Specific Chaperones for the Type VII Protein Secretion Pathway*§

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Abstract

Background: Pathogenic mycobacteria use the type VII secretion systems (T7SS) ESX-1 and ESX-5 to secrete virulence factors, but it is unknown how these systems recognize their cognate substrates.

Results: Pulldowns identified specific interactions between cytosolic components of ESX-1 and ESX-5 and subsets of cognate substrates.

Conclusion: T7SS substrates interact with associated cytosolic secretion system components.

Significance: Cytosolic chaperones contribute to system specificity in T7SS.

Mycobacteria use the dedicated type VII protein secretion systems ESX-1 and ESX-5 to secrete virulence factors across their highly hydrophobic cell envelope. The substrates of these systems include the large mycobacterial PE and PPE protein families, which are named after their characteristic Pro-Glu and Pro-Pro-Glu motifs. Pathogenic mycobacteria secrete large numbers of PE/PPE proteins via the major export pathway, ESX-5. In addition, a few PE/PPE proteins have been shown to be exported by ESX-1. It is not known how ESX-1 and ESX-5 recognize their cognate PE/PPE substrates. In this work, we investigated the function of the cytosolic protein EspG5, which is essential for ESX-5-mediated secretion in Mycobacterium marinum, but for which the role in secretion is not known. By performing protein co-purifications, we show that EspG5 interacts with several PE/PPE proteins and a PE/PPE complex that is secreted by ESX-5, but not with the unrelated ESX-5 substrate EsxN or with PE/PPE proteins secreted by ESX-1. Conversely, the ESX-1 parologue EspG1 interacted with a PE/PPE couple secreted by ESX-1, but not with PE/PPE substrates of ESX-5. Furthermore, structural analysis of the complex formed by EspG5 and PE/PPE indicates that these proteins interact in a 1:1:1 ratio. In conclusion, our study shows that EspG5 and EspG1 interact specifically with PE/PPE proteins that are secreted via their own ESX systems and suggests that EspG proteins are specific chaperones for the type VII pathway.

Bacterial pathogens use dedicated protein secretion systems to export virulence factors that interfere with cellular processes of the host to ensure bacterial survival, multiplication, and spread (1). Also, pathogenic mycobacteria, such as Mycobacterium tuberculosis, the causative agent of tuberculosis, secrete proteinaceous virulence factors. These bacteria are surrounded by a unique dierm cell envelope that requires a specialized secretion pathway, known as the ESX or type VII secretion pathway, to facilitate protein export (2).

M. tuberculosis contains five ESX clusters, designated ESX-1 to ESX-5. The most well studied locus, ESX-1, is crucial for the virulence of M. tuberculosis and secretes two virulence factors of the WXG100 protein family, i.e. EssA and EssB. In addition, ESX-1 secretes a number of proteins referred to as ESX-1 secretion-associated proteins (Esp) (3–8) and a few PE/PPE proteins (3, 7, 9). Pe/ppe genes, which are named after conserved Pro-Glu and Pro-Pro-Glu motifs near the N termini of their gene products, are present in high numbers in the genomes of several mycobacterial pathogens and are thought to contribute to mycobacterial virulence (10). Based on the presence of specific motifs in their C termini, the PE and PPE proteins are further divided into subfamilies, of which the PE_PGRS subfamily is the largest (11). Although some PE/PPEs are secreted via ESX-1, ESX-5 is responsible for the secretion of most of these proteins, including many PE_PGRS proteins (12–15). The ESX-5 locus is restricted to the genomes of slow growing mycobacteria. Importantly, all ESX substrates identified to date lack classical signal peptides, and the mechanism of substrate targeting is not yet fully understood (6, 16). Recently, we demonstrated that the C termini of PE proteins and several other ESX-1 substrates share a conserved YXXXD/E motif that is required for secretion. Intriguingly, this signal does not discriminate among the various ESX pathways (9).

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§ This article contains supplemental Table S1.

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The ESX clusters contain a number of conserved genes, termed ESX conserved components (ecc), which are required for secretion (8). In addition, there are components that are only present in one or a few ESX systems, one of which is EspG. EspG was originally thought to be specific for ESX-1, -2, and ESX-3, but a homologue with low similarity is also present in the ESX-5 locus. Disruption of this homologue in *Mycobacterium marinum* (mmar_2676) blocked secretion via ESX-5 (13) and affected intracellular levels of ESX-5 substrates (17). Here, we have investigated the role of MMAR_2676/EspG₃ in ESX-5 secretion, and we show that this protein interacts selectively with PE/PPE proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*M. marinum* WT strain E11 (18) and the ESX-5 mutants 7C1 (espG₃::tn) (13) and Mx2 (eccA₃::tn) (12) were grown and electroporated as described (14). *Escherichia coli* BL21(DE3), BL21(DE3) pLysS, DH5α, TOP10F, and XL10-Gold were grown at 37 °C in LB medium with shaking at 200 rpm, or on LB agar. When required, ampicillin was used at a concentration of 100 μg/ml, chloramphenicol at 30 μg/ml, kanamycin at 25 μg/ml, and hygromycin at 50 μg/ml for mycobacteria and 100 μg/ml for *E. coli*.

**Plasmid Construction**—PCRs were carried out with the Phusion High-Fidelity DNA polymerase (Finnzymes) using primers listed in supplemental Table S1.

The mmar_2672-2676 operon was amplified by PCR from *M. marinum* E11 genomic DNA using 5′ primer AbMm-Middle-H5-F and 3′ primer 1794-His-SpeI-R, which contains a His₆-encoding sequence and a SpeI restriction site. This fragment was cloned in the integrative pUC-Int-cat vector (13), resulting in pUC-Int-cat::Mm2672–76His, which was used to express C-terminally His-tagged EspG₃ under the same enzymes, resulting in pSMT3::PE68-1-encoding fragments, amplified with the 5′ NcoI site-containing primer pET68_F and either pET68noHis_R or pET68_R, both with HindIII sites, from pSMT3::PE35-HA-PPE68_1 (9) with primers pET35_F and pET35_R containing an NdeI and a KpnI site, respectively. This fragment was ligated into pET29b(+)::Rv2430–31c-His after Ndel/KpnI digestion, thereby replacing the rv2431c gene. Subsequently, the rv2430c-His fragment of this vector was substituted for either WT or His-labeled PPE68_1-encoding fragments, amplified with the 5′ NcoI site-containing primer pET68_F and either pET68noHis_R or pET68_R, both with HindIII sites, from pSMT3::PE35-HA-PPE68_1. This resulted in plasmids pET29b(+)::PE35-PE68_1 and pET29b(+)::PE35-PPE68_1-His. To generate the construct for expression of PE25 lacking the last 15 amino acids, together with C-terminally His-tagged PPE41, the pET29b(+)::Rv2430–31c-His vector was used as template in a nested PCR approach. A fragment containing the first RBS and the first 255 bp of rv2431c was amplified using primers pET-31cF and pET-31cR, which contained a PstI restriction site, and amplification fragment was digested with PstI and EcoRV and ligated into pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His. Similarly, a fragment coding for C-terminally His-tagged PPE41 without its last 20 amino acids was amplified from pSMT3::PE25-25c-HA-PPE41 with primers pET41_F and pET41d20C_R. Subsequently, replacement of the rv2430c-His fragment in pET29b(+)::Rv2430–31c-His as described above resulted in pET29b(+)::Rv2430–31c-His. Plasmids for *E. coli* expression of PE35 and PPE68_1, and with and without a C-terminal His tag, were constructed by first amplifying mmar_0185 from pSMT3::MmPPE35-HA-MmPPE68_1 (9) with primers pET35_F and pET35_R containing an Ndel and a KpnI site, respectively. This fragment was ligated into pET29b(+)::Rv2430–31c-His

For expression in *E. coli*, espG₃ and espG were cloned both with and without C-terminal His tag under control of the tac promoter in the pASK-IBA3c vector (IBA Gmbh). The genes were amplified using a 5′ primer containing an *E. coli* ribosomal binding site (RBS) and XbaI digestion site (1794-RBS-XbaI-F or EspG₃-RBS-XbaI-F) and a 3′ primer with HindIII digestion site (1794-R, 1794His-HindIII-R, or EspG₃His-HindIII-R). After digestion, the fragments were ligated into XbaI and HindIII-digested pASK-IBA3c, resulting in pLBA::EspG₃ or pLBA::EspG₃-His, pLBA::EspG₃-His. For expression of PE25 and C-terminally His₆-tagged PPE41 in *E. coli* a previously described construct was used in which the rv2431c and rv2430c genes were each placed behind a RBS, i.e. pET29b(+)::Rv2430–31c-His (19). To create an unlabeled version of PPE41, the rv2430c gene was amplified with primers pET41_F and pET41noHis_R containing NcoI and HindIII sites from pSMT3::PE25-25c-HA-PPE41 (9). This fragment was subsequently used to replace the rv2430c-His sequence in pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His, thereby generating pET29b(+)::Rv2430–31c-His.
Protein Co-purification Assays—Ni²⁺ purifications of His-labeled proteins expressed in *M. marinum* were performed by loading filtered soluble lysates on washed HisTrap HP columns (GE Healthcare) at 0.5 ml/min. Optionally, the filtered *M. marinum* lysates were precleared on HisTrap-Sepharose columns that had been stripped from Ni²⁺-NTA-agarose beads (Qiagen) for 1 h at room temperature. After washing the beads five times with phosphate buffer containing 20 mM phosphate, 150 mM NaCl, pH 8.0) containing 50 mM imidazole, bound proteins were eluted with 5 ml phosphate buffer containing 150 mM imidazole, and collected in 500-μl fractions. Wash and elution fractions were precipitated with 10% TCA. Alternatively, lysates of *M. marinum*-expressing proteins of interest were incubated with NiNTA-agarose beads (Qiagen) for 1 h at room temperature with head-over-head rotation. After washing the beads five times with phosphate buffer containing 20 mM imidazole, bound proteins were eluted by incubation with 100, 200, and 500 mM imidazole, respectively. Immunoprecipitation of HA-tagged proteins was performed using the HA Tag IP/Co-IP kit (Pierce).

For the *in vitro* pulldowns, soluble lysates of *E. coli* containing similar amounts of recombinant proteins were mixed with NiNTA-agarose beads (Qiagen) for 1 h at room temperature. Washing was performed as described above using a buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole (pH 8.0), and proteins were eluted using this buffer supplemented with 100 or 250 mM imidazole.

Cloning, Protein Expression, and Purification for Molecular Mass Measurements—espG₅ was cloned with an N-terminal His tag under control of the T7 promoter in the pETM-11 vector (EMBL). The gene was amplified using a 5’ primer containing a Ncol digestion site (AP-303) and a 3’ primer with Xhol digestion site (AP-304). After digestion, the fragments were ligated into Ncol and Xhol-digested pETM-11, resulting in pAP200. For expression of PE25 and C-terminally His-tagged PPE41 in *E. coli* a previously described construct was used in which the rv2431c and rv2430c genes were each placed behind a RBS (19). All proteins were produced in *E. coli* BL21(DE3) after induction with 0.2 mM isopropyl β-D-thiogalactopyranoside and overnight incubation at 20 °C. Cells were lysed by sonication in 25 mM HEPEs (pH 7.5), 150 mM NaCl supplemented with protease inhibitor mix (Serva). Lysates were cleared by centrifugation and subjected to standard immobilized metal ion affinity chromatography (IMAC) using HisTrap HP columns. After washing with 20 mM imidazole, recombinant proteins were eluted with a linear gradient of 20–250 mM imidazole in 25 mM HEPEs (pH 7.5), 150 mM NaCl. Relevant protein fractions were pooled and concentrated and subjected to protein treatment for tag removal. His tags were cleaved from EspG₅ by overnight incubation with tobacco etch virus protease at 4 °C. Protease and uncleaved protein were removed by a second IMAC step. In case of PE25/PPE41-His, biotinylated thrombin (Novagen) was used which was subsequently removed by streptavidin beads (Invitrogen) according to the manufacturer’s recommendations. All proteins were further purified to homogeneity by preparative size exclusion chromatography (HiLoad 16/600 Superdex 200 pg; GE Healthcare) in 20 mM HEPEs (pH 7.5), 150 mM NaCl. EspG₅ as well as PE25/PPE41 were concentrated using Vivaspin centrifugal concentrators (Corning) to a final concentration of 5.6 and 5.3 mg/ml, respectively. Protein concentrations were estimated by measurements of absorbance at 280 nm using theoretical molar absorption coefficients calculated by ProtParam.

Size Exclusion Chromatography-Tridetector Analysis—Proteins were analyzed with a Viscotek 305 tridetector (Malvern Instruments, Malvern, UK) which monitors light scattering, refractive index, and UV absorbance. This setup was connected to an analytical size exclusion column (Superdex 200 10/300 GL; GE Healthcare) equilibrated at 20 °C in 20 mM HEPEs (pH 7.5), 150 mM NaCl as running buffer. Flow rate was set to 0.5 ml/min, and the sample volume was 100 μl. Proteins were diluted to 1 mg/ml in running buffer prior to analysis. To detect heterotrimeric complex formation, EspG₅ was mixed with PE25/PPE41 at a 3-fold molar excess and incubated 30 min at 20 °C before injection. The provided OmniSEC software was used to acquire and evaluate all data. Molecular masses were estimated using refractive index combined with light-scattering data using BSA as an internal control using a refractive index increment with protein concentration (dn/dc) of 0.185 ml/g.

**SDS-PAGE, Immunoblotting, and Sera**—Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue G-250 (CBB; Bio-Rad), or transferred to nitrocellulose membranes by Western blotting. The membranes were incubated with mouse monoclonal antibodies directed against the conserved repeat of PE_PGRS proteins (mAb 7C4.1F7) (13), the influenza hemagglutinin epitope (HA.11; Covance), the C-terminal His₅ epitope (11922416001; Roche Applied Science), EssA (Hyb76-8; Statens Serum Institut, Copenhagen, Denmark), or GroEL2 (CS44; John Beilis, National Institutes of Health, Bethesda, MD, Contract AI-75320); or with rabbit polyclonal serum reactive against PPE41 (12), PPE68 (21), EspG₅, FtsH/Mmar_0752 (22), or EssN (Mtb9.9A) (23). Secondary horseradish peroxidase-conjugated goat anti-mouse IgGs

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4 E. N. G. Houben and W. Bitter, manuscript in preparation.
EspG5::tn mutant and pulldown experiment in *M. marinum*. A and B, immunoblots of equal amounts of whole cell pellets (P), whole cells treated with Genapol (Gp), Genapol surface extracts (Gs), and 2-fold excess of culture supernatants (S) from *M. marinum* WT strain E11, the isogenic espG5::tn mutant E12, and the mutant expressing espG5-His, all expressing PE25-HA/PE41, were probed with antibodies against EspG5, PE_PGRS, PPE41, HA tag, EssA, and the lysis control GroEL2. C, immunoblot analysis of total (T), soluble (Sol), and cell envelope (CE) fractions of *M. marinum* Mx2 (eccA5::tn) expressing EspG5-His. EspG5-His was detected using mAb directed against the His6 epitope, and cross-reactive polyclonal antiserum was used to detect the cytosolic protein MMAR_0752 and the inner membrane protein FtsH as controls for the fractionation. D, EspG5-His was purified from soluble lysates of Mx2 expressing EspG5-His from a plasmid (+) or the empty strain (−) on Ni-NTA beads. Unbound proteins (W), the last washing step (W), and proteins eluted with 100 mM (E1), 200 mM (E2), and 500 mM (E3) imidazole were separated by SDS-PAGE in a ratio of 1:20:10:10:10 and stained with CBB or probed with antiserum directed against the His-epitope immunoblot. Eluted EspG5-His is marked by an asterisk, and specific co-eluted proteins are marked by an arrowhead.

(A106PS; American Qualex) or goat anti-rabbit IgGs (611-1302; Rockland) were detected with ECL (Roche Applied Science or Pierce).

**Mass Spectrometric Analysis**—For analysis by nanoLC-MS/MS, protein lanes from CBB-stained gels were excised, prepared, and analyzed as described (7). Identified proteins were annotated using the Mycrobrowser website (24).

For MS/MS analysis, samples were prepared essentially as described (13) and analyzed by MALDI-TOF/TOF MS (AB Sciex TOF/TOF 5800). MS/MS spectra search was performed against the *M. marinum* FASTA database using Mascot software (Matrix Science). Searches were performed with a peptide mass tolerance of 0.15 Da and a fragment mass tolerance of 0.1 Da, while allowing a single site of miscleavage and oxidation of methionine as a variable modification. Only proteins with a score of >50 were considered significant.

**RESULTS**

EspG5 Interacts with PPE Proteins—To study the function of EspG5, we expressed C-terminally His-tagged EspG5 in the *M. marinum* espG5::tn mutant. Immunostaining with an antibody recognizing multiple PE_PGRS proteins showed that expression and (partially) secretion of these ESX-5 substrates were restored (Fig. 1A). Moreover, treatment of cells with the mild detergent Genapol to extract surface proteins (7) showed that surface localization of PE_PGRS proteins did not differ from the wild type strain (Fig. 1B). We also investigated secretion of heterologously expressed PE25 and PPE41. As previously shown, secretion of these proteins was markedly reduced in the espG5::tn mutant strain (9, 13). Importantly, secretion was restored to wild type levels upon complementation with EspG5-His (Fig. 1A). As controls we used the intracellular protein GroEL2 and the secreted ESX-1 substrate EssA. Together, these results show that EspG5-His is functional. Notably, EspG5 is not a secreted protein, as both EspG5 and EspG5-His were only detected in the cell pellet and not extracted with Genapol (Fig. 1, A and B). The subcellular localization of EspG5 was examined by a fractionation procedure, which showed that EspG5-His was exclusively present in the soluble fraction of the cells (Fig. 1C). As a control for successful fractionation an antisem that recognizes both the membrane protein FtsH and cytosolic MMAR_0752 was used.

To identify putative interacting partners of EspG5, a Ni-NTA pulldown was performed using membrane-free lysates of the espG5::tn mutant complemented with EspG5-His. However, although purification of EspG5 was successful, no specific interacting partners were detected (data not shown), perhaps because interactions are transient in the presence of a functional ESX-5 system. Therefore, this pulldown experiment was repeated using a different ESX-5 secretion mutant strain, i.e. eccA5::tn. Importantly, this strain is deficient in ESX-5-mediated secretion also after introduction of the EspG5-His construct (data not shown), and some of the ESX-5 substrates do accumulate in the cytosol (12). We therefore hypothesized that putative interactions between EspG5 and other proteins might be prolonged in this strain. Indeed, in two pulldowns, of which the second was optimized to reduce background binding to the beads (see “Experimental Procedures”), we could now observe protein bands that were exclusively present in the eluted fractions of the EspG5-His expressing strain (see Fig. 1D for the optimized pulldown). To identify these interacting proteins, total protein isolations with and without EspG5-His were subjected to nanoLC-MS/MS analysis. As also observed after SDS-PAGE analysis (Fig. 1D), proteins with the highest spectral counts were present in both samples and could therefore be
EspG5-His or both EspG5-His and a C-terminally HA-tagged EspG5. These results indicate that EspG5 specifically interacts with PE/PPE proteins, which are known substrates for the ESX-5 system and are underrepresented in mass spectrometry experiments. Of note, PE/PPE proteins often have a low number of trypsin recognition sites and are therefore notoriously underrepresented in mass spectrometry experiments. These results indicate that EspG5 specifically interacts with PE/PPE proteins.

To confirm this interaction a co-purification assay was performed using lysates of the eccA5::tn strain expressing EspG5-His or both EspG5-His and a C-terminally HA-tagged EspG5. Although a minor amount of PPE33-HA bound to the beads in the absence of EspG5-His, significantly more was co-purified with EspG5-His (Fig. 2A). The unrelated cytosolic control M. marinum MMAR_0752 was only present in the flow-through fraction (Fig. 2A). Furthermore, in the reciprocal pulldown using beads coated with HA mAb, both EspG5-His and endogenous EspG5 were specifically co-purified, whereas in control lysates both forms of EspG5 ended up in the flow-through (Fig. 2B). In conclusion, these data confirm that EspG5 interacts with PPE33.

EspG5 Interacts with PE/PPEs Secreted by ESX-5—The PE/PPE proteins that we identified as interacting partners of EspG5 all belong to the SVP subfamily. To test whether EspG5 also interacts with other PE/PPE proteins secreted via ESX-5 we selected the PE25/PPE41 complex, which is a well known heterodimeric substrate complex. To substantiate these results in vitro interaction studies were performed, using lysates of E. coli cells expressing either the PE25/PPE41 complex or C-terminally His-tagged EspG5. The soluble fractions were mixed, and the His-containing EspG5 protein was isolated using Ni-NTA agarose beads. Interestingly, two specific bands co-eluted with EspG5-His in the M. marinum eccA5::tn mutant, and a pulldown was performed. Immunoblot analysis of obtained fractions showed that PPE41 indeed specifically co-eluted with EspG5-His (Fig. 2C), indicating that EspG5 also interacts with PPE41.

To substantiate these results in vitro interaction studies were performed, using lysates of E. coli cells expressing either the PE25/PPE41 complex or C-terminally His-tagged EspG5. The soluble fractions were mixed, and the His-containing EspG5 protein was isolated using Ni-NTA agarose beads. Interestingly, two specific bands co-eluted with EspG5-His, with an apparent molecular mass of ∼24 kDa and ∼15 kDa, respectively (Fig. 3A, lanes 13–16). Immunoblot analysis identified the larger band as PPE41, whereas the apparent molecular mass of the smaller band corresponds to that of PE25, indicating that EspG5 interacts with the PE25/PPE41 complex. In a control purification using only the PE25/PPE41-containing lysate the majority of PPE41 was detected in the flow-through fraction (Fig. 3A, lanes 9–12). The interaction between EspG5 and PPE41 was confirmed in a reciprocal pulldown assay using C-terminally His-tagged PPE41 (Fig. 3A, lanes 1–8).

To analyze the stoichiometry of the complex formed by EspG5 and PPE41, we performed size exclusion chromatography in line with light-scattering and refractive index analysis. This revealed that EspG5 migrated as a single species with an estimated molecular mass of 32 kDa, indicating that the protein exists as a monomer in solution (Fig. 3B). Although the molecular mass of the PE25/PPE41 complex is similar to that of monomeric EspG5, PE25/PPE41 elutes slightly earlier from the gel filtration column. This is due to its tightly folded helical structure, resulting in an increased hydrodynamic radius in contrast to the apparent more globular nature of EspG5.

![FIGURE 2. EspG5 interacts with PPE33 and PPE41 in M. marinum.](image-url)

**TABLE 1**

| Accession number | Annotation | Molecular mass | Spectral counts (% amino acid coverage) |
|------------------|------------|----------------|---------------------------------------|
| MMAR_3661        | PPE38      | 37             | None + EspG5-His                      |
| MMAR_1460        | PPE33      | 47             | None + EspG5-His                      |
| MMAR_1514        | PPE31      | 38             | None + EspG5-His                      |

| Experiment 1 | Experiment 2 |
|--------------|--------------|
| None + EspG5-His | None + EspG5-His |
| 0             | 0             |
| 0             | 7 (16%)       |
| 5 (8%)        | 5 (11%)       |
| 4 (7%)        | 1 (3%)        |

*Annotated as their orthologues in M. tuberculosis H37Rv (24).*

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mixing of EspG5 with PE25/PPE41 complex, a peak was detected with a molecular mass of ~60 kDa, corresponding to a 1:1:1 heterotrimeric complex (Fig. 3B).

In addition to PE/PPE proteins, the ESX-5 system also secretes EsxN. However, our nanoLC-MS/MS analysis of proteins co-purified with EspG5-His did not reveal an interaction with EsxN. To determine whether this other class of ESX-5 substrates also interacts with EspG5, another in vitro pulldown assay was performed. EsxN forms a heterodimer with EsxM, and therefore both proteins were co-expressed from an E. coli expression vector. Although EspG5-His was efficiently pulled down, no co-purification of EsxN was observed (Fig. 3C), indicating that EspG5 specifically interacts with PE/PPE proteins.

EspG5 Does Not Interact with the Type VII Secretion Signal—We have previously shown that the flexible C terminus of PE25 contains a YXXX/E/D motif that is required for secretion (9). To investigate whether EspG5 interacts with this domain, in vitro pulldowns were carried out with E. coli lysates containing PE25 and PE25 lacking the C-terminal 15 amino acids. As shown by immunoblotting, EspG5 still co-purified with PPE41, indicating that binding of EspG5 does not depend on the YXXX/E/D motif (Fig. 3D). Using a similar approach we showed that also the flexible C-terminal domain of PPE41 is not essential for the interaction with EspG5 (Fig. 3D). Together, these experiments indicate that EspG5 interacts with the structured core domain of the PE/PPE complex.

FIGURE 3. EspG5 interacts specifically with ESX-5-dependent PE/PPE proteins in vitro. A, C, and D, SDS-PAGE/CBB and immunoblot analysis of fractions obtained by Ni-NTA co-purifications in mixed lysates of E. coli strains expressing recombinant mycobacterial proteins, or strains in which protein expression was not induced (-). In A and C the total input material (T), unbound proteins (FT), the final washing step (W5), and eluted proteins (E) are shown in a 1:1:4:4 ratio, and in C also the first washing step (W1) is included. In D eluates of in vitro pulldowns carried out in parallel are shown in equal amounts. Immunoblots, proteins were detected with sera directed against EspG5, PPE41, EsxN, or the His epitopes. Symbols label bands corresponding to EspG5 (*), PPE41 (#), PPE41(20C) (4), PE25 (●) and PPE68_1 (○). B, analytical gel filtration and Static Light Scattering of EspG5, with and without PE25/PPE41. Molecular masses derived from light scattering combined with refractive index analysis (right y axis) are plotted across the peaks as a function of elution volume. Molecular mass estimates of individual and complex components match the expected molecular mass of monomeric proteins within the expected error range.
EspG Homologues Specifically Bind Cognate PPEs—We have recently shown that PE35 and PPE68_1 are secreted by ESX-1 in M. marinum (7, 9). To determine whether EspG5 only recognizes PE/PPE substrates of ESX-5, we performed an in vitro pulldown using lysates of E. coli strains expressing EspG5 and C-terminally His-labeled PPE68_1. In parallel, EspG5 was incubated with soluble fractions of E. coli expressing PE25/PPE41-His or empty E. coli strains, as positive and negative controls, respectively. Interestingly, EspG5 did not co-elute with PPE68_1-His (Fig. 3D). This was again confirmed in a reciprocal in vitro pulldown assay using EspG5-His (Fig. 4A). Together, these results show that EspG5 does not interact with PPE proteins secreted via the ESX-1 pathway.

EspG5 is 22% identical at the amino acid level to the ESX-1-encoded protein EspG1, which has been suggested to affect the stability of PPE68 in M. tuberculosis (25). These facts led us to hypothesize that EspG1 interacts with PE/PPE proteins secreted via ESX-1. To investigate this, we performed in vitro pulldowns using lysates of E. coli expressing C-terminally His-tagged EspG1 mixed with lysates containing PE25/PPE41 or PE35/PPE68_1. In both reactions, a band of ~27 kDa was visible in CBB-stained gels, which was identified by immunoblotting as EspG1 (Fig. 4A). In the pulldown with PE25/PPE41 no additional specific bands were visible on protein gels, and only a weak band was detected with antibodies directed against PE35/PPE68_1 containing lysate a major specific band of ~37 kDa was detected, which was recognized by antibodies directed against M. tuberculosis PPE68 (Fig. 4A). These results show that whereas EspG5 specifically interacts with the ESX-5 substrates PE25/PPE41, the ESX-1-encoded EspG1 fails to associate with PPE41, but does interact with the ESX-1-substrate PPE68_1.

To confirm these observations, we introduced a plasmid encoding C-terminally HA-labeled PE35 and PPE68_1 in the M. marinum eccA5::tn mutant expressing EspG5-His. Notably, this strain is also affected in ESX-1 secretion, and ESX-1 substrates accumulate in the cytosol (13). In an HA immunoprecipitation assay, PPE68_1 co-purified with PE35-HA (Fig. 4B), showing that this PE/PPE couple indeed forms a complex. Of note, an additional band was visible just below the expected molecular mass of PPE68_1 in the anti-PPE68 immunoblot, which could represent endogenous PPE68_1. This immunoprecipitation also showed that PE35-HA does not interact with EspG5, which was also confirmed in a reciprocal pulldown (Fig. 4, B and C). However, CBB staining of the pulldown for PE35-HA did reveal specific co-purification of two proteins with an apparent molecular mass of ~36 and ~30 kDa (Fig. 4D). MS/MS analysis showed that the ~36-kDa product was indeed PPE68_1, whereas the ~30-kDa product was identified as (endogenous) EspG5. These results show that also in M. marinum the ESX-1 substrates PE35 and PPE68_1 interact specifically with EspG1. Taken together, we conclude that EspG5 and EspG1 are both specific chaperones for cognate PE/PPE proteins.

**DISCUSSION**

In this study, we show that the ESX-5-encoded MMAR_2676, which is required for ESX-5-mediated protein secretion (13, 14), interacts with cognate PE/PPE proteins. The similarity of MMAR_2676 to the EspG proteins encoded by other ESX clusters was previously considered too low for this protein to be a true EspG homologue (8). Here, we provide...
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FIGURE 5. Genetic organization of the ESX-1-like cluster and the separate pe-ppe-espG locus in N. farcinica.

compelling experimental evidence that despite this modest similarity, MMAR_2676 and EspG₁ are functionally conserved. Therefore, we propose that MMAR_2676 should be annotated as EspG₅.

Pulldown of His-labeled EspG₅ resulted in co-purification of PE and PPE proteins, but interestingly not of the ESX-5-encoded WXG100 proteins EsxM and EsxN. These results suggest that EspG₅ specifically interacts with members of the PE/PPE families. This finding seems to correlate with the composition of the different ESX loci, as all ESX loci that contain pe/ppe genes also have an espG homologue. Consistently, ESX gene clusters in close relatives of mycobacteria that lack pe/ppe genes, e.g. in Corynebacterium glutamicum and Streptomyces coelicolor, also lack an espG homologue (26). However, Nocardioidia farcinica has an additional ESX locus that contains both a pe/ppe gene couple and an adjacent espG-like gene, as well as a small pe-ppe-espG gene cluster at a distinct genomic location (Fig. 5). These findings together confirm the link between the espG and the pe/ppe families.

What is the function of the EspG proteins? Previous studies have indicated that inactivation of espG₅ or espG₁ abolishes secretion and/or affects the intracellular levels of some ESX substrates (13, 14, 25, 27). This suggests that EspG proteins play a role in expression, stability, and/or secretion of the substrates. As intracellular levels of constitutively expressed PE and PPE proteins were also markedly affected in an espG₅ mutant (14), it is unlikely that EspG₅ directly regulates the mRNA expression of the ESX-5 substrates. Rather, EspG₅ might act as a chaperone that maintains the substrates in a translocation-competent form, protects them from degradation prior to secretion, and/or serves as a targeting factor for ESX-5 secretion. We have recently shown that the PE/PPE proteins secreted via different type VII secretion systems share a conserved secretion signal, located in the C terminus of the PE proteins, but that this signal does not determine through which ESX system they are secreted (9). System specificity might be mediated through targeting of secreted effectors by specific chaperones, similar to the type III secretion pathway in Gram-negative bacteria. In type III secretion, several different classes of chaperones are distinguished, which have one or a few cognate substrates and function independently of ATP (28, 29). EspG proteins could function similarly. Consistently, deletion of the secretion signal-containing C terminus of PE25 did not affect the interaction with EspG₅. Inspection of the sequences of PE/PPE proteins and their cognate EspG chaperones does not immediately reveal residues or motifs that could explain the specificity of the interaction. Notably, EspG₁ and EspG₅ share low homology. It is plausible that the basis for the specificity is a structural motif in the EspG protein and/or structural core domain of the PE/PPE complex.

Our results are in contrast to the recent observation that knock-out of espG₅ in M. tuberculosis does not affect PPE41 secretion (15). However, these differences are not unique as functional differences between species have also been shown for EspG₁. This protein is necessary for ESX-1-mediated secretion of WXG100 proteins in M. marinum and Mycobacterium smegmatis but not in M. tuberculosis (25, 27, 30). In the last species, disruption of espG₁ did affect both secretion and stability of PPE68 (25). These observations suggest that EspG proteins can be (partly) redundant.

In conclusion, we propose that the EspG homologue of each ESX system recognizes its cognate PE/PPE proteins, maintains them in a stable conformation, and possibly also directs them to the membrane-embedded secretion machinery (Fig. 6). Subsequently, recognition of the YXXXE/E secretion motif by a component of the secretion machinery, potentially the FtsK/SpoIIE-like ATPase EccC (16), initiates translocation through the secretion channel. Future experiments will determine whether EspG₅ is able to deliver PE/PPE proteins to ESX-5 membrane components.

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