stocks in 95% ethanol, except AY9944, which is water soluble), and purified Shh-N or BMP7 (from H. Reddi) were added at the initiation of the cultures. Control explants were cultured with the maximum ethanol concentration used for drug treatments. All of the explants were cultured for 40 to 48 hours except for assays of Pax7 repression, which were cultured for 20 to 22 hours. Primary antibodies used were HNF3β (K2), Isl1/Isl2 (40.2D6), Pax7 (from S. Morton and T. Jessell), and HNK-1/N-CAM (Sigma Biosciences).
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19. Induction of HNF3β and Isl1 in midline explants was fully inhibited with 4.0 μM AY9944, 1 μM triparanol, and 1 μM cyclopamine. Intermediate doses of AY9944 (0.5 μM), triparanol (0.25 μM), and cyclopamine (0.25 μM) blocked induction of HNF3β while permitting induction of Isl1.
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22. Chick embryos were treated with ~10 μM jervine at stage 20 for 8, 16, 24, or 32 hours before fixation and stained with an antibody that recognizes Shh-N. No difference in the normal localization of Shh-N protein to the apical domain of floor plate cells or on the surfaces of commissural axons (*16*) was seen in treated embryos.
23. Cleavage of the His_6Hh-C protein was stimulated by 12 μM cholesterol, but this cleavage was neither stimulated nor inhibited by the addition of jervine, cyclopamine, or tomatidine, even at 350 μM.
24. Other sterols that participated in the reaction with similar efficiency to cholesterol are β-sitosterol, 5-androsten-3β-ol, and 7β-hydroxycholesterol. Sterols that participated with reduced efficiency are coprastan-3-ol, ergosterol, 4β-hydroxycholesterol, 19-hydroxycholesterol, 20α-hydroxycholesterol, 22(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 25-hydroxycholesterol. Epicholesterol, cholesterol acetate, α-ecdysone, 20-OH ecdysone, 3-keto-5-cholestene, and thiocholesterol (3(β)-thiol) were unable to participate in the reaction. The in vitro reaction thus requires an unhindered hydroxyl at the 3β position on a tetracyclic sterol nucleus, although neither the isooctyl side chain nor the number or positions of the double bonds in the sterol nucleus appear to affect autoprocessing critically.
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Inhibition of a *Mycobacterium tuberculosis* β-Ketoacyl ACP Synthase by Isoniazid

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Although isoniazid (isonicotinic acid hydrazide, INH) is widely used for the treatment of tuberculosis, its molecular target has remained elusive. In response to INH treatment, saturated hexacosanoic acid (C26:0) accumulated on a 12-kilodalton acyl carrier protein (AcpM) that normally carried mycolic acid precursors as long as C50. A protein species purified from INH-treated *Mycobacterium tuberculosis* was shown to consist of a covalent complex of INH, AcpM, and a β-ketoacyl acyl carrier protein synthase, KasA. Amino acid–altering mutations in the KasA protein were identified in INH-resistant patient isolates that lacked other mutations associated with resistance to this drug.

INH is a front-line drug of choice for the treatment of tuberculosis (*1*). Despite the apparent simplicity of its chemical structure, the mode of action of this drug is complex. INH is a prodrug that requires activation by the mycobacterial catalase-peroxidase enzyme (KatG) to an active form that then exerts a lethal effect on an intracellular target or targets (*2–4*). Because of physical and biochemical changes occurring coincident with INH toxicity, it has been proposed that the lethal effect lies in the biosynthetic pathway for mycolic acids (*2, 5–7*). The detrimental effect of INH on mycolic acid synthesis exactly parallels the time course of the loss of *Mycobacterium tuberculosis* viability and is accompanied by an accumulation of saturated hexacosanoic acid (C26:0), implicating this fatty acid as an intermediate in the biosynthetic pathway that produces mycolic acids (*8–11*).

The majority of INH-resistant clinical isolates become resistant by losing or altering KatG activity, not by mutation of the target of the activated prodrug (*12, 13*). Despite considerable effort, identification of the INH target in *M. tuberculosis* by genetic approaches has not been accomplished (*14*). However, a library of DNA fragments from a resistant strain of the fast-growing saprophyte *M. smegmatis* was used to isolate a putative target designated InhA (*15*). InhA is an NADH-dependent enoyl-[acyl carrier protein] (ACP) reductase with a chain-length specificity centering at 16 carbons (*16, 17*). Reconciling the catalytic function of InhA (reduction of an unsaturated fatty acid) with the observed biochemical correlate of toxicity (accumulation of a saturated fatty acid) has been extremely difficult. Furthermore, although this target is sufficient to induce resistance to 50 μg of INH per milliliter in *M. smegmatis*, the same constructs induce only low levels of resistance in *M. tuberculosis* (to 0.1 μg/ml, while KatG wild-type clinical isolates resistant to 1 to 2 μg/ml are commonly encountered) (*14*). Sequencing of clinical isolates

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of *M. tuberculosis* has revealed mutations in putative regulatory regions upstream of the *inhA* gene and potential coding sequence mutations that may be directly involved in INH resistance, but these occur only in a subpopulation of INH-resistant, wild-type catalase-peroxidase isolates (*13*, *18*–*21*). Thus, although the InhA protein may be involved in INH-resistance, it probably does not represent the target whose inhibition results in hexacosanoic acid accumulation, and mutations in InhA and KatG do not appear to be sufficient to account for all of the observed resistance (*14*).

To identify this enzymatic target, we examined two-dimensional gel electrophoretic protein profiles of *M. tuberculosis* in response to low-level INH treatment (1 μg/ml), a condition that induces the accumulation of hexacosanoic acid (Fig. 1A). Pulse-labeling with [^{35}S]methionine revealed two protein species of 12 and 80 kD that were significantly up-regulated. [^{14}C]Acetate labeling under the same conditions showed that the 12-kD protein accumulated with bound lipid (*22*). The NH_2-terminus of this protein was determined to be PVTQEEIIAGIAEIIEEVTGIEPSEIT (*23*). Pooling of two-dimensional gel fragments containing the 80-kD protein from 100 gels indicated that its NH_2-terminal sequence was identical to that determined for the 12-kD protein. Searching of the recently completed mycobacterial genome revealed that this sequence is present in *M. tuberculosis* only once (Rv2244) where it corresponds to the NH_2-terminus of a 12,492-dalton ACP.

ACPs are small proteins covalently modified by the attachment of phosphopantetheine that function to carry the growing fatty acyl chain between component enzymes of Type II fatty acid synthase (FAS) systems (*24*). The mycobacterial protein was similar to a family of ACPs with the highest identity to FabC from *Streptomyces glaucescens* (*25*). AcpM was purified to homogeneity by taking advantage of the solubility of ACPs in 80% ammonium sulfate and their insolubility upon subsequent acidification of such supernatants (Fig. 1B) (*26*). Native gel electrophoresis of whole-cell lysates showed that this small ACP accumulated after INH treatment (Fig. 1B, lanes 1 and 2) (*27*). Purified AcpM from INH-treated cells was saponified, and the methyl esters of the attached lipids were analyzed by reversed-phase thin-layer chromatography (TLC) revealing predominantly hexacosanoic acid, although fatty acids as small as palmitate were also observed in lower abundance (Fig. 1C, lane 4). Identical samples analyzed by argentation TLC confirmed that the accumulated fatty acids were fully saturated (*22*). AcpM isolated from untreated cells carried a broader range of fatty acids, with abundant species of 18, 28, and 50+ carbons (Fig. 1C, lane 3). This result suggests that AcpM is involved in a Type II FAS, which produces the meromycolate branch of full-length mycolic acids.

Treatment of *M. tuberculosis* with [^{14}C]INH resulted in the labeling of a protein whose two-dimensional electrophoretic mobility exactly matched that of the 80-kD protein (*22*). The 80-kD protein displayed considerable instability, particularly to neutral or basic conditions, but was not dissociated by reduction, heating, or detergent. Subsequent analysis by two-dimensional gel electrophoresis demonstrated that the 80-kD protein was a minor component of the total cellular protein pool, in spite of intense specific radiolabeling (*22*). Working entirely under neutral to moderately acidic conditions (pH 5 to 7), we purified the 80-kD protein species to near homogeneity from INH-treated *M. tuberculosis* H37Rv lysates (Fig. 2, A and B) (*28*). The purification was monitored by both [^{35}S]methionine labeling and immunoreactivity of an unlabeled parallel preparation with affinity-purified antipeptide sera specific for the NH_2-terminal sequence of AcpM. The purified material (lanes 4 of Fig. 2, A and B) was base-unstable and could be converted quantitatively to a 50-kD form by dialysis against a weak base (lanes 5). The 50-kD form retained radiolabel; however, it did not react with the AcpM antisera and no NH_2-terminal sequence could be obtained.

To establish the relationship between the 80-kD and 50-kD forms of this protein, we subjected excised gel slices of each protein to in situ trypsin digestion (*29*). High-performance liquid chromatography (HPLC) and radiolabeled-HPLC patterns of extracted tryptic peptides showed fragments common to both protein species and a fragment unique to the 80-kD form. The 80 kD-specific fragment (peak 3 in Fig. 3A) was sequenced and corresponded exactly to an internal fragment of AcpM (highlighted se-

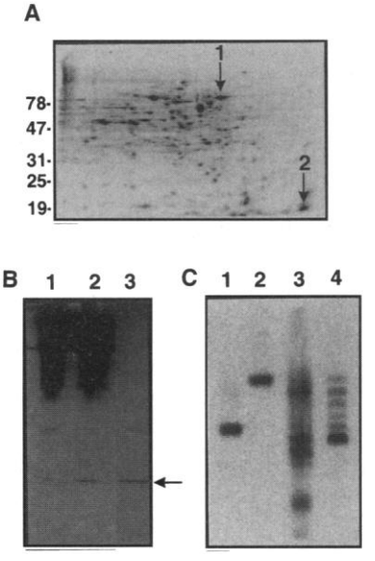

Fig. 1. INH-induced up-regulation of protein synthesis in *M. tuberculosis* H37Rv. (**A**) Two-dimensional gel electrophoresis of total soluble proteins from [^{35}S]methionine pulse-labeled, INH-treated *M. tuberculosis* H37Rv (INH at 1 mg/ml for 30 min, then pulse-labeling for 15 min). Arrow 1 indicates the 80-kD form, and arrow 2 indicates AcpM. Untreated samples are identical except for the absence of labeled species 1 and 2. Molecular size standards (in kilodaltons) are indicated on the left. The first-dimension isoelectric focusing is run from basic on the left to acidic on the right. (**B**) We performed a 15% native gel electrophoresis at pH 9.0 without SDS. Lane 1, untreated; Lane 2, treated with INH as in (**A**); Lane 3, purified AcpM (*26*). (**C**) Autoradiogram of a reversed-phase (KC-18) TLC plate of methyl esters of lipids saponified from purified AcpM; Lane 1, [^{14}C]tetracosanoic acid standard (C24:0); Lane 2, [^{14}C]palmitic acid standard (C16:0); Lane 3, methyl esters of lipids saponified from AcpM purified from untreated H37Rv; Lane 4, methyl esters of lipids saponified from AcpM from INH-treated H37Rv.

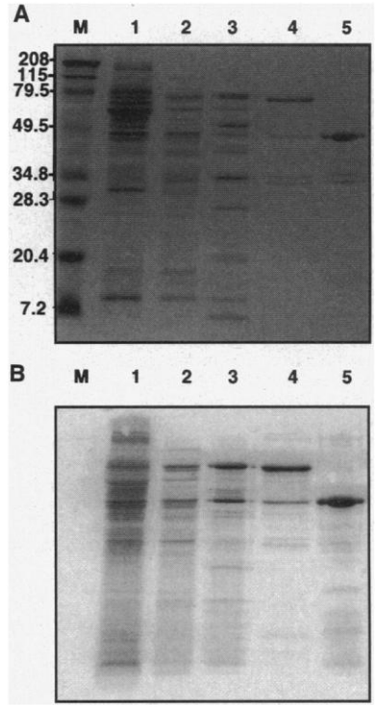

Fig. 2. Purification and labeling of the 80-kD up-regulated protein from H37Rv. (**A**) Purification of the 80-kD protein. M, molecular size markers (in kilodaltons), indicated to the left of the gel. Lane 1, crude cell lysate (*28*); Lane 2, 70% ammonium sulfate pellet; Lane 3, pooled fractions from Phenyl Sepharose HIC column; Lane 4, pooled fractions from Resource Q HPLC column; Lane 5, sample from Lane 4 dialyzed overnight against 10 mM NaOH at 4°C. (**B**) Same as (**A**) except the sample was labeled with [^{35}S]methionine (*28*).

quence in Fig. 3B). The five most abundant peptides, which were present in both the 80- and 50-kD polypeptide, each mapped to internal tryptic fragments of a single 43.3-kD polypeptide, KasA (Fig. 3B).

The precise structure of the 80-kD species that contains both AcpM and KasA is not known, although incorporation of [^{14}C]INH suggested a complex containing both proteins and INH. Matrix-assisted laser desorption/ionization mass spectrometric analysis of the purified 80-kD species showed no significant high-mass species, but instead revealed one dominant species at a mass of 12,598 atomic mass units (amu) (*22*). The mass of apoAcpM is 12,492 amu and that of the acylpyridine moiety of INH is 106 amu, suggesting that this fragment may correspond to a covalent complex of INH and AcpM (predicted mass: 12,598 amu). Inhibition of KasA function would be expected to stop fatty acid elongation and result in the accumulation of a saturated fatty acid precursor, the observed result of INH treatment.

Attempts to associate overexpression of AcpM and KasA with INH resistance in several mycobacterial species were uniformly unsuccessful (*30*). Subcloning experiments revealed that both AcpM and KasA were independently toxic when overexpressed at high levels. In the case of AcpM, this was an expected result, because overexpression of ACPs in other systems is lethal (*31*). This also accounts for the failure to obtain the target by standard cloning techniques, since AcpM toxicity would prevent isolation of the closely linked KasA. This observation further suggests that AcpM up-regulation in response to mycolate deprivation may be directly involved in INH toxicity.

To examine the potential involvement of KasA mutations in the development of INH-resistance in patient isolates of *M. tuberculosis*, the *kasA* gene was sequenced in entirety from a genetically diverse panel of INH-resistant and INH-susceptible strains. No mutations were identified in *kasA* in any of 43 INH-susceptible strains. In contrast, among 28 INH-resistant isolates, four were found to have amino acid altering mutations in the coding sequence of *kasA* (Table 1). In two of these strains, no alteration was found in other loci involved in INH resistance (including *katG*, *inhA*, *ahpC*) (*32*), while in the other two strains, a *katG* alteration was also found in codon 315. However, these two strains had a disproportionately high minimum inhibitory concentration, thereby suggesting an additive effect of the double mutation (*katG* plus *kasA*). From this limited data set, *kasA* mutations in patient isolates resistant to INH occur at a frequency of approximately 5 to 20%, similar to or in excess of the occurrence of mutations in *inhA* or *ahpC*, two other genes containing mutations associated with INH resistance. The extremely low frequency of occurrence of unselected nucleotide sequence alterations in *M. tuberculosis* (which are 1000 times less frequent than in organisms like *Escherichia coli*) (*33*) is presumptive evidence of involvement of these *kasA* mutations in INH resistance. This presumption is supported by the absence of *kasA* sequence variation in the sample of 43 INH-susceptible strains. Taken together, these results provide compelling evidence that *kasA* coding sequence alterations participate in the development of INH resistance in the course of human antituberculosis drug therapy.

These results have several implications for understanding INH resistance in *M. tuberculosis*. First, meromycolic acids are synthesized by a previously undescribed Type II FAS system for which AcpM is apparently the carrier for lipids up to 50 carbons in length. Second, KasA represents an important potential target for future development of therapeutics, because inhibition of its function appears to be lethal. Third, the marked up-regulation of AcpM and KasA accompanying the inhibition of mycolic acid synthesis implies the existence of a regulatory mechanism responsive to the levels of meromycolate or mycolate produced. Such a regulatory system could be

Table 1. Nucleotide and amino acid (*23*) changes in the coding sequence of *kasA* in INH-resistant clinical isolates of *M. tuberculosis*. Two of these strains (HN335 and HN93) have no alterations in KatG, InhA, or AhpC. The remaining two strains have KatG Ser^{315} changes and show a high minimum inhibitory concentration (>10 μg/ml) for INH.

Strain	Codon	*kasA* changes	
		Nucleotide	Amino acid
HN113	66	GAT → AAT	D → N
HN335	269	GGT → AGT	G → S
TB029	312	GGC → AGC	G → S
HN93	413	TTC → TTA	F → L

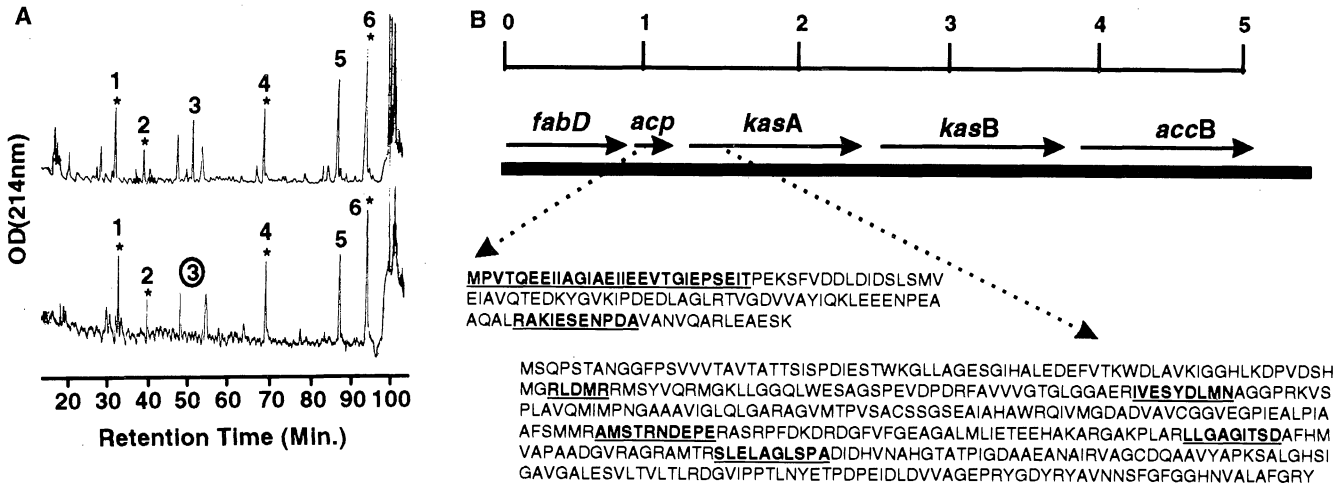

Fig. 3. Identification of the trimolecular complex of KasA, AcpM, and INH. (**A**) (Top trace) HPLC profile of the 80-kD protein (from the sample shown in lane 4 of Fig. 2, A and B) following in-gel tryptic digestion. Numbered peaks were sequenced, and asterisks indicate that the peptide was radiolabeled and contained methionine residues. (Bottom trace) Tryptic peptide profile of the 50-kD protein (lane 5 of Fig. 2, A and B) treated as above. OD, optical density measured at 214-nm wavelength. (**B**) Operon map of the AcpM/KasA genomic locus from cosmid MTCY427 (*23*). The top sequence corresponds to AcpM. The experimentally determined NH_2-terminal sequence is shown in bold and underlined, as is the internal tryptic peptide corresponding to peak number 3 in (A). The bottom sequence is KasA and bold and underlined peptides represent the experimentally determined sequences corresponding to peaks numbered 2, 4, 1, 6, and 5 in (A) in order of their appearance in the sequence.

exploited to construct reporter strains for the rapid screening of novel inhibitors of these critical constituents of the mycobacterial cell wall.

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30. The intact gene cluster was cloned as a Kpn I to Bsp HI fragment into pMV206H (*14*) which had been cut with Kpn I and Nco I. Transformation of this construct into either *M. smegmatis* or *M. tuberculosis* H37Rv and analysis of the resulting transformants by SDS–polyacrylamide gel electrophoresis (PAGE) showed that none of the encoded proteins appeared significantly up-regulated. *acpM* was cloned individually as an Sph I to Bam HI fragment into pMV261H, and the orientation was determined by restriction mapping. Expression in this construct is driven by the mycobacterial *hsp60* promoter. Transformation of this construct into either *M. smegmatis* or *M. tuberculosis* produced no colonies (<1 colony/μg of DNA) while transformation of the same gene in the opposite orientation produced between 1×10^3 and 1×10^6 colonies/μg of DNA. *kasA* was polymerase chain reaction–amplified using *Pwo* polymerase and was cloned into pMH29H, in which transcription is driven by a synthetic mycobacterial promoter sequence. Transformation of this construct into *M. smegmatis* and *M. tuberculosis* produced very small slowly growing colonies. These colonies overproduced KasA protein as demonstrated by SDS-PAGE and protein immunoblotting using affinity-purified antisera to a peptide segment of the KasA primary sequence. It was not possible to accurately assess the INH susceptibility of these colonies because of their impaired growth, but they did not appear to be significantly more resistant to INH than were controls.
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32. DNA was isolated from recent clinical isolates from the collection of J.M.M. HN113 was recovered from the sputum of a patient in Houston, TX, was resistant to INH at >10 μg/ml, and had the KatG Ser^{315} → Thr^{315} mutation. HN113 had nine copies of IS6110 and belonged to major genetic group 1 (*33*). Strain HN335 was recovered from a patient in Houston and showed INH resistance at a level of 1 to 2 μg/ml. This strain had no mutations in KatG, 14 copies of IS6110, and belonged to group 2. Strain TB029 was recovered from a patient in Japan and was resistant to >10 μg/ml of INH. It contained a KatG Ser^{315} → Asn^{315} mutation, four copies of IS6110, and belonged to group 1. Strain HN93 was recovered from a lymph-node biopsy of a Houston patient and showed INH resistance at 1 to 2 μg/ml. This strain had a single copy of IS6110 and also belonged to group 1. None of these strains had alterations in the *inhA* locus or upstream of *ahpC*.
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Axonal Swellings and Degeneration in Mice Lacking the Major Proteolipid of Myelin

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Glial cells produce myelin and contribute to axonal morphology in the nervous system. Two myelin membrane proteolipids, PLP and DM20, were shown to be essential for the integrity of myelinated axons. In the absence of PLP-DM20, mice assembled compact myelin sheaths but subsequently developed widespread axonal swellings and degeneration, associated predominantly with small-caliber nerve fibers. Similar swellings were absent in dysmyelinated *shiverer* mice, which lack myelin basic protein (MBP), but recurred in MBP*PLP double mutants. Thus, fiber degeneration, which was probably secondary to impaired axonal transport, could indicate that myelinated axons require local oligodendroglial support.

Proteolipid protein (PLP) is a four-helix–spanning membrane protein thought to stabilize the ultrastructure of central nervous system (CNS) myelin by forming the double-spaced intraperiod line (IPL), but neither PLP nor its splice isoform DM20 is required for spiral membrane wrapping and myelin compaction (*1*). Mutations of the X-linked PLP gene (*2*) cause Pelizaeus-Merzbacher disease (PMD) and spastic paraplegia–2 (SPG-2) in humans and related disorders in animal models, such as the *jimpy* (*jp*) mouse, characterized by premature death of oligodendrocytes and dysmyelination. However, the severe consequences of spontaneous PLP mutations are explained, at least in part, by the toxicity of the encoded

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