Identification of a MAP65 isoform involved in directional expansion of plant cells

Jordi Chan[a], Guojie Mao[a], Andrei Smertenko[b], Patrick J. Hussey[b], Mike Naldrett[c], Andrew Bottrill[c], Clive W. Lloyd[a,*]

[a] *Department of Cell and Developmental Biology, John Innes Centre, Norwich NR4 7UH, UK*
[b] *The Integrative Cell Biology Laboratory, School of Biological and Biomedical Sciences, University of Durham, South Rd., Durham DH1 3LE, UK*
[c] *Proteomics Facility, John Innes Centre, Norwich NR4 7UH, UK*

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Abstract MAP65 comprises a multigene family specific to plants. To see which isoform is utilised for the unique mechanism of cell expansion, uncomplicated by division structures, carrot cells were deprived of auxin whereupon they stopped dividing and elongated instead. During elongation, a MAP65 protein triplet reduced to a single band. Mass spectrometric analysis demonstrated that this corresponded to a single carrot cDNA; it also corresponded to the major protein previously shown to form filamentous cross-bridges between microtubules in vitro. This MAP65 isoform is concluded to have a major role in establishing the parallel microtubule arrays characteristic of cells undergoing directional expansion.

Key words: MAP65; Plant cytoskeleton; Cortical microtubule; Proteomics

1. Introduction

The cortical array of microtubules is an assembly, unique to plants, that helps to organise the plant cell wall over large areas of diffusely growing cell surface [1]. Despite the lack of fixed microtubule organising centres and the dispersion of microtubules over the cortex, microtubules are generally highly organised occurring in parallel groups with the same orientation being maintained over hundreds of micrometres. Microtubule-associated proteins (MAPs) are likely to play an important part, particularly the protein(s) that link microtubules side by side, converting individual microtubules into an unitary array and maintaining a minimal inter-microtubule spacing. In the electron microscope, fine lateral arms have been observed to maintain the spacing between microtubules [2]. MAP65, originally detected in tobacco cells [3], has been biochemically purified from carrot cells as a protein triplet on 1D sodium dodecyl sulphate (SDS) polyacrylamide gels and, when added to brain microtubules, shown to draw them into parallel groups [4]. Carrot MAP65 forms filaments that have a regular axial spacing along the microtubule backbone and, since these 25–30 nm side arms are the same dimension as seen in cells, it is likely that they are responsible for maintaining the regular organisation of the cortical array.

In tobacco [3,6] and carrot [5], polyclonal antibodies to MAP65 recognise all four microtubule arrays – the preprophase band, the spindle and the phragmoplast of dividing cells plus the interphase cortical array. However, since 'MAP65' contains multiple size variants it is possible that analysis of MAPs isolated from unsynchronised cell populations masks the expression of particular isoforms at particular stages of the cell cycle.

To investigate whether some or all of the proteins in the triplet are responsible for construction of the cortical array, we synchronised cells in interphase [7] and isolated MAPs from cells exclusively containing transverse groups of parallel microtubules. The triplet identified on blots of dividing populations reduced to a single band when cells were 100% in interphase. Mass spectral analysis showed it to belong to the most conserved group 1 of the diverse MAP65 family. A cDNA encoding this polypeptide was isolated. The predominant band from the highly purified MAP65 sample, which was previously shown to induce microtubules to form parallel groups in vitro [4], also matched this cDNA. We conclude that this particular gene product has a specific function in the parallel grouping of cortical microtubules.

2. Materials and methods

The carrot suspension [7] was grown in 0.44% (w/v) Murashige and Skoog medium, supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% (w/v) sucrose but without coconut milk. To arrest division and encourage cell elongation, cultures were washed by centrifugation and resuspended in medium minus 2,4-D. Protoplasts were then frozen and extracted in tubulin isolation buffer (5 mM imidazole HCl, pH 7.5, 3 mM $CaCl_2$, 1 mM dithiothreitol, 0.4 mM phenylmethylsulphonyl fluoride, 0.2 mM leupeptin and 6 mg/ml insoluble polyvinyl pyrrolidone) [8]. MAPs were affinity-purified from this extract by adding 0.5 mg/ml taxol-stabilised pig brain microtubules and taxol to 20 μM. The microtubule pellets were analysed by SDS–PAGE and Western blotting as described. Coomassie-stained bands were analysed by mass spectrometry.

Peptides generated from enzymatic digestion of each gel band were loaded at high flow rate onto a trapping column (0.3 mm i.d. × 1 mm, with 5 μm C18 100 Å PepMap packing, LC Packings, The Netherlands) and eluted through a reverse-phase capillary column (75 μm i.d. × 50 mm column, with 5 μm C18 100 Å PepMap packing) directly into the nano-electrospray ion source of a quadrupole time-of-flight (Q-TOF) 2 mass spectrometer (Micromass, Manchester, UK). The

*Corresponding author. Fax: (44)-1603-450022.
E-mail address: clive.lloyd@bbsrc.ac.uk (C.W. Lloyd).

Abbreviations: MAP, microtubule-associated protein; MALDI-TOF MS, matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry; Q-TOF-MS, quadrupole time-of-flight mass spectrometry

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resulting fragment ion spectra were assigned using the PepSeq de novo sequencing tool (Micromass).

Carrot mRNA was purified from total RNA from 5 day old carrot suspension culture cells using Oligotex resin (Qiagen, UK). The cDNA was synthesised using a ZAP-cDNA Synthesis kit (Stratagene, UK) according to the manufacturer's recommendations and cloned into the *Xho*I/*Eco*RI sites of the UniZAP XR vector. The library was packaged with Gigapack II Gold packaging extracts and amplified in XL-1 Blue MRF′ cells (Stratagene, UK). The titre of the primary library was estimated as 8.9×10^6 pfu/ml. This library was screened using an anti-MAP65 antibody [6] according to the Stratagene recommendations. A total number of 1.5 million plaques were screened. A positive clone with a 2 kb cDNA insert was recovered corresponding to an open reading frame of 576 amino acids as determined from a comparison with the sequence of NtMAP65-1 [6].

To check the types of MAP65 that can be isolated from suspension cells, neuronal taxol microtubules were added to an extract of *Arabidopsis* protoplasts. The presence of MAP65 in the microtubule pellets was confirmed by immunoblotting with anti-MAP65 antibodies. Protein bands were cut from SDS polyacrylamide gels, trypsinised and the tryptic peptide mass fingerprint, which was obtained by matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS), compared in silico to the theoretical fingerprints derived from cognate proteins in the *Arabidopsis* genome. Bands were searched with a mass error of 50 ppm. All reported protein identities had a minimum coverage by matching peptides of 20%.

3. Results and discussion

Dividing carrot cultures are characterised by small dense clumps of cells. Such a culture (7 days old) was taken as the zero point of the experiment (Fig. 1A, 0 days). By subculturing into medium deficient in the growth regulator, 2,4-D, the cells stopped dividing and over 4 days underwent rapid elongation to form tubular cells with zero mitosis (Fig. 1B, 4 days). In these elongated cells, the microtubules are known to be orientated perpendicularly to the direction of cell elongation [7].

In the dividing culture, three bands could be identified by immunoblotting microtubule spindowns with anti-MAP65 antibodies (Fig. 1C, 0 days) as previously described [4,5]. The upper band is a minor component but can be demonstrated by overloading the gel as shown in Fig. 1C, 0 days. It is not detected at lower protein loadings (Fig. 1D, 0 days) but the thick lower band seen at the heavier protein loading now resolves as a doublet (Fig. 1D, 0 days), as illustrated by the double arrowheads and as previously reported [5]. After 4 days in medium without the synthetic auxin, 2,4-D, the cells undergo significant cell elongation; the largest size variant is no longer expressed (Fig. 1C, 4 days) and the lower band now resolves as a single band at lower protein loadings (Fig. 1D, 4 days). This single band from elongated cells was excised and analysed by mass spectrometry (Q-TOF). In parallel with this, we isolated cDNA from a carrot suspension composed predominantly ($>97\%$) of interphase cells. The peptide sequences obtained from the single 'interphase' carrot band exactly matched those encoded by the cDNA (MLLQLEQECLDVYKRK; VGSPVVDESDLSLK and IPALVESLVAK). This suggests that the band is composed solely of protein derived from a single gene (EMBL Nucleotide Sequence Database, Accession No. AJ520103). Phylogenetic analysis showed that the protein encoded by the carrot cDNA was most similar to the group 1 MAP65 subfamily comprised of: three tobacco proteins (cac17794 [NtMAP65-1a], cac17795 [NtMAP65-1b], cac17796 [NtMAP65-1c]), and two *Arabidopsis* proteins (At5g55230 [MAP65-1], At4g26760 [MAP65-2]) (see [9]). For comparison, we analysed the microtubule spindowns from a dividing *Arabidopsis* cell suspension and identified three size variants of MAP65 by MALDI-TOF. The tryptic peptide mass fingerprints of two bands matched At5g55230 [MAP65-1] (suggesting post-translational modification, alternative splicing or proteolysis), and another matched At4g26760

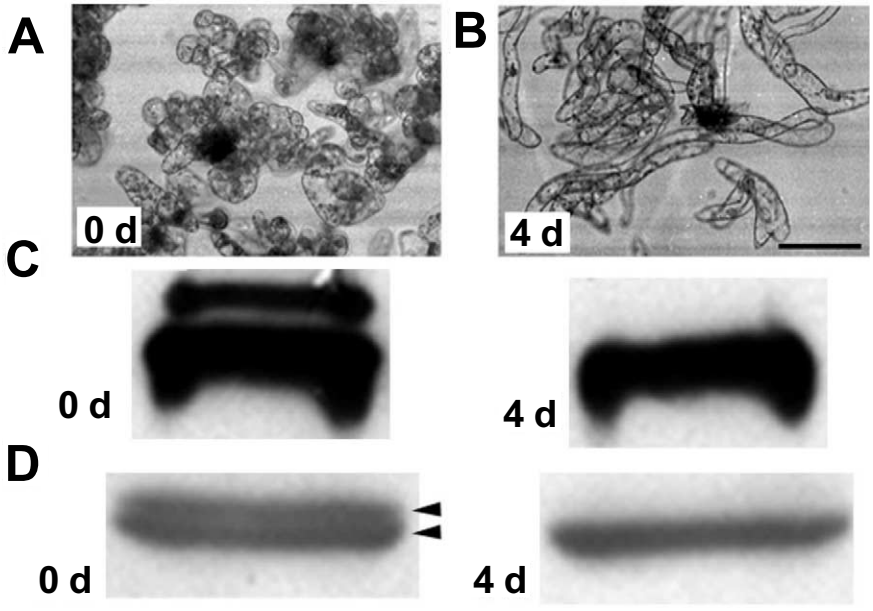

Fig. 1. The number of MAP65 isoforms is reduced in elongated cells. A: A dividing carrot suspension at zero time (0 days) contains clumps of rounded cells. B: After 4 days in medium without 2,4-D the cells stop dividing and become highly elongated. C: Immunoblots, using anti-MAP65 antibodies, of microtubule spindowns from cells at 0 and 4 days after removal of the growth hormone, 2,4-D. Gels in C were heavily overloaded to show the upper band at 0 days which disappears by 4 days. In D, gels were more lightly loaded in order to resolve the thick lower band shown in C, 0 d. In D, 0 d this band is resolved as a doublet (arrowheads). By 4 days without auxin, this resolves as a single band (D, 4 d).

[MAP65-2]. Therefore, both genes from the *Arabidopsis* suspension were, as for the carrot 'interphase' cDNA, most similar to the MAP65 group 1 subfamily.

Nine MAP65s are known from the *Arabidopsis* genome, and the rice genome database contains 14. It is probable that not all are expressed in undifferentiated cell suspensions: a selective pattern of expression is known for the tubulins in this carrot suspension [10]. Also, less abundant isoforms and those failing to bind microtubules in vitro might have escaped detection. Nevertheless, it seems clear that the number of MAP65 size variants that do bind microtubules in co-sedimentation assays is reduced as the cells stop dividing and undergo directional cell expansion. This suggests a cell cycle and/or hormonally regulated pattern of expression.

In our previous study of a highly purified carrot MAP65 fraction (purified from cytoskeleton preparations by microtubule affinity, salt elution then fractionation on sucrose density gradients) we isolated a 60, 62 and 68 kDa triplet of MAP65s [4]. In the present study we analysed this purified sample by Q-TOF. The 60 and and 62 kDa bands yielded sequences cognate to group 1 MAP65s. The 60 kDa band was most similar to the group 1 tobacco NtMAP65-1a [6] [LLKALANAR; QAELEDI]. The 68 kDa band did not fall into this group of most conserved MAP65s but was most similar to *Arabidopsis* At5g51600 [MAP65-3] [LLSMLEEYNILR; NQFSDVVKQLR]. Neither group of peptides is encoded by the isolated carrot cDNA. However, peptides from the 62 kDa band – like the single band from interphase carrot cells – were identical to the sequence predicted by the carrot cDNA [LALEEDKEQR; EPAELLDDMDNQIVK and LVNKIPALV].

Post-translational modification is expected to affect the pattern of size variants. However, this alone cannot explain the fact that the 60 and the 68 kDa bands yield peptides not encoded by the cDNA cognate to the 62 kDa band; this suggests, instead, an underlying cell cycle-regulated pattern of expression of multiple genes. Evidently, not all MAP65 genes utilised by dividing carrot cells are expressed when cells exit mitosis and undergo expansion in G0/G1.

It is significant that this 62 kDa species purified on sucrose density gradients [4] was by far the major component of the MAP65 triplet (c. 75%) and for that reason was concluded to be most likely responsible for the cross-bridging activity exhibited by the sample in vitro. The present study now confirms that the peptides derived from this predominant species match the isolated cDNA and therefore share an identity with the single protein band detected in elongated interphase cells. This strongly suggests that the MAP65 isoform encoded by the isolated cDNA is responsible for maintaining the parallelism of cortical microtubules during the rapid phase of cell elongation.

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