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Degradation-resistant protein domains limit host cell processing and immune detection of mycobacteria

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ABSTRACT

The *Mycobacterium tuberculosis* genome reveals a large family of glycine–alanine rich PE–PGRS proteins. Due to similarities with the glycine–alanine rich Epstein–Barr nuclear antigen 1, there has been interest in whether PE–PGRS proteins inhibit cellular processing and presentation via the major histocompatibility complex class I pathway. We investigated whether PE–PGRS proteins were resistant to ubiquitin–proteasome-dependent degradation and $CD8^+$ T cell recognition. Upon transient expression of ubiquitin fusion constructs of either full-length $Rv0978c^{PE-PGRS}$ protein or its PE domain in HeLa cells, the former was markedly less susceptible to proteasomal degradation. When peptides of varying glycine and alanine content from different PE–PGRS proteins were fused to the N-terminus of SIINFEKL peptide, the alanine-rich fusions elicited lower interleukin-2 responses in SIINFEKL-specific $CD8^+$ T cells, with corresponding decrease in lysis of cells presenting such peptides. When $CD8^+$ T cells from *Mycobacterium bovis* BCG-immunized mice were stimulated with either full-length PE–PGRS protein Rv3812 or its PE domain, the former exhibited a lower level of cytotoxicity against BCG-infected autologous macrophages. These results suggest that mycobacterium PE–PGRS proteins have domains that confer resistance to ubiquitin–proteasome-dependent protein degradation, and the bacteria may have an abundance of such proteins to evade immune detection and killing of mycobacterium-infected cells.

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb) infection, is an infectious disease of global importance. Whether in active disease or latent infection, chronicity of tuberculosis infection relates to the ability of Mtb to maintain a persistent intracellular existence in the host, despite immune surveillance (Flynn and Chan, 2001). It is therefore likely that the pathogen has evolved ways of evading immune detection. Here, we investigate how specific motifs in a large family of Mtb proteins subvert the host antigen processing mechanism to evade presentation to $CD8^+$ T lymphocytes via the MHC class I pathway.

One factor affecting proteolytic stability of proteins is the presence of a destabilizing N-terminal residue and an internal lysine (or lysines), which is the site of covalent conjugation with ubiquitin (Ub) or a poly-Ub chain. The N-end rule relates the *in vivo* half-life of a protein with the identity of its N-terminal amino acid residue (Varshavsky, 1996). This was uncovered by studying Ub fusions with a reporter green-fluorescent protein (GFP) (Bachmair et al., 1986; Dantuma et al., 2000b). The ubiquitin-specific proteases cleave ubiquitin off the fusion, and the N-terminal residue is exposed. If the N-terminal residue of GFP is a destabilizing amino acid like arginine (R), the fusion protein is targeted for ubiquitin–proteasome-dependent (UPD) proteolysis (Bachmair et al., 1986; Johnson et al., 1995). While Ub–R-GFP is highly unstable, the Ub–P-GFP fusion protein expressed transiently is more stable, and Ub–G-GFP is most stable.

Antigens from intracellular pathogens are degraded by the host cell proteasome and loaded onto MHC class I molecules for presentation to antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTLs). Degradation of endogenous proteins by UPD proteolysis is important for such antigen processing (Rock and Goldberg, 1999). Reduced processing and presentation will have a direct impact on CTL antigen recognition and their lytic activity against infected host cells.

Epstein–Barr virus nuclear antigen 1 (EBNA1) is involved in the maintenance of viral episomes in infected cells (Masucci and Ernberg, 1994). CTLs specific for EBNA1-peptides are present in high frequency during primary EBV infection (Blake et al., 1997), suggesting that EBNA1-expressing cells may be targeted for killing.

Abbreviations: Mtb, *Mycobacterium tuberculosis*; Ub, ubiquitin; UPD, ubiquitin–proteasome-dependent; CTL, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; EBNA, Epstein–Barr nuclear antigen; BCG, bacille Calmette–Guérin.

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However, EBNA-1 specific CTLs fail to recognize cells expressing endogenous full-length EBNA-1, due to a failure of antigen presentation correctable by the deletion of a Gly–Ala repeat domain (Blake et al., 1997). Insertion of the Gly–Ala repeat fragment of EBNA1 into a different EBV protein that is normally processed through the MHC class I pathway prevents the altered protein from antigen processing, and subsequently, affects antigen presentation (Levitskaya et al., 1995). The presence of the long internal Gly–Ala repeat also protects EBNA1 from UPD proteolysis *in vitro* (Levitskaya et al., 1997). EBV isolates contain different lengths of Gly–Ala repeats (Dillner et al., 1984), therefore, the Gly–Ala repeat is thought to play a role in immune evasion of EBV-infected cells by excluding an important potential target from antigen presentation.

Tuberculosis is also a chronic intracellular infection, thus Mtb could have similar ways of avoiding immune recognition. The Mtb genome suggests the existence of a large family of 61 PE–PGRS proteins, each with a conserved Pro-Glu (PE)-rich N-terminus and a C-terminus with polymorphic Gly-rich repetitive sequences (PGRS) (Cole et al., 1998). This family is unique to mycobacteria, but their function is not fully understood. PE–PGRS proteins and EBV nuclear antigens both contain extensive Gly–Ala repeats, and the length of the Gly–Ala repeat varies between different clinical isolates (Banu et al., 2002; Cole et al., 1998; Dillner et al., 1984). However, the Mtb proteins have relatively much fewer of such repeats than EBNA1, and the frequency of occurrence of Gly–Ala repeats in different PE–PGRS proteins within each Mtb strain is variable. It is unclear whether Mtb PE–PGRS proteins would also confer protection against antigen processing by the host proteasome in a similar manner. We focused this study on two members of the PE–PGRS family, encoded by *rv0978c* and *rv3812*. The PGRS domain of the Rv0978cp protein is Gly–Ala rich, in common with most of the PE–PGRS family members. *Mycobacterium marinum* orthologues of Rv3812p are required for persistence in granulomas and macrophages *in vivo* (Ramakrishnan et al., 2000). Our data suggest that the PGRS domain protects the PE domain from UPD degradation and that this influences $CD8^+$ T cell recognition of the protein.

2. Materials and methods

2.1. Plasmids

By sequential cloning into pcDNA3 (Invitrogen), three variants of pUb-X-2HA-eGFP, pUb-X-2HA-Rv0978c$_{1-92}$ (PE) and pUb-X-2HA-Rv0978c (PE–PGRS) were designed, each with codons for Gly, Pro or Arg in position 1 after ubiquitin within 'X'. *rv0978c* was amplified from genomic DNA of *M. tuberculosis* H37Rv.

2.2. In vitro transfection and Western blotting

HeLa cells (3×10^5) were transfected with 2 μg of each plasmid using Lipofectamine 2000 (Invitrogen). Sixteen hours after transfection, cells were trypsinized and lysed for 30 min on ice using cell lysis buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, and protease inhibitor cocktail from BD). Subsequently, the lysed cells were centrifuged and supernatants collected. In some cases, 10 μg/ml of proteasome inhibitor, carboxybenzyl-leucyl-leucinal (MG-132, Calbiochem) was added 4 h after transfection, and lysates harvested 12 h later. These lysates were immobilized on Hybond-P and probed with mouse monoclonal anti-GFP antibody (clone B-2, Santa Cruz) at 0.1 μg/ml, mouse anti-HA antibody (clone 12CA5) at 50 ng/ml, rabbit polyclonal anti-Ub antibody (Sigma) diluted 1:1000 or mouse anti-γ-tubulin (clone C-11, Santa Cruz) at 0.1 μg/ml, after prior blocking with 5% non-fat milk. Peroxidase-conjugated secondary antibodies were detected by chemiluminescence (ECL kit, GE Biosciences).

2.3. IL-2 response assay

The IL-2 secreting B3Z cytotoxic T cell clone specific for $OVA_{258-265}$ (SIINFEKL), an MHC class I (H-$2K^b$)-binding epitope of chicken ovalbumin, was used (Karttunen et al., 1992). The H-$2K^b$ dendritic cell clone DC2.4 (Shen et al., 1997) at 5×10^4 cells/well were pulsed with SIINFEKL-fusion peptides for 4 h, and co-cultured with B3Z cells (10 T cells:1 DC) for 14 h. Culture supernatants were tested for IL-2 production by ELISA (Biolegend).

2.4. Murine immunization

All experiments were approved by the NUS Institutional Animal Care and Use Committee. *Mycobacterium bovis* BCG (Pasteur strain) was prepared in phosphate buffered saline (1×10^6 in 100 μl), used to immunize BALB/c mice intraperitoneally, and 2 weeks later, splenic $CD8^+$ T cells were negatively selected by magnetic cell sorting (Miltenyi Biotec).

2.5. Cytotoxicity assay

$CD8^+$ T cells were seeded at 2×10^6 cells/ml, and stimulated in the presence of autologous macrophages for 48 h with 1 μg/ml of different recombinant proteins. The stimulated $CD8^+$ T cells were subsequently washed, and co-cultured with 2×10^4 BCG-infected autologous macrophages at an effector: target ratio of 10: 1 for 12 h. Cytotoxicity assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions. In some experiments, a similar cytotoxicity assay was also performed with B3Z cells as effector cells and peptide-pulsed C57Bl6 murine bone marrow-derived macrophages as the target cells.

2.6. Statistics

IL-2 responses to different peptides were compared using the Student t test. Percentage cytotoxicity between different antigen-stimulation groups (Fig. 5) was compared using the Mann–Whitney *U* test.

3. Results

3.1. Designing stable and unstable eGFP fusion proteins

A schematic diagram of the fusion proteins generated is shown in Fig. 1. We first generated three chimeric hemagglutinin (HA)-tagged ubiquitin–eGFP fusion proteins to study their proteolytic stability when expressed transiently in HeLa cells. At 16 h after transfection,

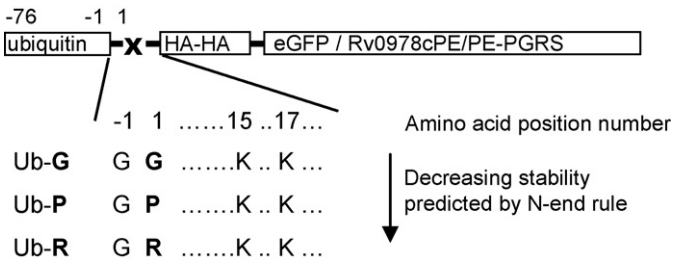

Fig. 1. Ubiquitin fusions for targeting eGFP or PE–PGRS proteins for proteasomal degradation. A schematic representation of the fusion constructs used, showing alternative N-terminus residues which would be exposed upon ubiquitin cleavage. This amino acid directly downstream of ubiquitin (G, P or R) is numbered as position 1. The lysine residues in positions 15 and 17 are potential poly-ubiquitination sites. HA: hemagglutinin polypeptide, included to facilitate protein purification and detection.