Feeling sugar-protein interactions using carbon nanotubes : a molecular recongition force microscopy study

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Chapter 2

An AFM study of FIN filaments: filaments in between the nuclei of dividing *Saccharomyces cerevisiae* (yeast) cells

Fin is a protein that is present in yeast cells. During cell division, Fin forms filaments that co-localize with microtubuli. Two questions were addressed in this chapter: Can Fin polymerize on its own, or does it use microtubuli, or possibly DNA or RNA as a template? Does Fin need ATP or GTP for polymerization?

By AFM imaging in air, we found that Fin can polymerize on its own, in the absence of tubulin, DNA and RNA. No GTP or ATP was needed for polymerization. We show that two different types of Fin filaments exist: a rigid filament, in which a periodic substructure was resolved, and a flexible filament. The rigid filament type has a subunit length of around 13 nm and a measured width of 9 nm. This measured width which corresponds closely to the measured width of 10 nm that was found with the electron microscope. Rigid filaments could be bundles or super-coils of single FIN filaments.

This Chapter is partly based on:

Martijn J. van Hemert, Gerda E.M. Lamers, Dionne C.G. Klein, Tjerk. H. Oosterkamp, H. Yde Steensma, and G. Paul van Heusden
The *Saccharomyces cerevisiae* Fin1 protein forms cell cycle-specific filaments between spindle pole bodies
PNAS **99** (2002), 5390-5393

2.1 Introduction

Filaments in between the nuclei in dividing cells of *Saccharomyces cerevisiae* (baker's yeast) were discovered in the yeast group at the Institute of Biology in Leiden. Several aspects such as the interaction between these Fin-filaments and well-studied microtubuli were examined.

From confocal fluorescence images, it is known that Fin protein is present in a non-filamentous form in small-budded yeast cells, and in a filamentous form in large budded yeast cells. Confocal fluorescence images now show that the Fin filaments co-localize with microtubuli, but no evidence for a direct interaction between the Fin proteins and Tub proteins was found. Electron microscopy studies demonstrated that Fin protein polymerizes in the absence of tubulin, DNA and RNA, and ATP nor GTP is required for polymerization. With the AFM we wanted to get more information on the structure of the Fin filaments, and several control experiments were done in order to check if the filaments were indeed composed of Fin, which had not been checked for the electron microscopy results.

In the next section, a short introduction to cell division of yeast is given, especially focusing on the role of microtubuli.

Yeast cell division

We studied cell division of *Saccharomyces cerevisiae* (baker's yeast), which is a small, single-celled fungus. Although yeast is a eukaryotic cell, cell division of yeast is different from normal eukaryotic cell division. Instead of cell enlargement during interphase

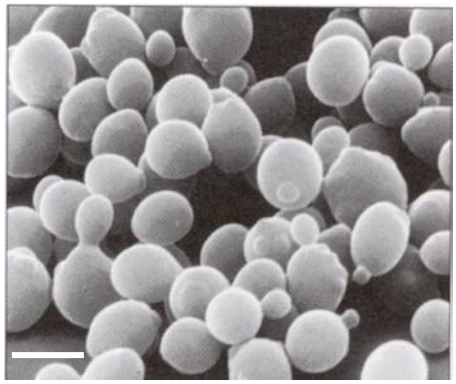


Figure 1 Scanning electron microscopy image of dividing yeast cells, with buds ranging from very small to almost the size of the mother cell. Scale bar is 10 µm. Copyright © 1998 from Essential Cell Biology by Bruce Alberts *et al.* Reproduced by permission of Garland Science/ Taylor & Francis Books, Inc.

(S-phase) when the chromosomes replicate, a bud emerges from the mother cell that will eventually become the daughter cell, as is shown in Figure 1.

When the bud is nearly as big as the mother cell, the nucleus divides (this happens later than in other eukaryotic cells), and two cells form after separation of the bud. At this point, the cell cycle starts over again. The role of microtubuli in cell division is described shortly in the next section.

Microtubuli

Like in other eukaryotic cells in the prophase of cell division, microtubuli form the mitotic spindle to enable the separation of the sister chromatids, such that the mother and daughter cell both get one copy of the chromosomes, as is illustrated in Figure 2. In addition

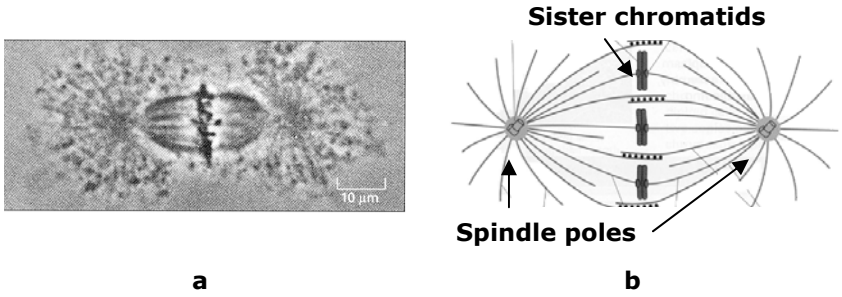


Figure 2 (a) Phase-contrast light microscopy image of a dividing cell during metaphase, clearly showing the chromosomes that are aligned between the two mitotic spindles (b) Schematic drawing of a mitotic spindle, showing the two spindle poles and the chromosomes in between. Copyright © 1998 from Essential Cell Biology by Bruce Alberts *et al.* Reproduced by permission of Garland Science/ Taylor & Francis Books, Inc.

to the well-known microtubuli, P. van Heusden *et al.* found another type of filament, which was called Fin1: filaments in between nuclei[1]. Fin1 filaments form directly after formation of the mitotic spindle, align with it, and disappear when the cell cycle is completed. In non-dividing cells, Fin1 proteins are present in the nucleus in a non-filamentous form. The next section gives a short overview of everything that is known up to now about the Fin1 protein.

Fin1 protein

Fin1 is a 30 kDa protein that mainly consists of α-helices, and putative coiled-coil regions. It has a remarkably high iso-electric point (pI) of 10, which means that it is a basic protein. The C-termini of Fin monomers show hydrophobic interaction. How the Fin monomers form a filament and what the structure (linear, helical,…) of a Fin filament is, is not known.

Confocal fluorescence microscopy

In order to find the localization of Fin relative to microtubules, localization of both Fin1 proteins and tubulin (Tub1) proteins in yeast cells was visualized using confocal fluorescent microscopy. Fin1 protein was tagged with cyano fluorescent protein (CFP) and Tub1 protein was tagged with green fluorescent protein (GFP). CFP was excited at 457 nm and emission was detected at 464-500 nm, and GFP was excited at 488 nm and emission was detected at 464-500 nm. In Figure 3, yeast cells that are in different stages of the cell cycle can be seen. Small-budded cells are at the start of cell division.

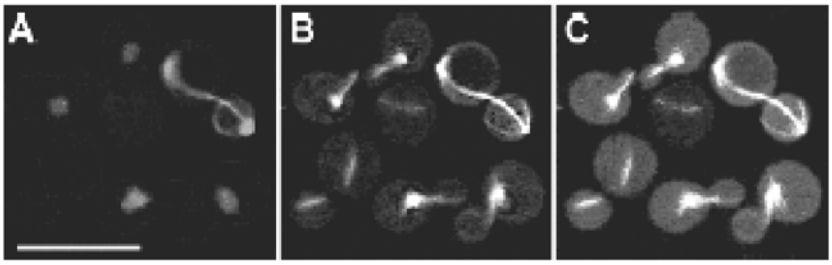


Figure 3 Confocal fluorescence images of yeast cells in different stages of cell division. (a) CFP labeled Fin, (c) GFP labeled tubulin (b) superposition of a and c. The scale bar is 10 μm. Images were made by Paul van Heusden en Gerda Lamers.

Figure 3 a shows the fluorescence of Fin1, and Figure 3 c shows the fluorescence of Tub1. In small-budded cells, CFP-Fin1 protein is non-filamentous, while GFP-Tub1 protein is present in small filaments. In Figure 3 b, Figure 3 a and c are superimposed, in order to show that Fin1 protein and Tub1 protein co-localize: In large-budded cells, both filaments of CFP-Fin1 protein (Figure 3 a, upper cells) and GFP-Tub1 protein Figure 3 c are present with apparent co-localization.

Electron microscopy

A drop of 10 µl containing 200 ng of Fin was put on a carbon grid, dried and stained with 2% phosphotungstic acid. With the electron microscope, images were taken and filaments with a width of around 10 nm were found, as is shown in Figure 4.

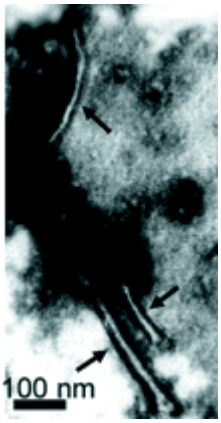


Figure 4 Electron microscopy image of negatively stained Fin filaments. Arrows point to the filaments. Image was made by Martijn van Hemert.

Goals

In order to learn more about the Fin1 protein and its polymerization process, we would like to answer two fundamental questions:

1. Can Fin1 polymerize on its own, or does it use microtubuli, or possibly DNA or RNA as a template?
2. Does Fin1 need ATP or GTP for polymerization?

With AFM in air, we were able not only to answer these questions, but we also found two different types of structure of the Fin1 filaments: a rigid and a flexible type.

2.2 Methods

Fin 1 preparation

Fin 1 monomers were isolated from yeast cells by vortexing the cells together with glass beads in an 8M Urea and 0.5 M NaCl solution, in order to open the cells and denature the proteins, which means that Fin filaments are not present anymore, only monomers. Fin1 proteins were separated using Ni-NTA-agarose affinity

chromatography, where Ni binds to a sequence of 6 histidines that were inserted in Fin1. After this, Fin1 proteins were dialyzed against low salt, in order to remove Urea. This renatures the protein, and filaments can possibly be formed. For single molecule studies with the AFM, it is crucial that the protein solutions that are used are extremely pure, especially when the impurities tend to absorb very well to mica. Otherwise it will be very hard to determine what kind of, and how many different proteins are present on the test surface.

Sample preparation

Fin is a basic protein (pI=10), which means that it is positively charged at neutral pH. This implies that the proteins can be immobilized electrostatically on freshly cleaved mica, which is negatively charged. A droplet of 10 µl containing 200 ng of Fin1 protein in 25 mM NaCl/5mM sodium phosphate buffer at pH 7.5 was applied on a freshly cleaved mica surface, rinsed with deionized water after 30 s and dried in a nitrogen flow.

Atomic force microscopy

To investigate if the Fin1 protein can polymerize on its own, without the help of tubulin, DNA, or RNA present, Fin filaments from yeast cells were broken down by denaturing the proteins using Urea as was described above, and subsequently the Fin1 protein was renatured before AFM investigation.

Two types of Fin1 filaments were found in AFM studies performed in air. A rigid filament was found, an example of which can be seen in Figure 5a, with a measured width of on average 9 nm, a height of 1.5 nm, and a subunit length of 13 nm. The measured width is a convolution between the tip diameter and the filament diameter, and this is one of the reasons that the measured width exceeds the measured height. Another reason is that the filament is attached to the surface, which influences the height-width ratio. Finally, the presence of a thin water layer reduces the measured height in AFM imaging in air. Also a flexible type of filament was found, of which an example is depicted in Figure 5b.

For the flexible filament type, we found a measured width of on average 9 nm, and a height of 0.6 nm. We found both types of filaments together on one sample surface (Figure 5 c), which implies that different filament formation is not concentration dependent[2].

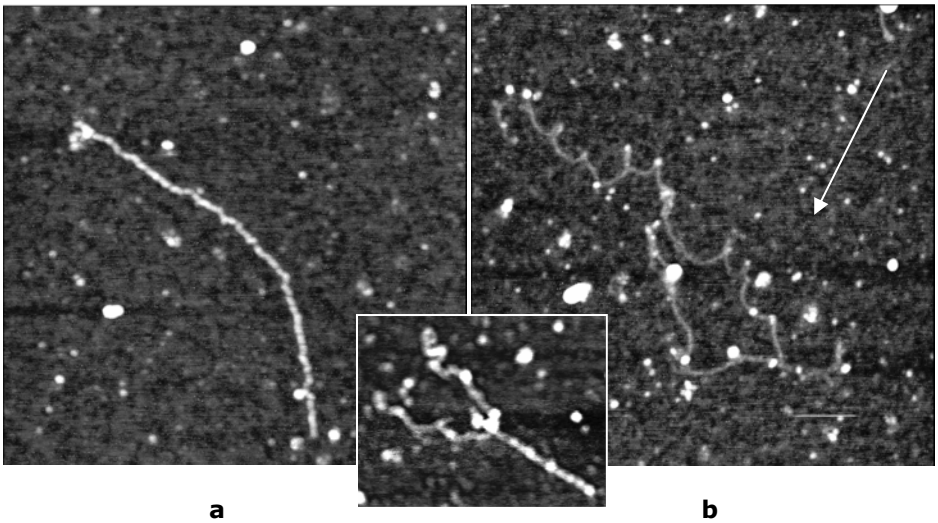


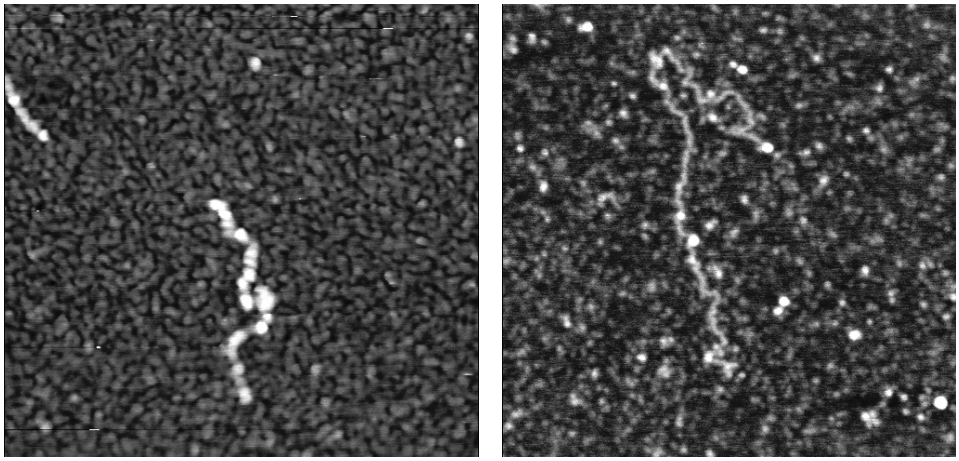
Figure 5 AFM image taken in air. Two different types of filaments are formed, a rigid filament (a), a flexible filament (b) and a combined filament (c). Scales are (a) 750 nm x 750 nm x 3 nm, (b) 900 nm x 900 nm x 2 nm, and (c) 550 nm x 430 nm x 2 nm. The arrow in (b) points into the direction of rinsing of the sample.

Control experiments

In order to make sure that the filaments that were imaged by AFM were not DNA or RNA, we added the enzymes DNAse (DNAse: Fin, 1:20), that breaks up DNA, and RNAse (RNAse: Fin, 1:20), which breaks up RNA, to the Fin solution. After incubation, we applied the mixture to a mica surface. In these positive control experiments, we still observed both types of filaments as is shown in Figure 6.

Another positive control was performed by filtering the Fin solution, using a 300 kDa filter. This filter removes DNA and RNA that are possibly present in the Fin solution. After incubation of the filtrate on mica we still observed both types of filaments, which implies that the filaments that we are imaging are not DNA or RNA.

As a negative control, we incubated Fin monomers in the presence of Urea, which means that the Fin proteins are denatured and no filaments should be able to form. Indeed, no filaments were found by AFM.



a b

Figure 6 AFM images taken in tapping mode in air of Fin filaments after treatment with (a) DNAse and (b) RNAse. The enzymes are clearly resolved against the background. Scales are (a) 580nmx 580nm x 3.8nm and (b) 800nmx 800nmx 3.5nm.

2.3 Discussion and conclusion

Care has to be taken when interpreting the AFM images, because we look at Fin that is immobilized on a surface, and immobilization changes the shape that the filaments have compared to their shape in solution. When a long and flexible polymer is immobilized from solution onto a surface, the transition from three to two dimensions implies loss of one degree of freedom. This results in a large reduction in the number of possible configurations that the polymer can access. Transport from solution onto a surface can happen relatively slowly, which is called equilibration, or relatively fast, called kinetic trapping.[3] Equilibration implies that the transport is determined only by diffusion. In this case, the shape of the immobilized filaments (2D) resembles the shape of the filaments in liquid (3D), and it is possible to extract valuable information about the polymer such as the persistence length.

But, as Fin is a basic protein and therefore positively charged at low pH, we expect that the protein immediately binds to the negatively charged mica through kinetic trapping. This makes it more difficult to interpret the images, because in addition to intrinsic conformations, also surface-induced conformations are present. For Figure 5b for instance, one could imagine that the filament landed on the surface at a few points first, and then the rinsing moved the filament into one direction (see the arrow in Figure 5 b), creating the form of a chain that is pinned at several points. In the case of kinetic

trapping, it is still possible to extract some information. The length of the subunits of a filament in 3D is the same as that in 2D.

To conclude: From the AFM images we learned that Fin can polymerize *in vitro* on its own, without the help of tubulin, without a DNA or RNA template, without ATP or GTP. It forms two types of filaments, a rigid and a flexible filament. The rigid filament type has a subunit length of around 13 nm and a measured width of 9 nm. This measured width which corresponds closely to the measured width of 10 nm that was found with the electron microscope.

Rigid filaments could be bundles or super coils of single FIN filaments. An important remark here is that charges on the FIN filaments could prevent them from aggregating. But, ions from the buffer could screen the charges on the filaments and in this way make aggregation or super coiling possible.

Although the presence of Fin filaments in yeast cells has been demonstrated, the role of Fin in the yeast cell is unclear up to now. Knock-out experiments show that yeast cell-division is unaltered in the absence of Fin. Maybe Fin can take over the function of the microtubuli in extreme conditions, such as abnormal pH values or temperature. Now that we have verified that FIN forms filaments on its own, FIN can indeed have a back-up function and take over the role of microtubuli. It would be interesting to see if for extreme temperatures or pH-values, yeast cells can still divide normally.

This chapter shows the value of AFM use with unmodified tips in air for the study of protein structure. However, study of structure-function relationships of proteins requires use of AFM in liquid. This will be discussed in the next chapter.

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[1] Hemert, M.J. van, Lamers, G.E.M., Klein, D.C.G., Oosterkamp, T.H., Steensma, H.Y., and Heusden, P.H. van. The Saccharomyces cerevisiae Fin1 protein forms cell cycle-specific filaments between spindle pole bodies. PNAS **99** 8 (2002), 5390-5393

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[3] Rivetti, C., Guthold, M., and Bustamante, C., Scanning force microscopy of DNA deposited onto mica: Equilibration versus kinetic trapping studied by statistical polymer chain analysis, J. Mol. Biol. **264** (1996), 919-932