from interacting with one partner to another during the interconversion between the DNA-binding and DNA-non-binding forms of IdeR. In view of these, the overlapping surface on the SH3-like domain used for its self-association and its interaction with the N-terminal domain might serve as a promising target for developing antituberculosis drugs.

Earlier models presumed that the SH3-like domain acts to stabilize the DNA-non-binding form of DtxR by interacting with a proline-rich region in its N-terminal domain, mainly based on NMR spectroscopy analysis of the structure of a truncation form of DtxR, in which the whole DNA-binding sub-domain and much of the dimerization sub-domain no longer exist (55). Given that the proline-rich region is an internal sequence that is an inseparable part of the N-terminal domain, interaction between the proline-rich region and the SH3-like domain would have to overcome an extraordinary spatial hindrance in the intact IdeR protein. In view of this obstacle, whether such interaction indeed occurs in the full-length DtxR/ IdeR has to be questioned.

In contrast to our observations, self-association was not observed for the separately expressed SH3-like domain of the DtxR protein when examined using non-denaturing PAGE (20). In view of this, the SH3-like domain of DtxR was subject to similar studies as we have performed for its counterpart in IdeR. Results of our chemical cross-linking analysis (see Fig. S2A) and protein fragment complementation assays (see Fig. S2B) demonstrate that the SH3-like domain of DtxR is also able to undergo self-association under both our in vivo and in vitro conditions. One likely reason for their failing to detect the occurrence of self-association for the SH3-like domain of DtxR is that the samples applied there were purified via a denaturation and renaturation process, such artificial handling might have undermined the successful self-association. It should be pointed out that the protein fragment complementation assay did not reveal any occurrence of interaction between the SH3-like domain of DtxR and that of IdeR in living M. smegmatis (as indicated by the failure of growth of co-transformed cells on spot 7, Fig. S2B), suggesting such self-association is highly species specific.

The eukaryotic SH3 domains, which share few similarities in amino acid sequences with the SH3-like domain of IdeR, have been known to interact with a proline-rich motif to mediate protein-protein interactions (56). The properties of the SH3-like domain unveiled here drove us to examine whether there is any literature reporting the occurrence of similar self-association for the SH3 domains of eukaryotic proteins. The effort dug out at least two likely cases, the Islet-brain 1 (a scaffold protein that participates in the organization of the JNK signaling pathway) and Eps8 (a substrate protein of several receptor and non-receptor tyrosine kinases), where the separately expressed SH3 domains were reported to undergo self-association (57, 58). Whether the SH3 domains in these two and likely other proteins also play the similar interaction switch (i.e., such self-association probably only occurs under certain unique physiological conditions for the intact proteins) as those revealed for the SH3-like domain in IdeR warrants further investigation.
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**FOOTNOTES**

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The abbreviations used are: Amp, ampicillin; Atc, anhydrotetracycline; DP, 2,2′-dipyridyl; DTT, dithiotheritol; Hyg, hygromycin; Kan, kanamycin; PDM, phenylenedimaleimide; TMP, trimethoprim.
Figure 1. The pair of constructed plasmids used for detecting protein-protein interactions in living mycobacterial cells (A) and a feasibility test of the system (B). (A) Schematic representation of the two plasmids, pLC_TetR1/2 and pLC3, carrying the DNA sequences encoding F[1,2] and F[3] fragments of mDHFR respectively. Linker, DNA encoding the flexible (Gly3Ser)3 fragment; MCS, a multiple cloning site; F_TetO, a hybrid promoter having two tandem tetO sequences (solid diamonds) integrated into the strong mycobacterial promoter P1; P_kmr, a strong mycobacterial promoter derived from the M. bovis BCG groEL2 gene; tetR, DNA sequence encoding the E. coli Tn10 Tet repressor (TetR) protein; oriM and oriE, the replication origins derived from mycobacteria and E. coli respectively; AttP, the phage attachment site that allows the integration of pLC3 plasmid into the genome of mycobacteria; int, DNA sequence encoding the integrase of the mycobacteriophage L5. The detailed sequences of the FLAG tag and the MCS site are shown at the bottom. (B) The M. smegmatis cells were co-transformed with pLC_Tet1/2-GCN4 and pLC3-GCN4 (the three spots at the top) or with pLC_Tet1/2-GCN4 and pLC3-IdER (the three spots at the bottom) before growing in the indicated selection media. Atc (anhydrotetracycline) will bind to and inactivate the transcription repression activity of TetR; TMP (trimethoprim) will inhibit the enzymatic activity of prokaryotic DHFR. The cells were grown on agar plates made of M63/glycerol minimal media, containing 100 µg/ml Amp, 25 µg/ml Kan and 50 µg/ml Hyg. TMP and Atc were added to a final concentration of 10 µg/ml and 50 ng/ml, respectively.

Figure 2. Three truncated forms of IdER (A) and the utilization of them to analyze the pairwise interactions between the various domains of IdER in living M. smegmatis cells (B). (A) Represented are the three truncated forms of M. smegmatis IdER protein, in which either the dimerization sub-domain, the SH3-like domain, or the N-terminal domain was deleted (shown below the line representing the full length IdER protein). (B) Survival analysis of M. smegmatis cells co-expressing the indicated pairs of fusion proteins on the selection media. Proteins or protein fragments expressed from the pLC_Tet1/2 plasmid (fused to mDHFR-F[1,2]) are indicated on the top and those expressed from the pLC3 plasmid (fused to mDHFR-F[3]) are indicated on the left. The selection media contains TMP (10 µg/ml), Ate (50 ng/ml), Amp (100 µg/ml), Kan (25 µg/ml) and Hyg (50 µg/ml), all added in the M63/glycerol minimal agar media. The yeast GCN4 protein was used here for both positive and negative controls, for its ability to self-associate (spot aA) but inability to interact with any form of IdER (spots aB, aC, aD, aE, bA, cA, dA and eA).

Figure 3. In vitro examination (A, B and C) and identification of the interaction surface for the self-association of SH3-like domain of IdER (D, E). (A) The elution profile of the separately expressed SH3-like domain of IdER (0.5 mg/ml) being subjected to a size-exclusive chromatography analysis. Indicated on the top are the elution positions of the molecular standards. (B) The cross-linking (with glutaraldehyde) results of the independently expressed SH3-like domain of IdER (1 mg/ml). The positions of the molecular standards are shown on the left; positions of the monomer and cross-linked dimer of the SH3-like domain are indicated on the right. (C) Non-denaturing pore gradient PAGE (6%-45%) analysis of the separately expressed SH3-like domain of IdER (1mg/ml) at a temperature of 4°C, with the electrophoresis time being indicated. The position of dimeric form in each lane was indicated with a **. (D) Western blotting detection of disulfide bond formation (thus producing a dimeric form) of the separately expressed SH3-like domain of IdER (presented in the whole soluble cell extract without any purification, see Experimental Procedures for details) each having a cysteine substitution at the indicated position, using the anti-His-tag monoclonal antibody. Results obtained under non-reducing (-DTT) and reducing (+DTT) conditions are shown at the top and bottom respectively. Blank represents the whole soluble cell extract sample with no SH3-like domain being over-expressed. (E) Locations of the positions 173, 192, 201, 202, 203, 218, 219 and 220 that resulted in disulfide bond formation when substituted by cysteine residues (indicated by red color) on the structure of the SH3-like domain alone (left) or on one subunit of the active form of the intact IdER (right) (PDB number: 1U8R, Ref. 17). The DNA-binding and the dimerization sub-domains are shown as pink and green color respectively.
Figure 4. The SH3-like domain self-associates in the inactive form but interacts with the N-terminal domain in the active form of the full-length IdeR. (A) SDS-PAGE analysis of the IdeR(C102D/H173C) protein after reacted with a cysteine-specific cross-linker (p-PDM at 500 μM) in the presence of a metal chelator (DP at 3 mM; lanes 8), or in the presence of metal ion (Co²⁺ at 200 μM; lane 7). The samples in lanes 6 and 5 represent the purified IdeR(C102D/H173C) protein with or without being treated with the cross-linking agent (neither Co²⁺ nor DP was added in both samples). Analyzed in lanes 1 to 4 was the cysteine-free IdeR(C102D) protein after being subjected to a parallel treatment. Position of the dimeric form of IdeR(C102D/H173C) resulted from the cross-linking reaction was indicated on the right. The position of a second minute amount of cross-linked product which was most likely resulted from the amino-targeted cross-linking, was indicated with a “*” on the right. (B) EPR spectrum of IdeR(C102D/H173C) (at 250 μM) that was spin-labeled with PROXYL as recorded at a temperature of 150K in the presence of DP (at 10 mM). The ratio of 0.58 (being larger than 0.4, a threshold value) for the amplitude of d1 to d is taken to demonstrate the occurrence of spin-spin interaction between the pair of PROXYL groups. The length of the bar shown at the bottom right represents the width of 10 Gauss (G). Shown in the inset is the EPR spectrum of the same sample recorded at a temperature of 300K. (C) SDS-PAGE analysis of a parallel cross-linking treatment for IdeR(C102D/E95C/K185C) with o-PDM (at 500 μM). Positions of the uncross-linked and intra-subunit cross-linked monomeric forms of IdeR(C102D/E95C/K185C) are indicated on the right.

Figure 5. Immobilization of the self-association of the SH3-like domain or the interaction between the SH3-like domain and the N-terminal domain generates converse effects for the operator-binding capacity of IdeR. (A, C) IdeR(C102D/H173C) (A) or IdeR(C102D/E95C/K185C) (C) at the indicated concentrations and with the disulfide bonds preformed under proper conditions (see Experimental Procedures), were either incubated directly (the +DTT lanes) or after the disulfide bonds being disrupted (the −DTT lanes) with a constant amount of the specific Oₘₚ₉₈ operator DNA (40 pmol, being labeled with the fluorescent group FAM at the 5’ end of one strand) for DNA-binding examination via EMSA. The DNA bands were visualized by scanning the gels with a Typhoon scanner (with the exciting wavelength at 488 nm). Positions of the free DNA and the DNA in complex with IdeR were indicated on the left. Metal ion Co²⁺ (at 200 μM) was added in the reaction mixture, the PAGE gel and the running buffer to keep IdeR in its active form for all assays. Existence of the expected disulfide bonds in IdeR(C102D/H173C) (B) and IdeR(C102D/E95C/K185C) (D) or their disappearance in the presence of DTT was demonstrated by SDS-PAGE analysis. Schematically depicted are the most likely patterns of domain-domain interactions and disulfide bond formations being found in the corresponding forms of the IdeR(C102D/H173C) or IdeR(C102D/E95C/K185C) protein.

Figure 6. Cleavage of the linker between the SH3-like domain and the N-terminal domain impairs both the DNA-binding and the metal ion-responsive transcription repression activities of IdeR. (A) Schematic representation of the IdeR (C102D), IdeR(C102D, thromA) (with a thrombin cleavage site incorporated into the linker region) and IdeR(C102D)ΔSH3 (with the SH3-like domain deleted) proteins, all containing a His-tag. The sequence of linker region and that of the integrated thrombin cleavage site are shown in detail. (B) Products of the IdeR(C102D, thromA) protein after being cleaved with thrombin (2 units per milligram of IdeR protein, at 37°C) for the indicated time (lanes 3-6) as analyzed by tricine-SDS-PAGE and visualized by Coomassie Blue staining. The Sample analyzed in lane 2 was the IdeR(C102D, thromA) protein without being cleaved by thrombin. Purified IdeR(C102D) (lane 1), IdeR(C102D)ΔSH3 (lane 7) and the SH3-like domain (lane 8) were included here simply to indicate the approximate positions of the intact or the cleaved products of the IdeR(C102D, thromA) protein. (C) Comparison of the DNA binding activities of the intact IdeR(C102D, thromA) (lanes 2-5), the cleaved IdeR(C102D, thromA) (lanes 7-10) and IdeR(C102D)ΔSH3 (lanes 12-15) as analyzed by EMSA. For the analysis of the three samples, the Oₘₚ₉₈ DNA was kept constant at 10 pmol, and proteins added at an increasing amount: 0.025 nmol (lanes 2, 7 and 12), 0.05 nmol (lanes 3, 8 and 13), 0.075 nmol (lanes 4, 9 and 14) and 0.1 nmol (lanes 5, 10 and 15). Here, the cleavage of IdeR(C102D, thromA) was performed by incubating the protein with thrombin at a concentration of 2 units /mg protein at 37°C for 4 hours. (D)
Relative activities of the luciferase expressed from the pPT-luc reporter plasmid (0.5 μg in each reaction) in the presence of the various forms of IdeR proteins (each 25 nmol) as measured by using the in vitro transcription/translation system. Metal free conditions were maintained by adding the metal chelator DP (50 μM) (open columns). To exclude the possible influence of DP on the in vitro transcription/translation reaction, level of the relative luciferase activity was calculated as the percentage of luciferase activity (in the presence of IdeR and 50 μM DP) in comparison with the luciferase activity measured in the presence of 50 μM DP but without adding IdeR. Each value represents the mean of triplicate independent experiments with standard deviations indicated. The cleaved IdeR(C102D, thromA) was prepared as in (C). Existence of the trace amount of thrombin alone hardly affected the expression level of the luciferase via the in vitro transcription/translation (data not shown).

Figure 7. Lengthening the linker between the N-terminal domain and the SH3-like domain impairs the metal ion-responsive transcription repression activities of IdeR. (A) Schematic representation of IdeR(C102D, 2XL) and IdeR(C102D, 4XL) with the lengthened linker sequences shown in detail. In IdeR (C102D, 2XL), the linker was lengthened by the insertion of an identical linker sequence (TPGVNTEVDS) of the M. smegmatis (M. sm) IdeR. In IdeR(C102D, 4XL), the linker was lengthened by the insertion of one copy of the linker sequences of M. smegmatis IdeR and two copies of the linker sequences (GPEPADDAN) of the M. tuberculosis (M. tb) IdeR. (B) SDS-PAGE analysis of the purified IdeR(C102D, 2XL) (lane 3) and IdeR(C102D, 4XL) (lane 4) proteins. IdeR(C102D) (lane 2) was included here as a control. (C) Metal ion-responsive repression activities of IdeR(C102D), IdeR(C102D, 2XL) and IdeR(C102D, 4XL) as measured by using the in vitro transcription/translation system against an increased amount of DP (performed as that for Fig. 6D). The concentrations of the IdeR proteins as well as the plasmid pPT-Luc were added at the same concentrations as described in Fig. 6 (D). Level of the luciferase activity was calculated as the percentage of luciferase activity (in the presence of IdeR and each indicated concentration of DP) in comparison with the luciferase activity measured in the presence of the same concentration of DP but without adding IdeR.

Figure 8. The transcription of the IdeR-controlled fxbA gene is up-regulated in M. smegmatis cells that heterologously over-express the SH3-like domain. The mRNA level of the fxbA gene in M. smegmatis cells transformed with the pLC_Tεβ1/2 plasmid expressing GCN4 (column A), wild type IdeR (column B), SH3-like domain of IdeR (column C) and IdeRΔ(1-74) (in which the DNA-binding subdomain was deleted; column D) was measured via quantitative PCR method and normalized against that of the constitutively expressed mspA gene in each RNA sample. The fxbA expression levels displayed in columns B to D were represented as the folds of that in column A. All transformed M. smegmatis cells were cultured in Middlebrook 7H9 medium (supplemented with 25 mg/ml Kan, 0.5% glycerol, 0.5% Tween 80 and 50 ng/ml Ate) at 37°C to late log phase. RNA extraction and quantitative PCR were performed as described in Experimental Procedures. Schematically illustrated at the bottom are the most likely patterns for each of the over-expressed IdeR variants (all being expressed as fused to the N-terminal of the mDHFR-F[1,2]) to interact with the endogenous IdeR of the M. smegmatis cells.

Figure 9. A heterologous over-expression of the SH3-like domain of IdeR delays the growth of M. tuberculosis H37Rv both in culture medium and in mice. (A) Colonies of M. tuberculosis H37Rv cells transformed with pLC3-IdeR(Δ1-140) (MtSH3) or pLC3-GCN4 (MtCtrl) after being cultured on Middlebrook 7H10 agar for 4 weeks. Both the SH3-like domain of IdeR and the GCN4 protein were expressed as fused to the N-terminal of the mDHFR-F[3]. (B) The Kaplan-Meier survival curves of BALB/c mice (n=8 for both groups) after being intravenously infected with 1X10⁷ Mt-SH3 (black thick line) or MtCtrl (grey thin line). Shown in inset are the mean weights of the mice (n=3 for each group) infected with Mt-SH3 (solid column) or MtCtrl (open column) measured at the 28th day after the initial infection. The error bars represent ± s. e. (C) and (D) The cfu counts of bacteria loads in spleens and lungs of mice at the 7th, 14th and 28th day after the initial infection with the same amount of Mt-SH3 (solid columns) or MtCtrl (open columns) as that used in panel (B). Each point represents mean of four replicates. Error bars represent as ± s. e. It should be noted that the scales of the Y-axes in (C) and (D) are different.
Figure 10. A proposed working model highlighting the modulatory roles of the SH3-like domain during the interconversion between the active and inactive dimeric forms of IdeR in response to the fluctuations of Fe$^{3+}$ levels in mycobacterial cells.
FIGURES

Fig. 1

(A)

(B)

- - + A.tc
- + + TMP

pLCtet1/2-GCN4; pLC3-GCN4
pLCtet1/2-GCN4; pLC3-Ider

1 2 3
Fig. 5

(A) IdeR(C102D/H173C) - 0.1 0.2 0.3 0.4 (nmol)

(B) N-terminal domain

(C) IdeR(C102D/E95C/K185C) - 0.1 0.2 0.3 0.4 (nmol)

(D) SH3-like domain

Inter-subunit immobilized
Unimmobilized

Free DNA

Dimer

Monomer

N-terminal domain

SH3-like domain

Unimmobilized

Intra-subunit immobilized

1 2 3 4 5 6 7 8 9

1 2 3

66kDa
45kDa
30kDa
20.1kDa

66kDa
45kDa
35kDa
28.5kDa
20.1kDa

DTT

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Fig. 6

(A) 

| N-terminal domain | SH3-like domain |
|-------------------|-----------------|
| IdeR(C102D)       | TPOVTKTEDVS     |
|                   | AAALEHHHHHH     |
| linker            |                 |
| IdeR(C102D, thromA) | LVPRGSPVVKTEDVS |
| Thrombin          | AAALEHHHHHH     |
| cleavage site     | linker          |
| IdeR(C102D)\:SH3 | AAALEHHHHHH     |

(B) 

Incubated with thrombin (hours)

(C) 

Intact IdeR(C102D, thromA)  Cleaved IdeR(C102D, thromA)  IdeR(C102D)\:SH3

(D) 

% control luciferase expression

1, 2  Intact IdeR(C102D, thromA)
3, 4  Cleaved IdeR(C102D, thromA)
5, 6  IdeR(C102D)\:SH3
Fig. 7

(A) N-terminal domain

| IdeR(C102D, 2XL) | IdeR(C102D, 4XL) |
|------------------|------------------|
| KTPGNTEDVSTP | KTPGNTEDVSTP |
| Linker (M.am) | Linker (M.am) |
| AAGLEH6 | AAGLEH6 |

SH3-like domain

| IdeR(C102D, 2XL) | IdeR(C102D, 4XL) |
|------------------|------------------|
| KTPGNTEDVSTP | KTPGNTEDVSTP |
| Linker (M.am) | Linker (M.am) |
| Linker (M.ab) | Linker (M.ab) |
| AAGLEH6 | AAGLEH6 |

(B) Western blot

(C) % control luciferase expression vs. Metal chelator (DP, µM)

- ▲ IdeR(C102D, 4XL)
- ◇ IdeR(C102D, 2XL)
- ▼ IdeR(C102D)
Fig. 8

Relative $f_{yb4}$ expression level (folds)

(A) GCN4
(B) IdeR<sup>wt</sup>
(C) IdeR-like domain
(D) IdeR<sup>Δ(1-74)</sup>

- N-terminal domain
- SH3-like domain
- mDHFR-F<sub>[1,2]</sub>

N-terminal domain with the DNA-binding sub-domain deleted
Fig. 9

(A) Mtb-SH3

(B) Mtb-Ctrl

(C) Fraction alive

(D) Mean weight of mice (g)

(C) CFU/spleen (X10^5)

(D) CFU/lung pair (X10^5)

(Days) 7 14 28

(C) Ctrl SH3

(D) Ctrl SH3
Fig. 10

Iron overload

Repression

Iron Starvation

De-repression

SH3-like domain
Iron-binding Site 1
Iron-binding Site 2
N-terminal domain in DNA-binding form
N-terminal domain in DNA-non-binding form
The SH3-like domain switches its interaction partners to modulate the repression activity of mycobacterial Iron-dependent transcription regulator (IdeR) in response to metal ion fluctuations
Chong Liu, Kai Mao, Meng Zhang, Zhaogang Sun, Weizhe Hong, Chuanyou Li, Bo Peng and Zengyi Chang

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