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ABSTRACT

Erp (Exported Repetitive Protein, Rv3810) protein of *Mycobacterium tuberculosis*, a member of a mycobacteria-specific family of extracellular proteins, is a crucial virulence factor which is secretory in nature with a canonical signal sequence. Although the calculated molecular weight of RvErp is ~26.0 kDa but it electrophoreses as ~36.0 kDa, therefore, RvErp is also known as P36 or Pirg. It consists of three domains: a highly conserved amino terminal domain, a central variable region consisting of P(G/A)LTS repeat motif and a conserved carboxy terminus domain rich in proline and alanine. The number and the fidelity of PGLTS repeats vary considerably among mycobacterial species. The C-terminus hydrophobic domain of RvErp anchors it to the membrane. RvErp is found to be up regulated during nutrient starvation conditions. RvErp contributes to cell wall structure and colony morphology. RvErp is reported to appear as doublet when *M. bovis* supernatants were probed with sera from infected cattle but appears as a single band when recombinant RvErp was over expressed and purified from *Escherichia coli*. Preliminary observations of our studies also indicate RvErp as either doublet or as multiple bands when over expressed in higher eukaryotic cells.

The deletion of Rv*Erp* gene of *M. tuberculosis* results in very low levels of multiplication in macrophage cell lines as well as in mouse model of infection indicating that RvErp somehow modulates the macrophages in such a way that favours the survival of mycobacterium. However, neither the details of the mechanisms of biological role of RvErp protein nor the factors responsible for its post translational modifications are known.

To gain insight into the function(s) and regulation of RvErp within macrophages, yeast two hybrid assay was attempted. RvErp devoid of its 22 amino acid long signal sequence (RvΔssErp) was used as "bait" and mouse macrophage cDNA library as "prey". The signal sequence of RvErp was deleted to qualify as bait. Y2H assay resulted in nine putative interacting protein partners. G-protein coupled receptor (GPCR) kinase 2 (GRK2) is one of them. GRK2 is a eukaryotic kinase with multiple functions. It desensitizes the receptors by phosphorylation i.e. receptors become unresponsive to stimuli.

GRK2 interacted directly with RvΔssErp *in vitro*, as confirmed by GST pull down assays. Co-immunoprecipitation (Co-IP) studies in CHOK1 fibroblast cell line confirmed that both RvErp as well as RvΔssErp (devoid of signal sequence) interacted with GRK2

in vivo. Interestingly, ΔssErp of *M. smegmatis* interacted weakly with GRK2 in comparison to RvΔssErp. While the deletion of amino terminus of RvErp (RvΔNErp) affected the interaction of RvΔssErp with GRK2 due to its localization into nucleus, the deletion of PGLTS and COOH domains of RvErp had no effect on the interaction with GRK2. Next, all the domains of GRK2 were found to interact coherently with RvΔssErp as no loss in interaction is seen with any of GRK2 deletion mutants used. Although, RvΔssErp was found to be co-localizing with GRK2 in the cytoplasm but RvΔNErp did not co-localize due to its nuclear localization.

Furthermore, RvΔssErp exhibited multiple bands at ~ 45.0 kDa with enhanced anomalous electrophoretic mobility upon over expression in mammalian cells. This could happen due to multiple post translational modifications of RvΔssErp. Besides, anamoly in mobility was abolished when C-terminus of RvΔssErp was deleted.

GRK2 is a kinase and our studies showed that RvΔssErp exhibited multiple bands; therefore, one of the most plausible options is phosphorylation of RvErp by GRK2. One of the Bioinformatics tools, Predikin Web Server also predicted GRK2 as one of the potential kinase of RvΔssErp. Therefore, *in vitro* kinase assay were deployed to study the GRK2 mediated phosphorylation of RvΔssErp. Although *in vitro* phosphorylation of purified RvΔssErp by GRK2 was found to be heparin dependent, phoshprylation of RvErp *in vivo* was heparin independent. Phosphoamino acid analysis demonstrated that GRK2 phosphorylated RvΔssErp at Serine residues. Moreover, loss of GRK2 mediated phosphorylation upon deletion of N-terminus of RvΔssErp and presence of only one spot on autoradiogram in peptide mapping by 2D-TLC peptide maps of RvΔssErp confirmed that GRK2 phosphorylates Serines located in N-terminus of RvΔssErp.

Continuous stimulation of β-Adrenergic receptor (β-AR, a GPCR) by its agonist, isoproterenol, leads to GRK2 dependent receptor internalization and desensitization of GPCR signalling, thus attenuation of cAMP production. Therefore, the effect of interaction of RvΔssErp with GRK2 on receptor internalization and cAMP production was examined. The increase of β-AR on the plasma membrane surface and increased cAMP production as a result of interaction of RvΔssErp with GRK2 indicated that RvΔssErp impairs GRK2 mediated internalization of β-AR. In sum, the interaction of RvΔssErp with GRK2 evades GRK2 mediated regulation of cell signalling responses.

The *in vivo* phosphorylation pattern of RvΔssErp in presence of heparin indicates that GRK2 is not the only kinase which can phosphorylate RvErp. Other eukaryotic

kinases could also phosphorylate RvErp. However, no other host kinase has been identified in Y2H assay. NetPhosK 1.0 and Scansite Servers, bioinformatics tools, were used to identify the probable kinases of host. These servers predicted p38 MAPK as another eukaryotic kinase which can phosphorylate RvΔssErp.

For the investigation of p38 mediated phosphorylation of RvΔssErp, first, the interaction of RvΔssErp with p38 was confirmed *in vivo*. p38 mediated phosphorylation of RvΔssErp were confirmed by *in vitro* kinase assays. Phosphoamino acid analysis demonstrated that p38 phosphorylated RvΔssErp predominantly at serine residues but threonines were also found to be phosphorylated. *In vitro* analysis of seven spots obtained on autoradiogram of peptide map of p38 mediated phospho-RvΔssErp, indicated that there are atleast four major target sites along with possibly three minor sites. The details of the interactions of p38 with RvΔssErp would be explored in future.

To conclude, we have identified RvErp to be a substrate for host kinases, namely GRK2 and p38. This is the first report showing the interaction of RvErp with host kinases and thus phosphorylation of RvErp at different types of amino acids. Furthermore, these studies also exhibit the subversion of GRK2 function by *M. tuberculosis* RvΔssErp protein. However, further studies are required to explore the significance and regulation of eukaryotic host mediated phosphorylation of RvΔssErp. Besides phosphorylation, other post translational modifications also need to be explored.