PapA1 and PapA2 are acyltransferases essential for the biosynthesis of the *Mycobacterium tuberculosis* virulence factor Sulfolipid-1

Pawan Kumar*, Michael W. Schelle*, Madhulika Jain[†], Fiona L. Lin*, Christopher J. Petzold[‡], Michael D. Leavell[‡], Julie A. Leary[‡], Jeffery S. Cox[†], and Carolyn R. Bertozzi*[§]

*Department of Chemistry and Howard Hughes Medical Institute, University of California, Berkeley, CA 94720; [†]Department of Microbiology and Immunology, University of California, San Francisco, CA 94143; and [‡]Department of Chemistry and Division of Molecular and Cellular Biology, Genome Center, University of California, Davis, CA 95606

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Mycobacterium tuberculosis produces numerous exotic lipids that have been implicated as virulence determinants. One such glycolipid, Sulfolipid-1 (SL-1), consists of a trehalose-2-sulfate (T2S) core acylated with four lipid moieties. A diacylated intermediate in SL-1 biosynthesis, SL₁₂₇₈, has been shown to activate the adaptive immune response in human patients. Although several proteins involved in SL-1 biosynthesis have been identified, the enzymes that acylate the T2S core to form SL₁₂₇₈ and SL-1, and the biosynthetic order of these acylation reactions, are unknown. Here we demonstrate that PapA2 and PapA1 are responsible for the sequential acylation of T2S to form SL₁₂₇₈ and are essential for SL-1 biosynthesis. *In vitro*, recombinant PapA2 converts T2S to 2'-palmitoyl T2S, and PapA1 further elaborates this newly identified SL-1 intermediate to an analog of SL₁₂₇₈. Disruption of *papA2* and *papA1* in *M. tuberculosis* confirmed their essential role in SL-1 biosynthesis and their order of action. Finally, the Δ *papA2* and Δ *papA1* mutants were screened for virulence defects in a mouse model of infection. The loss of SL-1 (and SL₁₂₇₈) did not appear to affect bacterial replication or trafficking, suggesting that the functions of SL-1 are specific to human infection.

pathogenesis | biochemistry | glycolipid | sulfation

The thick *Mycobacterium tuberculosis* (*M. tb*) cell wall consists of numerous glycolipids that are distinctive to the mycobacterial genus, including phosphatidylinositol mannosides, trehalose mycolates, and lipoarabinomannans (1). These molecules are essential for many of the characteristics that distinguish mycobacterial pathogenesis, such as the inhibition of phagosomal maturation, drug resistance, and alteration of the host immune response (2–6). A family of cell surface sulfated lipids (dubbed sulfatides) were identified in *M. tb* extracts and correlated to strain virulence (7–9). The most abundant sulfatide, termed Sulfolipid-1 (SL-1), consists of a trehalose core, four fatty acyl groups, and a sulfate ester (Fig. 1A) (10–13). Despite the discovery of SL-1 nearly 50 years ago, the biological function of the molecule is not known. Conflicting reports suggest a role for SL-1 in superoxide (O₂^{•−}) release from human neutrophils or monocytes, alteration of trehalose dimycolate toxicity, and inhibition of trehalose dimycolate-induced macrophage recruitment (14–19). The relevance of these studies to the physiological role of SL-1 in *M. tb* infection is debatable.

Although the role of SL-1 remains elusive, advances in genetics and metabolite analysis have sped the discovery of genes, proteins, and intermediates associated with SL-1 biosynthesis (20). Currently, three proteins are known to be involved in SL-1 assembly: Stf0, Pks2, and MmpL8. The sulfotransferase Stf0 sulfates trehalose at the 2-position, forming trehalose-2-sulfate (T2S), thereby initiating SL-1 biosynthesis (21). Meanwhile, the polyketide synthase Pks2 synthesizes the phthioceranol and hydroxyphthioceranol lipids that occupy the 6-, 6'-, and 3'-positions of SL-1 (Fig. 1A) (22). The proteins responsible for

transfer of the Pks2 products and the palmitoyl group to the T2S core, and the order in which these lipids are added, have not yet been defined.

Insight into the order of lipid addition came from characterization of the putative lipid transporter MmpL8 (23, 24). A mutant strain, Δ *mmpL8*, lacks SL-1 but accumulates the diacylated intermediate SL₁₂₇₈ (named for its observed mass) inside the cell (Fig. 1B). This intermediate possesses two of the four SL-1-associated lipids: a hydroxyphthioceranol group at the 3'-position and a palmitoyl group at the 2'-position (24). SL₁₂₇₈ was recently found to be an immunostimulant in human tuberculosis patients (25). The glycolipid is presented on the surface of *M. tb*-infected antigen-presenting cells by CD1b, a member of the MHC class I-like CD1 family. Intriguingly, the Δ *mmpL8* mutant, which lacks SL-1 but accumulates SL₁₂₇₈, shows attenuated virulence in mice (23, 24). By contrast, a Δ *pks2* mutant, which lacks both SL-1 and SL₁₂₇₈, is indistinguishable from WT *M. tb* in mice and guinea pigs (23, 26). These observations suggest that SL₁₂₇₈, and possibly other SL-1 intermediates, modulate *M. tb* pathogenesis.

In our effort to define the functions of *M. tb* sulfolipids, we sought to identify the machinery underlying conversion of T2S to SL₁₂₇₈. As a starting point we turned our attention to other *M. tb* lipid biosynthetic pathways for which the acyltransferases had been characterized. Recently, an acyltransferase that installs the lipid groups on the *M. tb* virulence factor phthiocerol dimycocerosate (PDIM) was identified as the polyketide synthase-associated protein PapA5 (27–29). This enzyme transfers a mycocerosyl group from the acyl carrier protein domain of the polyketide synthase Mas, which, like many polyketide synthases, including Pks2, lacks a thioesterase domain that would otherwise release the lipid (6, 30). *papA5* belongs to a family comprising four additional members (27, 31). Two of these, *papA2* and *papA1*, reside in proximity to other SL-1 genes in the *M. tb* genome (Fig. 1C). Given the similarity of the PDIM biosynthetic gene cluster to that of SL-1, we reasoned that the corresponding proteins, PapA2 and PapA1, may be involved in SL-1 biosynthesis.

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Abbreviations: *M. tb*, *Mycobacterium tuberculosis*; SL-1, Sulfolipid-1; T2S, trehalose-2-sulfate; PDIM, phthiocerol dimycocerosate; FT-ICR, Fourier transform ion cyclotron resonance; PCoA, ¹⁴C-palmitoyl-CoA.

[§]To whom correspondence should be addressed. E-mail: crb@berkeley.edu.

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Fig. 1. SL-1, SL₁₂₇₈, and genomic organization. (A) Structure of SL-1. (B) Structure of SL₁₂₇₈. (C) The genomic arrangement of the SL-1 biosynthetic cluster. *papA1* resides downstream of *pks2*, whereas *papA2* lies further downstream of *mmpL8*.

Here we demonstrate that PapA2 and PapA1 are responsible for the sequential acylation of T2S and are required for the synthesis of SL₁₂₇₈ and SL-1 *in vivo*. The order of addition of the two lipids found in SL₁₂₇₈ was first determined by using purified enzymes and synthetic substrates. These results were validated by disrupting *papA2* and *papA1* in *M. tb* and analyzing the metabolite profile of each mutant using high-resolution MS and radiolabeling. Finally, these mutants were evaluated for virulence defects in a mouse model of tuberculosis.

Results

***papA2* and *papA1* Reside in an SL-1 Biosynthetic Gene Cluster.** The genes encoding Pks2 and MmpL8 are clustered in the *M. tb* genome (Fig. 1C). Within the same cluster reside two genes annotated as polyketide synthase-associated proteins, *papA2* and *papA1* (31). As discussed above, the related gene *papA5* encodes an acyltransferase that esterifies phthiocerol with mycocerosic acids in the assembly of PDIM (27–29). *papA5* is associated with *mas*, the polyketide synthase responsible for mycocerosic acid synthesis. Similarly, *papA1* is associated with *pks2*, which encodes the enzyme that synthesizes the phthioceranic acids of SL-1. However, *papA2* is distal from *pks2* in the *M. tb* genome and is not directly associated with another polyketide synthase. This arrangement is unique among the *M. tb* *pap* genes, suggesting that PapA2 may function independent of a polyketide synthase. Further biochemical analysis of the interactions between the Pap proteins and their associate Pks proteins will be needed to validate these genetic implications.

PapA2 Is an Acyltransferase That Esterifies T2S. Given the unique genomic arrangement of *papA2*, we reasoned that PapA2 may be the acyltransferase that transfers the palmitoyl group to the 2'-position of T2S. To test this hypothesis we amplified *papA2* from *M. tb* H37Rv genomic DNA and expressed the protein in *Escherichia coli*. Purified PapA2 was then incubated with ¹⁴C-palmitoyl-CoA (PCoA) and either trehalose or synthetic T2S. The reaction mixtures were separated by TLC and imaged by phosphorescence. Hydrolysis of PCoA was seen in the reactions

containing PapA2 in the absence of another substrate. However, a unique product was observed in the reaction containing PapA2, PCoA, and T2S (Fig. 2A). Unsulfated trehalose was not a competent substrate for PapA2. Kinetic analysis of the PapA2 reaction containing T2S and PCoA showed substrate inhibition by PCoA, similar to that reported for PapA5 [supporting information (SI) Fig. 6]. The *K_m* value for T2S was determined to be 2.5 mM whereas the *K_m* for PCoA was 6.0 μM (Table 1). For comparison, PapA5 exhibits a *K_m* of 0.5 mM for 1-octanol and 4 μM for PCoA.

We then characterized the product of the PapA2 reaction by Fourier transform ion cyclotron resonance (FT-ICR) MS and NMR spectroscopy. Analysis of the crude PapA2 reaction by FT-ICR MS yielded a major ion with *m/z* = 659.30, corresponding to the exact mass of palmitoyl T2S (Fig. 2B). Fragmentation of the ion at *m/z* = 659.30 by tandem MS yielded peaks that were identical to those derived from chemically synthesized trehalose-2-sulfate-2'-palmitate (SI Fig. 7). NMR analysis of the PapA2 reaction product showed the presence of a palmitoyl group at the 2'-position of T2S (SI Fig. 8). These data demonstrate that PapA2 palmitoylates T2S at the 2'-position to form trehalose-2-sulfate-2'-palmitate (termed SL₆₅₉ based on its observed mass).

PapA1 Acylates SL₆₅₉, the Product of PapA2. We next reasoned that the PapA2 reaction product, SL₆₅₉, could be a substrate for PapA1. To test this hypothesis we expressed and purified PapA1 and then incubated the protein with PCoA and SL₆₅₉. A new product appeared only in the presence of all three reaction components (Fig. 2C). The palmitoyl group of SL₆₅₉ was found to be essential for modification by PapA1, as T2S was not modified. Further kinetic experiments showed substrate inhibition by SL₆₅₉ but not by PCoA (SI Fig. 9). The *K_m* of PCoA for PapA1 was much higher than that observed for PapA2, suggesting that PCoA is not the physiological substrate of the enzyme (Table 1). The related PapA5 acyltransferase has been shown to transfer mycocerosate directly from the polyketide synthase Mas (29). Thus, it is likely that PapA1's biological function is to

Fig. 4. Disruption of *papA2* or *papA1* does not alter the virulence of *M. tb* in mice. BALB/c mice were infected via aerosol with WT (filled squares), $\Delta papA2$ (open circles), or $\Delta papA1$ (open triangles) strains at an initial inoculum of ≈ 250 bacteria per lung. Growth in the lungs (A), spleen (B), and liver (C) was monitored by harvesting organs at 0, 10, 23, and 44 days after infection. Bacillary loads were determined by plating dilutions on solid media. Each data point represents the average of cfu from four to five mice, and error bars indicate the standard deviation from the means.

while retaining the ability to produce T2S. Similarly, the $\Delta papA1$ mutant was deficient in SL₁₂₇₈ synthesis but still produced T2S and SL₆₅₉. These data were corroborated by FT-ICR MS analysis of culture extracts. A peak with $m/z = 659.30$ was seen in both WT and $\Delta papA1$ *M. tb* extracts but was absent from the $\Delta papA2$ extracts (Fig. 3C). Also, the lipid envelope of SL₁₂₇₈ was observed in WT *M. tb* extracts but was absent from both the $\Delta papA2$ and $\Delta papA1$ extracts (SI Fig. 10). These data support our proposal that, in *M. tb*, T2S is first elaborated by PapA2 to form SL₆₅₉, which is then acylated by PapA1 with a (hydroxy)phthioceranoyl group, yielding SL₁₂₇₈. These results thus establish the order of the first two lipid additions in SL-1 biosynthesis.

Mutation of *papA2* or *papA1* Does Not Affect *M. tb* Virulence.

Previous studies have addressed the contribution of SL-1 to the virulence of *M. tb* in animal models. The SL-1-deficient $\Delta pks2$ mutant was previously screened in mice for virulence defects (23, 26). However, the mutation did not hinder replication of the bacteria in the lungs, livers, or spleens, indicating that SL-1 is not critical to infection in mice. Curious as to how $\Delta papA2$ and $\Delta papA1$ would fare *in vivo*, we infected BALB/c mice with the two mutants and assessed the ability of the bacteria to replicate in the lungs and traffic to the spleen and liver (Fig. 4). Similar to the $\Delta pks2$ mutant, the $\Delta papA2$ and $\Delta papA1$ mutants showed no virulence defect when compared with WT *M. tb*.

Discussion

The biochemical and genetic data presented here reveal the order of addition of the first two lipids to the T2S core of SL-1, as well as the acyltransferases responsible for their installation. Based on our current work and other recent studies, we propose the biosynthetic route for SL-1 shown in Fig. 5. The process begins with the installation of a sulfate group at the 2-position of trehalose by Stf0. PapA2 then installs the straight chain lipid found in SL-1 and is specific for T2S, further indicating that

Fig. 5. Proposed SL-1 biosynthetic pathway. (A) Trehalose is first sulfated by Stf0 to form T2S. PapA2 then acylates the 2'-position of T2S to form SL₆₅₉. A (hydroxy)phthioceranoyl group is then synthesized by Pks2 and transferred directly by PapA1 onto SL₆₅₉ to generate SL₁₂₇₈. (B) The diacylated SL₁₂₇₈ is then transferred by MmpL8 to the exterior of the cell where the final acylations occur.

sulfation of trehalose is the first step in SL-1 biosynthesis (21). The resulting SL₆₅₉ is then acylated by PapA1 with a (hydroxy)phthioceranoyl group, the product of Pks2 (22). Despite significant sequence similarity, PapA2 and PapA1 are very specific for T2S and SL₆₅₉, respectively, and their roles in SL-1 biosynthesis are not interchangeable. There remains uncertainty regarding the physiological acyl donor substrates of the two acyltransferases. Both enzymes are capable of using PCoA as a substrate *in vitro*, and this may be a physiological substrate for PapA2. However, we cannot rule out the possibility that PapA2's natural substrate is another acyl pantotheine-based cofactor, such as a pantotheinyl acyl carrier protein. Moreover, our genetic evidence points to PapA1 as a mediator of (hydroxy)phthioceranoyl group transfer, but its transfer from Pks2 may be direct or involve a (hydroxy)phthioceranoyl-acyl carrier protein intermediate. Resolution of these remaining questions will require advances in polyketide synthase enzymology, including the development of recombinant expression systems and *in vitro* biochemical assays.

The last characterized step in SL-1 biosynthesis is the transport of SL₁₂₇₈ to the outer leaflet of the cell by MmpL8. The enzymes

or processes responsible for the final acylation of SL₁₂₇₈ to SL-1 have yet to be determined. The proposed biosynthetic route indicates that the final lipid modifications are extracellular. This hypothesis is strengthened by the accumulation of SL₁₂₇₈ on the interior of the cell in the Δ mmpL8 mutant (23). However, the (hydroxy)phthioceranol groups at the 6- and 6'-positions of SL-1 are putatively synthesized intracellularly by Pks2, suggesting that these lipids are transported to the exterior of the cell and attached to SL₁₂₇₈ by an unknown acyltransferase (22). One proposed candidate for SL₁₂₇₈ elaboration is antigen 85, an extracellular acyltransferase involved in trehalose mycolate synthesis (23, 33). However, because of the dissimilarity between phthioceranic acid and mycolic acid, we feel that it is unlikely that this enzyme participates in SL-1 biosynthesis.

Another possibility is that PapA1 is responsible for all three (hydroxy)phthioceranol transfer reactions and that these occur intracellularly before transport by MmpL8. No direct evidence of this reaction has been observed, however. The low solubility of SL₁₂₇₈ (or SL₁₂₇₈ analogs) makes it difficult to address this possibility using *in vitro* biochemical assays. The polyketide synthases involved in PDIM biosynthesis sequester the lipids they produce, effectively solubilizing the lipid during biosynthesis (29, 34). Also, MmpL7 associates with the phthiocerol polyketide synthase PpsE and is proposed to coordinate PDIM biosynthesis (35). *In vivo*, MmpL8 may sequester SL₁₂₇₈ and present the 6- and 6'-positions for acylation by PapA1 or act as a scaffold for SL-1 synthesis, similar to MmpL7. In this scheme, MmpL8 would transport fully formed SL-1 to the capsular layer of the bacteria. Further biochemical and genetic experiments are necessary to determine which hypothesis is most likely.

The availability of reliable genetic techniques and the development of high-resolution metabolite analysis tools have sped our progress toward understanding the details of SL-1 biosynthesis. However, the lack of an observable phenotypic difference between SL-1-deficient strains and WT *M. tb* in the murine tuberculosis model has undermined efforts to assign a function to the metabolite. The activity of SL₁₂₇₈ as a CD1b-restricted T cell antigen is intriguing and may be relevant to human infection. However, mice do not possess a counterpart of CD1b (36, 37). Thus, efforts to elucidate the role of SL-1 and its intermediates in pathogenesis may ultimately be frustrated in this model. Indeed, other bacterial sulfated metabolites are known to have host-specific functions. The alfalfa symbiont *Sinorhizobium meliloti* and the rice pathogen *Xanthomonas oryzae* both produce sulfated metabolites that are sensed by their hosts but not required for viability in other environments (38–41). Until tuberculosis models that more closely reflect the human disease are developed, we may not be able to assign a role for many metabolites produced by *M. tb*, including SL-1.

Materials and Methods

Expression and Purification of PapA2 and PapA1. DNA sequences encoding *M. tb* PapA2 and PapA1 were amplified from the H37Rv genomic DNA by PCR, cloned into pET28a (Novagen, Madison, WI), and transformed into the *E. coli* strain BL21 for protein expression. Cells were grown to an OD₆₀₀ of 0.6 at 37°C, and protein expression was induced with 0.1 mM isopropyl- β -D-thiogalactoside for 15 h at 22°C. PapA1 was expressed with both N- and C-terminal His₆ tags, and PapA2 was expressed as a C-terminal His₆-tagged protein. Cells were harvested by centrifugation and resuspended in Ni-NTA followed by anion exchange chromatography using a HiTrap Q column on an AKTA FPLC system. Eluted protein was concentrated and frozen in 100 mM sodium phosphate buffer (pH 7.2; 1 mM DTT and 10% glycerol). Protein concentrations were measured using the Bradford assay. On the basis of the densitometry data, both proteins were determined to be >90% pure.

Reagents and Chemicals. The synthesis for SL₆₅₉ is described in *SI Text*. Trehalose-2-palmitate and T2S were synthesized as previously described (42–45). NMR and MS data were consistent with previous reports. PCoA (55 mCi/mmol) was purchased from ARC Radiochemicals (St. Louis, MO). The plasmid pET28a and Ni-NTA affinity resins were purchased from Novagen and Qiagen (Valencia, CA), respectively. All other chemicals were purchased from Sigma (St. Louis, MO) or Fluka (St. Louis, MO) and used without further purification.

Biochemical Characterization of PapA1 and PapA2. PapA1 or PapA2 (1 μ M) was incubated with 20 μ M PCoA and 1 mM trehalose or T2S in reaction buffer [100 mM sodium phosphate (pH 7.5), 1 mM DTT, and 10% glycerol] at 25°C for 60 min. Subsequently, the reactions were quenched by the addition of an equal volume of ethanol. The products were analyzed by TLC, eluting with 35:65 methanol:chloroform, and were quantified by using phosphorimaging followed by densitometry. Kinetic parameters for PapA2 and PapA1 were determined by obtaining the initial velocities of the reactions at varying substrate concentrations.

Tandem Reactions with PapA2 and PapA1. PapA2 (1 μ M) was incubated with 20 μ M unlabeled PCoA and 1 mM T2S in reaction buffer at 25°C for 4 h to generate trehalose-2-sulfate-2'-palmitate (SL₆₅₉) *in situ*. Subsequently, the enzyme was heat-killed by boiling the reaction mixture at 100°C for 5 min, followed by addition of 1 μ M PapA1 and 20 μ M PCoA. The reaction products were analyzed by TLC as described above.

FT-ICR MS Characterization of Reaction Products. PapA2 (1 μ M) was incubated with 100 μ M unlabeled PCoA and 1 mM T2S in 100 mM ammonium bicarbonate (pH 7.2) for 6 h at room temperature. In a separate reaction, 1 μ M PapA1 was incubated with 200 μ M synthetic SL₆₅₉ and 100 μ M unlabeled PCoA in 100 mM ammonium bicarbonate (pH 7.2) for 6 h at room temperature. The resulting reaction products and appropriate control reactions were analyzed by FT-ICR MS.

Construction of *M. tb* Mutants. *M. tb* cells (Erdman strain) were cultured in 7H9 medium supplemented with 10% oleic acid albumin dextrose catalase, 0.5% glycerol, and 0.05% Tween 80, or on 7H10 solid agar medium supplemented with 10% oleic acid albumin dextrose catalase and 0.5% glycerol. Hygromycin (50 μ g/ml) was used when necessary. The Δ papA2 and Δ papA1 mutant strains were created by homologous recombination using specialized transduction phages phmws103 and phmws113, respectively (46). These deletions replaced 1,171 bp of *papA2* (amino acids 39–429) and 1,064 bp of *papA1* (amino acids 103–457) with a hygromycin resistance cassette.

Biochemical Analysis of Sulfolipids. *M. tb* cultures were synchronized and grown to OD₆₀₀ = 0.8. Aliquots (10 ml) were resuspended in an equal volume of PBS supplemented with 1 mg/ml sodium acetate and labeled with 150 μ Ci of [³⁵S]sulfate for 18 h at 37°C. Cell pellets were extracted with chloroform:methanol (2:1) and analyzed by TLC, eluting with chloroform:methanol:water (60:12:1) or water:isopropanol:ammonium hydroxide (1:6:1) as the developing solvent. Radioactivity was quantified by using phosphorimaging followed by densitometry.

MS. All mass spectra were acquired on a Bruker Apex II FT-ICR MS equipped with a 7T superconducting magnet (Bruker Daltonics, Billerica, MA). Extracts of *M. tb* were ionized by using electrospray ionization (Analytica, Branford, CT) in negative ion mode and were infused at a rate of 2 μ l/min in 2:1 chloroform:methanol. The ions were accumulated in an external hexapole for 0.5–2 s (the time was adjusted to maximize ion signal without depleting spectral resolution) before being transferred to the

ICR cell for high-resolution analysis. Spectra were acquired by using XMass 5.0.10 (Bruker Daltonics), are composed of between 256,000 and 1 million data points, and are an average of between 32 and 128 scans. All spectra were internally calibrated by using known compounds.

Mouse Infections. Bacteria were grown to log phase, cup-sonicated by using a Branson (Danbury, CT) Sonifier 250 at 90% for 15 s, spun for 5 min at $40 \times g$ to remove clumps, and diluted to the desired inoculum in PBS. Bacteria were administered to BALB/c mice via nebulization for 15 min by using a custom-built aerosolization chamber (Mechanical Engineering Shops, University of Wisconsin, Madison, WI). For infections, bacterial suspensions with an $OD_{600} = 0.1$ were used, resulting in an initial seeding of ≈ 250 bacteria per mouse. Organs from infected mice

were homogenized and plated for analysis of cfu. Four or five mice were used per time point. All mice were housed and treated humanely as described in an animal care protocol approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Note. While this article was under review, Bhatt *et al.* (47) reported that *M. tb* mutants in *papA2* and *papA1* are deficient in SL-1 biosynthesis. The biochemical activities and biosynthetic order of the corresponding proteins were not reported.

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