Mycobacterial PE_PGRS Proteins Contain Calcium-Binding Motifs with Parallel β-roll Folds

Nandita Bachhawat1,*# and Balvinder Singh2

1 G.N. Ramachandran Knowledge Center for Genome Informatics, Institute of Genomics and Integrative Biology, Council of Scientific and Industrial Research, Delhi 110007, India; 2 Institute of Microbial Technology, Chandigarh 160036, India.

# Current address: Department of Bioinformatics, DAV College, Chandigarh 160012, India.

The PE_PGRS family of proteins unique to mycobacteria is demonstrated to contain multiple calcium-binding and glycine-rich sequence motifs GGXGXD/NXUX. This sequence repeat constitutes a calcium-binding parallel β-roll or parallel β-helix structure and is found in RTX toxins secreted by many Gram-negative bacteria. It is predicted that the highly homologous PE_PGRS proteins containing multiple copies of the nona-peptide motif could fold into similar calcium-binding structures. The implication of the predicted calcium-binding property of PE_PGRS proteins in the light of macrophage-pathogen interaction and pathogenesis is presented.

Key words: Mycobacterium tuberculosis, virulence factors, PE_PGRS, calcium-binding motif, parallel β-roll fold

Introduction

The PE/PE_PGRS multigene family unique to mycobacteria accounts for about 5% of the Mycobacterium tuberculosis genome. The family includes 38 PE-encoding genes and 61 PE_PGRS-encoding genes scattered throughout the genome, characterized by a relatively conserved NH2-terminus of ~110 amino acids (aa) followed by a COOH-terminal glycine-rich repeat region ranging from ~100 aa to over 500 aa in length (1, 2). Several PE_PGRS proteins have been demonstrated to be associated with the replication and survival of M. tuberculosis within macrophages of infected host tissues and are important for pathogenesis (3). One of the functions proposed for these proteins is that they are a source of antigenic variability to evade host immune responses (1). Other observations suggest the possibility that PE_PGRS proteins could either be cell surface constituents (adhesins) (4) that can influence bacterial cell structure (5) or could interfere with immune responses by inhibiting antigen processing (6). A recent study has shown that the evolution and expansion of the PE and PPE families is closely associated with the ESAT-6 (esx) gene cluster and has suggested a functional interdependence (7). As the ESAT-6 cluster encodes proteins involved in secretion and membrane pore formation, it is likely that the ESAT-6 gene cluster-encoded proteins might also be required for secretion of some of the PE family proteins.

Despite these reports, the precise function and underlying mechanism of action still remain unknown for this large family of proteins. The aim of this study was therefore to investigate all of the 61 PE_PGRS proteins in the PE subfamily to predict whether they carry any distinct function in M. tuberculosis. Attempts were made to determine their underlying mechanism of action, and an explanation was sought for answering why there are so many different PE_PGRS proteins in M. tuberculosis.

Results

Attempts to identify similar proteins in databases to provide insights into their functions have been difficult owing to the repetitive nature of these proteins. Similarity searches of PE_PGRS proteins invariably identify only other PE_PGRS family members or glycine-rich proteins such as those found in highly elastic plant cell wall proteins (8). To search specifically for non-mycobacterial microbial homologues that have so far not been identified, we analyzed the microbial genome databases (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) excluding...
Mycobacterium species (9). Using the widely investigated PE_PGRS protein Rv1818c as query and without masking the low complexity regions, one of the proteins revealed from the BLAST results was an RTX (repeat in toxin) toxin of Magnetococcus sp. MC-1 (COG2931), which is a related Ca\(^{2+}\)-binding protein. The Rv1818c protein has 42% identity over 100% stretch of its PGRS domain to the glycine-rich domain of the COG2931 protein (data not shown). Although this domain corresponds to only a small region (5.8%) of the large RTX protein, the fact that some PE_PGRS proteins were reported as surface-exposed adhesin-like molecules (5, 10) prompted us to examine this similarity more carefully.

The family of RTX toxins includes haemolysin, cyclolysin, leukotoxin, and metallopeptidase, and these proteins seem to have two properties in common. First they bind calcium, and second they contain a multiple tandem repeat of nona-peptides, GGXGXD/NXUX, where X = any amino acid and U = unpolar/large hydrophobic residue at the C-terminal end of each protein (11, 12). It was subsequently found that these sequence repeats constitute a Ca\(^{2+}\)-binding structure called a parallel \(\beta\)-helix or parallel \(\beta\)-roll and might participate in host cell binding (13). To examine whether Rv1818c also contains this nona-peptide repeat corresponding to a Ca\(^{2+}\)-binding motif, we searched for GGXGXD/NXUX as the calcium-binding motif and detected numerous such motifs in Rv1818c (Figure 1). Subsequently the presence of this motif was examined in all the 61 PE_PGRS proteins. Among the different PE_PGRS proteins grouped according to domains (not shown), 56 PE_PGRS proteins were found to contain this motif. The other five PE_PGRS proteins that lacked this motif were Rv0742, Rv0832, Rv0978c, Rv3652, and Rv3812. However, among the five proteins, Rv0832 (137 aa) may be frame-shifted in M. tuberculosis H37Rv to be fused with a protein of 749 aa encoded by Rv0833; Rv0978c is a protein of 331 aa that contains an unusually short (78 aa) PRGS motif; while Rv3652 (104 aa) may also be a frame-shifted PE_PGRS protein. All these proteins belong to both classical and non-classical types of PE_PGRS proteins. The maximum number of repeats was found in Rv3345c where 77 copies of this calcium-binding parallel \(\beta\)-helix or \(\beta\)-roll motif GGXGXD/NXUX were present (Table S1). A total of 911 such calcium-binding motifs, among

### Table

| Nine amino acid motifs | Inter-motif distance (amino acid) |
|------------------------|-----------------------------------|
| 168 G G A G G N V A S | 176 | – |
| 215 G G A G G N G G L | 223 | 39 |
| 243 G G A G G D G G L | 251 | 20 |
| 262 G G T G T N V T G | 270 | 11 |
| 273 G G A G G N G G L | 281 | 3 |
| 289 G G V G G D G V A | 297 | 8 |
| 364 G G A G G N A G L | 372 | 67 |
| 393 G G A G G N G G T | 401 | 19 |
| 438 G G A G G N G T G | 446 | 37 |

G G X G X/D/N X U X = Consensus

X = arbitrary amino acid, U = unpolar/large hydrophobic residue

**Fig. 1** The GGXGXD/NXUX calcium-binding motifs (bold) identified in Rv1818c and their alignments.
which 403 are GGXGXDXUX type and the remaining
508 are GGXGXNXUX type, were detected among
all the PE_PGRS family members. The number of
repetitive motifs varied according to the length of the
open reading frames (ORFs), and the inter-motif dis-
tance was not fixed. For example, Rv1818c (498 a)
contains 9 motifs and the inter-motif distance varies
from a minimum of 3 aa to as high as 67 aa (Figure 1).
This is unlike RTX toxins where the calcium-binding
motifs are not distributed so randomly. In this con-
text, it is worth mentioning that the U residues of
GGXGD/NXUX motifs in PE_PGRS proteins are
not always unpolar/large hydrophobic residues in na-
ture (Table S1). In more than 70% of these cases,
U = G. In the remaining cases, U could be any ar-
bitrary residue except for cystein (data not shown).
We also determined the secondary structure of all the
61 PE_PGRS proteins including Rv1818c to reaffirm
the presence of β-strands intercepted by coils in the
C-terminal PGRS domain, which is a characteristic of
parallel β-roll structure (data not shown).

To identify structurally important folds of
PE_PGRS proteins, we attempted to associate them
with structurally similar bacterial protein(s). Em-
ployment of the 3D-Jury system (14) to predict a
possible protein fold of known function, using the C-
terminal PGRS domain (382 aa) of Rv1818c as query
sequence, revealed a Ca^{2+}-binding fold with a sta-
tistically moderate score (Table S2). The highest fold
recognition scores were compiled next for the PGRS
domains of all 61 proteins (Table S2). Interestingly,
~70% of the total PE_PGRS proteins exhibited a
common fold to the C-terminal β-roll Ca^{2+}-binding
domain of Serratia marcescens metalloprotease [PDB
ID: 1SRP (15) and 1SAT (16)] and the alkaline pro-
tease of Pseudomonas aeruginosa IFO3080 [PDB ID:
1AKL (17) and 1KAP (18)], all of them belong to the
RTX family.

The Rv3344c protein with fold prediction data
greater than the confidence threshold limit set by
3D-Jury system (Table S2) was used subsequently to
generate an optimized 3D molecular model us-
ing the alkaline protease of P. aeruginosa (PDB ID:
1AKL)(17) as the template. It is important to men-
tion here that although according to the GenBank
report, the Rv3344c gene (Accession No. YP_177961)
might be a gene fragment that should be in-frame
with a following ORF (MTV016.45c), no frame-shift
was found when checked in BAC and cosmide clones
(as of GenBank latest update: 24-May-2007). The
MODELER program was used to create the model
(18, 19). The predicted model (Figure 2) was found
to adopt Ca^{2+}-binding parallel β-helix or parallel β-
roll structures (13) located at the turn of coil re-
gions. The models (18, 19) created for the PE_PGRS
proteins with 1AKL/1KAP/1SAT/1SRP (PDB IDs)
fold scores greater than 25.0 were also predicted to
hold calcium ions in the individual glycine-rich nona-
sequence motifs (data not shown).

Discussion

Our results presented here suggest that the highly ho-
logorous PGRS domain of the majority of the PE
subfamily proteins have calcium-binding motifs and
are therefore likely to be calcium-binding proteins.
Calcium-dependent adhesins are known to exist in the
soil bacterium Rhizobium species where their attach-
ment to the developing root hairs of leguminous plants
is considered to be the first step in the host-specific
infection process that leads to a nitrogen-fixing symbiosis (20). Analogous to the Rhizobium nodulation
genode nodO that encodes a Ca^{2+}-binding protein in-
volved in interactions with plant root cells in a Ca^{2+}-
dependent way, it is possible that a similar Ca^{2+}-
dependent PE_PGRS-mediated interaction may exist
in M. tuberculosis with host cells (4, 10).

The earliest interactions of M. tuberculosis with
macrophages are known to result in a number of alter-
ations in Ca^{2+} signaling events critical for phagosome
maturation (21–23). In macrophages, activation of
cytosolic Ca^{2+}-regulated enzyme Ca^{2+}/calmodulin-
dependent protein kinase II (CaMKII) is essential for the
phagosome-lyosome fusion (24). Other studies have demonstrated that M. tuberculosis blocks
this pathway via inhibition of sphingosine kinase, a
macrophage enzyme that increases cytosolic Ca^{2+} lev-
eels (22, 25). Furthermore, the maturation and the
acidification of mycobacterial phagosome could be
restored by artificially raising the cytosolic Ca^{2+} lev-
eels using Ca^{2+} ionophores (21) or receptor stimula-
tion with ATP (26). This has suggested that M. tu-
berculosis depletes internal Ca^{2+} stores in infected hu-
man macrophages (21, 26).

In the light of these observations, a role for Ca^{2+}-
binding PE_PGRS proteins could be envisaged as fol-
ows. The initial non-specific attachment of M. tu-
berculosis to the host alveolar macrophages via Ca^{2+}-
dependent PE_PGRS proteins will cause a sudden dip
in calcium concentration at the focal point of host
pathogen interaction (27, 28). Such an event would lead to a fall in the cytosolic Ca$^{2+}$ levels in macrophages, ultimately preventing phagolysosome fusion. We thus postulate that the initial host-pathogen interactions could play a very crucial role to the sensing and establishment of the bacilli’s intracellular pathogenesis.

The generalized calcium-dependent adhesion ability of PE_PGRS proteins does not rule out the possibility that these proteins have additional functions. Existence of “non-classical” type of PE_PGRS proteins (data not shown) with protein folds other than “conserved parallel $\beta$-roll calcium-binding folds” (Table S2) indicates that some PE_PGRS proteins might have yet undiscovered additional virulence-related functions that help the bacilli to survive in infected host (6).

Additionally, it will also be of worth to see if these initial PE subfamily protein(s)-host cell interactions can promote membrane cholesterol accumulation at the site of mycobacterial entry (29) that might eventually modulate the membrane depolarization event needed for the entry of external Ca$^{2+}$.

**Materials and Methods**

The genome information of *M. tuberculosis* strain H37Rv was retrieved from NCBI genome database as Accession NC_000962 (1). Subsequently, the re-annotated *M. tuberculosis* genome sequence was consulted from Camus et al (30). An indigenously developed algorithm was written in C++ language to extract all the 61 PE_PGRS ORF sequences.

To search specifically for non-mycobacterial microbial homologues of PE_PGRS proteins, we analyzed the microbial genome databases (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) excluding *Mycobacterium* species (9). Classification of all the 61 PE_PGRS proteins from *M. tuberculosis* H37Rv based on domain patterns was performed using the PROSITE database (31) at http://au.expasy.org/prosite/. Fold recognition data for these proteins
PGRS Proteins

PGRS proteins are cell surface constituents of Mycobacterium tuberculosis with potential calcium interacting with glycine-rich nonapeptide motifs. The calcium-complex structure was stabilized by energy minimization using the same software. The model of parallel \( \beta \)-roll structure of PGRS domain was generated by Insight II software (Accelrys Inc., San Diego, USA). The glycine-rich nona-peptide motifs was generated by Insight II software (Accelrys Inc., San Diego, USA). The calcium-complex structure was stabilized by energy minimization using the same software.

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Authors’ contributions

NB conceived and supervised the study, collected the data relating to the fold, created the models, and assisted in manuscript preparation. BS collected the data relating to the fold, created the models, and prepared the manuscript. Both authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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**Supporting Online Material**

Tables S1 and S2

http://www.imtech.res.in/bvs/PE-PGRS-Mtb/