Endocytosis-like protein uptake in the bacterium Gemmata obscuriglobus


Edited by Carl R. Woese, University of Illinois, Urbana, IL, and approved May 28, 2010 (received for review January 27, 2010)

Endocytosis is a process by which extracellular material such as macromolecules can be incorporated into cells via a membrane-trafficking system. Although universal among eukaryotes, endocytosis has not been identified in Bacteria or Archaea. However, intracellular membranes are known to compartmentalize cells of bacteria in the phylum Planctomycetes, suggesting the potential for endocytosis and membrane trafficking in members of this phylum. Here we show that cells of the planctomycete Gemmata obscuriglobus have the ability to uptake proteins present in the external milieu in an energy-dependent process analogous to eukaryotic endocytosis, and that internalized proteins are associated with vesicle membranes. Occurrence of such ability in a bacterium is consistent with autogenous evolution of endocytosis and the endomembrane system in an ancestral noneukaryote cell.

A major unsolved problem in biology is how the many unique characteristics of the eukaryote cell evolved, including endomembranes and their dynamic features, such as endocytosis. Endocytosis is known as a eukaryote-specific process by which cells internalize molecules from the plasma membrane and recycle them back to the surface or sort them to lysosomes for degradation. There is evidence that endocytosis must have been present in cells as far back as the last eukaryotic common ancestor (LECA) and in an ancestral noneukaryote cell.

eukaryotes | evolution | endocytosis | bacteria | planctomycetes

Gemmata obscuriglobus Cells Are Able to Internalize GFP via an Energy-Dependent Process. To investigate the possibility of an endocytosis-like mechanism in the planctomycete bacterium Gemmata obscuriglobus, we incubated cells with GFP and examined them via confocal laser scanning microscopy (CLSM). Remarkably, the protein was detected inside the cells (Fig. 1A), usually within 5 min. Location of GFP inside the cells rather than on the cell surface was indicated by the optical sectioning inherent with CLSM. The uptake of GFP seemed to be energy-dependent, because if incubated at 0 °C or in the presence of sodium azide, an inhibitor of respiration and oxidative phosphorylation, cells did not take up GFP (Fig. 1B and Fig. SI4). Temperature sensitivity of GFP uptake was also demonstrated by its inhibition at 37 °C (Fig. SI4), a temperature above the maximum growth temperature of G. obscuriglobus (24). ATP suppressed the inhibitory effect of sodium azide (Fig. 1B and Fig. SI8), confirming that GFP uptake is an energy-dependent process. The uptake of full-length GFP by cells of G. obscuriglobus was confirmed by Western blot analysis (Fig. 1C). As expected, nonplanctomycete bacterium Escherichia coli, acting as a negative control, did not take up GFP (Fig. 1C). Such an uptake process is saturable, as expected for a receptor-mediated process, the concentration of proteins at which the uptake process reaches saturation corresponding to ≈10 μg/mL (Fig. S2). This finding demonstrates the internalization of proteins in a bacterium. Internalization of intact external macromolecules, and more specifically proteins, has not been recorded in members of domains Bacteria or Archaea. Some bacteria, such as Lactococcus lactis, can use proteins only by degrading them at the cell surface using proteases and incorporating the resulting peptides (25). A 35-aa oligopeptide is so far the longest shown to bind to a bacterial receptor (26).

We next tested whether the mechanism of uptake is receptor mediated. Coincubation of GFP with unequal ratios of Cy3-labeled Ig resulted in dominant internalization of the protein in excess, whereas equal internalization was detected when a 1:1 ratio was used (Fig. S3). This implied that different proteins can be internalized by G. obscuriglobus and that they are competing for the same receptor.


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor. See Commentary on page 12739.

†T.G.A.L. and E.S. contributed equally to this work.

‡To whom correspondence should be addressed. E-mail: fuent@biosci.uq.edu.au.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1001085107.

www.pnas.org/cgi/doi/10.1073/pnas.1001085107

PNAS | July 20, 2010 | vol. 107 | no. 29 | 12883–12888

PNAS | 2010, vol. 107 | no. 29 | 12883–12888
the same surface receptor. Such assumption was further supported by the fact that an excess of BSA, ovalbumin, or GST greatly diminished uptake of GFP (Fig. 1D and Fig. S4). Additionally, plasmid DNA did not affect GFP uptake (Fig. 1D). A Cy3-labeled 25-mer oligonucleotide was not internalized by G. obscuriglobus (Fig. S5), demonstrating that in contrast to proteins, DNA is not internalized by this bacterium. Considered together, these results are consistent with a receptor-mediated protein uptake mechanism in G. obscuriglobus.

Proteins Internalized by G. obscuriglobus Are Compartmentalized and Degraded in the Paryphoplasm Cell Region. To gain more information about the mechanism of uptake, we investigated the subcellular localization of different proteins after their internalization. Coincubation of Cy5-labeled fluorescent streptavidin or Cy3-labeled Ig with GFP resulted in colocalization of the two distinct proteins to the same compartment (Fig. S6). The proteins were found to be restricted to an outer region of the cell cytoplasm, within the external cytoplasmic membrane indicated by SynaptoRed stain, and distinct from the DAPI-stained region containing DNA (Fig. 2A). Transmission electron microscopy (TEM) examination of thin sections of G. obscuriglobus cells preincubated with GFP defined this region as the paryphoplasm (Fig. 2B and schematic diagram Fig. 2C). This suggests a functional role for paryphoplasm as a protein-segregating compartment where exogenous proteins can be accumulated. We next tested whether the internalized proteins are proteolyzed in this compartment. Cells were incubated with DQ Green BSA (DQBSA), fluorescence of which appears only upon proteolysis, and with IgG-Cy3, the latter added shortly before examination and serving as a marker for the paryphoplasm. The fluorescent DQBSA proteolysis product colocalized with IgG-Cy3 in the paryphoplasm (Fig. S7A), illustrating the role of this compartment in degradation of internalized proteins. The confirmation that internalized proteins are degraded was obtained by first incubating G. obscuriglobus with a mouse IgG and then monitoring its fate—proteolysis of the Ig was detected by Western blot analysis (Fig. S7B). GFP was not suitable for this assay because it is highly resistant to proteolysis (27).

Fig. 1. G. obscuriglobus cells uptake proteins but not DNA in an energy-dependent process. (A) Bright field microscopy and CLSM images of G. obscuriglobus cells incubated with GFP (10 μg/mL) for 60 min. Left and Right microscopy images correspond to bright field and GFP fluorescence, respectively. Because of optical sectioning effects, only some of the cells in the clump positive for GFP signal can be visualized. (Scale bar, 4 μm.) (B) Time-course experiment with GFP alone (filled circles), with GFP and 1 mM sodium azide (open circles), or with GFP and both 1 mM sodium azide and 5 mM ATP (filled triangles). After washing of G. obscuriglobus cells, GFP fluorescence was quantitatively analyzed by microplate reader assay. Error bars represent the SD from three experimental replicates. (C) Western blot analysis showing uptake of full-length GFP (∼27 kDa) by G. obscuriglobus. Cells were incubated with GFP for 1 h. After washing, cell lysates were electrophoretically separated on an SDS gel (Left), then blotted and probed with an anti-GFP antibody (Right). Lane 1, E. coli DH5α incubated with GFP; lane 2, G. obscuriglobus incubated without GFP; lane 3, G. obscuriglobus incubated with GFP. (D) Competition uptake experiment with GFP, together with either BSA or DNA. G. obscuriglobus cells were incubated for 1 h with 10 μg/mL of GFP alone, with GFP and BSA (100 μg/mL), or with GFP and plasmid DNA (100 μg/mL). Cells were then washed and analyzed for fluorescence emission by microplate reader assay. Error bars represent the SD from three experimental replicates.

Fig. 2. Internalized proteins in G. obscuriglobus cells are localized to paryphoplasm. (A) G. obscuriglobus cells were incubated with GFP and then stained with DAPI and SynaptoRed. A GFP-containing region is seen in the cytoplasm bounded by the cytoplasmic membrane as defined by the SynaptoRed staining and is separated from the nuclear body (DAPI staining). (B) TEM image of a section of high-pressure frozen cryosubstituted cells of G. obscuriglobus, immunogold-labeled to detect GFP via anti-GFP antibody and secondary antibody conjugated with 10 nm colloidal gold. Gold particles (short arrows) labeling internalized GFP are only associated with paryphoplasm (P). Gold particles were excluded from both the double membrane-bounded nucleoid and the riboplasm. The riboplasm (R), fibrillar nucleoid (N), and intracytoplasmic membrane (ICM) are indicated. (Scale bar, 500 nm.) (C) Diagram representing the functional compartmentalization of G. obscuriglobus. N, nucleoid; NE, nuclear envelope; ICM, intracytoplasmic membrane; R, riboplasm; CM, cytoplasmic membrane; CW, cell wall.
Internalized Proteins Are Associated with Vesicles. We next used immunogold labeling of high-pressure frozen cells to investigate whether the internalized GFP was associated with any specific intracellular structures. Gold particles indicating presence of GFP in the parryphoplasm were often seen lining vesicle-like entities ca. 50–200 nm wide bounded by a single bilayer membrane (Fig. 3A, Insets 2–4). In some cases, gold particles were seen lining an infolding of the cytoplasmic membrane (Fig. 3B), perhaps representing an initial stage in the process of GFP internalization. An invagination of cytoplasmic membrane was also seen during uptake of HRP-conjugated antibody, the reaction product of which was surrounded by an infolding membrane showing continuity with the cytoplasmic membrane (Fig. 3C). These results suggest formation of vesicle structures in the cells allowing extracellular molecules to be internalized. Remarkably, proteins possessing structural features of eukaryotic MC proteins have been recently described in planctomyces (23). MC proteins are related to the clathrin and coat protein families, all members of which are associated with vesicle formation or membrane curvature, and some of which (e.g., clathrin) are necessary for receptor-mediated endocytosis. The similarity in secondary and tertiary structure between gp4978, an MC-like protein of *G. obscuriglobus*, and clathrin heavy chain of yeast is illustrated in Fig. S8. An antibody had been previously raised against *G. obscuriglobus* gp4978 (23). In our study this antibody reacted with vesicle-like structures within the parryphoplasm (Fig. 3D). In some cases, gp4978-reactive material was associated with cytoplasmic membranes apparently in the first stages of membrane invagination (Fig. 3D, Inset 3).

To confirm that the internalized proteins are associated with membranes and vesicles, *G. obscuriglobus* cells were lysed by sonication and subjected to subcellular fractionation. After centrifugation at 100,000 × g, GFP was detected in the insoluble pellet consisting of membranes, cell walls, and other insoluble cell components, but it was excluded from the supernatant (Fig. S9A, step 1). The GFP-positive fraction was then separated via a series of sucrose steps and linear density gradient ultracentrifugations (Fig. S9). The maximum GFP signal was found to be associated with one fraction of ca. 60% sucrose density (Fig. S9A, step 3).

TEM examination of this fraction demonstrated that it consisted of broken membranes and small membrane-bounded vesicles. Immunogold labeling showed that some membranes and vesicles in this preparation were positive for GFP (Fig. 4A) and MC-like protein (Fig. 4B). The association of GFP and MC-like protein with fractionated membranes from lysed cells is consistent with the invariable association of GFP with membranes in the parryphoplasm of sectioned *G. obscuriglobus* cells. It is unlikely that properly folded GFP could pass through a membrane pore because the size limit for molecules that can enter via bacterial membrane pores is much smaller [e.g., 600 Da for *E. coli* (28)]. It is also unlikely that GFP could pass through a membrane pore as an unfolded polypeptide because (i) this protein forms a highly stable structure of 30–40 Å diameter that is resistant to unfolding owing to a high folding/unfolding energy barrier (27, 29), and (ii) cytoplasmic components for protein secretion and/or associated ribosomal machinery are not present in the external milieu. However, to exclude the possibility of uptake of unfolded GFP through a membrane pore, we incubated *G. obscuriglobus* with a cross-linked protein (mouse IgG) that is expected to retain much of its tertiary structure during the uptake. This experiment demonstrated that the cross-linked protein was taken up by *G. obscuriglobus* and that it colocalized with MC-like protein in the same step gradient membrane fraction (Fig. S10). TEM of negatively stained fractions demonstrated their purity (Fig. S11).

In summary, the observed results are compatible with an endocytosis-like mechanism but not with a channel-mediated mechanism in which unfolding of proteins is needed for import. An endocytosis-like mechanism is also consistent with our immunogold labeling results showing both GFP protein and MC proteins in the parryphoplasm.
The vesicle membrane (49) have occurred more than once from this point of view. The event ample of a parallel evolutionary development of an analog of some bacteria during evolution from a common ancestor. It might have occurred only in Domain Eucarya members and some proteobacteria. Cytoplasmic membrane association with clathrins, distant enough to be revealed only by secondary and tertiary structure, is also consistent with the improbability of HGT as an explanation. It is also significant for consideration of potential origins of an endomembrane system that G. obscuriglobus possesses an analog of the endoplasmic reticulum, because the nuclear envelope is formed from endoplasmic reticulum in metazoans and most likely in other eukaryotes. In view of our findings with plantctomycetes, an endomembrane system and compartmentalized cell organization in an ancestral protoeukaryote could have developed without the need for contributions from cells of other Domains of life. Our results imply that fusion between Archaea and Bacteria is not necessary to account for complex cell structure; internal membrane systems could have developed within a simple cell and without involvement of fusion of unrelated cells. G. obscuriglobus may be viewed as a snapshot of a possible stage in the autogenous origin of eukaryotes. This bacterium may exhibit an ancestral stage in the evolution of endocytosis and represent a unique in vivo model for a major stage in the evolution of the eukaryotic cell. The nature and extent of the endocytotic machinery in this organism should now be investigated at both structural and functional levels.

**Materials and Methods**

**Material.** GFP was expressed from proviral vectors in tobacco leaves (38) and purified by using anion exchange chromatography. Sheep anti-mouse Ig-HRP was from Amersham Biosciences, Cy3-labeled Ig was from Sigma-Aldrich (catalog no. CS2036), and Cy5-labeled streptavidin was from GE Healthcare (catalog no. PA 92005). Cy3-DNA (Sigma-Proliq) was a 25-mer oligonucleotide (5′-ATGTTAGACAGGCGAGGAAGTGC-3′) labeled with Cy3 at the 5′ end and hybridized with its unlabeled antisense oligonucleotide (5′-ACAGCTCTTGCGCCCTTGTCCACAT-3′). Cy3 was from Molecular Probes. Mouse monoclonal anti-GFP antibody (Roche, catalog no. 1181460001) was used for dot-blot, and mouse monoclonal anti-GFP antibody (Clontech, catalog no. 632381) was used for immunogold labeling. Antibody to gp4978 was an affinity-purified (acid-elution) rabbit polyclonal antibody raised against recombinantly expressed and purified gp4978 protein of an ORF identified as a eukaryotic MC coamter protein (National Center for Biotechnology Information reference sequence: ZP_02732338). 12886 | www.pnas.org/cgi/doi/10.1073/pnas.1001085107 Lonhienne et al.

**Bacteria and Culture Conditions.** The plantctomycete G. obscuriglobus was grown on plates containing M1 agar medium (39) and incubated aerobically at 28 °C for 4–7 d. M1 agar medium plates were prepared as follows: after pouring and allowing agar to set, plates were dried for 1 h with lids open in a biohazard cabinet. After inoculation by streaking with a sterile plastic loop charged with G. obscuriglobus grown on the same M1 agar medium, plates were sealed with paraffilm before aerobic incubation. These standardized methods for medium preparation, inoculation, and incubation were necessary for optimal demonstration of protein uptake. E. coli strain DH5α was cultured on plates containing LB medium incubated at 37 °C for 16 h; plates were prepared in the same way as for M1 agar plates.

**Uptake Experiments.** Protein uptake by G. obscuriglobus cells is dependent on pH, with optimum uptake occurring at pH 7.5. No protein uptake was ob-
servable at pH 5.8 or pH 8.8; therefore, for uptake experiments we used 10 mM Tris, pH 7.5, referred to as "incubation buffer" (IB). For all uptake experiments, cells were picked up directly from M1 agar plates using a 10-μL sterile plastic loop and resuspended in 50 μL of IB. In uptake experiments with GFP, Cy3-labeled Ig, Cy5-labeled streptavidin, and HRP-conjugated antibody, the final concentration of the proteins was 10 μg/mL, except if specified otherwise. After addition of proteins, cells were incubated at 28 °C for 60 min, except where specified otherwise. Cy3-DNA and Cy3 were added to the cells at a final concentration of 2 μM.

CLSM, TEM, and Image Analysis. After incubation with fluorescent proteins, 5 μL of cell suspension was placed on a coverslip and the coverslip inverted onto a glass slide to make a wet mount for CLSM. A Zeiss LSM510 Meta (Carl Zeiss) confocal laser scanning microscope was used with 10× dry, and 20× water-immersion objectives or with a 100× oil-immersion objective. Fluorescent molecules were visualized with an argon laser and detected with band-pass filters, with settings according to the manufacturer's recommendations. All high-pressure freeze and cryosubstituted sections were viewed using a JEOL 1010 transmission electron microscope operated at 80 kV. Images were captured using a SoftImaging Megaview III digital camera. The resulting files were annotated and resolution adjusted for final image production using Photoshop CS.

Time-Course Experiments. All experiments were carried out in triplicate using the same batch of G. obscuriglobus cells. Cells were resuspended in 2.5 mL of IB and aliquoted in three Eppendorf tubes, each containing 800 μL of cell suspension. Sodium azide (1 mM) was added 15 min before addition of GFP. ATP (5 mM) was added simultaneously with GFP. The samples were continuously mixed on a vertical rotating wheel. Aliquots of 100 μL from each tube were sampled at the indicated times and immediately cooled on ice. Upon completion of the experiment, all samples were centrifuged for 2 min at 5 °C at maximum speed (20,000 × g). After careful removal of the supernatant, cells were resuspended in 200 μL of ice-cold IB and centrifuged again. The supernatant was removed, and cells were resuspended in 100 μL of ice-cold IB. The cells were transferred into a black 96-well plate (Greiner Cellstar), and GFP fluorescence was measured using a fluorescence plate reader, POLARStar OPTIMA (Imgen Technologies) (excitation filter set to A-405, emission filter set to S20).

Competition Experiments. The experiments were carried out in three replicates using the same batch of G. obscuriglobus cells. Cells grown on plates were resuspended in 450 μL of IB and aliquoted in four Eppendorf tubes, each containing 100 μL of cell suspension. One of the aliquots was not incubated with GFP and served to monitor the fluorescence background. BSA (100 μg/mL) or plasmid pET-15b DNA (100 μg/mL) were added simultaneously with GFP (10 μg/mL) to cell suspensions, and samples were continuously mixed on a vertical rotating wheel. After 90 min of incubation, the samples were immediately cooled on ice. The samples were then treated in the same way as for the time-course experiment. The reported fluorescence data were obtained by subtracting background values corresponding to autofluorescence of cells.

Detection of Proteins by Dot and Western Blots. Total extracts of G. obscuriglobus or E. coli were resolved by SDS/PAGE (4–20% gradient gel) or dot-blotted and characterized by Western blot analysis using Alexa Fluor 680 goat anti-mouse antibody (Molecular Probes) as secondary antibody. Detection was done by using an Odyssey infrared imaging system (LI-COR).

High-Pressure Freezing and Cryosubstitution. Bacteria cultures were high-pressure frozen with liquid nitrogen using a Leica EMFRACT 2 high-pressure freezer. The frozen samples were kept and stored in a 2-mL tube containing liquid nitrogen before cryosubstitution was carried out. The frozen samples were transferred to a microfuge tube containing 0.2% uranyl acetate and 5% H2O2 in acetone and cryosubstituted in a Leica AFS at −85 °C for 48 h, warmed up to −50 °C at 3 °C/h, and washed 2 × 20 min in acetone at −50 °C. Cryosubstituted samples were embedded in Lowicryl HM20 resin by infiltration with 50% Lowicryl HM20 for 2 h, 75% for 2 h, and 3 × 100% for 12 h. Finally the samples in Lowicryl HM20 were polymerized under UV for 48 h at −50 °C and 48 h at 20 °C.

The samples containing Lonhienne et al. Ultratrace-sectioned using a Leica Ultratrace Ultramicrotome UC61. The cut sections were placed onto formvar-carbon-coated copper grids. Sections were stained with 5% uranyl acetate in ethanol for 20 s and then with 1% lead citrate for 20 s. After immunolabeling, sections were incubated with 1% glutaraldehyde for 2 min before staining.

Immunolabeling. Ultrathin sections of high-pressure frozen and cryosubstituted G. obscuriglobus and E. coli cells on formvar-carbon-coated copper grids were floated onto drops of block solution containing 0.2% (wt/vol) fish skin gelatin, 0.2% (wt/vol) BSA, 200 mM glycine, and 1× PBS on a sheet of Parafilm, and treated for 1 min at 150 W in a Biowave microwave oven. For detection of GFP, the grids were then transferred onto 8 μL of primary antibody, mouse monoclonal anti-GFP antibody (Clontech, catalog no. 632381) diluted 1:25 in blocking buffer, and treated in the microwave at 150 W, for 2 min with microwave on, 2 min off, and 2 min on. The grids were then washed on drops of Block solution three times and treated in the microwave at 150 W each time for 1 min before being placed on 8 μL of goat anti-mouse IgG (H+L) antibody conjugated with 10 nm gold (British Biocell International, catalog no. EM GAM10) diluted 1:50 in Block solution and treated in the microwave at 150 W, for 2 min with microwave on, and 2 min off. Then grids were washed three times in 1× PBS, each time being treated for 1 min each in the microwave at 150 W, and four times in water for 1 min each in the microwave at 150 W. The grids were examined via transmission electron microscope either not stained (for quantitation of gold particles) or stained with uranyl acetate and lead citrate after treatment with 1% glutaraldehyde. A negative control was performed, with no antibody of any type used in place of the primary antibody. Two replicates per species were performed for the immunogold labeling experiment. For detection of Gemmata MC-like protein, we used anti-gp4978 as primary antibody and goat anti-mouse IgG for secondary antibody conjugated with 10-nm gold, for detection of the primary antibody. High-pressure frozen G. obscuriglobus cells preincubated with GFP were used for statistical analysis of GFP and MC-like protein distribution in different cellular compartments (Fig. S12).

HRP Cytochemistry. Cells of G. obscuriglobus were grown, collected, and incubated with HRP-conjugated rabbit anti-mouse antibody as described for GFP uptake experiments. After 1 h of incubation, cells were washed three times with IB by repeated centrifugations in a microfuge and resuspensions of the pellet, and the final pellet was resuspended in IB containing 1.8% H2O2 and 0.1 mg/mL of DAB. After 5 min of incubation, cells were washed three times with IB, and the final pellet was high-pressure frozen for electron microscopy and processed as described above; resulting sections were not stained before examination. A negative control was used, whereby cells were incubated in IB only in place of HRP-conjugated rabbit anti-mouse antibody, with all other steps identical to above.

ACKNOWLEDGMENTS. We thank Michael P. Rout at The Rockefeller University (RU) (New York) for donation of gp4978 antibody, arranging use of bioimaging at RU during a visit of J.A.F. to his laboratory, and valuable discussions and hospitality; Alison North of RU and members of the Rout laboratory, especially Ben Timney and Jaclyn Tetenbaum-Novatt; Donoghue and Vitalia Sagulenko for use of bioimaging at RU during a visit of J.A.F. to his laboratory, and the School of Biological Sciences at Rice University (RU) (New York) for donation of gp4978 antibody, arranging use of bioimaging at RU during a visit of J.A.F. to his laboratory, and valuable discussions and hospitality; Alison North of RU and members of the Rout laboratory, especially Ben Timney and Jaclyn Tetenbaum-Novatt; Rob Sullivan (Queensland Brain Institute, St. Lucia, Australia) for providing fluorophore-labeled proteins; Peter O’Donoghue and Vitalia Sagulenko for reading the manuscript and providing comments; Icon Genetics (Princeton, NJ) for providing viral vectors allowing expression of GFP in tobacco, from which the GFP used in this study was derived; and the School of Biological Sciences at The University of Queensland for providing a fluorescence plate reader. J.A.F. was supported by Australian Research Council (ARC) Discovery Project Grant DP0881485 and E.S. by a fellowship funded by the same ARC Discovery Project grant. T.G.A.L. was supported by a fellowship funded by ARC funding to B.J.C. D.P.D. was supported by the European Molecular Biology Laboratory, J.D.F. was supported by National Institutes of Health Grant F32 GM082029.


Supplementary figures

A

<table>
<thead>
<tr>
<th>0°C</th>
<th>28°C</th>
<th>37°C</th>
<th>Sodium-azide</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>GFP</th>
<th>GFP + sodium azide</th>
<th>GFP + sodium azide + ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure S1
Figure S2
Figure S3

IgG-Cy3:GFP
1:20

IgG-Cy3:GFP
20:1

IgG-Cy3:GFP
1:1
Figure S4
Figure S5
Figure S6
Figure S7

(A) DQBSA  IgG-Cy3  merge

(B) Post-incubation time

<table>
<thead>
<tr>
<th>kDa</th>
<th>IgG</th>
<th>0h</th>
<th>2h</th>
<th>16h</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure S9
Figure S10
Figure S11
Fraction 7, step gradient

Fraction 9, 20%-60% gradient

Fraction 10, 20%-60% gradient

Figure S12
Supporting Information
Lonhienne et al. 10.1073/pnas.1001085107

SI Materials and Methods

Material. Ovalbumin was from Sigma-Aldrich (catalog no. A-5503). GST was expressed from expression vector pGEX-KG (1) and purified by affinity chromatography on a glutathione-agarose column according to the manufacturer’s instruction (Scientifix). Mouse monoclonal anti-GFP IgG (Roche; catalog no. 11814460-001) was used for dot-blots, Western blots, protein degradation, and protein cross-linking experiments, as an immunochemically detectable molecule for uptake. Alexa Fluor 680 goat anti-mouse antibody (Molecular Probes) was used as secondary antibody. DQ Green BSA was obtained from Invitrogen.

DQ Green BSA Proteolysis Experiment. Cells were incubated with DQ Green BSA (DQBSA; 0.02 μg/μL) in incubation buffer (IB) for 3 h and washed once with IB. The pellet was then resuspended in IB containing IgG-Cy3 (0.02 μg/μL), and cells were incubated for a further hour. After washing once and resuspending in IB, cells were examined by confocal laser scanning microscopy (CLSM).

For control experiments (see Insets), DQBSA (0.02 μg/μL) in IB was incubated with 0.002 μg/μL of trypsin (positive control) or without trypsin (negative control) for 1 h, and solutions were examined by CLSM with the same settings as used for monitoring DQBSA in *Gemmata obscuriglobus* cells.

Fractionation Experiments. Cells were collected from two to three M1-agar plates, washed once, and resuspended in 500 μL of bt-DMSO buffer (10 mM bis-Tris (pH 7.5), 0.1 mM MgCl2, and 20% DMSO) supplemented with 10 μL of protease inhibitor mix (Protease Inhibitor Mixture Set 3; Merck), 10 μg of DNase, and 10 μg of RNase. Cells were then sonicated using a Branson Sonifier 250, and unbroken cells were spun down in a microfuge at 5,000 × g for 10 min. Supernatant was centrifuged at 100,000 × g for 10 min. Supernatant was then resuspended in 500 μL of bt-DMSO buffer, loaded onto a five-step sucrose gradient (Fig. S9), and centrifuged in an SW60 rotor on a Beckman Coulter tabletop ultracentrifuge (Optima TLX). The pellet was resuspended in 500 μL of bt-DMSO buffer, loaded onto a five-step sucrose gradient (Fig. S9), and centrifuged in an SW60 rotor on a Beckman Coulter L8-60M ultracentrifuge at 215,000 × g for 4 h. Fractions were collected from the bottom of the tube, and each of them contains ~500 μL of material. Five microliters from each fraction were used for dot-blot analysis for presence of GFP. Fraction 3 (positive for GFP) was centrifuged using the same rotor and ultracentrifuge at 100,000 × g, and the pellet resuspended in 500 μL of bt-DMSO was loaded onto a 20–60% sucrose/bt-DMSO gradient. After centrifugation using the same rotor and centrifuge for 16 h at 215,000 × g, fractions were collected from the top (~400 μL each). GFP-positive fraction 10 from the 20–60% sucrose/bt-DMSO gradient was used as a load material for a 40–70% sucrose/bt-DMSO gradient centrifugation using the same conditions. This fractionation was performed in a similar way as the previous step. A schematic diagram of the fractionation experiments is presented in Fig. S9D. Quality of fractionation experiments was monitored by transmission electron microscopy using uranyl acetate-stained samples (Fig. S11).

Saturation Experiment. The experiment was carried out in three replicates using the same batch of *G. obscuriglobus* cells. Cells were grown on plates and prepared as described for experiments testing GFP uptake in the main text (Materials and Methods). *G. obscuriglobus* cells were resuspended in 850 μL of IB and aliquoted into eight Eppendorf tubes, each containing 100 μL of cell suspension. IB (1.9 mL) containing different concentrations of GFP was added to cell suspensions to reach a final concentration of GFP ranging from 2 to 40 μg/μL (Fig. S2). Samples were continuously mixed on a vertical rotating wheel. After 2 h of incubation at 28 °C, samples were immediately cooled on ice and centrifuged for 2 min at 5 °C at maximum speed (20,000 × g). After careful removal of the supernatant, cells were resuspended in 200 μL of ice-cold IB and centrifuged again. The supernatant was removed, and cells were resuspended in 100 μL of ice-cold IB. The cells were transferred into a black 96-well plate (Greiner Cellstar), and GFP fluorescence was measured using a fluorescence plate reader, POLARStar OPTIMA (Imgen Technologies) (excitation filter set to A-485, emission filter set to 520).

Competition Experiment. The experiment was carried out in three replicates using the same batch of *G. obscuriglobus* cells. Cells grown on plates were resuspended in 350 μL of IB and aliquoted in three Eppendorf tubes, each containing 100 μL of cell suspension. One of the aliquots was not incubated with GFP and served to monitor the fluorescence background. GFP (10 μg/mL) alone, GFP (10 μg/mL) and ovalbumin (100 μg/mL), or GFP (10 μg/mL) and GST (100 μg/mL) were added to cell suspensions, and samples were continuously mixed on a vertical rotating wheel. After 90 min of incubation at 28 °C, samples were immediately cooled on ice. The samples were then centrifuged and treated in the same way as for the saturation experiment. The reported fluorescence data were obtained by subtracting background values corresponding to autofluorescence of cells.

Testing Proteolysis of Mouse IgG (Anti-GFP Antibody) in *G. obscuriglobus* Cells. Cells grown on plates were resuspended in 350 μL of IB containing 1 μg/mL of mouse IgG. After 1 h incubation at 28 °C, cells were washed three times with 1 mL of IB buffer and resuspended in 350 μL of IB buffer. Aliquots of 100 μL were sampled immediately after resuspension and after 2 h and 16 h incubation at 28 °C. Incubation was terminated by cooling on ice. Cells were centrifuged and resuspended in 50 μL of SDS/PAGE loading buffer, and the lysate was loaded onto a polyacrylamide gel. Proteins contained in 10 μL of each sample were resolved by SDS/PAGE (4–20% gradient gel) and characterized by Western blot analysis using Alexa Fluor 680 goat anti-mouse antibody (Molecular Probes). Detection was performed by using an Odyssey infrared imaging system (Li-COR).

Cross-Linking of Mouse IgG (Anti-GFP Antibody) and Testing Uptake of Cross-Linked Ig. Mouse IgG (20 μg) was incubated in 100 μL of 10 mM Tris-HCl, pH 7.5, containing 1% formaldehyde for 1 h at room temperature. Formaldehyde solution was then substituted with 10 mM Tris-HCl, pH 7.5, using Vivasin 500 concentrators (3 kDa exclusion limit; GE Healthcare). Cells grown on plates (*G. obscuriglobus* or *Escherichia coli*) were resuspended in 50 μL of IB containing ~5 μg/mL of cross-linked mouse IgG. After 1 h of incubation at 28 °C, cells were washed three times with 0.5 mL of IB buffer and resuspended in 50 μL of SDS/PAGE loading buffer. The samples were resuspended in sample buffer without mercaptoethanol and loaded onto the SDS/PAGE gel, without heating, so as not to disrupt formaldehyde-linked bonds. Proteins were resolved by SDS/PAGE (4–20% gradient gel) and visualized by Western blot analysis using Alexa Fluor 680 goat anti-mouse antibody (Molecular Probes). Detection was performed by using an Odyssey infrared imaging system (Li-COR).

Mass Spectrometry. Anti-GFP IgG treated with or without formaldehyde was digested overnight with trypsin (1:25) in 25 mM ammonium bicarbonate. Samples were desalted and concentrated using a C18 ZipTip (Millipore) and spotted to a MALDI target plate with...
0.5 μL alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/mL CHCA/25 mM diammonium citrate/50% acetonitrile/0.1% TFA). Spectra were analyzed on a Voyager MALDI-TOF instrument in reflectron mode, across the mass range 700–4,000 m/z using a minimum of 500 shots per spectra. Data were processed with DataExplorer.

Quantification of gold particles was carried out using iTEM software from Olympus Soft Imaging.


Fig. S1. The uptake of GFP by *G. obscuriglobus* cells is energy dependent. (A) *G. obscuriglobus* cells incubated with GFP for 1 h at 0 °C, 28 °C, or 37 °C, or cells treated with 1 mM sodium azide 15 min before addition of GFP and incubation for 1 h at 28 °C. Top and bottom frames correspond to GFP fluorescence and bright field, respectively. (B) *G. obscuriglobus* cells were incubated with GFP (Left), preincubated with sodium azide for 15 min before addition of GFP (Middle), or preincubated with sodium azide before addition of GFP and ATP (Right). Cells were incubated for 1 h at 28 °C before CLSM. Top and bottom frames correspond to GFP fluorescence and bright field, respectively. (Scale bars, 50 μm.)

Fig. S2. Saturation experiment demonstrating concentration limit for protein uptake. *G. obscuriglobus* cells were incubated for 1 h with different concentrations of GFP. Cells were then washed and analyzed for fluorescence emission by microplate reader assay. Error bars represent the SD from three experimental replicates.

Fig. S3. Competition experiment using microscopy detection demonstrating that GFP competes with other proteins for uptake. *G. obscuriglobus* cells were incubated with mixtures of both Cy3-labeled Ig and GFP at ratios 1:20, 20:1, and 1:1 (1 unit corresponds to 10 μg protein/mL). (Scale bar, 5 μm.)

Fig. S4. Competition experiment using plate reader fluorescence detection demonstrating that GFP competes with other proteins for uptake. *G. obscuriglobus* cells were incubated with GFP (10 μg/mL) only, with both GFP (10 μg/mL) and ovalbumin (100 μg/mL), or with both GFP (10 μg/mL) and GST (100 μg/mL) for 90 min. Cells were then washed and analyzed for fluorescence emission by microplate reader assay. Error bars represent the SD from three experimental replicates.

Fig. S5. *G. obscuriglobus* cells do not uptake DNA. Cells were incubated with Cy3-labeled DNA (Left) or Cy3 dye alone (Right) before CLSM; only Cy3 dye alone stained the cells. (Scale bar, 10 μm.)
Fig. S6. Internalized proteins colocalize in G. obscuriglobus cells. CLSM of G. obscuriglobus cells incubated with mixtures of both Cy5-labeled streptavidin and GFP or Cy3-labeled Ig and GFP, at equimolar concentrations (10 μg protein/mL). Cy5-labeled streptavidin and GFP (Upper) or Cy3-labeled Ig and GFP (Lower) were coincubated with G. obscuriglobus cells for 1 h at 28 °C. Streptavidin-Cy5 and IgG-Cy3 indicate the signals for Cy5 and Cy3 only, GFP indicates signal for GFP fluorescence, and “merge” indicates merger of both Cy5 and GFP or Cy3 and GFP signals, respectively; the merge images show colocalization of the corresponding proteins. (Scale bar, 2 μm.)

Fig. S7. Degradation of proteins in paryphoplasm of G. obscuriglobus cells. (A) DQBSA is proteolyzed in G. obscuriglobus cells. Before CLSM, cells were incubated with DQBSA for 3 h at 28 °C, washed, and incubated for another hour with IgG-Cy3 (to indicate position of the paryphoplasm by the IgG-Cy3 taken up by cells). Frames correspond to DQBSA, IgG-Cy3, and merged fluorescence. The merged image shows colocalization of fluorescent DQBSA degradation product and IgG-Cy3. Insets correspond to controls for DQBSA proteolysis in absence of cells: Inset 1 illustrates absence of fluorescence for DQBSA alone; Inset 2 illustrates fluorescence of DQBSA digested by trypsin. (Scale bar, 2 μm.) (B) Mouse IgG (anti-GFP antibody) is proteolyzed in G. obscuriglobus cells. Cells were preincubated with mouse IgG for 1 h at 28 °C, washed, and further incubated at 28 °C. After the indicated incubation times, cells were washed, and the lysates were analyzed by SDS/PAGE under nonreducing conditions to better visualize degradation products and via Western blot using an anti-mouse antibody. Lane 1, mouse IgG (control); lane 2, 0 h of incubation; lane 3, 2 h of incubation; lane 4, 16 h of incubation. The band (arrow) representing IgG heavy chain (the sample was run in nonreducing conditions) is markedly decreased after 2 h and totally disappears after 16 h.

Fig. S8. Alignment and secondary structure comparison of gp4978 and yeast clathrin heavy chain (Uniprot ID: P22137- CLH_YEAST) from Saccharomyces cerevisiae. Protein profiles were aligned with the hhalign program available from the HHPred package (1). Secondary structures (α-helices and β-strands) were predicted with the PSI-PRED program (2) and are represented as pink cylinders (α-helices) and cyan arrows (β-strands) above and below the aligned sequences, respectively. Where regions containing either β-sheets or α-helices conform to domains corresponding to β-propellor or α-solenoid domains, the predicted (for gp4978) and known (for clathrin) domains of these β-propellers and α-solenoids are highlighted by a gray shading of the secondary structures.

Fig. S9. GFP cofractionates with membrane coat (MC)-like protein gp4978. (A) Diagram of fractionation of G. obscuriglobus cells preincubated with GFP. G. obscuriglobus cells were sonicated, and the insoluble material was separated by differential centrifugation (step 1). The insoluble material from step 1 was further fractionated via sucrose density gradient centrifugations (steps 2, 3, and 4). Dot-bLOTS from successive fractionations were performed with mouse IgG to detect GFP-positive fractions. A Western blot with mouse IgG was carried out to confirm the dot-blot results of the last fractionation (step 4). As an example a Western blot with fractions 2 and 9 is shown; M, molecular mass marker. Confirming dot-blot results, GFP was present in fraction 9 and largely absent in fraction 2. (B) Dot-blot and Western blot of fractions from the 40–70% sucrose density gradient centrifugation run corresponding to step 4, using anti-gp4978 antibody. Western blot result shows a reacting band of ≈125 kDa corresponding to the expected length of the MC-like protein gp4978.

Fig. S10. Uptake of cross-linked mouse IgG (anti-GFP antibody) by *G. obscuriglobus* cells. (A) Western blot analysis showing uptake of cross-linked mouse IgG. Cells were incubated with mouse IgG for 1 h. After washing, cell lysates were electrophoretically separated on an SDS gel (left), then blotted and probed with an anti-mouse antibody (right). Lane 1, non-cross-linked mouse IgG (anti-GFP antibody); lane 2, cross-linked mouse IgG; lane 3, *E. coli DH5α* cells preincubated with cross-linked mouse IgG; lane 4, *G. obscuriglobus* cells preincubated with cross-linked mouse IgG; lane 5, *G. obscuriglobus* cells not incubated with cross-linked mouse IgG. Note that the whole cross-linked IgG molecule of 150 kDa seen in lane 2 control is also present in lane 4, showing the protein incorporated by *G. obscuriglobus* cells. (B) Colocalization of cross-linked mouse IgG with MC-like protein gp4978 in the same step gradient membrane fraction derived from lysed *G. obscuriglobus* cells that had been incubated with mouse IgG (Upper). Dot-blot of fractions of a sucrose step gradient centrifugation using anti-gp4978 antibody (anti-MC-like protein) and anti-mouse antibody (to detect mouse IgG) (Lower). Below is a Western blot of fraction 3 from the sucrose step gradient centrifugation run seen in the upper panel, using anti-mouse antibody, showing reacting bands corresponding to cross-linked mouse IgG. Lane A, mouse IgG; lane B, cross-linked mouse IgG; lane C, fraction 3. (C) Confirmation of successful cross-linking of IgG by mass spectrometry. MALDI-TOF spectra of IgG treated with or without formaldehyde and digested with trypsin. Upper: Addition of 12 Da to mass at 1649 m/z to give 1,661 m/z, indicating the addition of formaldehyde to the protein. Lower: Presence of a new peak (arrow) in the presence of formaldehyde at 2,313 m/z, due to the cross-linking of two or more peptides by formaldehyde.

Fig. S11. Transmission electron micrographs of negatively stained preparations of fractions from density gradient centrifugations. Fraction 7 (Top) from the step gradient (negative for reaction with anti-GFP antibody via Western blot) contains mostly flagella as well as membranous debris; fraction 9 (Middle) from the 20–60% continuous gradient (negative for reaction with anti-GFP antibody via Western blot) contains mainly membrane sheets covered with characteristic pore-like structures; fraction 10 (Bottom) from the 20–60% continuous gradient (positive for reaction with anti-GFP antibody via Western blot) contains mostly membrane vesicles and small pieces of broken membranes. When this fraction 10 was further subjected to another sucrose density gradient fractionation (30–70%) it resulted in only one visible band, corresponding to fraction 9 positive for anti-GFP antibody reactivity. (Scale bars, 50 nm.)

Fig. S12. Distribution of gold particles detecting GFP and gp4978 MC-like protein in the paryphoplasm and the nonparyphoplasm regions of the *G. obscuriglobus* cells. Histogram of the average density of anti-GFP (A) and anti-gp4978 (B) gold labeling (gold particles/μm²) in the paryphoplasm and in the nonparyphoplasm remaining region (“rest of cell”) of the *G. obscuriglobus* cells. Error bars shown are 95% confidence intervals of the mean.