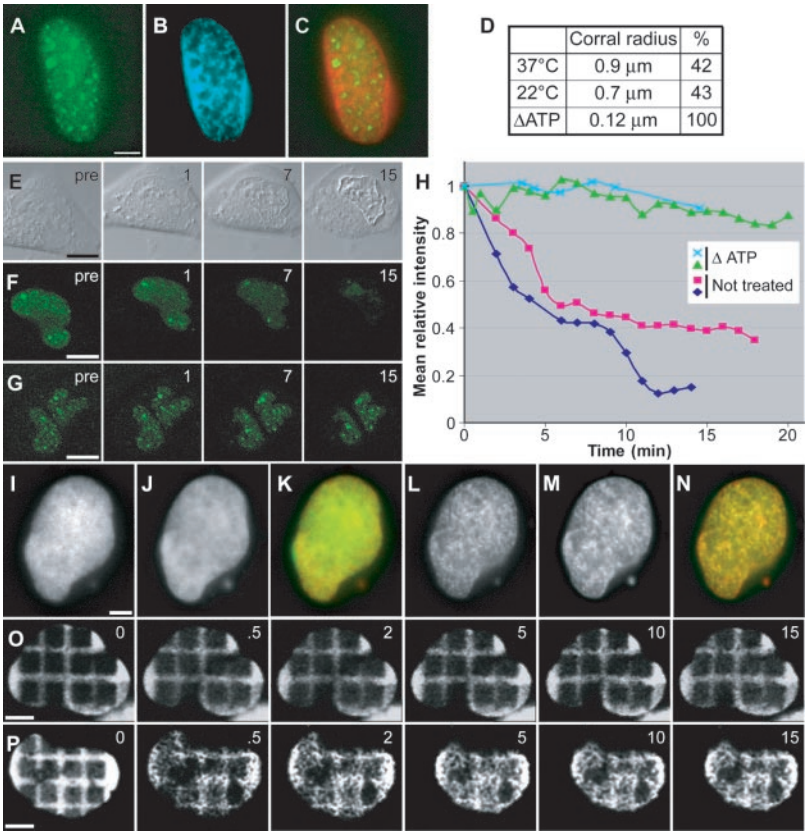
Fig. 3. Effect of energy depletion on mRNP movement. (**A** to **C**) A transfected and transcriptionally induced cell, 10 min after energy depletion. (A) Concentrated YFP-MS2 nuclear mRNPs; (B) Hoechst DNA stain; (C) Merge of YFP-MS2 mRNPs from (A) in green and Hoechst from (B) in red, showing segregation into two domains [movies S12 to S14 (*5*)]. (**D**) Table summarizing the corral radius measured for corralled particles tracked at 37°C, 22°C, and ATP-depleted cells. %, percent of corralled particles from total tracked. (**E** to **G**) Cells treated with 0.02% Triton (pre, before Triton; 1, 7, and 15 s, after Triton). (E) Differential interference contrast image. (F) YFP-MS2 labeled mRNPs are lost from the nucleus during permeabilization (time in minutes). (G) Same as (F), but the cells were first energy depleted and then permeabilized, and mRNP loss is slower. (**H**) Plots of the mean fluorescence intensity over time in the nucleus of permeabilized cells, treated as in (F) (red and dark blue lines) or as in (G) (green and light blue lines). (**I**) Cell expressing H2B-YFP and (**J**) counterstained with Hoechst. (**K**) Merge of (I) and (J). (**L** to **N**) The same cell 10 min after ATP depletion, showing the formation of nuclear subdomains. Bar, 5 μm. [movies 15 to 17 (*5*)]. (**O**) Time course of a cell transfected with H2B-YFP in which squares have been photobleached to form a grid (times are in minutes). (**P**) Same time course as in O, but ATP was depleted at time 0 min, showing major changes in chromatin structure together with nuclear shrinkage [movie S18 (*5*)]. Bar, 5 μm.



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Protein Kinase G from Pathogenic Mycobacteria Promotes Survival Within Macrophages

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Pathogenic mycobacteria resist lysosomal delivery after uptake into macrophages, allowing them to survive intracellularly. We found that the eukaryotic-like serine/threonine protein kinase G from pathogenic mycobacteria was secreted within macrophage phagosomes, inhibiting phagosome-lysosome fusion and mediating intracellular survival of mycobacteria. Inactivation of protein kinase G by gene disruption or chemical inhibition resulted in lysosomal localization and mycobacterial cell death in infected macrophages. Besides identifying a target for the control of mycobacterial infections, these findings suggest that pathogenic mycobacteria have evolved eukaryotic-like signal transduction mechanisms capable of modulating host cell trafficking pathways.

Pathogenicity of mycobacteria is tightly linked to their survival in host macrophages. Normally, phagocytosed microorganisms are rapidly transferred from phagosomes to lysosomes and are then degraded. However, pathogenic mycobacteria resist lysosomal delivery and survive within macrophages inside mycobacterial phagosomes (*1*, *2*). Mycobacterial phagosomes are characterized by the absence of lysosomal markers such as lysosomal associated membrane proteins (LAMP) and mature lysosomal hydrolases (*3*–*5*). Phagosomes harboring mycobacteria also contain markers for the early endosomal pathway (*6*, *7*), have a reduced amount of the vacuolar proton-translocating adenosine triphosphatase, and retain the Trp-Asp (WD) repeat–containing protein coronin 1 (also known as TACO or P57) that prevents fusion of phagosomes with lysosomes (*8*–*11*).

How mycobacteria interfere with phagosome-lysosome fusion is not understood (*12*–*14*). Several lipid moieties arrest phagosomal maturation by interfering with traffic from the Golgi complex (*15*, *16*), but

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the mycobacterial proteins responsible for interfering with lysosomal delivery remain unknown (*17*).

Modulation of host cellular trafficking pathways may be influenced by signal transduction molecules expressed by pathogenic bacteria. The *Mycobacterium tuberculosis* genome encodes 11 eukaryotic-like serine/threonine kinases (*18*, *19*). Mammalian protein kinase Cα is most closely related to protein kinase G (PknG) (*20*). PknG consists of a kinase domain flanked by a large N- and C-terminal domain of unknown function (fig. S1A). In contrast to most other mycobacterial serine/threonine kinases, PknG does not contain a transmembrane domain and is predicted to be located in the bacterial cytoplasm (*21*). Immunoblotting of *M. bovis* Bacille Calmette-Guérin (BCG) or *M. smegmatis–pknG* with antibody to PknG revealed an 85-kD band that was not present in the nonpathogenic mycobacterium *M. smegmatis* (fig. S1B). Analysis of the kinase activity showed that PknG was autophosphorylated, and phosphorylation was blocked in the presence of chelerythrine, similarly to the activity of protein kinase Cα (fig. S1C).

To analyze the contribution of PknG to inhibition of lysosomal transfer of *M. bovis* BCG within macrophages, we deleted the *pknG* gene (*22*). No PknG was found in the deletion strain (*M. bovis* BCGΔ*pknG*), whereas PknG was readily detected in the cytoplasm of wild-type cells (Fig. 1A) (fig. S2). The *pknG* gene was found to be dispensable for mycobacterial growth; no differences were observed either in cell morphology or in the growth of the wild-type and mutant *M. bovis* BCG (Fig. 1, A and B).

Intracellular trafficking of mycobacteria lacking *pknG* was analyzed after infection of macrophages with wild-type *M. bovis* BCG or *M. bovis* BCGΔ*pknG*. Virtually all (~95%) *M. bovis* BCGΔ*pknG* resided within LAMP-positive vacuoles, whereas wild-type *M. bovis* BCG was largely present in LAMP-negative phagosomes (Fig. 1C). To independently analyze the intracellular location of *M. bovis* BCGΔ*pknG*, we infected macrophages with wild-type *M. bovis* BCG or *M. bovis* BCGΔ*pknG* and then performed cell fractionation with the use of organelle electrophoresis (*9*, *23–25*). *M. bovis* BCGΔ*pknG* was present in organelles that were largely resolved in lysosomal fractions, in contrast to the wild-type bacteria that were transferred to nonlysosomal organelles (Fig. 1D).

The rapid lysosomal transfer of mycobacteria lacking PknG could result in their intracellular killing by bactericidal activities present in the lysosomes. When *M. bovis* BCG or *M. bovis* BCGΔ*pknG* was internalized into macrophages for 1 hour, followed by a chase in normal medium, *M. bovis* BCG readily proliferated within macrophages, whereas the *pknG* deletion mutant failed to grow (Fig. 1E); this finding suggests a crucial role for PknG in the survival of mycobacteria within macrophages.

The nonpathogenic mycobacterium *M. smegmatis* that does not express PknG was readily transferred to lysosomes upon entry into macrophages (Fig. 2, A and B). To investigate whether expression of PknG could modulate intracellular trafficking of *M. smegmatis*, we infected macrophages with wild-type or PknG-expressing *M. smegmatis*. Whereas for wild-type *M. smegmatis* the vast majority of bacteria was found within lysosomes, as judged by their colocalization with LAMP, expression of PknG prevented lysosomal transfer (Fig. 2, A and B). Cell fractionation confirmed that *M. smegmatis* was efficiently transferred to lysosomes as expected (*26*, *27*), but the bacteria expressing PknG were largely resolved in nonlysosomal fractions (Fig. 2C).

The ability of PknG to prevent lysosomal transfer suggested that it may affect survival of bacteria that have been internalized in macrophages. Control *M. smegmatis* was rapidly cleared by macrophages, but survival of *M. smegmatis* expressing PknG

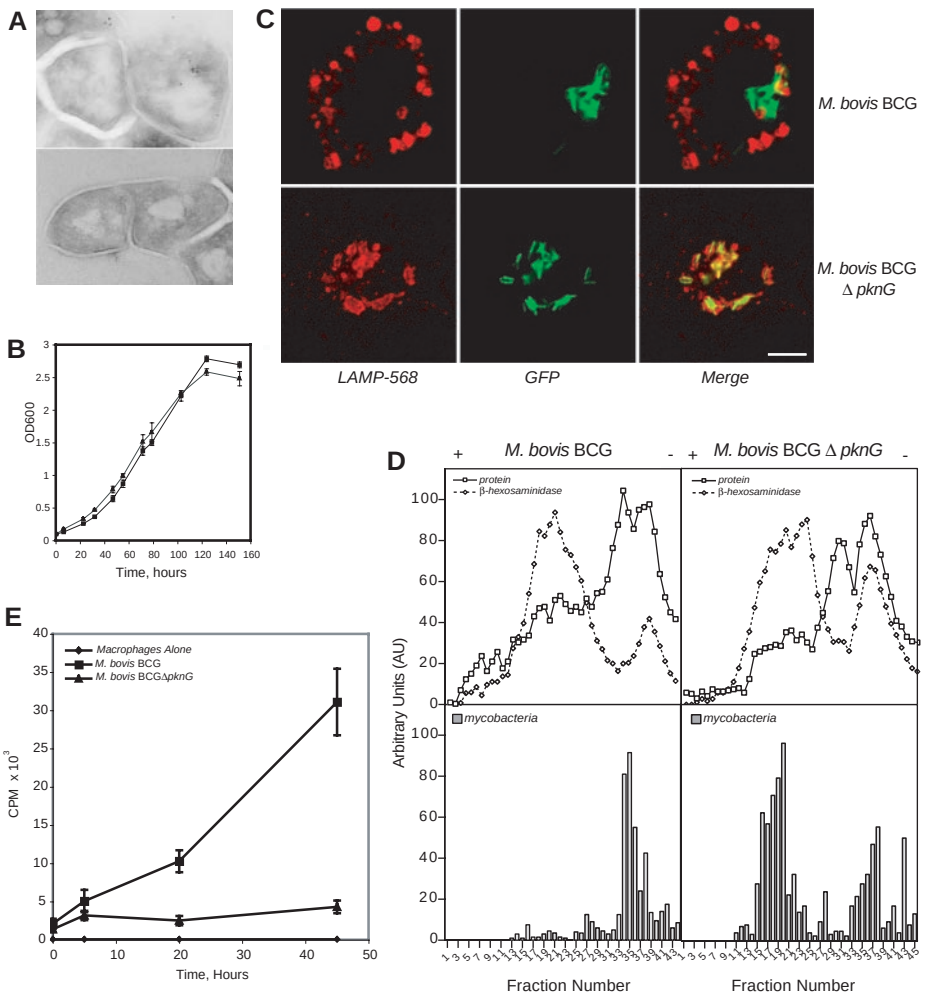

Fig. 1. Characterization of mycobacteria lacking protein kinase G. (**A**) Morphological characterization of *M. bovis* BCG and *M. bovis* BCGΔ*pknG* by immunocytochemistry. Magnification, ×31,000. (**B**) Growth of *M. bovis* BCG (▲) and *M. bovis* BCGΔ*pknG* (■) in 7H9/OADC medium. (**C**) Macrophages were infected with *M. bovis* BCG or *M. bovis* BCGΔ*pknG* expressing green fluorescent protein (GFP) for 1 hour, followed by a 2-hour chase. Cells were processed as described in (*22*) and stained with antibody to LAMP followed by Alexa Fluor 568 (LAMP-568). Scale bar, 10 μm. (**D**) Macrophages were incubated for 3 hours with *M. bovis* BCG or *M. bovis* BCGΔ*pknG*, homogenized, and subjected to organelle electrophoresis. The distribution of organelle-specific markers and the amount of bacteria per fraction were determined. The peaks around fraction 38 represent lymphocytes present in the preparation that were not homogenized. (**E**) Survival of *M. bovis* BCG or *M. bovis* BCGΔ*pknG* after a 1-hour infection followed by chase for the indicated times. Quantitation was performed by tritiated uracil incorporation. Shown are mean values (±SD) of six to eight determinations.

was significantly enhanced (Fig. 2D). Thus, the expression of PknG in *M. smegmatis* is sufficient to prevent lysosomal transfer and prolong intracellular survival.

To investigate whether the kinase activity of PknG is essential for its activity in avoiding lysosomal transfer, we analyzed the phenotype of mycobacteria expressing a kinase-dead mutant of PknG [$Lys^{181} \rightarrow$ Met; $PknG^{K181M}$ (*28*)]. When *M. smegmatis–pknG*^{K181M} was internalized in macrophages, lysosomal trafficking readily occurred similar to that seen with wild-type *M. smegmatis*, indicating that the kinase activity of PknG was required to prevent phagosome-lysosome fusion. When macrophages were infected with *M. bovis* BCG-*pknG*^{K181M}, virtually all bacilli were transferred to lysosomes despite normal viability of the bacteria (Fig. 3, A to C). Infection of macrophages with *M. bovis* BCG-*pknG*^{K181M} resulted in efficient killing of the intracellularly residing bacteria, in contrast to wild-type or PknG-overexpressing bacteria (Fig. 3D). Thus, the kinase activity is essential for the capacity of PknG to prevent lysosomal delivery and to mediate mycobacterial survival within macrophages.

Modulation of phagosome-lysosome fusion by PknG could be mediated through phosphorylation of host proteins after secretion within the macrophage. Although PknG does not contain an identifiable N-terminal signal sequence, many pathogenic microorganisms including *Mycobacterium* spp. have evolved alternative secretion pathways (*29*, *30*). To analyze secretion of PknG, we infected macrophages with either live or heat-killed *M. bovis* BCG; after preparation of phagosomes and solubilization, PknG was present in the pellet as well in the soluble fraction prepared from phagosomes harboring viable bacteria, but not after internalization of killed mycobacteria (Fig. 3E). In membrane fractions prepared from macrophages infected with live as well as killed mycobacteria, PknG was readily detectable (Fig. 3E). Also, the kinase-inactive mutant was secreted and detected in the cytosol when macrophages were infected with live, but not with killed, *M. smegmatis-pknG*^{K181M} (Fig. 3F). Moreover, immunocytochemistry revealed localization of PknG within phagosomes as well as in the cytosol of macrophages that had internalized live (Fig. 3G), but not heat-killed, mycobacteria (Fig. 3H). Thus, PknG is actively secreted by mycobacteria after uptake into macrophages and acquires access to the macrophage cytosol.

The involvement of the serine/threonine kinase PknG in intracellular trafficking of mycobacteria suggests that inhibition of PknG could alter the intracellular behavior of the mycobacteria. To search for potential specific inhibitors, we screened a compound library against glutathione *S*-transferase–PknG. A tetrahydrobenzothiophene was identified that specifically inhibited the kinase activity of PknG, with a median inhibitory concentration IC_{50} = 0.39 μM (AX20017; Fig. 4, A and B) as opposed to IC_{50} = 10 to 100 μM for eight other mycobacterial serine/threonine kinases (table S1). The inhibitor blocked the kinase activity of purified PknG but had no effect on the kinase activity of protein kinase Cα (Fig. 4C). The PknG-specific inhibitor did not affect macrophage growth characteristics at concentrations up to 20 μM (fig. S3), nor the growth of luminescent *M. tuberculosis* (Fig. 4D); these findings suggested that the PknG inhibitor had no direct effect on mycobacterial viability in culture, consistent with the phenotype of the mycobacterial strains lacking the *pknG* gene. Thus, this screen identified an inhibitor specific for PknG without any adverse effect on macrophages or mycobacterial viability outside host cells.

The presence of the inhibitor during infection of macrophages with mycobacteria caused a dose-dependent increase in the lysosomal localization of the mycobacteria (Fig. 4, E and F). When the intracellular trafficking of mycobacteria in the presence of AX20017 was analyzed by cell fractionation, virtually all intracellularly

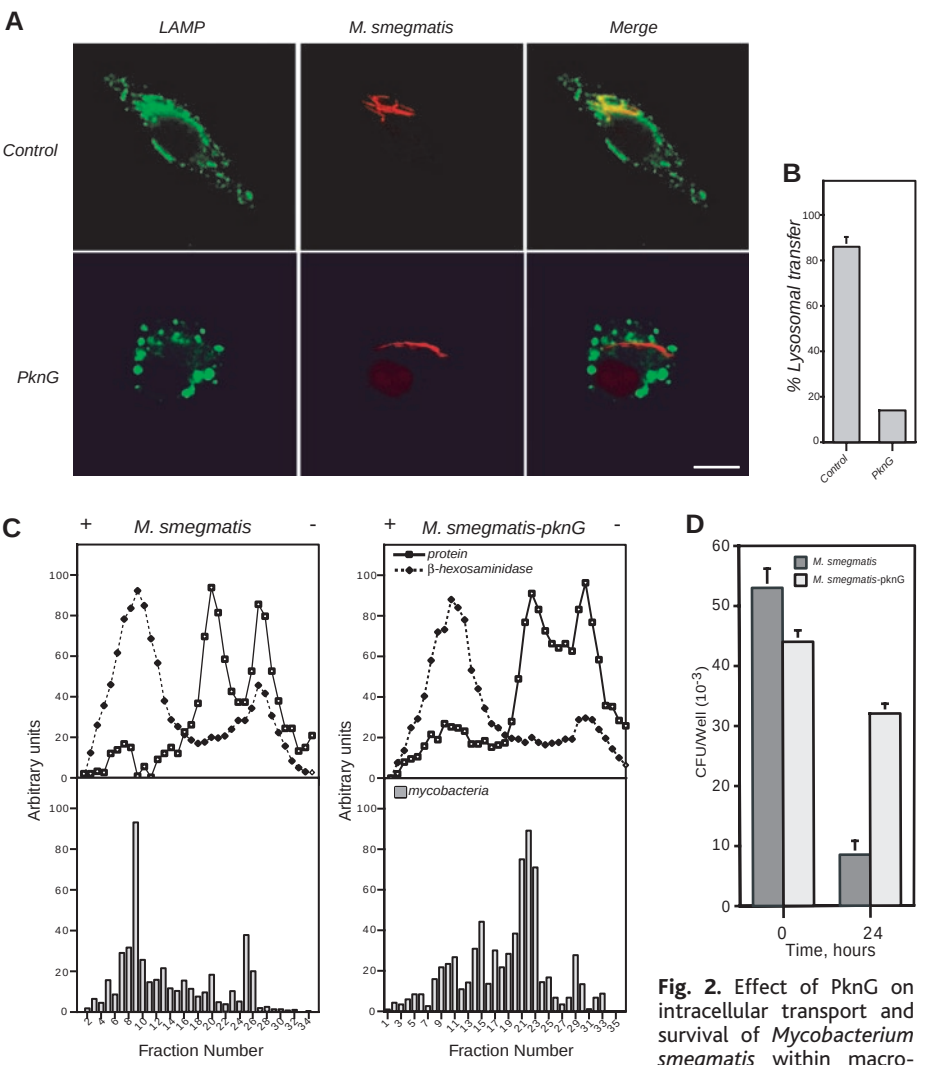

Fig. 2. Effect of PknG on intracellular transport and survival of *Mycobacterium smegmatis* within macrophages. (**A**) Macrophages were incubated with *M. smegmatis* or *M. smegmatis–pknG* for 1 hour, followed by a 2-hour chase, fixed, permeabilized, and stained with antibody to LAMP followed by Alexa Fluor 488. Bacteria were visualized with propidium iodide. Scale bar, 10 μm. (**B**) For quantitations, cells containing mycobacteria in LAMP-positive vacuoles were scored. Results are mean values (±SD) from three determinations (n = 50). (**C**) Macrophages were incubated for 3 hours with *M. smegmatis* (left) or *M. smegmatis–pknG* (right), homogenized, and subjected to organelle electrophoresis. (**D**) Macrophages (J774) were infected with *M. smegmatis* expressing PknG, or *M. smegmatis* transformed with plasmid (pSD5) alone for 3 hours, washed, and incubated for the times indicated before lysis and determination of colony-forming units (CFU).

residing bacteria were resolved in lysosomal fractions (Fig. 4G). The growth of both *M. bovis* BCG and *M. tuberculosis* was inhibited in a dose-dependent manner inside macrophages (Fig. 4, H and I). Thus, chemical targeting of PknG resulted in a phenocopy of deletion of the *pknG* gene; the bacteria were rapidly transferred to lysosomes and killed within infected macrophages.

Our data show that the eukaryotic-like serine/threonine PknG from mycobacteria mediates the survival of mycobacteria within macrophages by blocking lysosomal delivery, providing a remarkable example of the coevolution of pathogenic mycobacteria with their mammalian host cells. Eukaryotic-like serine/threonine kinases have now been found to be encoded by a number of bacterial genomes (*31*, *32*). Most of these genes may have been lost in prokaryotes during evolution because they are not needed for bacterial growth (*33*). Perhaps the expression of the serine/threonine kinase PknG has been maintained by pathogenic mycobacteria because of the selective advantage of avoiding phagosome-lysosome fusion concomitant with mycobacterial survival.

Specific inhibitors of protein kinases have been successfully developed for therapeutic usage against a variety of diseases (*34*, *35*). The compound that we have identified represents a promising candidate for the development of a class of drugs that would target intracellularly residing mycobacteria and allow the macrophage to carry out its innate bactericidal activity by shuttling these bacteria to lysosomes.

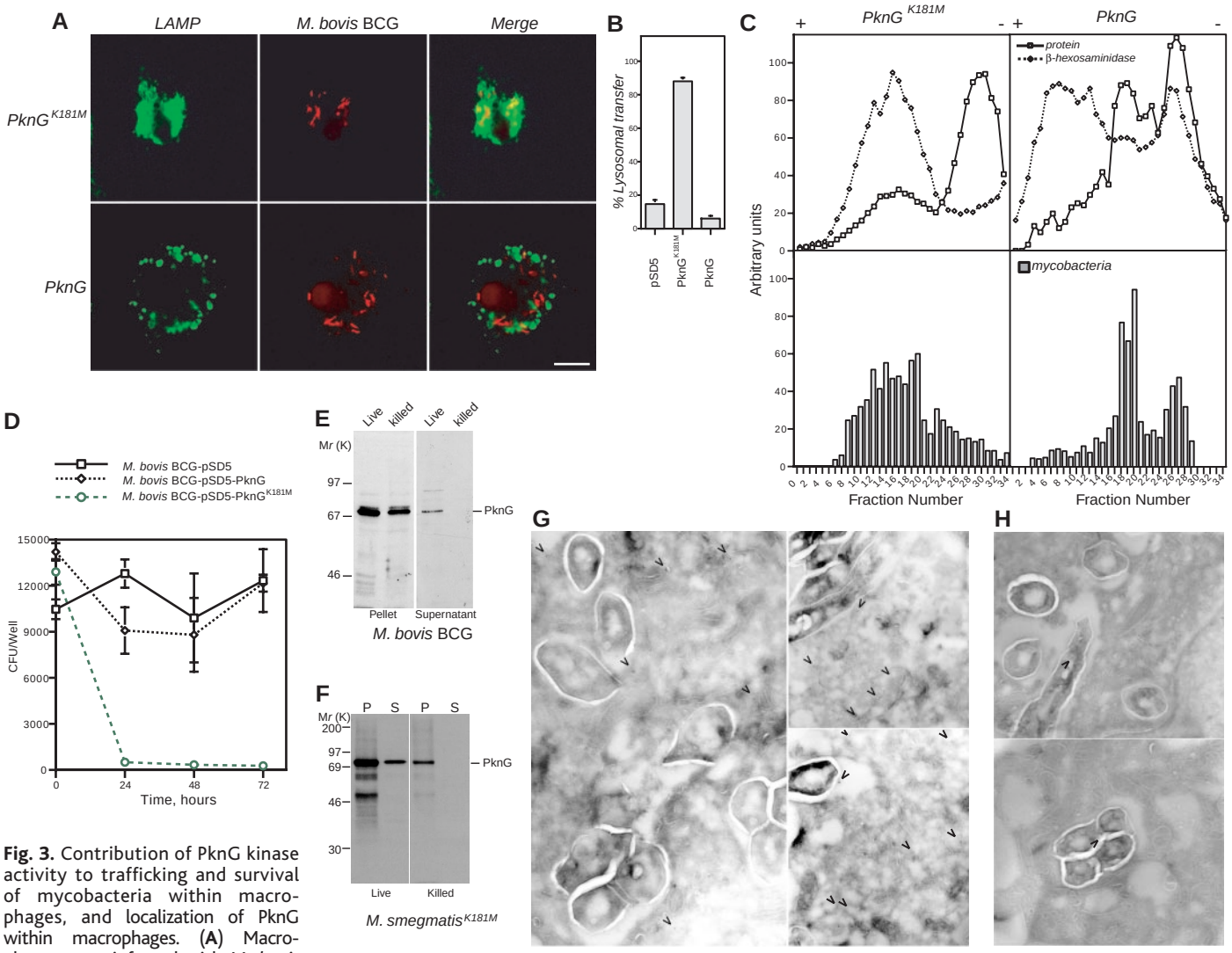

Fig. 3. Contribution of PknG kinase activity to trafficking and survival of mycobacteria within macrophages, and localization of PknG within macrophages. (**A**) Macrophages were infected with *M. bovis* BCG expressing PknG^{K181M} or PknG for 1 hour, followed by a 2-hour chase, fixed, permeabilized, and stained with antibody to LAMP followed by Alexa Fluor 488. Scale bar, 10 μm. (**B**) For quantitations, cells containing mycobacteria in LAMP-positive vacuoles were scored. Results are mean values (±SD) from three determinations (n = 50). Scale bar, 10 μm. (**C**) Macrophages were incubated for 3 hours with *M. bovis* BCG-*pknG*^{K181M} or *M. bovis* BCG-*pknG*, homogenized, and subjected to organelle electrophoresis. (**D**) Survival of *M. bovis* BCG, *M. bovis* BCG-*pknG*, or *M. bovis* BCG-*PknG*^{K181M} in macrophages after macrophage infection with *M. bovis* BCG transformed with the indicated plasmids for 3 hours. Data are from triplicates. (**E** and **F**) Macrophages were infected with live or killed *M. bovis* BCG (E) or *M. smegmatis-PknG*^{K181M} (F) for 16 hours, after which phagosomes were isolated and lysed by saponin treatment. Bacteria were separated by sedimentation (E). Alternatively, cytosol was separated from mycobacterial phagosomes by sedimentation (F). Proteins were separated by SDS–polyacrylamide gel electrophoresis followed by immunoblotting with antiserum to PknG. (**G** and **H**) Macrophages were infected with live (G) or killed (H) mycobacteria for 6 hours, after which cells were prepared for immunocytochemistry. PknG was detected with antiserum to PknG followed by 10 nm gold coupled to goat antirabbit antibodies. Arrowheads indicate the location of gold particles. Magnifications: left, ×17,000; upper middle, ×18,900; lower middle, ×14,200; right, ×14,500.

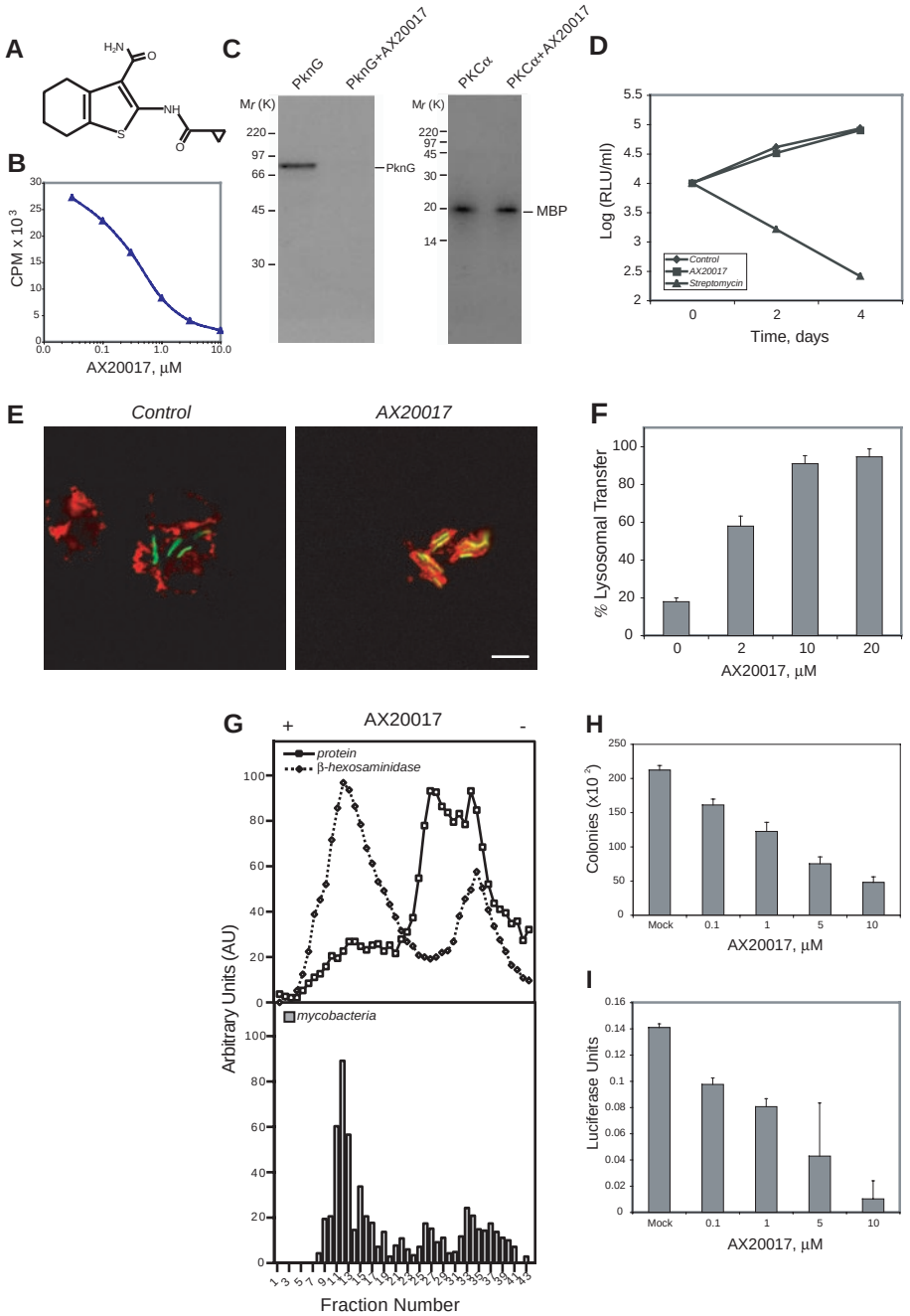

Fig. 4. Chemical inhibition of PknG. (**A**) Structure of AX20017. (**B**) Determination of IC_{50} value. (**C**) PknG or protein kinase Cα (0.1 μg) were assayed for kinase activity in the absence or presence of AX20017 (100 μM). (**D**) Activity of AX20017 on mycobacteria grown in vitro. *Mycobacterium tuberculosis* expressing the luciferase gene was inoculated in Sauton's medium with 5% fetal calf serum alone or containing AX20017 (10 μM) or streptomycin (10 μM). (**E**) Macrophages were infected with *M. bovis* BCG for 1 hour, followed by a 2-hour chase, in the absence (left) or presence (right) of AX20017 (20 μM), fixed, permeabilized, and stained with antibody to LAMP followed by Alexa Fluor 568. Scale bar, 10 μm. (**F**) For quantitation, cells containing mycobacteria in LAMP-positive vacuoles were scored. Results are mean values (±SD) from three determinations ($n = 50$). (**G**) Macrophages were incubated for 3 hours with *M. bovis* BCG, homogenized, and subjected to organelle electrophoresis. (**H** and **I**) Macrophages were infected with *M. bovis* BCG (H) or *M. tuberculosis* expressing the luciferase gene (I) and infection was allowed to proceed for 3 hours, after which cells were washed and treated with the indicated concentrations of AX20017 for 24 hours before lysis of the cells and determination of viable intracellular mycobacteria by analyzing CFU (H) or luciferase activity (I).

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Supporting Online Material

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References

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