NOTES

Characterization of a Molecular Chaperone Present in the Eukaryotic Flagellum

Jessica Shapiro,† Jessica Ingram,‡ and Karl A. Johnson*
Department of Biology, Haverford College, Haverford, Pennsylvania 19041

Received 8 April 2005/Accepted 22 June 2005

Chlamydomonas flagella contain a molecular chaperone now identified as HSP70A, a major cytoplasmic isoform. HSP70A synthesis is upregulated by deflagellation, and its distribution in the flagellum overlaps with the IFT kinesin-II motor FLA10. HSP70A may chaperone flagellar proteins during transport, participating in the assembly and maintenance of the flagellum.

The multiple compartments of the eukaryotic cell contain Hsp70 molecular chaperones that participate in the folding, targeting, assembly, and maintenance of the proteome (2, 9, 27). We previously found a putative flagellar Hsp70 in the green alga Chlamydomonas reinhardtii (1). Eukaryotic flagella are highly specialized, motile structures thought to contain only proteins directly involved in their assembly and function (6, 10, 17, 21). We identified this flagellar protein as Chlamydomonas HSP70A, a cytoplasmic chaperone, and examined its expression and localization within the context of the flagellum.

Molecular identification of flagellar Hsp70. The flagellar protein recognized by the pan-Hsp70 monoclonal antibody MA3-006 (Affinity BioReagents, Denver, CO) (1) was isolated from the matrix fraction of Chlamydomonas flagella (strain CC-1690; Chlamydomonas Genetics Center, Durham, NC) (26) by binding to ATP-agarose (Sigma, St. Louis, MO) (25), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Following tryptic digestion on a blot, matrix-

![Biochemical purification of flagellar Hsp70. Chlamydomonas flagellar matrix extracts (lanes 1) were incubated with ATP-agarose beads, and the depleted extract (lanes 2) was separated from bound material (lanes 3) by centrifugation; lanes 4 contain a 10-fold-heavier loading of the contents of lanes 3. Panel A shows a silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel loaded with equivalent fractions. Panel B shows an immunoblot of a gel identical to that used in panel A, probed with the monoclonal antibody MA3-006 originally used to identify the flagellar chaperone (1).](image1)

![RT-PCR analysis of HSP70A upregulation during flagellar assembly. Total RNA samples isolated from cells bearing full-length flagella (not deflagellated [NDF]) and cells regenerating flagella 30 min after pH shock deflagellation (DF) were subjected to RT-PCR using primers specific for mRNAs encoding (A) alpha tubulin, (B) beta tubulin, (C) cytoplasmic HSP70A, (D) the ribosomal protein S14, (E) a G protein beta subunit-like protein, or (F) chloroplast HSP70B. RT-PCR products were examined at five-cycle intervals, and examples were chosen to accurately compare the kinetics of product formation and, thus, initial mRNA abundances.](image2)
assisted laser desorption–time of flight mass spectrometry (Keck Protein Chemistry Laboratory, University of Massachusetts Medical School, Worcester, MA) uniquely identified the protein as HSP70A (13, 24), matching 9 of 11 peptide masses covering 26.2% of the protein. HSP70A is one of seven Chlamydomonas Hsp70 family members (20) and bears the carboxy-terminal EEVD invariably conserved in cytoplasmic isoforms (8).

**Regulation of HSP70A during flagellar assembly.** Genes encoding flagellar proteins typically are transcriptionally upregulated during organelar assembly (6, 10, 17, 21). Total RNA-samples were isolated (QIAGEN, Valencia, CA) from cells (CC-3941) bearing full-length flagella and cells with half-length, actively regenerating flagella (30 min after pH shock deflagellation). Reverse transcription (RT)-PCR (Titan; Roche, Indianapolis, IN) was performed using mRNA-specific primers (MWG, High Point, NC; for alpha-tubulin, GCCGGTATCC AGGTGGGCAATG and GATCAGCTGCTCGGGGTGG AAC; for beta-tubulin, CTGGAGCGCATCAACGTGTA C TTC and CCTGGGAACCGTGGACGCA; for HSP70A, CATACGGCAGTCTCGCGGACAGCT and CTGGGAACCGTGGACGCA; for CRY1, CCCCAGAGGAGGTG TGTAATGGGCAATG and AGTGGCGTGGGACGCTCGGAC; for CBLP, GCCCTGCTGCTGAACTGCT and CGACCAGCAGT CTTGGCGTTGTCG; and for HSP70B, CGAGCAGCTGCTGAACTGCT and CGACCAGCAGT CTTGGCGTTGTC) in five-cycle increments. Products were sized on agarose gels and documented using an AlphaImager (AlphaInnotech, San Leandro, CA). HSP70A mRNA levels increased strongly during flagellar assembly along with tubulin mRNAs (Fig. 2) (22), while constitutively expressed messages for ribosomal protein S14 (14) and G protein beta subunit-like protein (18) remained unchanged. However, HSP70B mRNAs, encoding a chloroplast-localized isoform (5), were also elevated, suggesting that HSP70A upregulation may be part of a whole-cell stress response to deflagellation (22).

**Localization of HSP70A.** To study the cellular distribution of HSP70A, affinity-purified antipeptide antibodies were generated (Research Genetics, Huntsville, AL) to its unique carboxy terminus (PSGGSGAGPKIEEVD) (13, 24). These antibodies reacted specifically with a 70-kDa protein on immunoblots of total cell protein (TP) and purified flagellar protein (FL) that was also recognized by MA3-006 (1) (Fig. 3). Based upon a yield of 10 μg of flagellar protein per 10⁶ cells (26), flagella contain approximately 10% of the cellular HSP70A pool. In
contrast, antibodies to chloroplast-localized HSP70B show a strong reaction to a cell body protein not present in flagella. Recent global characterization of the flagellar proteome by mass spectrometry also identified HSP70A as a Chlamydomonas flagellar protein (G. Pazour, N. Agrin, and G. B. Witman, unpublished data; cited at http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=chlre2&tid=H11005155023).

In addition, an epitope-tagged HSP70A allele was constructed. Complementary short oligonucleotides (TCGACTA CCCCCTACGACGTCCCGGACTACGCGAGGAGG TGGACTAA and TCGATTAGTCCACCTCCTCGCCGGT AGTCGCGGAGCTGCGTAGGGGTAG) encoding a hemagglutinin (HA) epitope (7) and retaining the highly conserved terminal sequence EEVD (8) were synthesized, annealed, and cloned into a SalI site in the HSP70A gene (13, 24) to append the carboxy-terminal sequence YPYDVPDYAGEEVD. This construct was cotransformed into Chlamydomonas cells (CC-2929) (14) with a selectable marker. HSP70A::HA was detected in roughly 10% of transformants by using the anti-HA monoclonal antibody 12CA5 (Roche). Several clones with stable, high levels of expression were characterized. Ectopic expression of HSP70::HA did not have gross effects on either growth or motility. On immunoblots using MA3-006 (which binds an internal sequence unchanged by epitope addition), HSP70A and HSP70A::HA were present at similar levels in transformant total cell protein and flagellar protein samples (Fig. 3). Note that HSP70A and HSP70A::HA can be distinguished immunologically using antipeptide and antiepitope antibodies, respectively, because of the carboxy-terminal placement of the epitope.

Immunofluorescent localization (11) of HSP70A confirmed that the chaperone was abundant in cell body cytoplasm and present in flagella (Fig. 4). Within flagella, wild-type HSP70A was distributed in a discontinuous, punctate fashion and concentrated in flagellar tips (Fig. 4). Previous localizations with MA3-006 (1) showed primarily tip labeling; the difference may be due to improvements in imaging technology (push-processed film versus a cooled charge-coupled-device camera) and antibody affinity. HSP70A::HA was distributed along the flagellum like the wild-type protein, although accumulation in the flagellar tip was less evident. Interestingly, in HSP70A::HA, the epitope coincides with a cochaperone binding site (8), implicating regulatory interaction and substrate release in its accumulation in the tip.

**Colocalization of HSP70A and the IFT kinesin-II FLA10.** The flagellar distribution of HSP70A is very similar to that of the components of the intraflagellar transport (IFT) system (3, 17, 19) responsible for shuttling unassembled axonemal pro-
teins from the cell body to the flagellar tips. Immunofluorescent colocalization (11) of HSP70A (using the rabbit antipeptide antibodies; red channel) and the anterograde IFT centrin colocalization (11) of HSP70A (using the rabbit antipeptides from the cell body to the flagellar tips. Immunofluorescence analysis, although the stoichiometry of labeling (indicated by yellow hues in red-green overlays) varied considerably (Fig. 5D and E). This variation suggests that HSP70A is not an integral part of the IFT machinery but is instead carried by IFT cargo. Despite considerable effort, we have been unable to identify specific HSP70A interactors by cofractionation, native gel electrophoresis, immunoprecipitation, cross-linking, or immunoelectron microscopy approaches, although flagellar Hsp70 was reported to copurify with IFT complexes on sucrose gradients in one study (15; see also reference 4). Within the flagellum, HSP70A may be interacting with many different molecular targets (redistributing as conditions change), which is consistent with the generalist strategy of Hsp70 involvement in protein folding, transport, and repair observed in other systems (2, 9, 27). Hsp70 proteins have also been implicated directly in cargo release from kinesin-driven anterograde fast axonal transport (23), suggesting that HSP70A may assist with both folding and delivery of flagellar proteins. Our molecular identification of this molecular chaperone opens the door to further investigation of its roles in flagellar assembly/disassembly and maintenance.

We thank Christoph Beck, Paul Lefebvre, and Elizabeth Harris for sharing Chlamydomonas strains and plasmids, John Leszyk for assistance with the matrix-assisted laser desorption–time of flight analysis, Marina del Rios for help with Chlamydomonas transformation, Geraldine Sheir-Neiss for technical assistance, John Butler for laboratory support, several reviewers for their constructive comments, and Wayne Rasband and Christopher Philip Mauer for making available the ImageJ program and RGO2 colocalization plug-in, respectively.

This work was supported by a fellowship (to J.S.) from the Howard Hughes Institute for Undergraduate Biological Sciences Medical Research Program (to Haverford College), the Haverford College Provost’s Office, and NSF MCB-9506236 and MCB-9982733 (to K.A.J.).

REFERENCES
1. Bloch, M. A., and K. A. Johnson. 1995. Identification of a molecular chaperone in the eukaryotic flagellum and its localization to the site of microtubule assembly. J. Cell Sci. 108:3541–3545.
2. Bukau, B., and A. L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366.
3. Cole, D. G. 2003. The intraflagellar transport machinery of Chlamydomonas reinhardtii. Traffic 4:435–442.
4. Cole, D. G., D. R. Diener, A. L. Himelblau, P. L. Beech, J. C. Fuster, and J. L. Rosenbaum. 1998. Chlamydomonas kinesin-II-dependent intracellular transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons. J. Cell Biol. 141:993–1008.
5. Drubin, D. G., M. Schroda, and C. F. Beck. 1996. Light-inducible gene HSP70B encodes a chloroplast-localized heat shock protein in Chlamydomonas reinhardtii. Plant Mol. Biol. 31:1185–1194.
6. Dutcher, S. K. 1995. Flagellar assembly in two hundred and fifty easy-to-follow steps. Trends Genet. 11:398–404.
7. Field, J., J. I. Nakawa, D. Brock, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a Ras-responsive adenyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. Mol. Cell. Biol. 8:2155–2165.
8. Freeman, B. C., P. P. Myers, R. Schumacher, and R. I. Morimoto. 1995. Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with Hsp70. EMBO J. 14:2121–2129.
9. Frydman, J. 2001. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu. Rev. Biochem. 70:603–647.
10. Johnson, K. 1995. Keeping the beat: form meets function in the Chlamydomonas flagellum. Bioessays 17:847–855.
11. Johnson, K. A. 1998. The axonemal microtubules of the Chlamydomonas flagellum differ in tubulin isoform content. J. Cell Sci. 111:331–342.
12. Kozmynski, K. G., P. L. Beech, and J. L. Rosenbaum. 1995. The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. J. Cell Biol. 131:1517–1527.
13. Muller, F. W., G. L. Igoi, and C. F. Beck. 1992. Structure of a gene encoding heat-shock protein HSP70 from the unicellular alga Chlamydomonas reinhardtii. Gene 111:165–173.
14. Nelson, J. A., E. P. B. Saeverede, and P. A. Lefebvre. 1994. The CRT7 gene in Chlamydomonas reinhardtii is essential for flagellar assembly in Chlamydomonas. Mol. Biol. Cell 6:793–772.
15. Rosenbaum, J. L., and G. B. Witman. 1990. A Chlamydomonas gene encodes a G protein beta subunit-like polypeptide. Mol. Gen. Genet. 231:443–452.
16. Scholey, J. M. 2003. Intraflagellar transport. Nat. Rev. Mol. Cell Biol. 4:813–825.
17. Schloss, J. A. 1990. A Chlamydomonas gene encodes a G protein beta subunit-like polypeptide. Mol. Gen. Genet. 221:443–452.
18. Silhout, C. D., and P. A. Lefebvre. 2001. Assembly and motility of eukaryotic cilia and flagella. Lessons from Chlamydomonas reinhardtii. Plant Physiol. 127:1503–1507.
19. Stoeck, V., M. P. Samanta, W. Tongprasit, and W. F. Marshall. 2005. Genome-wide transcriptional analysis of flagellar regeneration in Chlamydomonas reinhardtii identifies orthologs of ciliary disease genes. Proc. Natl. Acad. Sci. USA 102:3703–3707.
20. Tsai, M. Y., G. Morfini, G. Szebenyi, and S. T. Brady. 2000. Release of kinesin from vesicles by hsc70 and regulation of fast axonal transport. Mol. Biol. Cell 11:2161–2173.
21. von Gromoff, E. D., U. Treier, and C. F. Beck. 1998. Three light-inducible heat shock genes of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA 95:10093–10098.
22. Welch, W. J., and J. R. Feramisco. 1985. Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. Mol. Cell. Biol. 5:1229–1232.
23. Wilman, G. B. 1986. Isolation of Chlamydomonas flagella and flagellar axonomes. Methods Enzymol. 134:280–290.
24. Young, J. C., J. M. Barral, and F. Ulrich Hartl. 2003. More than folding: localized functions of cytosolic chaperones. Trends Biochem. Sci. 28:541–547.