Chapter 4
Results
4.1 Yeast Two Hybrid Screening

4.1.1 Introduction

Proteins are one of the basic building blocks in a cell responsible for carrying out diverse functions. The physical associations between proteins, that is protein-protein interactions are essential in all biological pathways. The protein interaction studies can predict the functional relationships between interacting proteins. The Yeast two hybrid (Y2H) assay was first developed by Fields and Song (1989). Since then it has been extensively used to study protein-protein interactions. The Y2H assay has a clear advantage over the classical biochemical or genetic methods of protein interaction studies, as it provides a living system with close resemblances to eukaryotic cell with cDNA of a gene as a minimum requirement to initiate screening (Sobhanifar, 2003). The Y2H assay is a relatively quick, easy, flexible and high throughput preliminary screening technique, for straight-forward study of protein interactions (Sobhanifar, 2003).

*Mtb* has the ability to modulate the infected host cells through various mechanisms and protein–protein interactions between host and *Mtb* proteins could be one of them. The availability of entire *Mtb* genome sequence (Cole et al., 1998) has led to the identification of several mycobacterial virulence determinants. The cell wall associated Erp protein of *Mtb* is one of them. The Erp protein devoid of signal sequence has been used as bait while mouse macrophage cDNA library has been used as prey for Y2H assay.

4.1.2 The prey (mouse macrophage cDNA)

The Lambda Uni-ZAP XR insertion vector with mouse macrophage cDNA library was procured from Stratagene. The titer of ExAssist helper phage and the Uni-ZAP XR mouse macrophage cDNA library were checked and found to be at par with the recommended titer (Fig. 7). The pBluescript SK(-) phagemid was excised from the UNI-ZAP XR vector following the mass excision protocol provided by Stratagene. The titer of the excised phagemids in SOLR cells was found to be ~ 7.8 x 10^7 cfu/ml (Fig. 8A). Upon restriction digestion of the phagemids with EcoRI and XhoI, DNA fragments ranging from ~0.0 kb to ~10.0 kb; having pBluescript SK(-) phagemid backbone at ~3.0 kb was observed (Fig. 8B). Mouse macrophage cDNA of ~0.5 kb to 2.5 kb size were excised and cloned into pADGAL4-2.1 (Y2H assay prey vector) as mentioned in materials and methods.
Fig. 7: Plaques produced by ExAssist helper phage (Panel A) and the Uni-ZAP XR mouse macrophage cDNA library (Panel B) at $10^{-5}$ dilution, respectively.

Fig. 8: Mass excision of pBluescript SK(-) phagemid with cDNA from the UNI-ZAP XR vector. (Panel A) Excised pBluescript SK(-) phagemid with mouse macrophage cDNA library in SOLR cells. (Panel B) pBluescript SK(-) phagemid with mouse macrophage cDNA library. Lane 1: 1.0 kb DNA ladder; Lane 2: EcoRI and XhoI digest of mouse macrophage cDNA library in pBluescript SK(-).
4.1.3 The bait Δsserp of *Mtb* H37Rv

The Δsserp (*erp* gene devoid of 22 amino acid long signal sequence) gene of size ~786 bp was PCR amplified from the *Mtb* H37Rv genomic DNA (Fig. 9A), cloned into pGEMT vector (Fig. 9B) and subsequently sub-cloned into pBDGAL4cam (Fig. 9C, Bait vector) as described in materials and methods. The pBDGAL4camΔsserp on digestion with PstI exhibited a single band at ~7.2 kb size representing a linearised vector having cloned gene in it (Fig. 9C, Lane 2), while on digestion with Sall an insert on ~786 bp was visible thus confirming that the inset Δsserp has been cloned in correct orientation (Fig. 9C, Lane 3).

4.1.4 Y2H assay of pBDGAL4camΔsserp as bait with mouse macrophage cDNA library in pADGAL4-2.1 (~0.5 to 2.5 kb, as prey)

4.1.4.1 Auto-activation test of GAL4ΔssErp fusion protein (bait)

The auto-activation test was done to check whether GAL4ΔssErp (ΔssErp protein fused with the binding domain of GAL4) fusion protein qualifies the criteria to be “bait” for Y2H assay. Y2H system of Stratagene is based on the expression of the HIS3 and lacZ reporter gene. HIS3 reporter gene may have leaky expression, so the results must be further confirmed with the LacZ assay or filter lift assay. YRG2 yeast strain with either pBDGAL4 or pBDGAL4camΔsserp were streaked onto SD His⁻ selection media at very high density. YRG2 (pBDGAL4camΔsserp) yeast cells were not able to grow on SD His⁻ selection, but yeast with pBDGAL4 phagemid were able to grow on it. Filter lift assay showed that yeast expressing pBDGAL4 (full length GAL4 protein) turned blue while the YRG2 expressing pBDGAL4camΔsserp (ΔssErp protein fused with the binding domain of GAL4) did not (Fig. 10). This experiment showed that the bait fusion protein (BDGAL4ΔssErp) was not able to auto-activate the expression of the reporter genes (HIS3 or LacZ) alone; hence it was confirmed that ΔssErp was not a transcription factor, thus, qualifying for its use as bait in Y2H assay (Fig. 10).

4.1.4.2 Y2H assay

The steps of Y2H assay were followed as described in the materials and methods. Two independent screening experiments with GAL4ΔssErp as bait and mouse macrophage cDNA (~0.5 kb to ~2.5 kb size) in pADGAL4-2.1 as prey were performed. In both the experiments a total of 26 blue colonies (His⁺-LacZ⁺) were obtained which turned blue in second filter lift assay. Out of these 26 colonies, only 22 His⁺-LacZ⁺ clones yielded
pADGAL4-2.1 plasmid DNA with different inserts. These 22 plasmids on restriction digestion with EcoRI and XhoI showed that the vector backbone of pADGAL4-2.1 (~ 7.6 kb) and inserts of various sizes were present in all the clones (Fig. 11A & 11B).

Fig. 9: Cloning of Δsserp gene into pBDGAL4cam vector. (Panel A) Lane 1: 1.0 kb DNA ladder; Lane 2: PCR amplified Δsserp gene. (Panel B) Lane 1: 1.0 kb DNA ladder; Lane 2: pGEMTΔsserp Sall digest. (Panel C) Lane 1: 1.0 kb DNA ladder; Lanes 2 & 3: pBDGAL4camΔsserp PstI and Sall digest, respectively.

Fig. 10: Auto-activation test of GAL4 and GAL4ΔssErp fusion protein. YRG2 yeast with pBDGAL4 and pBDGAL4camΔsserp phagemids were streaked onto SD His' plate and then filter lifted for LacZ assay. Arrow shows the position of these streaks.
4.1.5 Sequence alignment studies

Out of 22 clones that were obtained from Y2H experiments only 19 clones were sent for sequencing, leaving behind the three clones that showed three bands at the insert position (Fig. 11A, Lane 19; Fig. 11B, Lane 1 & 2). These clones could have been obtained due to ligation of three inserts in a single vector. The sequences were analysed manually and by using softwares mentioned in the materials and methods. Homology search showed nine independent full-length and partial His\(^+\)-LacZ\(^+\) gene sequences that aligned with known mouse macrophage genes; these sequences were designated by us as 101, 103, 107, 109, 111, 113, 115, 116 and 118 (Fig. 12), while other 10 sequences either aligned with clone contigs or genome sequences with lots of stop codons. The 101 was found to be spt2 (Serine Palmitoyl Transferase, subunit 2).

4.1.6 PCR amplification of His\(^+\)-LacZ\(^+\) sequences

Out of nine genes only eight genes were successfully PCR amplified (Fig. 13A, 13B & 13C) from excised pBluescript SK(-) phagemid with mouse macrophage cDNA library. The gene 107 was not amplified either due to very large size of the gene or lack of representation in the cDNA library.
Results

>gi|3343768|ref|NM_011479.2| Mus musculus serine palmitoyltransferase, long chain base subunit 2 (optic2), mRNA length=3755

Score = 839 bits (1825), Expect = 0.0
Identities = 380/380 (100%), Positives = 380/380 (100%), Gaps = 0/380 (0%)
Frame = +2/+1

Query 14
ATYYGAFATGNKPALVQGCEQILDEHLNASSLVGLGARSGCIGKLLEKL

Subject 748
ATYYGAFATGNKPALVQGCEQILDEHLNASSLVGLGARSGCIGKLLEKL

Query 194
LKDANVYQPPTRPWRKLILIGEYMMGEGIVRPEVIALKKKAYFLYDEASIGA

Subject 928
LKDANVYQPPTRPWRKLILIGEYMMGEGIVRPEVIALKKKAYFLYDEASIGA

Query 374
LGPSGQRQWVDPGLDPEOOPMGTFKSSFAGSGVGIGGKELIDYLRSHSAYAVATS

Subject 1108
LGPSGQRQWVDPGLDPEOOPMGTFKSSFAGSGVGIGGKELIDYLRSHSAYAVATS

Query 554
SPPMEQIITSMMEIQGQCSSTGLKEQQLATPERYLLRKLFFGFIIYGYEDSPVPA

Subject 1288
SPPMEQIITSMMEIQGQCSSTGLKEQQLATPERYLLRKLFFGFIIYGYEDSPVPA

Query 734
MLYMPAKIGAFGREMLKRNIGVVVVGFPATPIIESRARFCLSAAHTKEILDALICIDEV

Subject 1468
MLYMPAKIGAFGREMLKRNIGVVVVGFPATPIIESRARFCLSAAHTKEILDALICIDEV

Query 914
GDLQQEYSLRVIPIPLDPDFDETTYEETED*AFVLVRPGFFLPQCSVAFLSQQEQPHF

Subject 1648
GDLQQEYSLRVIPIPLDPDFDETTYEETED*AFVLVRPGFFLPQCSVAFLSQQEQPHF

Query 1094
SDHFMKTFLKLVATTPF 1153

Subject 1828
SDHFMKTFLKLVATTPF 1887

Fig. 12: Sequence alignment using tBlastX software (www.ncbi.nlm.nih.gov) of 101 or mouse Serine Palmitoyl Transferase 2.

Fig. 13: PCR amplification to obtain full length of genes found to be interacting with Δasper in Y2H assay. (Panel A) Lane 1: 1.0 kb DNA ladder; Lanes 2-5: PCR product of genes 109, 113, 116 and 118, respectively. (Panel B) Lane 1: 1.0 kb DNA ladder; Lanes 2-4: PCR product of genes spt2, 103 and 115, respectively. (Panel C) Lane 1: PCR product of genes 111; Lane 2: 1.0 kb DNA ladder.

4.2 GST Pull down assay
4.2.1 Introduction

The Y2H system is a powerful genetic method to screen cDNA libraries to identify putative protein–protein interactions. A screening with bait may yield many clones, including ones that are not biologically relevant and which needs to be eliminated. At times,
it is possible to determine which clones are relevant to the activity of the bait protein based on database searches. However, database searches alone do not reveal which clones should be further investigated. Hence, evaluation of each clone with an alternative assay must be performed to confirm the interaction of prey with the bait protein. GST pull-down assays have been used to identify either new interacting protein partners for a known protein or to confirm the interaction between a protein pair that has been discovered by Y2H or CoIP (Ren et al., 2003). In a GST pull-down assay, the purified "bait" protein expressed as a GST fusion is immobilized onto glutathione-coupled resin. Once bound, the bait is incubated with either purified protein or cell lysate to "pull down" the "prey" protein. Here, GST pull-down assay has been used to find out if purified recombinant SPT2 (bait) and purified recombinant AssErp (prey) protein pair interact directly with each other under *in vitro* condition.

4.2.2 Cloning, over-expression and purification of Asserp

4.2.2.1 Cloning of Asserp in pQE30 vector

The Asserp gene was cloned into pQE30 vector as described in materials and methods. The right clone of pQE30-Asserp on digestion with SalI exhibited an insert "Asserp" at ~786 bp (Fig. 14, lane 2), while on digestion with PstI gave a single band at ~4 kb of combined vector and insert (Fig. 14, Lane 3).

![Fig. 14: Cloning of Asserp into pQE30 vector](image)

Fig. 14: Cloning of Asserp into pQE30 vector. Lane 1: 1.0 kb DNA ladder; lane 2: pQE30-Asserp SalI digest; Lane 3: pQE30-Asserp PstI digest.

4.2.2.2 Over-expression and purification

The recombinant plasmid pQE30-Asserp was over expressed in M15 cells with best induction condition of 0.5 mM IPTG and 30 °C temperature for 6 hrs. The over expressing
cells were sonicated and the recombinant ΔssErp protein was purified from the soluble extracts of M15 cells using Ni-NTA column as described in materials and methods. The purity of Ni-NTA affinity purified ΔssErp protein was found to be > 90 % pure with some contaminating protein bands appearing above 36 kDa and degradation product visible below it (Fig. 15A). Western blotting of the Ni-NTA purified ΔssErp protein with anti-His Antibodies showed a desired ΔssErp band below 43 kDa marker and an intense band along side with the 72 kDa marker (Fig.15B).

The dialysed ΔssErp was further purified using anion exchange column (Q sepharose) as mentioned in materials and methods. It was observed that the passage of dialysed ΔssErp protein through Q sepharose, resulted in binding of all the high molecular weight protein impurities and non specific binding of a small portion of ΔssErp protein to the column (Fig. 16A). Most of the purified ΔssErp protein came out with the flow through (Fig. 16A; Lane FT). On washing the column with dialysis buffer containing 50 mM NaCl resulted in further elution of the desired ΔssErp protein bound non specifically (Fig. 16A; Lanes 1 & 2). While, impurities and very little desired ΔssErp protein came out of the column when washed with dialysis buffer containing 100 mM, 200 mM and 400 mM NaCl, respectively (Fig. 16A; Lanes 3 - 7). The yield obtained was ~ 2-3 mg/l of culture. The purified Erp protein has a calculated molecular weight of ~ 28 kDa but was seen between ~ 34 kDa to 43 kDa stained markers (Fig. 15B) and at ~ 35 kDa in unstained marker position (Fig. 16B).

4.2.3 Generation of polyclonal antibodies against ΔssErp protein in rabbit

Polyclonal antibodies were raised against recombinant ΔssErp protein in the rabbit and its antiserum was used to perform ELISA. The titer of the anti ΔssErp antibody on 11th day after giving 2nd booster dose was found to be 1:50,000. These antibodies were able to pick the endogenous level of Erp protein in the lysates of Mycobacterium tuberculosis, M bovis, M smegmatis at 1:25,000 dilution by western blotting (Fig. 17).

4.2.4 Cloning, over-expression and purification of GST-SPT2 fusion protein

The spt2 gene was cloned into pET41a vector as described in materials and methods. The correct clone on digestion with BamHI gave a band of ~1.1 kb (Fig. 18; Lane 2). The pET41a-spt2 plasmid was over expressed in BL21(DE3) host with an optimum expression condition of 1 mM IPTG and 30 °C temperature for 6 hrs. It was observed that most of the fusion protein was found to be insoluble (data not presented). The western blotting of GST-
SPT2 with anti His antibodies showed that a very small amount of GST-SPT2 fusion protein (just above 95 kDa marker) was available in the soluble fraction (Fig. 19A). Hence, this small soluble fraction of the GSTSPT2 protein was purified using GST sepharose column (Fig. 19B) as explained in materials and methods. The purified fraction showed impurities and the degradation products of GSTSPT2 protein (Fig. 19B; Lane 1).

**Fig. 15: Ni-NTA purification of ΔssErp protein.** (Panel A) Lane M: Unstained protein molecular weight marker; Lane L: Load; Lane FT: flow through; Lane W: Wash buffer with 10 mM imidazole; Lanes 1-6: Elution fractions of ΔssErp protein. (Panel B) Lane M: Prestained protein molecular weight marker; Lane 1: Western blotting of Ni-NTA purified ΔssErp with Anti-His antibodies.

**Fig. 16: Q sepharose purification of ΔssErp protein.** (Panel A) Lane M: Unstained protein molecular weight marker; Lane L: Ni-NTA purified ΔssErp dialysed in dialysis
buffer with 50 mM NaCl i.e. load; Lane FT: flow through obtained from the column; Lanes 1 & 2: The 7th and 8th fractions from wash with 50 mM NaCl containing dialysis buffer; Lane 3: The 7th fraction from wash with 100 mM NaCl containing dialysis buffer; Lanes 4 & 5: The 7th and 8th fraction from wash with 200 mM NaCl containing dialysis buffer; Lanes 4 & 5: The 7th and 8th fraction from wash with 400 mM NaCl buffer. (Panel B) Purified ΔssErp protein after final purification and concentration.

![Image](image_url)

**Fig. 17:** Lysates from different mycobacterial species probed with 1:25,000 dilution of Anti-ΔssErp antibody. Lanes 1-3: Lysate from *Mtb*, *M. bovis* and *M. smegmatis*, respectively. The arrow shows the position of Erp protein.

![Image](image_url)

**Fig. 18:** Cloning of *spt2* into pET41a vector. Lane 1: 1.0 kb DNA ladder; Lane 2: pET41a-*spt2* BamHI digest.
Fig. 19: Over-expression and purification of GST-SPT2 protein. (Panel A) Lanes 1-3: Western blotting of GST-SPT2 protein in lysate from BL21(DE3) cells at 37 °C, 30 °C and 25 °C with Anti-His antibodies. (Panel B) Lane M: Molecular weight marker; Lanes 1-6: Elution fractions 1 to 6 of GST-SPT2 protein in elution buffer with 20 mM reduced glutathione; Lane W: Wash buffer 1X PBS; Lane FT: flow through; Lane L: Load.

4.2.5 GST-Pulldown of ΔssErp using GST-SPT2 protein

The GST-Pull down assay was performed as described in the materials and methods. Equimolar concentration of purified recombinant ΔssErp, GST-SPT2 and GST proteins were added to the GST sepharose beads as described in the Fig. 20. The GST-SPT2 was able to bind to the GST sepharose beads which inturn was able to pull-down ΔssErp protein as observed in lane 3 (Fig. 20). On the other hand the GST protein alone was not able to pull-down ΔssErp protein (Fig. 20; lane 2). The ΔssErp protein did not exhibit any non specific binding with the GST sepharose beads (Fig. 19; lane 5). It was clearly observed in the present experiment that ΔssErp interacts directly and specifically to SPT2 under in vitro condition and this interaction did not involve other proteins.
4.3 Co-Immunoprecipitation
4.3.1 Introduction

The Y2H assay was used as a preliminary screen to fish out the probable interacting protein partners of ΔssErp, while GST-Pull down assay was used to confirm the Y2H results under in vitro condition. But the biggest question remained unanswered was: whether these two proteins interact with each other under in vivo conditions? A positive result in Y2H assay or in vitro GST-Pull down indicates that the two proteins has the ability to interact. Co-immunoprecipitation is one such technique which has been used to prove that the two proteins interact with each other under in vivo condition.

4.3.2 Cloning of Δsserp and spt2 into mammalian expression vectors

The cloning of Δsserp and spt2 genes into pcDNA2aFLAG and pcHA vectors respectively, has been described in materials and methods. On screening the pcDNA2AFLAG-Δsserp clones with HindIII + XbaI an insert of ~ 846 bp was observed in
the right clones (Fig. 21A, Lanes 3, 4, 6 & 7). A band of ~1.9 kb was observed on digestion of pcHA-spt2 with BamHI which confirmed the presence of spt2 in correct orientation (Fig. 21B, Lane 2).

![Fig. 21: Cloning of Δsserp into pcDNA2aFLAG and spt2 into pcHA mammalian expression vector. (Panel A) Lane 1: 1.0 kb DNA ladder; Lanes 2 & 5: Vector self ligation on digestion with HindIII + XbaI; Lanes 3, 4, 6 & 7: Right clones with pcDNA2AFLAG-Δsserp digested with HindIII + XbaI. (Panel B) Lane 1: 1.0 kb DNA ladder; Lane 2: pcHA-spt2 digested with BamHI.](image)

4.3.3 The ΔssErp protein interacts with SPT2 in BHK21 cells

The transient transfection of BHK21 cell line and the co-immunoprecipitation experiments were done as explained in the materials and methods. The FLAG-ΔssErp protein was immunoprecipitated by the Anti-FLAG beads which in turn was able to co-precipitate SPT2 protein at ~ 63 kDa position (Fig. 22, Lane 3, M2 FLAG IP panel). These observations clearly demonstrated that SPT2 interacts with ΔssErp protein under in vivo conditions also.

The recombinant ΔssErp protein purified from M15 bacterial cells exhibited a single band at ~ 36 kDa position (Fig. 16B). When same gene was cloned in a mammalian expression vector and over-expressed in BHK21 cell line, we obtained a doublet of ΔssErp protein at ~ 36 kDa which was reproducible in all the experiments (Fig. 22). No abnormality in Δsserp gene cloned into pcDNA2aFLAG vector was observed on DNA sequencing. The presence of two bands of FLAG-ΔssErp may be attributed to the post translational modification in the BHK21 cells.
Results

**Fig. 22:** Western blots showing interaction of SPT2 with ΔssErp. The BHK21 cells were co-transfected with FLAG-ΔssErp and HA tagged SPT2 protein expressing constructs. 20 hr post transfection the cells were lysed and subjected to co-immunoprecipitation with M2 FLAG antibodies. Co-immunoprecipitates and the lysates were electrophoresed on SDS-PAGE and transferred to nitrocellulose. The top panel was blotted for SPT2 with Anti-HA antibodies, while the central panel was blotted for ΔssErp using Anti-Erp antibodies and the lowest panel was probed with Anti-actin antibodies to show equal loading. Constructs transfected are indicated at the top left of the panel and the proteins are mentioned at the right.

### 4.3.4 Time kinetics to check the stability of interaction between SPT2 and ΔssErp

The BHK21 cells co-transfected with constructs expressing HA-SPT2 and FLAG-ΔssErp proteins either in combination or alone. The transfected cells were lysed at 20 hr, 36 hr and 48 hr post transfection and processed for co-immunoprecipitation. It was observed that the expression of HA-SPT2 and FLAG-ΔssErp proteins decreased from 20 hr to 36 hr and could not be detected at 48 hr post transfection (Fig. 23, lanes 3, 4 and 5 of lysate panel). At all the three time points the FLAG-ΔssErp protein was able to co-precipitate HA-SPT2 (Fig. 23, lanes 3, 4 and 5 of IP panel). A very slight increase in interaction between HA-SPT2 and FLAG-ΔssErp proteins were exhibited at 36 hrs post transfection (Fig. 23, lane 4, IP panel). Even though the HA-SPT2 protein was not visible in the lysate at 48 hrs...
post transfection, but the corresponding signal for HA-SPT2 was seen in the IP panel (Fig. 23, Lane 5).

4.3.5 Full length Erp protein interacts with SPT2 under in vivo condition

The full length erp gene was cloned into pcDNA2aFLAG vector as explained in the materials and methods. Upon restriction digesting the pcDNA2aFLAGerp clone with EcoRI an insert of ~ 852 bp was observed in the correct orientation (Fig. 24, Lane 2). All the previous protein interaction studies were performed with ΔssErp protein. To rule out the possibility of any anomalous result due to the deletion of signal sequence, we attempted to compare the binding capacity of full-length FLAG-Erp to HA-SPT2 with that of FLAG-ΔssErp. Although the level of expression of FLAG-Erp was weaker than FLAG-ΔssErp, but FLAG-Erp was successfully able to co-immunoprecipitated HA-SPT2 as observed in the IP panel (Fig. 25, Lane 3 of Anti-HA blot).

![Western blot showing stability of protein interaction between SPT2 and ΔssErp at different time points.](image)

Fig. 23: Western blot showing stability of protein interaction between SPT2 and ΔssErp at different time points. The BHK21 cells were co-transfected with FLAG-ΔssErp and HA-SPT2 expressing constructs. The cells were lysed at 20 hr, 36 hr and 48 hr post transfection and subjected to immunoprecipitation with M2 FLAG beads. The co-immunoprecipitates and lysates were used for immunobloting studies. The top panel was blotted for SPT2 using Anti-HA antibodies, the central panel was blotted for ΔssErp using Anti-Erp antibodies and the lower panel was blotted for Actin with Anti-Actin antibodies.
Fig. 24: Cloning of *erp* into pcDNA2aFlag vector. Lane 1: 1.0 kb DNA ladder; Lane 2: pcDNA2aFLAGerp digested with EcoRI.

Fig. 25: Full length FLAG-Erp protein interacts with HA-SPT2. The lysates from BHK21 cells co-transfected with different constructs of FLAG-Erp or FLAG-ΔssErp and HA-SPT2 were subjected to Anti-FLAG beads. The co-immunoprecipitates and lysate samples were resolved on SDS-PAGE and transferred to nitrocellulose. The top panel was blotted for SPT2 using Anti-HA antibodies, the central panel was blotted for ΔssErp using Anti-Erp antibodies and the lower panel was blotted for Actin with Anti-Actin antibodies. Constructs transfected are indicated at the top and the proteins are mentioned at the right.

4.3.6 PCR based deletion mutants of Δsserp and its cloning in pcDNA2aFLAG vector

Primers were designed to generate four PCR based deletion mutats of Δsserp gene. These mutants were designed on the basis of the fact that the Erp protein has three domains;
the amino terminus, the carboxy terminus and the central PGLTS repeat domain. The amino and the carboxy terminus are conserved in different mycobacterial species but the central PGLTS region is highly variable. The fourth mutation was based on the bioinformatics analysis (motif scan) showing Src kinase binding site under high stringency. The above deletion mutants were code named by us as ΔNerp, ΔCerp, ΔPGLTSerp and Δsrcerp, respectively (Fig. 26A). These mutants were first cloned into the cloning vector and then into mammalian expression vector, pcDNA2aFLAG as described in materials and methods (Fig. 26B). Upon restriction digestion of pcDNA2aFLAGΔNerp, pcDNA2aFLAGΔPGLTSerp, pcDNA2aFLAGΔCerp and pcDNA2aFLAGΔsrcerp clones with HindIII + XhoI an insert of ~645 bp, ~636 bp, ~489 bp and ~816 bp, respectively were observed confirming the correct orientation of the clones (Fig. 26B, Lanes 3, 4, 5 and 6).

4.3.7 Amino terminus domain of ΔssErp is involved in interaction with SPT2

To further analyse which domain of FLAG-ΔssErp is involved in the interaction with HA-SPT2, four deletion mutants of Δsserp were constructed and examined for its interaction with HA-SPT2 (Fig. 27). All the four constructs of Δsserp were co-transfected along with spt2 constructs in BHK21 cells. Interestingly, there was loss of interaction between FLAG-ΔNErp protein and HA-SPT2 (Fig. 27, FLAG IP Lane 3), while ΔCErp, ΔPGLTSErp and ΔSrcErp showed interaction with SPT2 (Fig. 27, FLAG IP lanes 4, 5 and 6). The lysates blot shows equal expression level of HA-SPT2 (Fig. 27, Lanes 1 to 6 of the lysates). It is well reported that the Erp protein shows a mobility shift from ~28 kDa to ~36 kDa. The relative mobility shift was observed for ΔNErp and ΔPGLTSErp deletion but this shift was abolished in case of ΔCErp deletion; emphasizing the fact that carboxy terminus might be responsible for the mobility shift (Fig. 27, Lanes 2, 3, 4 and 5).

4.3.8 M. smegmatis ΔssErp does not interact with SPT2

Now obvious query that arises is whether Erp from non-pathogenic mycobacteria interacts with SPT2? In order to answer this M. smegmatis Δsserp (msΔsserp) was cloned into pcDNA2aFLAG as described in materials and methods. A band of ~852 bp was seen upon restriction digestion of pcDNA2aFLAGmsΔsserp with HindIII + EcoRI (Fig. 28, Lane 2). The co-immunoprecipitation was performed from lysates of BHK21 cell line either transfected with msΔsserp or Δsserp alone or in combination with spt2. It was observed that M. smegmatis ΔssErp do not interact with SPT2 (Fig. 29, Lane 3, FLAG IP blot). Even
though MsΔssErp protein was larger in size than ΔssErp (Mtb), it was observed that the MsΔssErp protein moved faster than ΔssErp protein of Mtb H37Rv Fig. 29, Lanes 1, 3, 4 and 5 of Erp panel).

Fig. 26: Deletion mutants of ΔssErp. (Panel A) Schematic representation of Erp and its deletion mutants. From top to bottom, the ΔssErp (devoid of 22 amino acid signal sequence), ΔNErp (devoid of 22 amino acid signal sequence and 67 amino acid long NH2 domain), ΔPErp (devoid of 22 amino acid signal sequence and 70 amino acid long PGLTS repeat domain), ΔCErp (devoid of 22 amino acid signal sequence and 119 amino acid long COOH domain) and ΔSrcErp (devoid of 22 amino acid signal sequence and 10 amino acid long SRC domain). (Panel B) Cloning of various deletion mutants of Δsserp gene (as mentioned
in Panel A), into pcDNA2aFLAG vector. Lane 1: 1.0 kb DNA ladder; Lane 2: pcDNA2aFLAGΔsserp; Lane 3: pcDNA2aFLAGΔNerp; Lane 4: pcDNA2aFLAGΔPerp; Lane 5: pcDNA2aFLAGΔCerp and Lane 6: pcDNA2aFLAGΔsrcerp vector digested with HindIII + XhoI.

Fig. 27: Western blots showing binding of protein SPT2 with different deletion mutants of ΔssErp. The lysates from BHK21 cells co-transfected with different deletion mutants of ΔssErp along with SPT2 were subjected to anti-FLAG beads. IP and the lysates were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The top panel was blotted for SPT2 using Anti-HA antibodies, the central panel was blotted for ΔssErp and its deletion mutants using Anti-Erp antibodies and the lower panel was blotted for Actin with Anti-Actin antibodies. Constructs transfected are indicated at the top, position of molecular mass standards in kDa are shown at the left and the proteins are mentioned at the right.
**Fig. 28:** Cloning of msΔsserp in pcDNA2aFLAG vector. Lane 1: 1.0 kb DNA ladder; Lane 2: restriction digestion of pcDNA2aFLAGmsΔsserp with HindIII and EcoRI.

**Fig. 29:** *M. smegmatis* ΔssErp does not interact with SPT2. The lysates from BHK21 cells transfected with either ΔssErp or MsΔssErp alone or in combination with SPT2 were subjected to Anti-FLAG beads. Co-immunoprecipitates and the lysates were resolved on SDS-PAGE and transferred to nitrocellulose. The top panel was blotted for SPT2 using Anti-HA antibodies, the central panel was blotted for ΔssErp from either *Mtb* H37Rv or *M. smegmatis* using Anti-Erp antibodies and the lower panel was blotted for Actin with Anti-Actin antibodies. Constructs transfected are indicated at the top left and the proteins are mentioned at the right of the panel.
Results

4.3.9 PCR based deletion mutants of spt2 and its cloning in pcHA vector

Protein sequence analysis of SPT2 by InterProScan and ScanProsite did not reveal any specific domain or motif apart from a very long Serine palmitoyltransferase 2 domain covering entire stretch of the protein and a 10 amino acid long pyridoxal phosphate attachment site (374 – 383 amino acids). Hence the spt2 gene was deleted serially from N-terminus to C-terminus. Four deletion mutants of spt2 namely, spt2ΔN (1-399bp), spt2ΔM1 (400-799bp), spt2ΔM2 (800-1199bp) and spt2ΔC (1200-1670bp) were generated using PCR as described in materials and methods (Fig. 30A). The pcHA clones with the three deletion mutants of spt2 (namely ΔN, ΔM1 and ΔC) were screened with BamHI for the right clones. A band of ~1.5 kb from pcHA spt2ΔN, ~1.5 kb from pcHA spt2ΔM1 and a band of ~1.9 kb from pcHA spt2ΔC were obtained upon restriction digestion with BamHI (Fig. 30B, Lanes 2, 3 and 5) confirming the correctness of the clones. The clone pcHA spt2ΔM2 upon restriction digestion with BamHI showed a mobility shift of ~1670 bp in the pcHA vector confirming the presence of spt2ΔM2 gene (Fig. 30B, lane 4).

4.3.10 Carboxyl terminus of SPT2 is responsible for its interaction with ΔssErp

To find out the domain of SPT2 responsible for its interaction with ΔssErp, four deletion mutants were tested for their binding ability to ΔssErp. All the four mutants constructs of spt2 were transfected along with Δsserp construct in BHK21 cell line and subjected to immunoprecipitation using FLAG beads. It was observed that HA-SPT2ΔN, HA-SPT2ΔM1 and HA-SPT2ΔM2 proteins were co-immunoprecipitated by FLAG-ΔssErp (Fig. 31, Lanes 1, 2 and 3 of FLAG IP panel). Hence there was no effect of serial selections of 1-399 amino acids on its interaction with FLAG-ΔssErp. SPT2ΔC was not co-immunoprecipitated by FLAG-ΔssErp (Fig. 31, Lane 4 of FLAG IP panel). Therefore, the carboxyl terminus of SPT2 appeared to be responsible for its interaction with ΔssErp.
Fig. 30: Deletion mutants of spt2. (Panel A) Schematic representation of wild type SPT2 and its four deletion mutants. The first three deletion mutants SPT2ΔN, SPT2ΔM1 and SPT2ΔM2 were devoid of 133 amino acids. While the fourth deletion mutant SPT2ΔC was devoid of 161 amino acids. (Panel B) Cloning of deletion mutants of spt2 gene into pcHA vector. Lane 1: 1.0 kb DNA ladder; Lanes 2, 3, 4 and 5: BamHI digests of pcHASpt2ΔN, pcHASpt2ΔM1, pcHASpt2ΔM2 and pcHASpt2ΔC, respectively.
Fig. 31: Western blots showing interaction of ΔssErp with different deletion mutants of SPT2. The BHK21 cells were co-transfected with different deletion mutants of spt2 and Δsserp. The lysates were co-immunoprecipitated with anti-Flag beads. The co-immunoprecipitates and the lysates were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The top panel was blotted for HA-SPT2 using Anti-HA antibodies, the central panel was blotted for FLAG-ΔssErp using Anti-Erp antibodies and the lower panel was blotted for Actin using Anti-Actin antibodies. Constructs transfected are indicated at the top, position of molecular mass standards in kDa are shown at the left and the proteins are mentioned at the right.

4.4 Localization of Erp, ΔssErp and ΔNH2Erp in CHO K1 cell line

4.4.1 Introduction

Confocal microscopy has been extensively used to study the sub cellular localization of various proteins within the cells. Therefore, plasmids coding of CFP tagged Erp, ΔssErp, ΔNErp and YFP tagged SPT2 fusion proteins were transiently transfected either alone or in combination for determination of co-localization.

4.4.2 Cloning and expression in BHK21 cell line

The erp, Δsserp, ΔNerp and spt2 genes were cloned into pECFPN1 or pEYFPN1 vector so that the fluorescent tag is towards carboxyl terminus of these proteins as described
in the materials and methods. The inserts for erb and Δsarp genes were obtained at ~ 855 bp and ~ 786 bp upon digestion with HindIII + Sall (Fig. 31A, lane 2) and XhoI + Sall (Fig. 31A, lane 3), respectively, thus confirming the right orientation of the clone. A band of ~ 594 bp on digestion with EcoRI + XhoI exhibited that the pECFPN1ΔNerp was the right clone (Fig. 31B, lane 2). The spt2 gene was cloned into pEYFPN1 vector at EcoRI + XhoI site. A band of ~ 594 bp on digestion with BamHI confirmed that pEYFPN1spt2 is the correct clone (Fig. 31C, lane 2). The Erp, ΔssErp and ΔNH2Erp fusion with CFP were characterised by western blotting. The Erp-CFP, ΔssErp-CFP and ΔNerp-CFP were readily detectable with anti-GFP antibodies. On comparison of molecular mass of these constructs to that with the untagged proteins revealed an expected shift of ~ 27 kDa due to the presence of the CFP tag at the end of these fusion proteins (Fig. 32A, lanes 1, 2 & 3). A similar shift of ~ 27 kDa, the molecular weight of YFP was detected for SPT2-YFP (~ 90 kDa) tagged fusion protein when probed with anti-GFP antibodies (Fig. 32B).

Fig. 31: Cloning of erb, Δsarp, ΔNer and spt2 genes into pECFPN1 and pEYFPN1 vector, respectively. (Panel A) Lane 1: 1.0 kb DNA ladder; Lane 2: pECFPN1erb digested with HindIII & Sall; Lane 3: pECFPN1Δsarp digested with XhoI & Sall. (Panel B) Lane 1: 1.0 kb DNA ladder; Lane 2: pECFPN1ΔNerp digested with EcoRI & XhoI. (Panel C) Lane 1: 1.0 kb DNA ladder; Lane 2: pEYFPN1spt2 digested with BamHI.
Fig. 32: Western blotting with Anti-GFP antibodies showing the expression of CFP tagged Erp, ΔssErp, ΔNErp and YFP tagged SPT2 fusion protein in BHK21 cell line. (Panel A) Lanes 1, 2 and 3: Erp-CFP (arrow 1), ΔssErp-CFP (arrow 2) and ΔNErp-CFP (arrow 3) fusion proteins expressed in BHK21 cell line, respectively. The arrow heads shows the respective positions of the fusion proteins. (Panel B) Lanes 1: SPT2-YFP fusion proteins expressed in BHK21 cell line. The arrow head shows the position of SPT2-YFP fusion protein.

4.4.3 Erp and ΔssErp localises in Endoplasmic Reticulum (ER)

Plasmids expressing CFP tagged Erp, ΔssErp and ΔNErp proteins were transfected into CHO K1 cells and confocal slides were prepared after staining with ER tracker red dye as described in materials and methods. No interference between the fluorescence of CFP and ER tracker red dye was observed with the filter settings used as described in materials and methods. Confocal studies revealed that both Erp-CFP, ΔssErp-CFP localized with ER tracker red dye that specifically stains ER. No signal of Erp-CFP and ΔssErp-CFP were observed in the nucleus. The major signal of ΔNErp-CFP protein was present in the nucleus with a considerable loss in its ability to localize with ER tracker red dye. The localization pattern of Erp protein was observed to be altered in CHO K1 cells when amino acids (1-67) were deleted at the amino terminus of Erp (ΔNErp) but it remained unchanged when 22 amino acid long signal sequence was deleted (Fig. 32).
4.4.4 Co-localization of Erp, ΔssErp and ΔNErp with SPT2

Confocal images of CHO K1 cells co-transfected with SPT2-YFP expressing construct and different CFP tagged Erp constructs were taken as described in materials and methods. It was observed that Erp-CFP and ΔssErp-CFP proteins were exclusively localized to the cytoplasm. As expected the SPT2-YFP protein was localized around the nucleus in the endoplasmic reticulum. The Erp-CFP and ΔssErp-CFP proteins were found to be co-localized with SPT2-YFP (Fig. 33). This co-localisation was severely affected in cells expressing ΔNErp-CFP and SPT2-YFP proteins (Fig. 33). The ΔNErp-CFP was found to be present predominantly in the nucleus. The patterns observed in the co-localization slides were consistent in all the fields. No cross-talk between the fluorescence signals of CFP and YFP tagged proteins were observed with the filter settings used as described in materials and methods.
Fig. 32: Localization of Erp, ΔssErp and ΔNErp tagged with CFP. Confocal images of CHO K1 cells transfected with the CFP fusion constructs and ER tracker red dye as indicated towards left side of the panel. The images were captured in Ziess LSM 510 META with conditions for CFP and ER tracker red dye as mentioned in materials and methods. The CFP tagged Erp constructs are seen in blue colour while ER tracker red dye is seen in red colour.
**Fig. 33: Co-localization of Erp, ΔssErp and ΔNErp with SPT2.** CHO K1 cells were transfected with the CFP tagged Erp, ΔssErp and ΔNErp and YFP tagged SPT2 fusion constructs either in combination or alone as indicated on left side of the figure. The images were captured in Nikon A1R confocal microscope with conditions for CFP and YFP as mentioned in the materials and methods. The CFP tagged Erp constructs is in blue colour while YFP tagged SPT2 is red in colour.
4.5 Biological significance of SPT2 interaction with ΔssErp

4.5.1 Introduction

Protein-protein interaction regulates a range of cells metabolic processes. Similar rules also apply to our newly found interaction between ΔssErp and SPT2. The protein SPT2 is an enzymatic sub-unit of heterodimeric enzyme Serine palmitoyl transferase (Hanada, 2003).

4.5.2 ΔssErp of Mtb increases the activity of SPT in a dose dependent manner

To investigate the probable biological role of a direct interaction between ΔssErp protein of Mtb and SPT2, SPT activity assay was performed in BHK21 cells as described in the materials and methods. The treatment of BHK21 whole cell lysate with recombinant ΔssErp protein exhibited a dose dependent increase in condensation of Palmitoyl CoA and L-U-(14C-serine) resulting in an increased formation of 3-ketosphinganine (Fig. 34). An increase in endogenous SPT2 activity from BHK21 cell line was seen on treatment of 10 pg to 10 ng ΔssErp protein followed by a decline in the activity from 100 ng to 1 μg ΔssErp protein (Fig. 34). Approximately ~4.5 fold increase (10 ng ΔssErp protein) in the activity of endogenous SPT was observed in comparison to the untreated lysate from BHK21 fibroblast cells (Fig. 34).

4.5.3 ΔNErp protein does not modulate the activity of endogenous SPT

To investigate the effect of ΔNErp protein on endogenous SPT activity, we cloned ΔNerp gene in pQE30 vector, over expressed and purified the protein as described in materials and methods. The right clone of pQE30-ΔNerp on digestion with EcoRI + Sall exhibited an insert of “ΔNerp” at ~594 bp (Fig. 35A, Lane 7). The purified and concentrated ΔNErp protein was electrophoresed on SDA-PAGE (Fig. 35B, Lane 2). A single band of ΔNErp protein was obtained which electrophoresed below ~34 kDa marker when blotted with Anti-Erp antibodies (Fig. 35C). Upon treatment of BHK21 whole cell lysate with 10 ng of recombinant ΔssErp an increase in SPT activity was observed as expected but this increase was abolished when ΔNErp protein was used for the treatment (Fig. 35D).
Fig. 34: Purified ΔssErp protein modulates the activity of endogenous SPT from fibroblast in a dose dependent manner. Lysate from BHK 21 cells were treated with recombinant ΔssErp protein (10pg to 1μg) and SPT2 activity assay was performed. This experiment was repeated more than three times with reproducible results.
Results

Fig. 35: Purified δNErp protein does not increase the activity of endogenous SPT. (Panel A) Cloning. Lane 1: 1.0 kb DNA ladder; Lanes 2 to 7: Vector self ligation on digestion with EcoRI + Sall; Lane 8: Right clone with pQE30-δNerp digested with EcoRI + Sall; (Panel B) Expression. Lane 1: Pre-stained protein molecular weight marker; Lane 2: Purified concentrated δNErp protein. (Panel C) Western blotting of purified δNErp with Anti-Erp antibodies. (Panel D) SPT activity assay. Lysate from BHK 21 cells were treated with either 10 ng of recombinant AssErp or δNErp protein and SPT2 activity assay was performed. The SPT assay was repeated thrice with reproducible results.