Protein Methylation in Full Length *Chlamydomonas* Flagella

Roger D. Sloboda[1, 2]* and Louisa Howard[3]

[1]Department of Biological Sciences
Dartmouth College
Hanover, New Hampshire 03755

[2]The Marine Biological Laboratory
Woods Hole, Massachusetts 02543

[3]Dartmouth Electron Microscope Facility
Dartmouth College
Hanover, New Hampshire 03755

*Corresponding author: rds@dartmouth.edu, 603-646-2377 (fax: 603-646-1347)

Running head: Methylated flagellar proteins

Key words: flagella, protein methylation, *Chlamydomonas*, immunogold EM

ABSTRACT

Post-translational protein modification occurs extensively in eukaryotic flagella. Here we examine protein methylation, a protein modification that has only recently been reported to occur in flagella (Schneider et al. 2008). The cobalamin (vitamin B12) independent form of the enzyme methionine synthase (MetE), which catalyzes the final step in methionine production, is localized to flagella. Here we demonstrate, using immunogold scanning electron microscopy, that MetE is bound to the outer doublets of the flagellum. Methionine can be converted to S-adenosyl methionine, which then serves as the methyl donor for protein methylation reactions. Using antibodies that recognize symmetrically or asymmetrically methylated arginine residues, we identify three highly methylated proteins in intact flagella: two symmetrically methylated proteins of about 30 and 40 kDa, and one asymmetrically methylated protein of about 75 kDa. Several other relatively less methylated proteins could also be detected. Fractionation and immunoblot analysis shows that these proteins are components of the flagellar axoneme. Immunogold thin section electron microscopy indicates that the symmetrically methylated proteins are located in the central region of the axoneme, perhaps as components of the central pair complex and the radial spokes, while the asymmetrically methylated proteins are associated with the outer doublets.

INTRODUCTION

In 1992, six years after Bill Brinkley became editor-in-chief of *Cell Motility and the Cytoskeleton* following the untimely death of founding editor Robert D. Allen, Johnson and Rosenbaum (1992) demonstrated that tubulin and the radial spokes of *Chlamydomonas* flagella are delivered to the distal tip of the flagellar axoneme where assembly of the organelle occurs. Very shortly thereafter, the process of intraflagellar transport (IFT) was first observed in the Rosenbaum laboratory at Yale (Kozminski et al. 1993). IFT is characterized by the rapid, bidirectional movement of molecular motors and their associated cargo proteins back and forth along the length of cilia and flagella. IFT is necessary for organelle assembly and maintenance because IFT transports materials to the distal tip, the site of organelle growth and turnover, and returns components back to the cell body for degradation or recycling (Iomini et al. 2001; Kozminski et al. 1995). Analysis of mutants with defects in the process has provided abundant evidence that IFT plays an essential role not only in the morphogenesis of cilia and flagella but also in their maintenance. IFT is essential for numerous cellular and developmental processes that depend of flagellar or ciliary assembly, including mating in *Chlamydomonas*, sensory transduction, development of left-right asymmetry, vision, developmental signaling, and chemosensory behavior (see reviews by Blacque et al. 2008; Pan et al. 2005; Pedersen and Rosenbaum 2008; Sloboda and Rosenbaum 2007).

Currently, the basic mechanism of IFT is well understood. Two plus end directed motors, kinesin-2 (Kozminski et al. 1995) and OSM 3 (Snow et al. 2004), move particles in the anterograde direction (toward the tip); dynein 2 (Pazour et al. 1999; Porter et al. 1999; Signor et al. 1999) moves particles from the tip to the base in the retrograde direction. The particles themselves consist of the molecular motors and the IFT particle polypeptides used in the attachment of the motors to the cargo

being transported. The motors and IFT particle proteins have been isolated, cloned and sequenced (see Cole (2003) for review]. However, the exact mechanism by which IFT is regulated and how flagellar components are assembled onto or disassembled from the flagellar tip remains unclear.

Mechanisms that evolved to regulate IFT might require one or more posttranslational modifications of the IFT motor proteins, IFT complex A and/or B polypeptides, or related proteins. Such modifications could regulate the relative activities of the motors themselves or their interactions with IFT complex A or B polypeptides and cargo. Indeed, there are numerous examples of posttranslational modifications of flagellar proteins. Tubulin itself is phosphorylated (Piperno and Luck 1976) as are five radial spoke proteins (Piperno et al. 1981), several membrane/matrix proteins (Bloodgood 1992) and the alpha heavy chain of outer arm dynein (King and Witman 1994). When flagella are labeled in vivo by incubation of cells in the presence of ^{32}P-orthophosphate, more than 80 axonemal phosphoproteins can be resolved on 2-D gels (Piperno et al. 1981). In addition, the phosphorylation of flagellar proteins has been observed to change as flagellar activity changes (Bloodgood and Salomonsky 1994). Recent evidence suggests protein phosphorylation (and protein methylation) may play key roles in flagellar length control and/or stability. For example, several protein kinases have been implicated in the process of length control. A MAP kinase encoded by the LF4 gene, when mutated, results in flagella that are longer than normal (Berman et al. 2003), while the functions of two NIMA related kinases in *Chlamydomonas* are related to flagellar length control, flagellar severing, and cell cycle progression (Bradley and Quarmby 2005; Mahjoub et al. 2002). Another kinase, GSK3, is associated with *Chlamydomonas* flagella and is involved in length control (Wilson and Lefebvre 2004), and an aurora kinase translocates into flagella during gamete activation (Pan and Snell 2000) and is also involved in flagellar length control and flagellar excision (Pan et al. 2004). In vertebrates, aurora kinase is localized to the basal body of the primary cilium where it

phosphorylates HDAC6, a tubulin deacetylase, leading to disassembly of the primary cilium (Pugacheva et al. 2007).

In contrast to phosphorylation, observations related to flagellar protein methylation are less numerous, as this modification has only recently been reported in flagella. Specifically and only during flagellar resorption, four axonemal proteins become asymmetrically dimethylated, indicating a role for this modification in flagellar disassembly (Schneider et al. 2008). This modification occurs on arginine residues and involves the dimethylation of one of the two guanidino nitrogens of a target arginine residue; hence it is an asymmetric dimethylation. Protein methylation requires S-adenosyl methionine (SAM) as the methyl donor. The cobalamin (vitamin B12) independent form of the enzyme that produces methionine (methionine synthase, MetE) is present in the axoneme fraction of flagella (Schneider et al. 2008). The enzyme S-adenosyl methionine synthase, which produces SAM, is present in the membrane-matrix fraction of flagella (Pazour et al. 2005). Finally, the genome of *Chlamydomonas* encodes a class I protein arginine methyl transferase capable of methylating arginine residues, and the flagellar proteome has identified several proteins with this activity (Pazour et al. 2005). Thus, all of the components of a protein methylation pathway are likely to be present in flagella. Here, we examine full-length flagella for the presence of protein methylation activity, identify three methylated proteins in full-length flagella, and localize these proteins, and the enzyme MetE, in the axoneme.

MATERIALS AND METHODS

Cells and Antibodies

Chlamydomonas reinhardtii strain CC125, (wild type, mt+) were grown in 250 mL Erlenmeyer flasks containing 125 mL of sterile TAP medium (Gorman and Levine 1965) at 23°C on a cycle of 14 hours of light and 10 hours of dark, for four days, with continuous aeration. Antibodies to *Chlamydomonas* MetE were raised to a specific peptide (residues 667-684), characterized, and affinity purified as previously described (Schneider et al. 2008). Antibodies to symmetric dimethylated arginine (Sym11) and asymmetric dimethylated arginine (Asym24) were from Millipore. Antibodies to IFT139 were generously provided by Joel Rosenbaum and Dennis Diener (Yale University). These antibodies were raised using purified IFT particles as the immunogen, followed by the selection of cell lines secreting antibodies specific for IFT139 (see (Cole et al. 1998).

Preparation of the Membrane-Matrix and Axoneme Fractions of Flagella

Following flagellar isolation (Schneider et al. 2008), samples were adjusted to 2 mM pefablock (a protease inhibitor) and 0.5% Nonidet P-40 and rocked gently for 30 minutes at room temperature to extract the membrane lipids and release the membrane plus matrix proteins. The insoluble axonemes were collected at 12,000 RPM for 15 minutes at 4°C in the Sorvall SS34 rotor. The supernatant, containing the membrane-matrix proteins, was removed and the axonemes were suspended in HMDEK.

Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was performed using the buffer formulations of Laemmli (1970) on 4 - 10% acrylamide gradient gels containing 2 - 8M urea. The gels were stained with Coomassie Blue R - 250 (25% isopropanol, 10% acetic acid, 0.1% Coomassie Blue R - 250) overnight and then destained in 10% glacial acetic acid, 10% isopropanol. Immunoblot detection of methylated antigens was carried out as described previously (Schneider et al. 2008).

Immunocytochemistry and Fluorescence Microscopy

Clean coverslips were coated with 0.1% polyethyleneimine (PEI) in water for five minutes, washed with three changes of distilled water, and air dried. Coverslips were coated the day of an experiment. Samples of flagella were placed on a PEI coated coverslip for 10 minutes at room temperature. The coverslips were then placed in methanol or acetone at -20°C for 20 min. The coverslips were removed from the fixative, air dried, rehydrated in Tris buffered saline (TBS) (20 mM Tris, 0.9% NaCl, pH 7.4) and placed in blocking buffer (5% milk in TBS + 0.02% NaN_3) overnight at 4°C. The coverslips were then incubated for two hours at room temperature in primary antibodies diluted 1:200 in blocking buffer. The coverslips were washed three times in TBS for ten minutes each and then incubated with goat anti-mouse or goat anti-rabbit antibodies (Southern Biotechnology Associates, Inc.) diluted 1:1000 in blocking buffer for one hour at room temperature. The coverslips were then washed three times in TBS for ten minutes each, mounted on slides using Prolong Gold antifade reagent (Molecular Probes) and left in the dark overnight at room temperature. The slides were then sealed with clear nail polish prior to viewing.

Samples were viewed with a Zeiss Axioskop 2 mot plus microscope using a 63x/1.4 NA plan apochromatic objective under software control via MetaMorph (Molecular Devices).

Images generated by MetaMorph were manipulated for further assembly into figures using software written in MatLab (Schneider et al. 2008). No alterations of figure contrast or content from that represented by the original micrographs were performed, except for the cropping and lettering that occurred during the assembly of the figures.

Transmission Electron Microscopy

For transmission electron microscopy, flagella were isolated as previously described (Schneider et al. 2008). The final flagellar pellet was then overlaid with one ml of 4% paraformaldehyde in HMDEK (10 mM Hepes pH 7.5, 1mM DTT, 25 mM KCl, 0.5 mM EDTA, 5 mM $MgSO_4$). The samples were placed at room temperature for one hour and at 4°C overnight. Next, the samples were dehydrated and embedded in LR White, sectioned, and placed on Ni grids. Sections in LR white were processed for immunogold electron microscopy as follows. First, the grids were floated on a drop of PBS (4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.4) for 15 minutes and then on a drop of blocking buffer (2% BSA, 0.1% gelatin, 0.05% Tween-20 in PBS) for 30 minutes at room temperature. The grids were then placed on a drop of primary antibody in blocking buffer for two hours at room temperature. The samples were washed by floating on drops of PBS three times for five minutes each and then placed onto drops of secondary antibody (goat anti-rabbit labeled with 25 nM gold particles, from Electron Microscopy Sciences), diluted 1:20 in blocking buffer, for 60 minutes at room temperature. The grids were again washed three times in PBS and then twice in distilled water for five minutes each and air dried. Finally, the grids were stained with 2% aqueous uranyl acetate for seven min followed by Reynold's lead citrate for a maximum of 10 seconds. Samples were then viewed at 100kV using a JEOL TEM1010.

Immunogold Scanning Electron Microscopy

Immunogold scanning EM with a field emission gun (FEG-SEM) was carried out as previously described (Sloboda and Howard 2007) with the exception that here, intact cells instead of isolated flagella were used. To work with intact cells the procedure was modified as follows. Cells suspended in HMDEK were placed onto round coverslips (12 mm diameter) that had been previously coated with PEI as described above under Immunocytochemistry and allowed to adhere for 10 min. The coverslips were rinsed by dipping them sequentially in three beakers containing HMDEK and then inverted onto a drop of HMDEK containing 0.05% Nonidet P-40. After 30 seconds of extraction, the coverslips were rinsed as before and then floated on drops of antibody exactly as described for immunogold transmission EM above. After the wash step following the second antibody, the coverslips were floated on a solution of 2% glutaraldehyde in HMDEK for one hour, rinsed, and air dried. The samples were then critical point dried and coated with 2-3 nm of osmium as described (Sloboda and Howard 2007) and viewed at 15 kV with a FEI XL-30 field emission gun scanning EM using a spot size of 3. This generates a scan probe having a diameter of 1.7 nm. The instrument was operated both in conventional mode (imaging secondary electrons; see Figure 1) as well as in backscatter mode (which provides a clear image of the gold particles at high contrast; see Figure 2).

RESULTS

Methionine synthase (MetE) in the intact flagellum

MetE is the enzyme that synthesizes methionine from homocysteine. This enzyme is present in the flagellar proteome and recently Schneider et al. (2008) showed that this enzyme is associated with the axoneme fraction isolated from *Chlamydomonas* flagella. In addition, the amount of MetE present in flagella increases both during flagellar regeneration and flagellar shortening (Schneider et al, 2008). Figure 1A shows an intact *Chlamydomonas* cell with its characteristic two flagella viewed by scanning EM. Figure 1B shows another cell that was attached to a coverslip, extracted with 0.05% NP-40 for 30 seconds, and then prepared for immunogold scanning electron microscopy. Images were generated using an instrument with a field emission gun (FEG-SEM) as reported previously for isolated flagella (Sloboda and Howard 2007). When treated briefly with detergent as described here, the flagellar membrane lipids do not extract globally, *i. e.* they do not extract along the entire flagellar length at once. Rather, lipid extraction begins first at the distal end of the flagellum and then moves progressively toward the base as the length of time in the detergent increases. In figure 1B, the distal half of the flagellar membrane of this cell has been extracted, while the proximal half of the membrane has not, and is thus still relatively intact. This is indicated by the 'refractile' appearance of the proximal half of the flagellum as compared to the distal half. Figure 1C shows the apical tip of an extracted *Chlamydomonas* flagellum in which the outer double microtubules have begun to splay apart. This is characteristic of the tips of *Chlamydomonas* flagella after detergent extraction (Dentler and Rosenbaum 1977). This phenomenon can also be noted in the lower power image of the flagellum in Figure 1B, as well as in the enlarged

inset in Figure 1B. In the higher power image of Figure 1C, the nine individual outer doublets can be resolved, as well as the comparatively twisted central pair complex. These are numbered 1-9 and CP, respectively, in Figure 1C.

Higher power views of extracted flagellar tips labeled with 25 nm gold particles, indicating the presence of the enzyme MetE, are shown in Figure 2, panels A - D. MetE antibodies are bound to some, but not all, of the axonemal outer doublets, and there does not appear to be a regular or a defined pattern to this association. These observations confirm the immunofluorescence data previously reported (Schneider et al. 2008) and extend that information by showing that MetE is associated not only with the axoneme, determined previously by immunoblotting (Schneider et al. 2008), but also more specifically with the outer doublets themselves. In order to increase the number of flagella that attach and spread on the polyethylene imine coated coverslips, we used pf18 cells for this analysis, as the beating of wild type cells tended to complicate the attachment of the flagella. The pf18 mutation results in complete loss of the central pair apparatus, resulting in flagella that do not beat. Hence it is not possible from these data to determine if MetE is also associated with the central pair. Note also that in the FEG-SEM images of Figure 2, which are generated by backscattered electrons, no gold particles are apparent in the background, attesting to the specificity of the labeling shown here.

Protein methylation in full-length flagella

The enzyme MetE functions in the terminal step in methionine biosynthesis, generating methionine from homocysteine. S-adenosyl methionine (SAM, or AdoMet) is then produced from methionine by SAM synthase (Figure 3). SAM functions as the methyl donor for protein

methylation reactions, which occur most often on lysine or arginine residues. There are three structural classes of SAM-dependent methyltransferases (Bedford 2007), and the class I enzymes modify arginine, producing three different modifications of the arginine guanidino R group: monomethyl arginine (MMA), symmetric dimethyl arginine (sDMA), and asymmetric dimethyl (aDMA) arginine. Class I enzymes are thus protein arginine methyl transferases (PRMTs). Previously, Schneider et al. (2008) showed that asymmetric dimethylation of arginine residues occurs on four axonemal proteins, but only when the flagellar are induced to resorb. Immunofluorescence studies of resorbing flagella have revealed a punctate staining pattern due to these asymmetrically dimethylated flagellar proteins (Schneider et al. 2008). This pattern of staining is similar, but not identical, to the punctate staining of IFT particles.

When immunoblots of samples of full-length flagella are probed with antibodies to symmetric dimethyl arginine, the presence of two polypeptides can be detected in intact, full-length flagella and in the axoneme fraction, but not in the membrane/matrix fraction (Figure 4, Sym11 antibodies). These proteins migrate at relative molecular masses of approximately 30,000 and 40,000; a third protein that is much less reactive with the Sym11 antibodies can be observed migrating at a relative molecular mass of about 33,000. By comparison, asymmetrically dimethylated arginine can be detected clearly in a polypeptide migrating with a relative molecular mass of approximately 75,000 in samples of full-length flagella (Figure 4, Asym24 antibodies). These Asym24 antibodies also detected several proteins in full-length flagella that migrate at 250 kDa or larger; these proteins are much less reactive with the Asym24 antibodies than is the 75 kDa protein, however. Finally, note that the 75 kDa protein is one of four proteins the asymmetric methylation of which greatly increases during flagellar resorption (Schneider et al. 2008).

Based on relative migration, the symmetrically dimethylated polypeptides are clearly different than the asymmetrically dimethylated proteins that are produced during flagellar resorption, which are 56 – 75 kDa in size (see Schneider et al. 2008). The two symmetrically methylated proteins are tightly bound to the axoneme, because they are not removed by various extraction procedures, including 10 mM Mg-ATP, 0.5M NaCl, or 10 mM Mg-ATP plus 0.5M NaCl (Figure 4). Like antibodies to asymmetrically dimethylated arginine residues, however, antibodies to symmetrically dimethylated arginine residues label isolated flagella in a punctate pattern (Figure 5). As mentioned above, this pattern of labeling is reminiscent of IFT particle staining. However, the two classes of proteins are clearly not always coincident (compare the staining pattern of IFT 139 [green], an IFT complex A polypeptide, with that of symmetric dimethyl arginine [red] in the same flagella of Figure 5). This observation, coupled to the extraction and immunoblot data presented above (Figure 4), indicates that the two symmetrically methylated proteins present in full-length flagella are components of the axoneme and are not IFT particle related. This conclusion is confirmed by the following electron microscopic data.

Localization of methylated proteins by transmission electron microscopy

To define more closely the localization of these symmetrically dimethylated proteins in the axoneme we used thin section immunogold electron microscopy. Figure 6 shows a low power image of a field of sectioned flagella that has been labeled with antibodies to symmetric dimethyl arginine followed by 25 nm gold labeled secondary antibodies. Two flagella are labeled with a gold particle, and this labeling is specific to the axoneme, as no gold particles can be observed in regions of this section (or other sections, see following figures) not occupied by a flagellum. Figure 7 shows a collage of flagellar sections (including the two from Figure 6) in

which gold particles label the axoneme. The gold labels either the central region of the axoneme or the inner aspect of the outer double array. Hence, it appears that the symmetrically methylated proteins are localized inside the outer doublet array, perhaps components of the radial spokes (see Discussion).

By contrast, asymmetric protein methylation in full-length flagella (Figure 4) occurs on one or more proteins associated with the outer doublet microtubules (Figure 8). Gold particles in this collage of cross sections are associated only with the outer doublets, occasionally with their outer surfaces. No gold particles identifying asymmetrically methylated residues were observed in the central region of the axoneme. These data indicate that a protein, most likely the ~75 kDa polypeptide, containing asymmetrically methylated residues is associated with a different axonemal component than either of the two symmetrically methylated polypeptides identified in the immunoblot of Figure 4.

DISCUSSION

In this report we have extended here the catalog of known flagellar proteins containing posttranslational methylation modifications. Using antibodies to asymmetrically dimethylated arginine residues we have identified a single, major methylated protein in full-length flagella that carries this modification. Most likely, this 75 kDa protein is the same one noted previously in studies of protein methylation activity during flagellar resorption (Schneider et al. 2008). During resorption, four proteins in the range of 50 – 75 kDa become methylated. Three of these proteins cannot be detected in full-length flagella. The fourth, migrating at 75 kDa, was reported by Schneider et al. (2008) to be present in full length flagellar. This protein is either present in full-length flagella at a lower stoichiometry to the other three and/or it contains fewer methyl modifications as compared to the same protein in resorbing flagella (see Schneider et al. 2008).

By contrast, two other proteins in full-length flagella have been shown here to contain symmetric, dimethyl arginine residues. These proteins have masses of approximately 30 kDa and 40 kDa, and their identities are as yet unknown. However, it is interesting to note a previous report (Multigner et al. 1992) in which histone H1 was shown to play a role in stabilizing the axoneme of sea urchin flagella. Given the extensive literature on the role of histone methylation in transcriptional activation and repression, and a growing interest in posttranslational modification via protein methylation (Bedford and Richard 2005), it is intriguing to speculate on the identity of the smaller of the two symmetrically methylated flagellar proteins identified here. Perhaps the smaller of these two proteins is histone H1. We are currently in the process of purifying these proteins to enable their unambiguous identification.

The observation of methylated proteins in the flagellum suggests, although it does not prove, that the enzymes for these modifications are resident in the flagella. Alternatively, flagellar proteins could be methylated in the cell body and then transported into the flagellum. Several lines of evidence suggest the enzymatic machinery for protein methylation is resident in the flagellum, however. Protein methylation requires a source of methionine, which is converted to S-adenosyl methionine, and the latter is used as the methyl donor. MetE, the enzyme that synthesizes methionine from homocysteine is a member of the flagellar proteome (Pazour et al. 2005) and has been localized to full length flagella by immunofluorescence and to the flagellar axoneme of full length flagella by immunoblotting (Schneider et al. 2008). Here, we show at the resolution of the electron microscope that MetE is bound to the outer doublets (Figure 2). Finally, the methylation of four proteins dramatically increases upon the induction of flagellar resorption (Schneider et al. 2008), suggesting a role for this modification in destabilizing key protein-protein interactions required for flagellar stability. Alternatively, methylation may promote the association of disassembling flagellar components with the retrograde IFT apparatus.

Methylation of arginine residues can take several forms, and two of these have been noted in the data reported here. Symmetric dimethyl arginine (sDMA) results when each of the two guanidino nitrogens of arginine is modified with a single methyl group. By contrast, asymmetric dimethyl arginine (aDMA) results when one of the two guanidino nitrogens carries two methyl groups. Methyl modification of arginine residues is catalyzed by protein arginine methyl transferases (PRMTs) which are Class I methyltransferases that use S-adenosyl methionine as the methyl donor (there are three Classes of SAM-dependent methyl transferases). Class I methyl transferases (the PRMTs) modify arginine residues, and there are

four types of Class I PRMTs (Bedford 2007). Type 1 PRMTs produce aDMA while type II PRMTs produce sDMA. Thus, it would appear that the flagella of *Chlamydomonas* must contain both type 1 and type 2 PRMTs if in fact the methylation of the proteins noted here is carried out within the flagellum. From a genomic perspective, there are six genes in the *Arabidopsis* genome with homology to PRMTs, and one of these is a Class I PRMT (accession # NP_563720) that his significant similarity to an as yet uncharacterized *Chlamydomonas* gene (accession # XP_001702822). These PRMTs from *Arabidopsis* and *Chlamydomonas* share key sequence features, including the conserved methyl transferase region I motif (Lin et al. 1996) at residue 66 (^{66}VLDVGSGTG in *Chlamydomonas*; in *Arabidopsis* this sequence is ^{74}VLDVGTGSG). Although the flagellar proteome contains a number of methyltransferase-like enzymes, the identity and localization of the enzymes responsible for the methylations reported here are currently unknown. Relative to the pathway outlined in Figure 3, however, vitamin B12 (cobalamin) independent methionine synthase (MetE) is an axonemal component (Figure 2); SAM synthase and AdoHcy hydrolase are present in the membrane-matrix fraction of the flagellar proteome (Pazour et al. 2005) and are thus, by contrast, most likely soluble components of the axoneme. Perhaps the binding of MetE to the axoneme helps to ensure an even distribution of this activity along the length of the flagellum.

We noticed a slight but consistent difference in the immunogold localization of proteins with sDMA modifications as compared to aDMA modifications (see Figures 7 and 8). The two proteins containing the sDMA modification appear to be restricted to the interior of the axoneme (Figure 7), while the protein containing the aDMA modification appears to be localized to the outer doublets (Figure 8). Recall that there are at least two different flagellar polypeptides carrying sDMA modifications that reacted strongly with the Sym11 antibodies

(Figure 4) and that are contributing to the localization seen in Figure 7. The Sym11 antibodies recognize specifically sDMA modified arginine residues, not the proteins themselves. In some images the localization appears to be more central in the axoneme (Figure 7, panels A, H, I) while in other images the labeling appears to be closer to the outer doublets (Figure 7, Panels B – G), but still within the boundary of the outer doublet array. Thus, it is very likely, though not proved by these data, that one of the sDMA containing polypeptides is localized in the region of the central pair complex and another with the radial spokes, perhaps where the spokes bind to the outer doublets. Resolution of this hypothesis will have to await the identification of these polypeptides and the availability of specific antibodies to them.

Finally, what can be said concerning resolution with respect to the localization of specific antigens via indirect (*i. e.* primary plus secondary antibodies) immunogold thin section electron microscopy? IgG molecules have the following dimensions: 14.5 nm x 8.5 nm x 4 nm (Lee et al. 2002; Silverton et al. 1977). A single 25 nm gold particle, including the mass of the secondary antibody bound to it, has an effective diameter of ~50 nm, because each gold particle is surrounded by a halo of IgG molecules (see Sloboda and Howard, 2007, inset to Figure 6b) bound to the gold particle by their Fc tails with their antigen combining sites projecting outward. Thus, the maximum distance a gold particle might be from the actual antigen in the sample would be ~30 nm, or the sum of the lengths of the primary and secondary antibodies. This distance is slightly greater than the diameter of a single microtubule in cross section (or the diameter of the gold particle itself).

With respect to the data from Figure 7, then, antibodies to sDMA residues detect an antigen that likely resides inside the outer doublet array. In sDMA labeled samples, some gold particles are associated with the center of the axoneme, as demonstrated by the labeling in

Figure 7 (panels A, H, and I), while other gold particles are closer to the inside aspect of the outer doublets (panels B – G). Given the resolution noted in the preceding paragraph, it is reasonable to conclude that the sDMA modified proteins may be components of the central pair apparatus and the radial spokes. With respect to the aDMA containing proteins, localization of gold particles was always observed either coincident with the outer doublet microtubules (Figure 8, panels A – G) or within one gold particle diameter of them (panels H, I). Thus, although the aDMA modified proteins are neither α- nor β-tubulin, as demonstrated by the immunoblot data shown in Figure 4, they may be components of the dynein arms, given that the aDMA antigen is localized to the outer doublets (Figure 8).

ACKNOWLEDGEMENTS

We thank Megan Ulland and Rita Werner-Peterson for their sequential, and expert, technical assistance with this project. This work was supported by NIH DK071720 (rds) and NSF MCB 0418877 (rds). Finally, rds would like to thank Bill Brinkley for his leadership of and dedication to this journal and, most importantly, for his friendship and support over the past three decades.

LITERATURE CITED

Bedford MT. 2007. Arginine methylation at a glance. J Cell Sci 120(Pt 24):4243-6.

Bedford MT, Richard S. 2005. Arginine methylation an emerging regulator of protein function. Mol Cell 18(3):263-72.

Berman SA, Wilson NF, Haas NA, Lefebvre PA. 2003. A novel MAP kinase regulates flagellar length in Chlamydomonas. Curr Biol 13(13):1145-9.

Blacque OE, Cevik S, Kaplan OI. 2008. Intraflagellar transport: from molecular characterisation to mechanism. Front Biosci 13:2633-52.

Bloodgood RA. 1992. Calcium-regulated phosphorylation of proteins in the membrane-matrix compartment of the Chlamydomonas flagellum. Exp Cell Res 198(2):228-36.

Bloodgood RA, Salomonsky NL. 1994. The transmembrane signaling pathway involved in directed movements of Chlamydomonas flagellar membrane glycoproteins involves the dephosphorylation of a 60-kD phosphoprotein that binds to the major flagellar membrane glycoprotein. J Cell Biol 127(3):803-11.

Bradley BA, Quarmby LM. 2005. A NIMA-related kinase, Cnk2p, regulates both flagellar length and cell size in Chlamydomonas. J Cell Sci 118(Pt 15):3317-26.

Cole DG. 2003. The intraflagellar transport machinery of Chlamydomonas reinhardtii. Traffic 4(7):435-42.

Cole DG, Diener DR, Himelblau AL, Beech PL, Fuster JC, Rosenbaum JL. 1998. Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons. J Cell Biol 141(4):993-1008.

Dentler WL, Rosenbaum JL. 1977. Flagellar elongation and shortening in Chlamydomonas. III. structures attached to the tips of flagellar microtubules and their relationship to the directionality of flagellar microtubule assembly. J Cell Biol 74(3):747-59.

Gorman DS, Levine RP. 1965. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. Proc Natl Acad Sci U S A 54(6):1665-9.

Iomini C, Babaev-Khaimov V, Sassaroli M, Piperno G. 2001. Protein particles in Chlamydomonas flagella undergo a transport cycle consisting of four phases. J Cell Biol 153(1):13-24.

Johnson KA, Rosenbaum JL. 1992. Polarity of flagellar assembly in Chlamydomonas. J Cell Biol 119(6):1605-11.

King SM, Witman GB. 1994. Multiple sites of phosphorylation within the alpha heavy chain of Chlamydomonas outer arm dynein. J Biol Chem 269(7):5452-7.

Kozminski KG, Beech PL, Rosenbaum JL. 1995. The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with flagellar membrane. J. Cell Biol. 131:1517-1527.

Kozminski KG, Johnson KA, Forscher P, Rosenbaum JL. 1993. A motility in the eukaryotic flagellum unrelated to flagellar beating. Proc Natl Acad Sci U S A 90(12):5519-23.

Lee KB, Park SJ, Mirkin CA, Smith JC, Mrksich M. 2002. Protein nanoarrays generated by dip-pen nanolithography. Science 295(5560):1702-5.

Lin WJ, Gary JD, Yang MC, Clarke S, Herschman HR. 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. J Biol Chem 271(25):15034-44.

Mahjoub MR, Montpetit B, Zhao L, Finst RJ, Goh B, Kim AC, Quarmby LM. 2002. The FA2 gene of Chlamydomonas encodes a NIMA family kinase with roles in cell cycle progression and microtubule severing during deflagellation. J Cell Sci 115(Pt 8):1759-68.

Multigner L, Gagnon J, Van Dorsselaer A, Job D. 1992. Stabilization of sea urchin flagellar microtubules by histone H1. Nature 360(6399):33-9.

Pan J, Snell WJ. 2000. Regulated targeting of a protein kinase into an intact flagellum. An aurora/Ipl1p-like protein kinase translocates from the cell body into the flagella during gamete activation in Chlamydomonas. J Biol Chem 275(31):24106-14.

Pan J, Wang Q, Snell WJ. 2004. An aurora kinase is essential for flagellar disassembly in Chlamydomonas. Dev Cell 6(3):445-51.

Pan J, Wang Q, Snell WJ. 2005. Cilium-generated signaling and cilia-related disorders. Lab Invest 85(4):452-63.

Pazour GJ, Agrin N, Leszyk J, Witman GB. 2005. Proteomic analysis of a eukaryotic cilium. J Cell Biol 170(1):103-13.

Pazour GJ, Dickert BL, Witman GB. 1999. The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. J Cell Biol 144(3):473-81.

Pedersen LB, Rosenbaum JL. 2008. Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. Curr Top Dev Biol 85:23-61.

Piperno G, Huang B, Ramanis Z, Luck DJ. 1981. Radial spokes of Chlamydomonas flagella: polypeptide composition and phosphorylation of stalk components. J Cell Biol 88(1):73-9.

Piperno G, Luck DJ. 1976. Phosphorylation of axonemal proteins in Chlamydomonas reinhardtii. J Biol Chem 251(7):2161-7.

Porter ME, Bower R, Knott JA, Byrd P, Dentler W. 1999. Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in Chlamydomonas. Mol Biol Cell 10(3):693-712.

Pugacheva EN, Jablonski SA, Hartman TR, Henske EP, Golemis EA. 2007. HEF1-dependent Aurora A activation induces disassembly of the primary cilium. Cell 129(7):1351-63.

Schneider MJ, Ulland M, Sloboda RD. 2008. A protein methylation pathway in Chlamydomonas flagella is active during flagellar resorption. Mol Biol Cell 19(10):4319-27.

Signor D, Wedaman KP, Orozco JT, Dwyer ND, Bargmann CI, Rose LS, Scholey JM. 1999. Role of a class DHC1b dynein in retrograde transport of IFT motors and IFT raft particles along cilia, but not dendrites, in chemosensory neurons of living Caenorhabditis elegans. J Cell Biol 147(3):519-30.

Silverton EW, Navia MA, Davies DR. 1977. Three-dimensional structure of an intact human immunoglobulin. Proc Natl Acad Sci U S A 74(11):5140-4.

Sloboda RD, Howard L. 2007. Localization of EB1, IFT polypeptides, and kinesin-2 in Chlamydomonas flagellar axonemes via immunogold scanning electron microscopy. Cell Motil Cytoskeleton 64(6):446-60.

Sloboda RD, Rosenbaum JL. 2007. Making sense of cilia and flagella. J Cell Biol 179(4):575-82.

Snow JJ, Ou G, Gunnarson AL, Walker MR, Zhou HM, Brust-Mascher I, Scholey JM. 2004. Two anterograde intraflagellar transport motors cooperate to build sensory cilia on C. elegans neurons. Nat Cell Biol 6(11):1109-13.

Wilson NF, Lefebvre PA. 2004. Regulation of flagellar assembly by glycogen synthase kinase 3 in Chlamydomonas reinhardtii. Eukaryot Cell 3(5):1307-19.

FIGURE LEGENDS

Figure 1: Scanning electron microscopy of an intact *Chlamydomonas* cell and its two flagella. Panel A: An intact cell prepared for FEG-SEM without any prior experimental manipulation. Panel B: A cell extracted for 30 seconds with 0.05% NP-40. Note that the flagellar membrane has not been extracted along its entire length; the proximal 2/5 is still intact. Rather, extraction proceeds in the distal to proximal direction along the flagellum with increasing extraction time. The inset in this panel shows the tip of the flagellum at a higher magnification (2.25x that of panel B). Panel C: An extracted flagellum that has splayed completely apart at the tip. The numbers identify nine outer doublets, but are not meant to be specific for outer doublets as identified in cross sections. CP = central pair. Panels A – C are conventional SEM images generated by secondary electrons. The scale bar in A = 5 μm; B = 2 μm; C = 500 nm.

Figure 2: Four representative FEG-SEM images of extracted flagella labeled for immunogold (25 nm) detection of MetE. These images were generated by backscattered electrons. Operation in the backscatter mode clearly shows the gold particles as white dots. The scale bar = 500 nm.

Figure 3: Diagram of the relationship of a subset of the enzymes that catalyze methionine metabolism and protein methylation. MetE (vitamin B12 independent methionine synthase, EC 2.1.1.14) is an axonemal component (Figure 2), while SAM synthase and AdoHcy hydrolase are components of the membrane-matrix fraction (Pazour et al. 2005). The flagellar localization of PRMT (protein arginine methyl transferase) is currently unclear, as the flagellar proteome contains a number of proteins with sequence similarity to methyl transferases.

Figure 4: Immunoblot of whole flagella probed with antibodies to symmetric dimethyl arginine (Sym11, left) and asymmetric dimethyl arginine (Asym24, right). Flag = whole flagella; MM (or M) = membrane matrix fraction; Axo (or Ax) = axoneme.

Figure 5: Immunofluorescence localization of proteins carrying symmetrically dimethylated arginine residues and IFT 139, an IFT complex A polypeptide, in isolated flagella. In the collage of images shown here, IFT139 is imaged in green and symmetric dimethyl arginine in red; colocalization is in yellow.

Figure 6: Localization of symmetric, dimethyl arginine (sDMA) residues by immunogold transmission electron microscopy. Shown here is a low power field of sectioned flagella to indicate the specificity of labeling and the total absence of non-specific labeling, the latter as evidenced by the lack of gold particles where there are no flagella.

Figure 7: A collage of representative images of flagella labeled with sDMA antibodies (including the two images shown in the previous figure), here revealed by 25 nm gold particles, indicating the presence of proteins modified with symmetric dimethyl arginine. The gold in panels A, H, and I is localized more centrally in the axoneme, while in panels B – G the gold label is associated with the inner aspect of the outer double array.

Figure 8: Localization of asymmetric, dimethyl arginine (aDMA) residues by immunogold transmission electron microscopy. The collage (Panels A – I) shows that aDMA is absent from the center of the axoneme and instead appears to be associated with the outer doublets.

Figure 1A-C
Sloboda and Howard

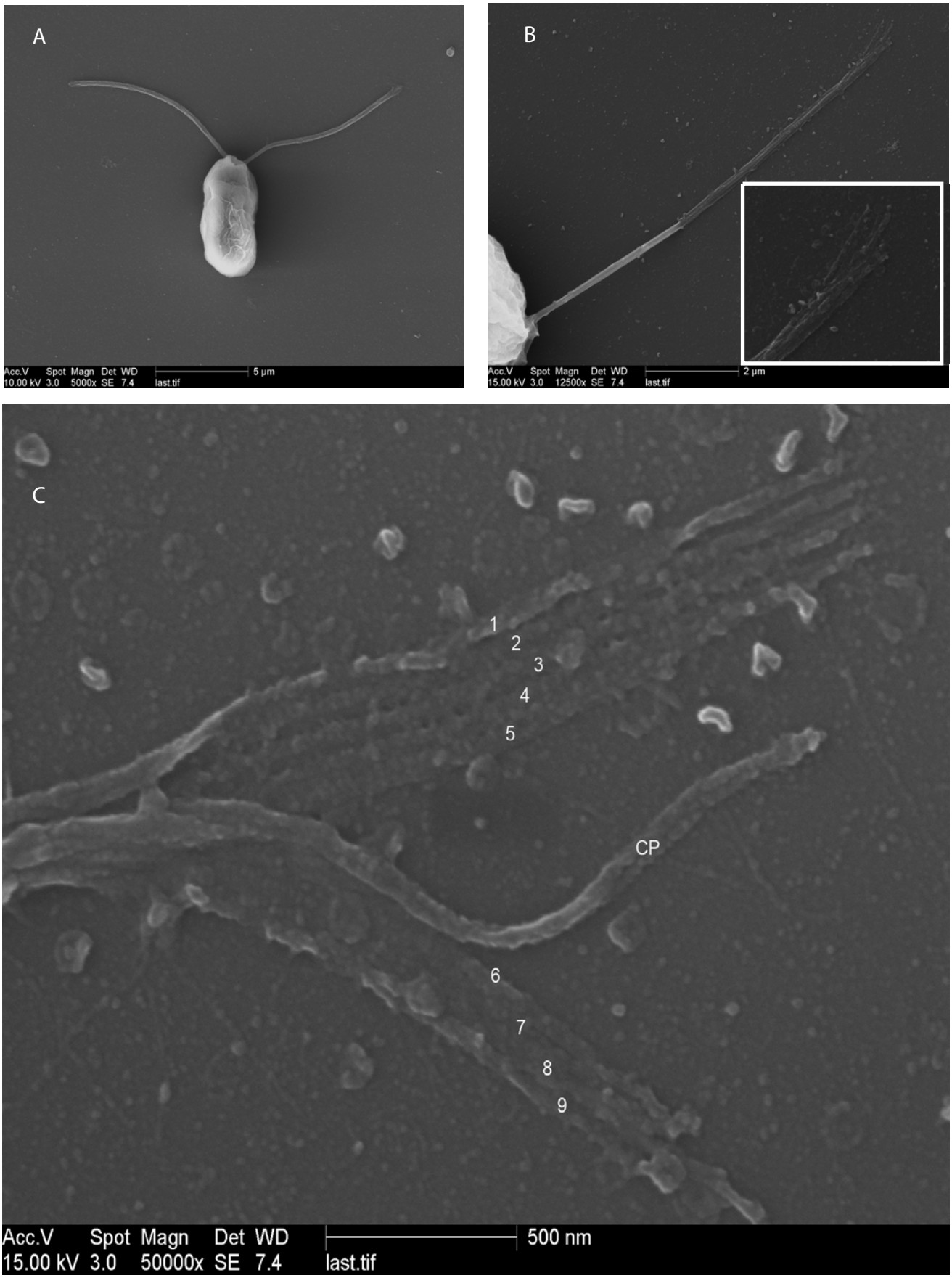

Figure 2A-D
Sloboda and Howard

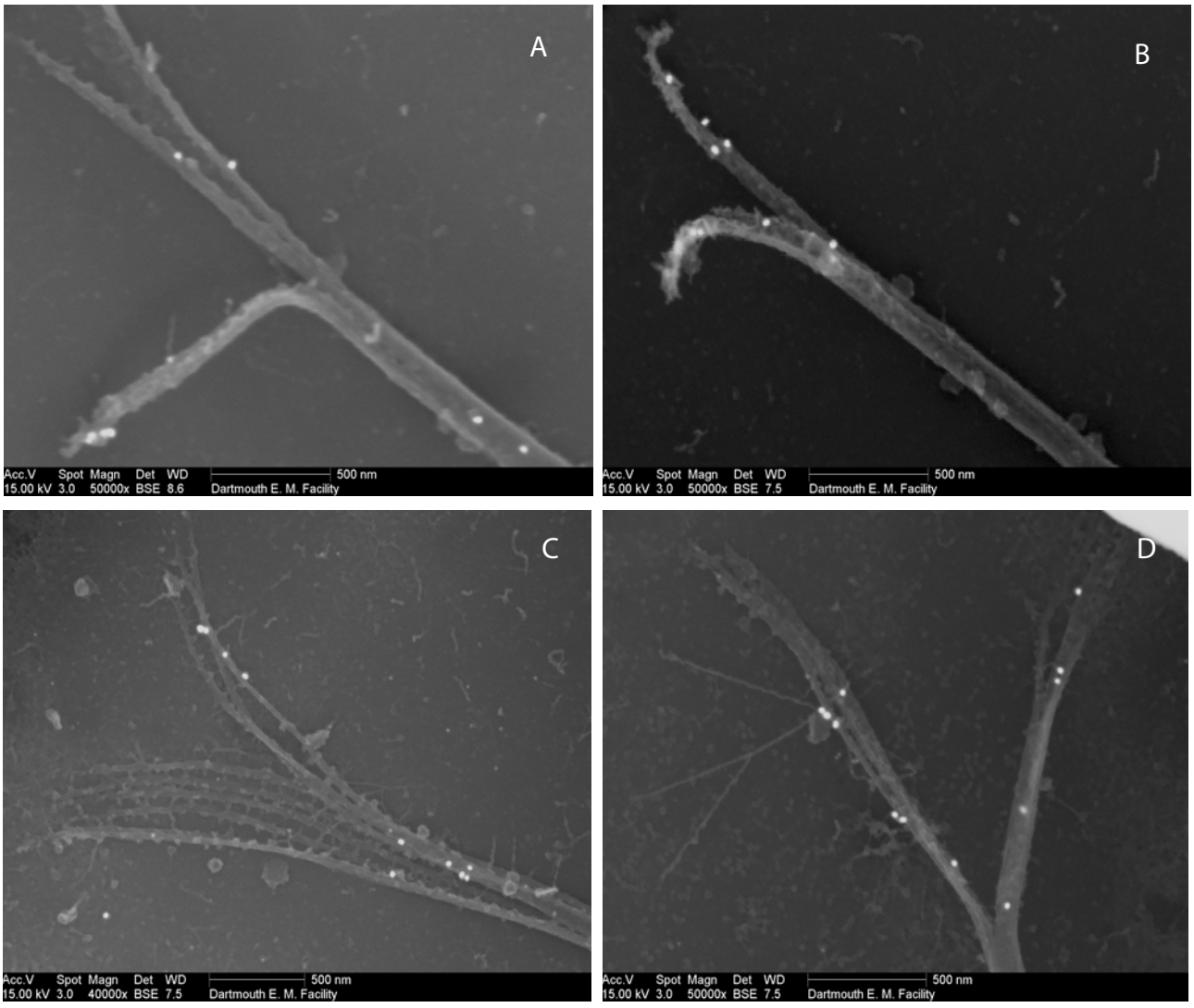

Figure 3
Sloboda and Howard

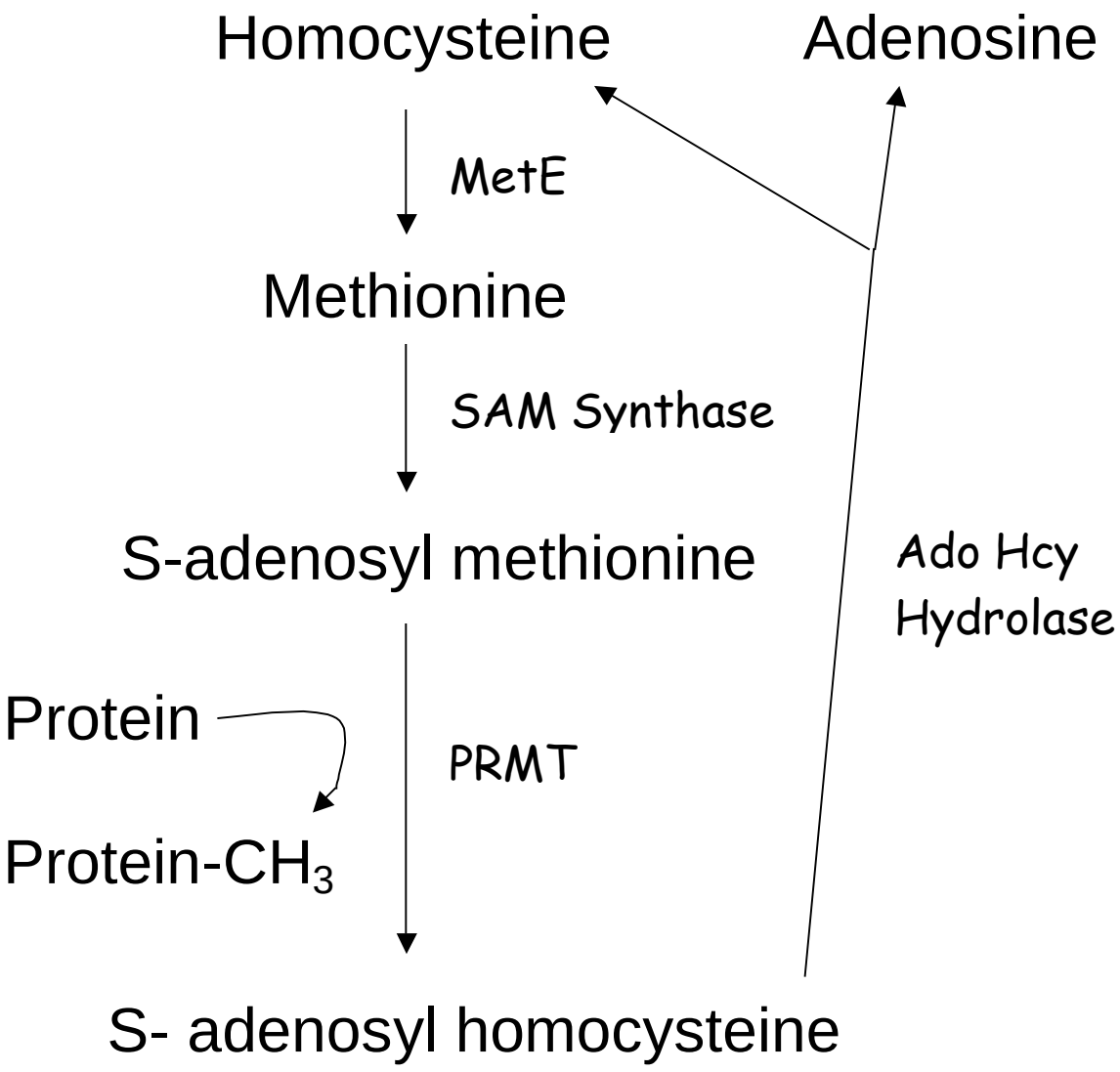

Figure 4
Sloboda and Howard

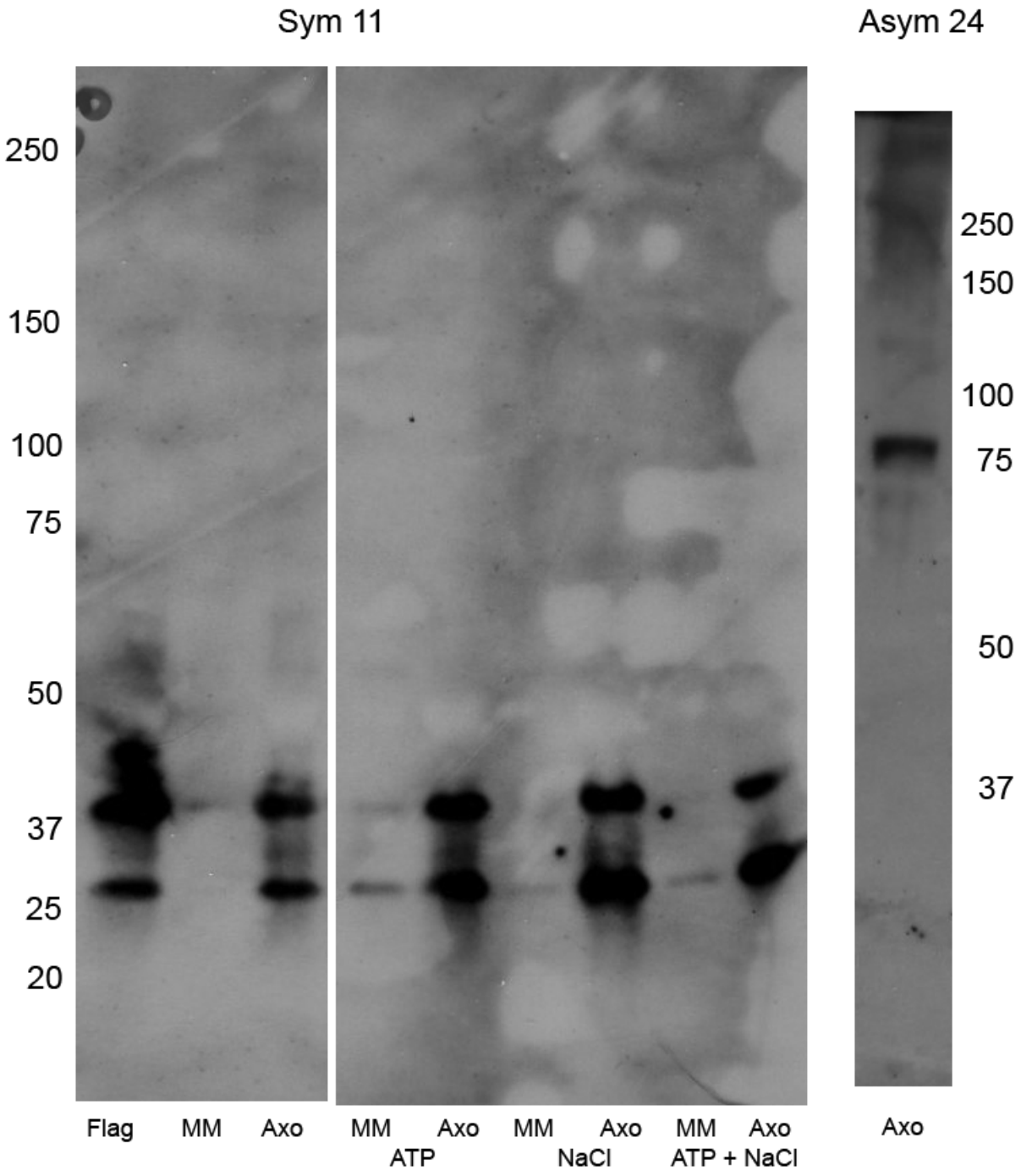

Figure 5
Sloboda and Howard

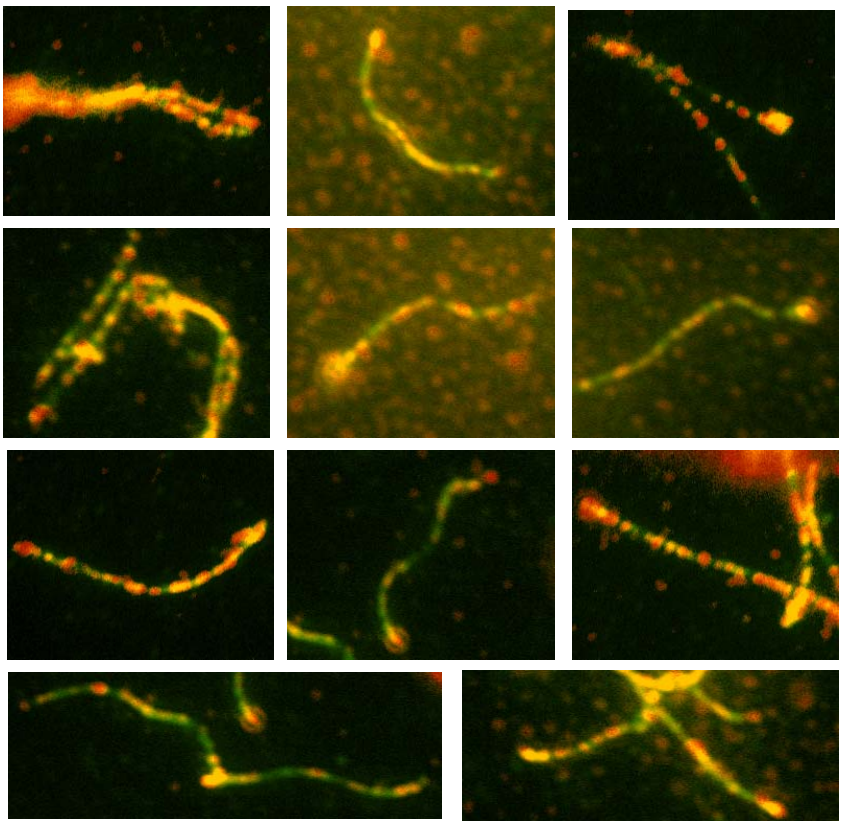

Figure 6
Sloboda and Howard

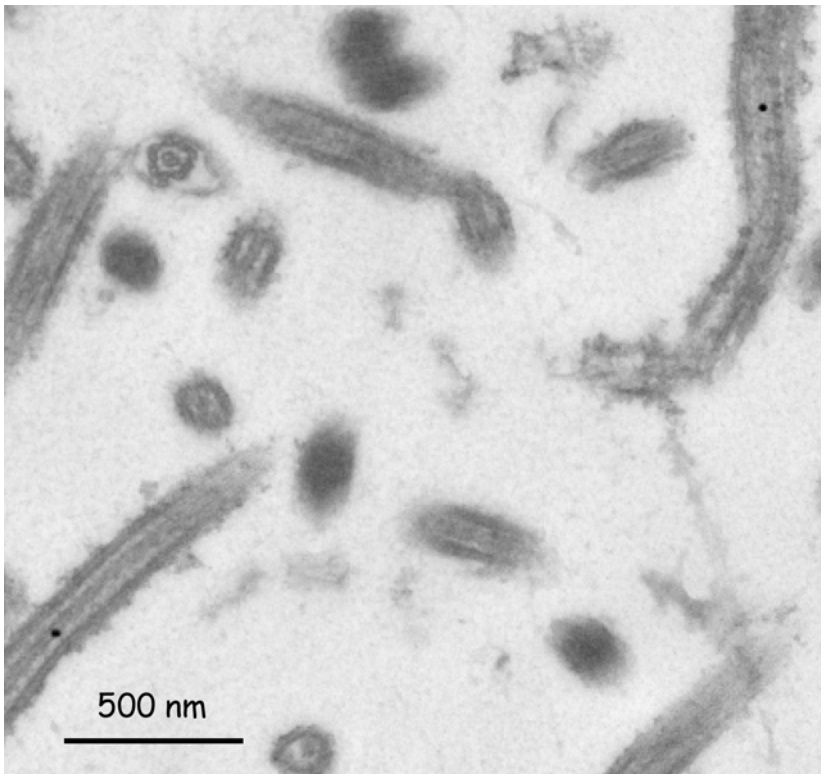

Figure 7
Sloboda and Howard

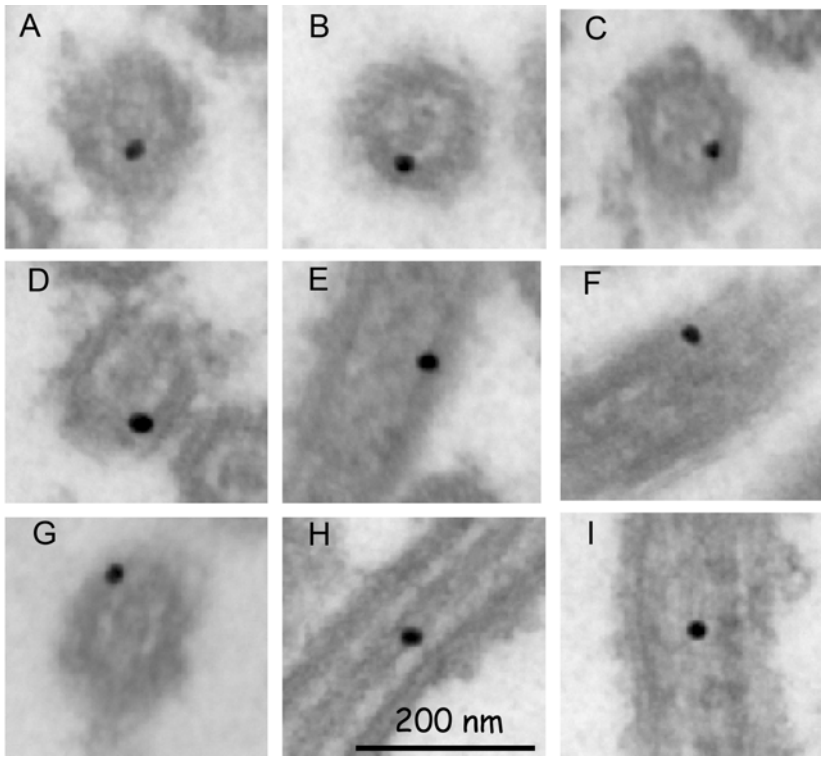

Figure 8
Sloboda and Howard