

Characterizing Components of the *Dictyostelium* centrosome

Dissertation
der Fakultät für Biologie der
Ludwig-Maximilians-Universität
München

vorgelegt von
Christine Zoglmeier, geb. Daunderer
aus München
November 2001

Ehrenwörtliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfsmittel angefertigt habe.

Christine Zoglmeier

Schliersee, November 2001

Dissertation eingereicht: 15.11.2001

Tag der mündlichen Prüfung: 05.07.2002

Erstgutachter: Dr. habil. Birgit Wetterauer

Zweitgutachter: Prof. Dr. Thomas Cremer

Sondergutachter: Prof. Dr. Manfred Schliwa

Teile dieser Arbeit wurden bereits veröffentlicht:

Originalarbeiten:

Gräf, R., Daunderer, C. and Schliwa, M. (1999). Cell cycle-dependent localization of monoclonal antibodies raised against isolated *Dictyostelium centrosomes*. *Biol. Cell* 91, 6: 471-477.

Gräf, R., Daunderer, C. and Schliwa, M. (2000). *Dictyostelium* DdCP224 is a microtubule-associated protein and a permanent centrosomal resident involved in centrosome duplication. *J. Cell Sci.* 113, 10: 1747-1758.

Daunderer, C., Schliwa, M. and Gräf, R. (2001). *Dictyostelium* centrin-related protein (DdCrp), the most divergent member of the centrin family, possesses only two EF-hands and dissociates from the centrosome during mitosis. *Eur. J. Cell Biol.*, in press.

Daunderer, C. and Gräf, R. (2001). Molecular analysis of the cytosolic *Dictyostelium* γ -tubulin complex. *Eur. J. Cell Biol.*, submitted.

Übersichtsartikel:

Daunderer, C., Schliwa, M. and Gräf, R. (1999). *Dictyostelium discoideum*: a promising centrosome model system. *Biol. Cell* 91: 313-320.

Gräf, R., Brusis, N., Daunderer, C., Euteneuer, U., Hestermann, A., Schliwa, M. and Ueda, M. (2000). Comparative structural, molecular and functional aspects of the *Dictyostelium discoideum* centrosome. *Curr. Top. Dev. Biol.* 49: 161-185.

Tagungsbeiträge:

Daunderer C., Schliwa M. and Gräf R. (1999). Cytosolic γ -tubulin complexes in *Dictyostelium discoideum*. *Mol. Biol. Cell* (Supplement) 10S: 18a

Die vorliegende Arbeit wurde von September 1997 bis Juni 2001 im Labor von Prof. Dr. Manfred Schliwa angefertigt und durch Dr. Ralph Gräf betreut. Die Vertretung vor der Fakultät wurde von Dr. habil. Birgit Wetterauer übernommen. Gefördert wurde die Arbeit durch die Deutsche Forschungsgemeinschaft.

CONTENTS

SUMMARY	4
ZUSAMMENFASSUNG	5
ABBREVIATIONS	7
1. INTRODUCTION	9
1.1 Centrosomes in different organisms	9
1.2 The essential centrosomal protein γ -tubulin and its cytosolic binding partners	11
1.3 The centrosome-associated EF-hand protein centrin	13
2. MATERIALS AND METHODS	15
2.1 Materials	15
2.1.1 Reagents	15
2.1.2 Antibodies	15
2.1.3 Enzymes	15
2.1.4 Antibiotics	15
2.1.5 Media	16
2.1.5.1 Media for cultivation of <i>D. discoideum</i>	16
2.1.5.2 Media for cultivation of <i>E. coli</i>	16
2.1.6 Buffers and solutions	17
2.1.7 Vectors	18
2.1.8 Bacterial and <i>D. discoideum</i> strains	18
2.1.9 Computer programmes	19
2.1.10 Other materials	19
2.2 Molecular biology methods	19
2.2.1 Agarose gel electrophoresis	19
2.2.2 DNA extraction from agarose gels	19
2.2.3 Determination of DNA and RNA concentration	19
2.2.4 Preparation of plasmid DNA	20
2.2.5 Polymerase chain reaction (PCR)	20
2.2.6 Reverse transcription-PCR (RT-PCR)	20
2.2.7 Oligonucleotides	21
2.2.8 DNA cleavage with restriction enzymes	21
2.2.9 Phosphatase treatment	22
2.2.10 Blunting of sticky ends with T4 DNA polymerase	22
2.2.11 Ligation of DNA into plasmid vectors	22
2.2.12 Preparation of electrocompetent <i>E. coli</i> cells	22

2.2.13 Electrotransformation of <i>E. coli</i> cells	22
2.2.14 Identification of transformed clones in <i>E. coli</i>	23
2.2.15 Preparation of chromosomal DNA from <i>D. discoideum</i>	23
2.2.16 Transformation and cloning of <i>D. discoideum</i>	23
2.2.17 Isolation of polyadenylated RNA from <i>D. discoideum</i>	24
2.2.18 Electrophoresis of RNA and Northern blotting	24
2.2.19 Southern blotting	25
2.2.20 Radioactive labelling of DNA probes, hybridization and detection	25
2.2.21 Digoxigenin labelling of DNA probes, hybridization and detection	25
2.2.22 Construction of a subgenomic library enriched in DdSpc98 sequence	26
2.2.23 Screening of cDNA and genomic libraries	26
2.2.24 Construction of the vectors for myc/6xHis -tagging of endogenous γ -tubulin and DdCrp	27
2.2.25 Construction of bacterial expression vectors	28
2.3. Biochemical and immunological methods	28
2.3.1 SDS-Polyacrylamide gel electrophoresis (PAGE)	28
2.3.2 Coomassie staining	29
2.3.3 Colloidal Coomassie staining	29
2.3.4 Methanol/chloroform precipitation of proteins	29
2.3.5 Western blots and immunostaining	30
2.3.6 Determination of protein concentration	30
2.3.7 Purification of bacterially expressed proteins	30
2.3.7.1 Purification of bacterially expressed His-DdCrp	30
2.3.7.2 Purification of bacterially expressed, MBP-tagged proteins	31
2.3.7.3 Purification of myc/6xHis -tagged cytosolic γ -tubulin complexes from <i>D. discoideum</i>	31
2.3.8 Preparation of whole cell extracts, nuclei and centrosomes from <i>D. discoideum</i>	32
2.3.9 Gel filtration	33
2.3.10 Antigen preparation and immunizations	33
2.3.11 Covalent coupling of antibodies and purified proteins to NHS-activated sepharose	33
2.3.12 Affinity purification of antisera	34
2.3.13 Immunoprecipitation	34
2.4 Cell biological methods	35
2.4.1 Cultivation and preservation of <i>D. discoideum</i>	35
2.4.2 Cultivation and preservation of <i>E. coli</i>	35
2.4.3 Indirect immunofluorescence microscopy on whole <i>D. discoideum</i> cells and isolated centrosomes	35
2.4.4 Confocal microscopy	36
2.4.5 Electron microscopy	36

3. RESULTS	37
3.1 Investigation of cytosolic γ -tubulin complexes in <i>D. discoideum</i>	37
3.1.1 Cloning of DdSpc97 and DdSpc98	37
3.1.2 Sequence analysis of DdSpc97 and DdSpc98	42
3.1.3 Southern and Northern blot analysis of DdSpc98 and DdSpc97	45
3.1.4 Investigation of the subcellular distribution of DdSpc97 and DdSpc98	46
3.1.4.1 Generation of polyclonal antibodies and GFP-tagged mutants	46
3.1.4.2 Western blots, immunofluorescence microscopy and electron microscopy	47
3.1.5 Generation of a mutant stably expressing myc/6xHis -tagged γ -tubulin	52
3.1.6 Gel filtration of purified γ -tubulin complexes	54
3.1.7 Purification and identification of the components of <i>D. discoideum</i> γ -tubulin complexes	56
3.2 Investigation of the <i>D. discoideum</i> homologue of centrin	59
3.2.1 Cloning of DdCrp	59
3.2.2 Southern and Northern blot analysis of DdCrp	60
3.2.3 Sequence analysis of DdCrp	61
3.2.4 Investigation of the subcellular distribution of DdCrp	64
3.2.4.1 Generation of polyclonal antibodies raised against DdCrp	64
3.2.4.2 Subcellular distribution of DdCrp on Western blots and in immunofluorescence microscopy	65
3.2.4.3 Generation of a <i>D. discoideum</i> mutant expressing myc/6xHis tagged DdCrp	68
3.2.4.4 Cell cycle dependent changes in the localization of DdCrp	69
4. DISCUSSION	72
4.1 Investigation of cytosolic γ -tubulin complexes in <i>D. discoideum</i>	72
4.1.1 Tagging of endogenous γ -tubulin with a myc/6xHis tag	72
4.1.2 The size and stability of <i>D. discoideum</i> γ -tubulin complexes	72
4.1.3 The composition of the prevailing <i>D. discoideum</i> γ -tubulin complex	74
4.1.4 The possible significance of γ -tubulin complexes in <i>D. discoideum</i>	75
4.2 Investigation of the <i>D. discoideum</i> homologue of centrin	78
4.2.1 DdCrp as the most divergent member of the centrin family	78
4.2.2 DdCrp lacks two of the four EF-hand consensus motifs	79
4.2.3 Cell-cycle dependent changes of DdCrp localization	79
4.2.4 Possible implications for DdCrp	81
5. CONCLUSION	82
6. REFERENCES	83
LEBENS LAUF	91
DANKSAGUNGEN	92

SUMMARY

In this work three functionally important centrosomal proteins from *Dictyostelium* were identified, cloned and characterized. Furthermore, the nature and composition of cytosolic γ -tubulin complexes in *Dictyostelium* was investigated.

One of the three proteins that were cloned was *Dictyostelium discoideum* centrin-related protein (DdCrp), which turned out to be the most divergent member of the centrin family. Most strikingly it lacks two out of four centrin-specific EF-hand consensus motifs, whereas a number of other centrin-specific sequence features are conserved. Southern and Northern blot analysis and the data from *Dictyostelium* genome and cDNA projects suggested that DdCrp is the only centrin isoform present in *Dictyostelium*. Immunofluorescence microscopy with anti-DdCrp antibodies revealed that the protein is localized to the centrosome, to a second, centrosome-associated structure close to the nucleus and to the nucleus itself. Confocal microscopy resolved that the centrosomal label is confined to the corona surrounding the centrosome core. In contrast to other centrins striking cell-cycle dependent changes were monitored in the localization of DdCrp. The centrosomal and centrosome-associated label disappeared during prometaphase, most likely in concert with the dissociation of the corona at this stage, whereas the intensity of the nuclear label was unaltered.

To investigate the size and composition of cytosolic γ -tubulin complexes, which are thought to serve as a pool for recruitment of microtubule-nucleating sites to the centrosome, a mutant *Dictyostelium* cell line was created. In this mutant the endogenous copy of the γ -tubulin gene was replaced by a version of the gene carrying a C-terminal myc/6xHis tag. γ -Tubulin complexes isolated from this mutant by affinity purification were generally much smaller than the large γ -tubulin ring complexes (γ TuRCs) found in higher organisms. The stability of *Dictyostelium* γ -tubulin complexes depended strongly on the purification conditions, with a striking stabilization of the prevailing small complex under high salt conditions. In the course of the investigation of cytosolic γ -tubulin complexes two proteins, DdSpc97 and DdSpc98, were identified and cloned. Homologues of these proteins have been found to be components of γ -tubulin ring complexes of higher organisms and of the small Tub4p complex present in *S. cerevisiae*. Both proteins localized to the *Dictyostelium* centrosome throughout the cell cycle and were also present in a cytosolic pool. It could be demonstrated that the prevailing small complex present in *Dictyostelium* consists of DdSpc98 and γ -tubulin, whereas DdSpc97 does not associate. *Dictyostelium* is thus the first organism investigated so far where the three proteins do not interact stably in the cytosol.

Both the unusual composition of cytosolic *Dictyostelium* γ -tubulin complexes and the striking differences of DdCrp to all other centrins may be attributed to the distinct structure and duplication mode of the *Dictyostelium* centrosome.

ZUSAMMENFASSUNG

Im Rahmen dieser Arbeit wurden drei funktionell bedeutsame Proteine des Zentrosoms von *Dictyostelium* identifiziert, kloniert und charakterisiert. Außerdem wurde die Art und Zusammensetzung von cytosolischen γ -Tubulin-Komplexen in *Dictyostelium* untersucht.

Eines der klonierten Proteine ist das Centrin-Homolog von *Dictyostelium*, DdCrp (*Dictyostelium* centrin-related protein). Es konnte gezeigt werden, dass DdCrp das am wenigsten konservierte Mitglied der Centrin-Familie ist. Insbesondere fehlen in DdCrp zwei der vier Centrin-typischen, Ca^{2+} -bindenden EF-Hand Motive, wohingegen eine Reihe anderer Centrin-spezifischer Sequenzmerkmale konserviert sind. Southern und Northern Blot Untersuchungen sowie die Daten des *Dictyostelium* Genomprojekts und von verschiedenen cDNA Projekten wiesen darauf hin, dass DdCrp die einzige Centrin-Isoform in *Dictyostelium* ist. Immunfluoreszenzaufnahmen mit Antikörpern gegen DdCrp zeigten, dass DdCrp am Zentrosom, aber auch an einer zweiten, mit dem Zentrosom assoziierten Struktur am Zellkern und schließlich im Zellkern selbst lokalisiert ist. Durch konfokale Mikroskopie konnte gezeigt werden, dass sich die Lokalisation im Zentrosom auf die Corona beschränkt, die den Zentralkörper des Zentrosoms umgibt. Im Gegensatz zu anderen Centrinen wurden bei der Lokalisation von DdCrp zellzyklusabhängige Veränderungen beobachtet: die zentrosomale und die Zentrosom-assoziierte Markierung verschwanden während der Prometaphase, was vermutlich mit der Abdissoziation der Corona zu diesem Zeitpunkt zusammenhängt. Die Markierung im Zellkern blieb dagegen unverändert.

Um die Größe und Zusammensetzung von cytosolischen γ -Tubulin-Komplexen zu untersuchen, die vermutlich ein Reservoir für die Bildung von Mikrotubuli-Nukleationsstellen darstellen, wurde eine *Dictyostelium*-Mutante erzeugt. In dieser Mutante war das endogene γ -Tubulin-Gen durch eine am C-Terminus mit einem myc/6xHis-tag versehene Version ersetzt worden. γ -Tubulin-Komplexe, die aus dieser Mutante aufgereinigt werden konnten, waren deutlich kleiner als die γ -Tubulin Ringkomplexe, die in höheren Organismen gefunden wurden. Die Größe der γ -Tubulin-Komplexe in *Dictyostelium* variierte stark in Abhängigkeit von den Aufreinigungsbedingungen. Erstaunlicherweise wurde der hauptsächlich vorkommende kleine Komplex durch Hochsalzbedingungen stabilisiert. Im Verlauf der Analyse von γ -Tubulin-Komplexen wurden die Proteine DdSpc97 and DdSpc98 identifiziert und kloniert. Homologe dieser Proteine sind Bestandteile der γ -Tubulin-Ringkomplexe höherer Organismen beziehungsweise der kleinen Tub4p Komplexe aus der Bäckerhefe. Beide Proteine waren in *Dictyostelium* während des gesamten Zellzyklus am Zentrosom lokalisiert und waren auch in cytosolischen Formen zu finden. Es konnte gezeigt werden, dass der in *Dictyostelium* hauptsächlich vorkommende γ -Tubulin-Komplex aus DdSpc98 und γ -Tubulin besteht, wohingegen DdSpc97 kein Bestandteil des Komplexes ist. *Dictyostelium* ist demzufolge der erste Organismus, in dem die drei Proteine nicht stabil im Cytosol interagieren.

Sowohl die ungewöhnliche Zusammensetzung des *Dictyostelium* γ -Tubulin-Komplexes, als auch die auffälligen Abweichungen von DdCp zu anderen Centrinen hängen vermutlich mit der besonderen Struktur und dem einzigartigen Duplikationsmechanismus des *Dictyostelium*-Zentrosoms zusammen.

ABBREVIATIONS:

aa	Amino acid
ATP	Adenosine-5'-triphosphate
BCIP	Bromo-chloro-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
C'-	Carboxy terminal
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
Dd	<i>Dictyostelium discoideum</i>
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DdCaM	<i>Dictyostelium discoideum</i> calmodulin
DdCrp	<i>Dictyostelium discoideum</i> centrin related protein
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
ds	Double stranded
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N'-tetraacetic acid
EM	Electron microscopy
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
γ TuRC	γ -Tubulin ring complex
γ TuSC	γ -Tubulin small complex
H ₂ O	Distilled water
His-tag	Histidine tag
IPTG	Isopropyl- β -D-thiogalactopyranoside
kbp	Kilo base pairs
kDa	Kilo Daltons
MOPS	Morpholinopropanesulfonic acid
MT	Microtubules
MTOC	Microtubule organizing center
MW	Molecular weight
N'-	Amino terminal
NBT	Nitroblue-tetrazolium chloride
OD	Optical density
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCM	Pericentriolar material
PCR	Polymerase chain reaction
pfu	Plaque forming units
PIPES	Piperazine-N, N'-bis-[2-ethanesulfonic acid]
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SPB	Spindle pole body
TBE	Tris/borate/EDTA

TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N, N, N, N'-tetramethylenediamine
Tris	Tris-hydroxymethyl-ammoniummethane
Triton-X-100	t-Octylphenoxypolyethoxyethanol
Tween 20	Polyoxyethylene-sorbitanemonolaureate
U	Units
v/v	Volume per volume
w/v	Weight per volume
wt	Wild-type
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1.1 INTRODUCTION

1.1 Centrosomes in different organisms

More than one century ago Theodor Boveri first recognized the fundamental role of the centrosome in the cell division cycle (Boveri, 1887). According to his observations in dividing *Ascaris* eggs he proposed that the centrosome serves as "the proper cell division center", that it divides once per cell cycle and is responsible for the establishment of the mitotic apparatus. Centrosomes turned out to display remarkably different morphologies in other cell types. Therefore, the term "microtubule organizing centre" (MTOC) was suggested by Pickett-Heaps (1969) to describe the diverse structures with microtubule nucleating activity present in different cell types. Except for a few cell types, e. g. higher plant or female meiotic cells, most eukaryotic cells possess a defined centrosome, which is responsible for the organization of their microtubule cytoskeleton. Thus there must be evolutionary constraints keeping cells from dispensing with this organelle and reflecting the individual requirements of the respective cell type. It is conceivable that there are differences in the dynamics of the microtubule cytoskeleton between cells with a rigid cell boundary (e. g. plant cell wall) and motile, amoeboid cells and differences between unicellular and multicellular organisms.

Certainly the most familiar centrosomal morphology is the centriolar centrosome of mammalian cells, which consists of a pair of barrel-shaped centrioles surrounded by a cloud of pericentriolar material (PCM) (reviewed in Kellogg et al., 1994) (Fig. 1A). Microtubules emanating from the centrosome are anchored in the PCM, which is the actual site of microtubule nucleation. A second, well-studied type of MTOC is the yeast spindle pole body (SPB), an acentriolar, disc-like trilaminar structure embedded in the nuclear envelope (reviewed in Winsor and Schiebel, 1997) (Fig. 1B). Nuclear microtubules emanate from the inner and cytoplasmic microtubules from the outer of the three plaques. Due to its excellent genetic and biochemical accessibility many centrosomal proteins have been first characterized in the *Saccharomyces cerevisiae* system.

Fundamental insight has been gained from a comparative analysis of these two thoroughly studied types of centrosome. Due to the phylogenetic divergence of yeast and mammals and the structural differences of their MTOCs, components conserved between the two organisms are undoubtedly of fundamental importance for microtubule organization and were thus not dispensed within the course of evolution. However, proteins from *Saccharomyces cerevisiae* are often highly aberrant, even if they are well conserved in other organisms (reviewed in

Dauderer et al., 1999). Therefore, important conserved proteins might not be identified when restricting the search to yeast and mammals.

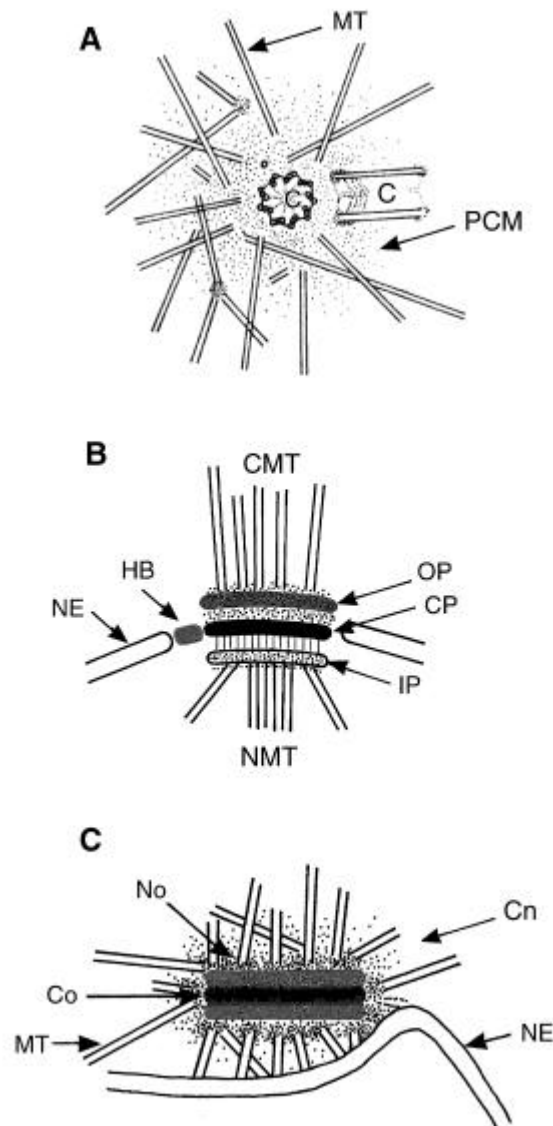


Fig. 1 Different centrosomal morphologies (Diagrammes adapted from Kalt and Schliwa, 1993). **(A)** The typical mammalian centrosome with two barrel shaped centrioles (C), which are oriented perpendicular to each other. Microtubules (MT) emanate from the amorphous pericentriolar material (PCM) clustering around the centrioles. **(B)** The *Saccharomyces cerevisiae* spindle pole body consists of three main layers, the outer plaque (OP) facing the cytosol, the inner plaque (IP) facing the nucleus and the central plaque (CP) which is embedded in the nuclear envelope (NE). Cytoplasmic microtubules (CMT) emanate from the outer and nuclear microtubules (NMT) from the inner plaque. A small structure, called half-bridge (HB) is found next to the inner plaque, which is thought to be the precursor of the duplicating spindle pole body. **(C)** The box-shaped *Dictyostelium* centrosome consists of a three-layered core structure (Co), which is surrounded by an amorphous corona (Cn). Electron-dense nodules (No) are found in the corona, from which microtubules (MT) radiate. The centrosome is linked to the nuclear envelope (NE) via a strong, fibrous linkage, but is not embedded in the membrane.

The cellular slime mould *Dictyostelium discoideum*, a well established model organism for the investigation of cell development and motility (reviewed in Maeda et al., 1997), may successfully complement research on the yeast SPB and centriolar centrosomes. Its compact, acentriolar centrosome (Fig. 1C), also termed nucleus-associated body (NAB) because of its tight linkage to the nucleus (Moens, 1976; Omura and Fukui, 1985; Roos, 1975), may be viewed as representative for a large group of "lower" organisms, including many fungi and protists. It consists of a matchbox-shaped, three-layered core structure, which is surrounded by a corona consisting of amorphous material (Moens, 1976; Omura and Fukui, 1985; Roos, 1975). In interphase cells 30-40 microtubules emanate from electron-dense nodules embedded in the corona, which is therefore thought to correspond to the PCM found in other cell types (Euteneuer et al., 1998).

Recently important advances have been made that will help to establish this interesting centrosome type, which differs from both the yeast and mammalian MTOCs, as a promising centrosome model system. For example the structural changes and time course of the *Dictyostelium* centrosome cycle (see DISCUSSION, Fig. 34) were resolved on a thus far unprecedented level using a combination of electron microscopy and live observation of *Dictyostelium* mutants with GFP-labelled centrosomes (Ueda et al., 1999). Important steps towards a biochemical characterization were the establishment of an isolation protocol for *Dictyostelium* centrosomes (Gräf et al., 1998) and the subsequent generation of a range of anti-centrosomal monoclonal antibodies (Gräf et al., 1999). Furthermore, sequence information is increasingly becoming available from the *Dictyostelium* genome sequencing project and cDNA projects, which will be of great value for the identification of new centrosomal components. In fact, in the past few years seven different *Dictyostelium* centrosome components could be cloned with the aid of these prerequisites.

1.2 The essential centrosomal protein γ -tubulin and its cytosolic binding partners

One of the most thoroughly investigated centrosomal components is the protein γ -tubulin, which is ubiquitously found at MTOCs of all eukaryotes (reviewed in Joshi, 1994; Pereira and Schiebel, 1997). Genetic studies demonstrated that γ -tubulin is essential for the organization of the microtubule skeleton (Horio et al., 1991; Martin et al., 1997; Oakley et al., 1990; Sobel and Synder, 1995; Sunkel et al., 1995) and antibody inhibition experiments in vertebrates implicated γ -tubulin in microtubule nucleation (Felix et al., 1994; Joshi et al., 1992).

In all centrosome types investigated so far γ -tubulin is found at the sites of microtubule nucleation, where it specifically associates with microtubule minus ends: In animal

centrosomes it localizes to the PCM, in the yeast SPB to the outer and inner plaque and in the *Dictyostelium* centrosome it is found in the electron-dense nodules embedded in the corona (Euteneuer et al., 1998). In animal centrosomes γ -tubulin has been shown to be present in large, ring-shaped complexes, which are anchored in the PCM. The diameter of these complexes has approximately the size of a microtubule (25 nm) (Moritz et al., 1995; Vogel et al., 1997; Zheng et al., 1995) and intriguing models have been proposed to explain how these complexes, called γ TuRCs (γ -tubulin ring complexes), serve to nucleate microtubules (Erickson and Stoffler, 1996; Zheng et al., 1995). γ TuRCs are also found in substantial amounts in the cytoplasm of oocytes or early embryonic cells (Stearns and Kirschner, 1994; Vogel et al., 1997; Zheng et al., 1995). In many species oocytes do not possess any centrosomes prior to fertilization. They store large amounts of centrosomal proteins, including pre-assembled γ TuRCs, in their cytoplasm, because there is no synthesis of centrosomal material during the first rapid cell divisions until the early blastula stage. These oocytes are therefore a good source for purifying and investigating cytosolic γ -tubulin complexes. γ TuRCs have sedimentation coefficients of about 30S and were shown to consist of six or more proteins in addition to γ -tubulin, most of which have been cloned by now (Fava et al., 1999; Gunawardane et al., 2000b; Martin et al., 1998; Murphy et al., 1998; Oegema et al., 1999; Tassin et al., 1998; Zhang et al., 2000). In *Saccharomyces cerevisiae* no ring-shaped γ TuRCs seem to be present, neither at the SPB nor in cytoplasmic complexes. Instead, the yeast γ -tubulin homologue Tub4p was found to form small cytoplasmic complexes (6 S) with only two other proteins, Spc97p and Spc98p (Geissler et al., 1996; Knop et al., 1997). These small complexes interact directly with receptor molecules at the SPB: Spc110p at the inner plaque and Spc72p at the outer plaque (Knop and Schiebel, 1997; Knop and Schiebel, 1998). No other structural proteins seem to be required for the nucleation of microtubules at the yeast SPB. Interestingly, small complexes (γ -TuSCs) consisting of γ -tubulin and homologues of Spc97p and Spc98p were also found in higher organisms, e.g. *Drosophila* embryos, and were shown to be subunits of the larger γ -TuRCs (Oegema et al., 1999). When purified under high-salt conditions γ -TuRCs dissociate into salt-stable small γ -TuSCs and the remaining γ -TuRC-components. It is important to note that isolated γ -TuSCs have a small, but measurable microtubule nucleating activity, which is about 150-fold lower per mole of complex compared to isolated γ -TuRCs (Oegema et al., 1999). This suggests that the ring complex is not essential for microtubule nucleation, but is a specially adapted structure enhancing the efficiency of nucleation.

In this study the size and composition of cytosolic γ -tubulin complexes in *Dictyostelium* was investigated, whose centrosomal morphology differs from both the yeast and animal system.

The aim was to determine whether *Dictyostelium* γ -tubulin interacts with several proteins, similar to animal γ -TuRCs, or with only two proteins, similar to yeast Tub4p complexes, or whether it possibly assembles into completely different complexes. Due to the unique structural features of the *Dictyostelium* centrosome solving this issue may be of great interest for the discrimination between species-specific structural or essential microtubule-nucleating proteins, respectively.

1.3 The centrosome-associated EF-hand protein centrin

The ubiquitous centrosomal protein centrin was the first centrosomal protein that was characterized on the molecular level (Baum et al., 1986). It is a small, calcium-binding, calmodulin-related EF-hand protein (Schiebel and Bornens, 1995) and was discovered in the flagellar apparatus of the unicellular green alga *Chlamydomonas reinhardtii* (Huang et al., 1988). Since then homologues of the protein have been found in a variety of organisms, including other green algae (Bhattacharya et al., 1993; Ko et al., 1999), protozoa (Brugerolle et al., 2000; Levy et al., 1996; Madeddu et al., 1996; Meng et al., 1996), yeast (Baum et al., 1986), plants (Hart and Wolniak, 1999; Zhu et al., 1992) and vertebrates (Errabolu et al., 1994; Lee and Huang, 1993; Middendorp et al., 1997; Ogawa and Shimizu, 1993). So far in all cell types possessing a discrete centrosomal structure centrins were found to be associated with this structure, but additional subcellular localizations have been demonstrated for some organisms. In green algae, for example, centrin is a component of the basal bodies, but also of the distal fibers linking the two basal bodies, the flagellar roots linking the flagellar apparatus to the nucleus and the stellate fibers in the transition zone between basal body and flagellar axoneme (McFadden et al., 1987; Salisbury, 1998; Wright et al., 1985). In higher plants, where no structured MTOC can be found, centrin homologues are not localized at mitotic spindles, but at the developing cell plate (DeVecchio et al., 1997). Nevertheless, sequence conservation among the members of the centrin family is very high, ranging from 80-90% amino acid identity among vertebrates (Errabolu et al., 1994; Lee and Huang, 1993; Middendorp et al., 2000; Ogawa and Shimizu, 1993) to 50-70% amino acid identity between vertebrates and lower organisms (Baum et al., 1986; Hart and Wolniak, 1999; Zhu et al., 1992). The high degree of conservation and the ubiquity of the protein in all species investigated so far suggest that centrin is essential for proper cell function. However, the exact role of the protein is not quite clear. Centrin-based fibers in *Chlamydomonas* contract upon Ca^{2+} binding and seem to be responsible for orienting and segregating basal bodies (Taillon et al., 1992; Wright et al., 1989; Wright et al., 1985). The contractile fibers are also important

for microtubule severing during flagellar excision (Sanders and Salisbury, 1989; Sanders and Salisbury, 1994). Mutational analysis of the *Saccharomyces cerevisiae* homologue of centrin, Cdc31p, revealed that the protein is essential for cell viability. Temperature-sensitive cdc31 mutants arrest their cell cycle at the restrictive temperature with single, enlarged spindle pole bodies and G2 DNA content, but without a satellite (Byers and Goetsch, 1975). The appearance of the satellite at the cytoplasmic side of the half bridge, the site of Cdc31p localization (Spang et al., 1993), is normally the first sign of SPB duplication. Cdc31p is therefore thought to be involved in the initiation of spindle pole body duplication.

In animal cells, most of the centrin is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles (Paoletti et al., 1996). In humans three centrin genes (HsCen1 to 3) have been identified. HsCen1 and 2 seem to be more closely related to algal centrins, whereas HsCen3 is more similar to yeast Cdc31 (Middendorp et al., 1997). It is conceivable that the different centrins have different cellular functions. For HsCen3 a role in centrosome duplication was suggested (Middendorp et al., 2000), whereas HsCen2 seems to be involved in other cell division events (Paoletti et al., 1996). The three isoforms are also differentially expressed in epithelial cells: all three isoforms are expressed during cell differentiation, but only isoform 2 and 3 are expressed during cell proliferation (Laoukili et al., 2000). Taken together, despite their strong sequence conservation the cellular tasks different centrins fulfill seem to be manifold.

In this work the centrin homologue of *Dictyostelium discoideum* was identified and investigated, which turned out to be an especially divergent member of the centrin family. The protein shows an unusual localization and its sequence is so aberrant that it may have to be classified as a centrin-related protein (DdCrp). But since no other centrin gene has been found in *Dictyostelium* so far it is most likely at least functionally conserved and acts like a "real" centrin in the cell.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Unless stated otherwise chemicals were obtained from Sigma-Aldrich (Deisenhofen), Merck (Darmstadt), Carl Roth (Karlsruhe), Difco (Augsburg), Serva (Heidelberg) and Boehringer Mannheim (Mannheim) and were of p. a. quality.

2.1.2 Antibodies

Anti γ -tubulin, rabbit antiserum	(Euteneuer et al., 1998)
Anti-DdSp97, rabbit antiserum	This work
Anti-DdCrp, rabbit antiserum	This work
Anti-DdCP224, mAb 4-148	(Gräf et al., 1999)
Anti-Digoxigenin	Boehringer Mannheim
Goat anti-rabbit IgG, coupled to calf intestinal phosphatase	Sigma
Goat anti-mouse IgG, coupled to calf intestinal phosphatase	Sigma
Goat anti-rabbit IgG, coupled to Cy3 or FITC	Dianova
Goat anti-mouse IgG, coupled to Cy3 or FITC	Dianova
Goat anti-mouse IgG Alexa 488	Molecular probes, Inc.
Goat anti-mouse IgG Alexa 468	Molecular probes, Inc.
Anti c-myc, mAb 9E10	(Evan et al., 1985)
Anti-GFP, mAb 264-236-1	Chemicon

2.1.3 Enzymes

All restriction enzymes and buffers were purchased from New England Biolabs.

Other enzymes are listed together with the method they have been used for.

2.1.4 Antibiotics

Blasticidin S	ICN Biochemicals
Geneticin (G418)	Sigma
Penicillin/Streptomycin	Sigma

2.1.5 Media

2.1.5.1 Media for cultivation of *D. discoideum*

AX medium (Claviez et al., 1982)

14.3 g/l peptone (Oxoid), 7.15 g/l yeast extract (Oxoid), 50 mM maltose, 3.5 mM Na₂HPO₄, 3.5 mM KH₂PO₄, pH 6.7.

HL-5c medium

5 g/l yeast extract (Difco), 2.5 g/l bacto tryptone (Difco), 2.5 g/l casein peptone (Merck), 5 g/l proteose peptone (Oxoid) 10 g/l glucose, 1.2 g/l KH₂PO₄, 0.35 g/l Na₂HPO₄, pH 6.5.

Soerensen buffer (Malchow et al., 1972)

14,6 mM KH₂PO₄, 2 mM Na₂HPO₄, pH 6.0.

Phosphate agar plates

15 g/l bacto-agar in Soerensen buffer.

SM-agar plates

10 g/l peptone (Oxoid), 1 g/l yeast extract (Oxoid), 10 g/l glucose, 9 g/l bacto-agar, 16 mM KH₂PO₄, 5.7 mM KH₂PO₄, 4 mM MgSO₄, pH 6.5.

2.1.5.2 Media for cultivation of *E. coli* (all from Sambrook et al., 1989)

LB-medium

10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.4.

SOB-medium

20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.55 mM KCl.

SOB-MM-medium

SOB-medium supplemented with 20 mM glucose, 10 mM MgSO₄, 10 mM MgCl₂.

NZYM agar plates

10 g/l caseine (hydrolyzed), 5 g/l NaCl, 5 g/l yeast extract, 2 g/l MgSO₄, 15 g/l agar.

SM agar plates

9 g/l agar, 10 g/l peptone, 50 mM glucose, 1 g/l yeast extract, 4 mM MgSO₄, 16 mM KH₂PO₄, 5.7 mM K₂HPO₄.

SM buffer

100 mM NaCl, 8 mM MgSO₄, 50 mM Tris/HCl pH 7.5, 0.01% gelatin.

Top-agar

10 g/l caseine (hydrolyzed), 5 g/l NaCl, 5 g/l yeast extract, 2 g/l MgSO₄, 7 g/l agarose.

LB-amp medium

LB medium with 100 µg/ml ampicillin.

LB-agar plates

1,5% agar in LB.

LB-amp agar plates

LB-agar plates with 100µg/ml ampicillin.

2.1.6 Buffers and solutions

Buffers and solutions not listed below are described together with the method they have been used for.

100 x Denhardt's reagent

2% Ficoll 400, 2% Polyvinylpyrrolidone, 2% BSA.

PHEM-buffer (Schliwa et al, 1982):

60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9.

TE-buffer

10 mM Tris/HCl, 1 mM EDTA, pH 8.0.

20 x SSC

3 M NaCl, 0.3 M Na-citrate.

10 x TBE

890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.3.

PBS

70 mM Na₂HPO₄, 30 mM KH₂PO₄, 150 mM NaCl, pH 7.0.

TBS

10 mM Tris/HCl, pH 7.4, 150 mM NaCl.

Urea sample solution

9 M urea, 10% SDS, 5% 2-mercaptoethanol.

5 x Laemmli sample buffer

625 mM Tris/HCl, pH 6.8, 25% sucrose, 10% SDS, 0.025% bromophenolblue, 10% 2-mercaptoethanol.

SDS running buffer

25 mM Tris/HCl, pH 8.3, 0.1% SDS (w/v), 192 mM glycine.

2.1.7 Vectors

pQE30	Qiagen
pMalc2	New England Biolabs
pUCBsrÄBam	(Adachi et al., 1994)
pγrsGFP	(Ueda et al., 1997)

2.1.8 Bacterial and *D. discoideum* strains

E. coli XL-1 blue (Stratagene) was used for cloning and screening of cDNA libraries.

E. coli LE392 (NEB) was used for screening of cDNA libraries.

Klebsiella aerogenes (Williams and Newell, 1976) was used for the cultivation of *D. discoideum*.

SOLR (Stratagene) was used for *in vivo* excision.

D. discoideum strain AX2-214 (axenically growing derivative of the isolate NC-1, Raper, 1935).

2.1.9 Computer programmes

Winword 6.0 and 8.0 (Microsoft)

Adobe Photoshop 5.5, Apple Works 5.0, MacDraw Pro 1.5, NIH image 1.6.2 , Phylip Phylogeny package version 3.5 (Felsenstein, 1993), (all Macintosh)

Unix GCG-package (University of Wisconsin Genetics Computer Group)

Leica TCS-NT confocal imaging system.

2.1.10 Other materials

Talon ® His-affinity resin	Clontech
Ni-NTA His-affinity resin	Qiagen
NHS sepharose 4B	Pharmacia
Hybond N Nylon membrane	Amersham Pharmacia Biotech
Nitrocellulose BA85	Schleicher &Schüll
X-ray film X-omat	Kodak
Membrane for dialysis	Biomol

2.2. Molecular biology methods

2.2.1 Agarose gel electrophoresis

The separation of DNA fragments according to their size was usually performed using gels with 1% agarose in TAE buffer. Samples were mixed with 1/5 vol of 6 x TAE loading dye (10 mM Tris/HCl pH 8.0, 50 mM Na-EDTA pH 8.0, 1% SDS, 30% glycerol, 0.1% bromophenolblue) before loading. Gels were run with 5 V/cm and subsequently stained with 1 µg/ml ethidium bromide in TAE for 20 min. Bands were detected by UV-illumination and documented with the Eagle Eye II system (Stratagene, Heidelberg).

2.2.2 DNA extraction from agarose gels

Bands were excised, transferred to sterile Eppendorf vials, weighed and purified with Qia Quick columns (Qiagen) following the instructions of the manufacturer.

2.2.3 Determination of DNA and RNA concentration

DNA and RNA concentration in solutions was determined by measuring the A_{260} of the diluted sample after calibration of the photometer with a buffer control. An A_{260} of 1.0 corresponds to 50 µg/ml of DNA and to 40 µg/ml of RNA (Sambrook et al., 1989). DNA

concentration in ethidium-bromide-stained agarose gels was estimated by comparing band intensities with a molecular weight marker.

2.2.4 Preparation of plasmid DNA

Plasmid DNA was prepared from overnight cultures using the Qia Spin Prep kit for small scale preparations and the Qiagen Plasmid Midi kit (both Qiagen, Hilden, Germany) for cultures up to 100 ml.

2.2.5 Polymerase chain reaction (PCR)

For the analytical amplification of DNA fragments (e.g. colony screening) normal Taq polymerase (various manufacturers) was used for PCR. 25 μ l reactions contained 20 mM dNTP mix (5 mM for each nucleotide), 25 pmol 5' and 3'-primer, 1 U Taq polymerase and 2.5 μ l 10 x PCR buffer (100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 0.1% (w/v) gelatin). Bacterial cells, λ -phages, cDNA or plasmid DNA were used as template. Prior to amplification the reaction was denatured at 94°C for 2 min. (or 5 min. if bacteria or λ -phages were used as template). Generally 30 amplification cycles (denaturing at 94°C for 30 s, annealing at 45-60°C for 45 s, elongation at 72°C for 30-120 s) were performed. The annealing temperature of the primers was calculated as $4 \times (\text{number of G/C residues}) + 2 \times (\text{number of A/T residues}) - 3$.

1 min elongation time per 1000 bp was allowed. For preparative and very long PCRs the Expand high fidelity PCR system (Boehringer Mannheim) was used according to the instructions of the manufacturer.

2.2.6 Reverse transcription -PCR (RT-PCR)

This method was used to amplify cDNA fragments flanked by known sequences (e.g. genomic DNA). 1 μ g polyadenylated RNA was mixed with 1 μ g of a specific reverse primer in a total of 16 μ l, denatured at 70°C for 5 min and slowly cooled to 42°C to ensure specific annealing. Reverse transcription was initiated by addition of 5 μ l 5 x M-MLV buffer (Promega), 2 μ l dNTP-mix (5 mM each), 1 μ l RNasin inhibitor (Boehringer Mannheim) and 1 μ l M-MLV reverse transcriptase (Promega) and carried out for 1 h at 42°C. 2.5 μ l of the reverse transcription reaction were used as template for subsequent amplification by PCR using a specific primer pair.

2.2.7 Oligonucleotides

MycHis+: GGATCCGTTGATGGTGGTGAACAAAAATTAATTTTCAGAAGA
 AGATTTACTCGAGCATCATCATCATCATTAATCTAGA
 MycHis-: CCTAGGCAACTACCACCACTTGTTTTTAATTAAGTCTTCT
 TCTAAATAATGAGCTCGTAGTAGTAGTAGTAATTAGATCT
 γ mh rev: TGCTCGAGTAATAAATCTTC
 γ 8: CCAAGAGTAATCGATTCA
 γ 2: TGAAGTTTTACGTACAGATTC
 λ for: GGTGGCGACGACTCCTGGAGCCCCG
 Tn5neo: CGAACTGCAGGAGTGGGGAG
 Cen for: ATGAAAACATAAAACGTGT
 Cen rev: TCAATATATCTTTTTCCA
 CenforBam: TATATAGGATCCATGAAAACGTGT
 CenrevSac: TATATAGAGCTCTCAATATATCTTTTTCCA
 Cenfor3Eco: TATATAGAATTCATGAAAACATAAAACGTGT
 CenrevPst: TATATAGCAGTTCAATATATCTTTTTCCA
 SSC444-1 TTGAATCTGATCTTGCTTCACCT
 SSC444-2: TATAACCAGAGCCATCTCT
 CenfEco: TATGAATTC AATTTCAAACGAACAAATCC
 CenrBam: TATGGATCCATATATCTTTTTCCAATAT
 S97f2Bam: AAAGGATCCATTGAATATTCAAAGAATG
 S97r2Pst: TATCTGCAGTTAACTTGATGAAGAAGTCTTATTC
 S97fBam: AAAGGATCCATGACAACCCAACCAAC
 S97r3Pst: TATCTGCAGTTATTGATTACATTTCTAACAAC
 S98f5Sal: TACGCGTCGACTAATTACAGCAA
 S98r*Bam: CGGGATCCAATGGATTTAAATCTT
 98-14: ACGCCAAATCGAAGCCAACG
 98-f2: TCAACATTTGTACAGATACCA

2.2.8 DNA cleavage with restriction enzymes

Restriction digests were performed using the buffer system and temperature recommended by the manufacturer. Completion of the digests was analyzed on agarose gels. Chromosomal DNA (e.g. for Southern blots) was incubated with constant agitation for at 6-8 h using at least 100 U of enzyme for 10 μ g DNA in a volume of 250 μ l. Fresh enzyme was added after 3-4 hours.

2.2.9 Phosphatase treatment (Sambrook et al., 1989)

To prevent religation of linearized vectors with compatible ends 5' phosphate groups were removed by treatment with calf intestinal phosphatase (CIP). 2.5 µg linearized vector DNA were incubated in a 25 µl reaction in 1 x CIP buffer (50 mM Tris/HCl, pH 9.0, 1 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM spermidin) or NEB buffer 2-4 with 1 U CIP for 30 min at 37°C. The reaction was terminated by heating to 65°C for 10 min and the DNA was subsequently purified on an agarose gel.

2.2.10 Blunting of sticky ends with T4 DNA polymerase

Both 3' and 5' overhangs can be blunted with T4 DNA polymerase in the presence of nucleotides. 1 µg DNA were incubated in a 20 µl reaction in 1 x T4 DNA polymerase buffer (33 mM Tris-acetate, 66 mM K-acetate, 10 mM Mg-acetate, 0.5 mM DTT, 0.1 mg/ml BSA) containing dNTPs (0.5 mM of each nucleotide) with 1 U T4 DNA polymerase at 15°C for 20 min. The reaction was terminated by extraction with phenol/choloroform and DNA was purified on a Superdex 200 HR spin column or an agarose gel.

2.2.11 Ligation of DNA into plasmid vectors

DNA fragments were ligated with T4 DNA ligase in a total volume of 10 µl at 16°C for 16 h using the buffer system supplied by the manufacturer. For sticky end ligations the molar ratio of vector : insert was about 1:2, for blunt end ligations about 3:1. The concentration of DNA fragments was estimated from the band intensities on analytical agarose gels. Blunt end ligation of PCR fragments was performed with the Sure Clone ligation kit (Pharmacia) following the instructions of the manufacturer.

2.2.12 Preparation of electrocompetent *E. coli* cells

1 l LB-medium was inoculated with 5 ml of an overnight culture of the desired *E. coli* strain (usually XL1-blue) and grown to an OD₆₀₀ of 0.5. Cells were sedimented (3000 rpm, JA 14.1 rotor, 10 min, 4°C) and gently resuspended in cold, sterile H₂O. After three washes with cold H₂O (in sterile Falcon tubes) cells were washed in cold 10% glycerol and finally resuspended in a total volume of 2 ml 10% glycerol. After aliquotting (50 µl) into sterile Eppendorf cups cells were immediately frozen in liquid nitrogen and stored at -70°C.

2.2.13 Electrotransformation of *E. coli* cells

Electrocompetent cells were thawed on ice, mixed with 1-5 µl of DNA and transferred to a precooled sterile electroporation cuvette (distance between the electrodes: 2 mm). After one

pulse (2.5 kV, 25 mF, 200) 1 ml of cells were resuspended in 1 ml SOB-MM medium, gently agitated for 45 min and plated on LB-agar plates.

2.2.14 Identification of transformed clones in *E. coli*

Whenever possible bacterial colonies were selected and transferred with a sterile pipet tip into a PCR reaction tube containing the pre-pipetted reaction mixture and screening PCR was immediately performed. An aliquot of the cells still adhering to the tip was transferred to LB-agar plates and incubated at 37°C overnight to have a backup of positive clones. Alternatively, plasmid DNA was prepared from 5 ml overnight cultures, digested with appropriate restriction enzymes and analyzed on an agarose gel to identify successful transformants.

Whenever a blue/white colony selection was possible (e. g. using pBluescript) agar plates were treated with 60 µl IPTG solution (50 mM) and 40 µl X-Gal solution (20 mg/ml in dimethylformamide) prior to plating the transformants and only white colonies were picked.

2.2.15 Preparation of chromosomal DNA from *D. discoideum*

$1-2 \times 10^8$ cells of an axenically growing culture were washed twice with cold H₂O, and the cell pellet was resuspended in 50 ml lysis buffer (10 mM Mg-acetate, 10 mM NaCl, 30 mM HEPES, pH 7.5, 10% sucrose, 2% Nonidet P40). Cells lysed upon this treatment and nuclei were sedimented at 6000 g (JA 20 rotor, 7500 rpm, 10 min, 4°C), resuspended in SDS-lysis buffer (TE buffer with 0.7% SDS) and supplemented with 100 µl proteinase K solution (14,7 mg/ml). After 2-3 h incubation at 60°C the lysate was carefully extracted with an equal volume of phenol/chloroform (Sambrook, 1989) until the upper phase was clear (2-4 times). DNA was precipitated by addition of 1/10 volume of 2 M Na-acetate (pH 5.2) and 2 volumes of ethanol and the white threads of DNA were fished with a glass hook. DNA was washed in 70% ethanol, air-dried and dissolved in 200-500 µl of TE buffer.

2.2.16 Transformation and cloning of *D. discoideum*

Electroporation

Dictyostelium cells were grown to a density of $2-3 \times 10^6$ cells/ml, harvested and washed once in cold Soerensen buffer and twice in cold electroporation buffer (50 mM sucrose, 10 mM KH₂PO₄, pH 6.1). Cells were resuspended in cold electroporation buffer at a final density of 1×10^8 cells/ml, mixed with 15-30 µg of plasmid DNA and transferred to a precooled, sterile electroporation cuvette (distance between electrodes 4 mm). After two pulses (1.0 kV, 3 µF) in an electroporation device (Gene pulser, Biorad) cells were transferred to a sterile tissue

culture dish for a 15 min recovery period at room temperature. After supplementation with a $MgCl_2/CaCl_2$ solution (final concentration 1 mM each) cells were gently agitated for another 15 min at room temperature. Finally cells were resuspended in 50 ml HL-5c medium and distributed into a 24-well plate. After a 24 h recovery period without antibiotics the respective antibiotic (4 $\mu\text{g/ml}$ Blastidicin S (ICN) or 10 $\mu\text{g/ml}$ G418 (Sigma)) was added and the cells were incubated for 8-14 days until colonies of resistant cells appeared.

Cloning of transformants

Transformants were resuspended with a sterile pipette, a droplet of the cell suspension was transferred to a coverslip and cells were examined by immunofluorescence microscopy (section 2.4.4). If cells with the desired label were found, different concentrations of the remaining cells were plated on SM agar plates together with a dense suspension of *Klebsiella aerogenes* cells. After 2-3 days incubation at 21°C feeding plaques appeared and transformants were lifted with a sterile toothpick from the edges and transferred to a 24-well plate with HL-5c medium containing Blastidicin S (4 $\mu\text{g/ml}$) or G418 (10 $\mu\text{g/ml}$) and a Penicillin/Streptomycin solution (Sigma).

2.2.17 Isolation of polyadenylated RNA from *D. discoideum*

Polyadenylated RNA (mRNA) was prepared with the QuickPrep mRNA micro kit (Amersham-Pharmacia) according to the instructions of the manufacturer. The yield was consistently 7-8 μg of mRNA per 1×10^7 cells and mRNA was precipitated in 2-4 aliquots with 1/10 vol 2 M K-acetate and 2 vol of ethanol and glycogen and stored at -70°C until use. Precipitated mRNA was recovered by 15 min centrifugation at 14000 rpm (Beckman CS-15R centrifuge, F2402 rotor), washed with 70% ethanol in DEPC-treated water, air-dried and dissolved in DEPC-treated water.

2.2.18 Electrophoresis of RNA and Northern blotting

A method described by Liu and Chou (1990) was used, in which RNA treated with a denaturing sample buffer is electrophoresed on a native agarose gel. Under these conditions RNA stays denatured for at least 3 h, which is sufficient for many applications.

5 μg of mRNA were denatured with the same volume of 2 x denaturing buffer (2 x TBE, pH 8.3, 13 % w/v Ficoll, 0.01 % bromophenol blue) at 65°C for 10 min. Samples were loaded on a 1.5 % agarose gel in DEPC-treated TBE buffer and electrophoresed with 2 V/cm. An RNA size marker (NEB) electrophoresed on the same gel was excised, stained with ethidium bromide and photographed next to a ruler.

After electrophoresis the gel was equilibrated in 10 x SSC for 30 min and blotted onto a nylon membrane in 20 x SSC by capillary transfer. The blot was air-dried and the RNA cross-linked by UV-illumination for 1.5 min.

2.2.19 Southern blotting

10 µg of genomic DNA digested as described in section 2.2.8 were electrophoresed on a 0.8% agarose gel in TE buffer at 1-2 V/cm. The gel was stained with ethidium bromide and photographed next to a ruler. The DNA was denatured by agitating the gel 3 x 15 min in 1.5 M NaCl/0.5 M NaOH. The gel was then neutralized by 3 x 15 min washes in 1 M Tris/Cl pH 7.4/1.5 M NaCl. After equilibration of the gel in 10 x SSC DNA was transferred to a nylon membrane in 10 x SSC by capillary transfer. The blot was air-dried and DNA cross-linked by UV-illumination for 1.5 min.

2.2.20 Radioactive labelling of DNA probes, hybridization and detection

DNA probes for the hybridization of Northern and Southern blots were synthesized with the "prime it" labelling kit (Stratagene) following the instructions of the manufacturer. This method involves hybridization of random oligonucleotides to a single strand DNA probe and synthesis of the complementary strand by the Klenow fragment, incorporating α -³²P-labelled ATP. Nucleotides that had not been incorporated were removed by centrifuging through a Sephadex-G50 column.

The probe was denatured for 5 min at 100°C prior to hybridization and mixed with about 10 ml hybridization buffer (50% formamide, 1% Na-laurylsarcosinate, 0.1% SDS, 4 mM EDTA (pH 7.2), 0.12 M Na-phosphate buffer (pH 6.8), 2 x SSC, 4 x Denhardt's reagent). The nylon membrane was pre-hybridized with hybridization buffer for 1 h at 37°C before addition of the DNA probe. Hybridization was performed at 37°C over night and the filter washed 3-5 times in hybridization wash buffer (same composition as hybridization buffer but without Denhardt's reagent) at 37°C until radioactivity measured in the discarded wash buffer had reached background levels. The membrane was wrapped in cling foil and exposed on an X-ray film for 1-5 days at -70°C with an intensifying screen.

2.2.21 Digoxigenin labelling of DNA probes, hybridization and detection

For non-radioactive detection DNA probes were labelled with DIG using the DIG DNA labelling and detection system (Boehringer Mannheim). DIG-labelled DNA probes were synthesized by PCR, using a 20 x dNTP mix (see section 2.2.5) that had been supplemented with an equal volume of 10 x DIG DNA labelling mix (same total dNTP concentration). The

labelled probe, which migrates noticeably slower than the corresponding unlabelled PCR fragment, was purified on an agarose gel. The probe was either eluted from the gel slice or the molten gel slice was directly used for hybridization.

Nylon filters with cross-linked DNA were pre-hybridized in Easy Hyb solution (Boehringer Mannheim) at 37°C for 1 h and were then supplemented with the freshly denatured DIG-labelled probe (at least 50 ng/100 cm² filter surface, concentration estimated from the band intensity on ethidium bromide stained gels). Hybridization was performed at 37°C over night and the filters were washed twice with high salt buffer (2 x SSC, 0.1% SDS) for 5 min at room temperature and twice with low salt buffer (0.1 x SSC, 0.1% SDS) for 15 min at 65°C. Filters were equilibrated with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 min and incubated with anti-digoxigenin antibodies coupled to alkaline phosphatase (1:5000 dilution in maleic acid buffer containing 1% blocking reagent) for 30-60 min at room temperature. Unbound antibodies were removed by three 10 min washes in maleic acid buffer and the probe was detected by NBT/BCIP colour detection (see section 2.3.5)

2.2.22 Construction of a subgenomic library enriched in DdSpc98 sequence

Because DdSpc98 was very poorly represented in cDNA libraries subgenomic libraries enriched in DdSpc98 sequence were constructed. To achieve this, Southern blots of genomic DNA digested with different restriction enzymes were probed with a 609 bp, PCR-generated ³²P labelled DdSpc98 probe (base position 276 to 885 of clone JAX4a195a03, using primers 98-5 and 98-12) to identify fragments of suitable length for cloning into expression vectors. Suitable fragment sizes (approx. 2 and 7 kb) were obtained for *Eco*RI fragments (see section 3.1.1). They were excised from preparative 0.8% agarose gels loaded with 12 µg *Eco*RI digested genomic DNA, eluted and purified with Qia Quick columns (Qiagen, Hilden, Germany). The eluted *Eco*RI fragments were ligated into λZAPII-*Eco*RI arms and packaged *in vitro* (GigapackIII, Stratagene, Amsterdam, Netherlands) following the instructions of the manufacturer.

2.2.23 Screening of cDNA and genomic libraries

For the screening of λZAP libraries XL1blue cells and for λgt11 libraries LE392 cells were grown overnight in LB-medium containing 10 mM MgSO₄/0.02% maltose, harvested and resuspended in 10 mM MgSO₄ at an OD₆₀₀ of 1.0. 600 µl of the bacterial suspension were infected with 60000-70000 pfu of the λ-phage library and agitated for 20 min at 37°C. The suspension was plated with 12-13 ml of warm Top agar on NZYM agar plates (Ø 9 cm). The plates were incubated at 37°C for 4-6 h at 37°C until plaques had grown to a diameter of

about 1 mm. Plates were allowed to cool at 4°C before nylon filters cut to the size of the plates were briefly placed onto the plaques. The position of the filters was labelled by piercing with a needle. The filters were placed with the phage side up on Whatman 3MM filters soaked with denaturing buffer (1.5 M NaCl/0.5 M NaOH) for about 5 min to lyse the phages and to denature the DNA. The filters were then neutralized by treating them the same way with neutralizing buffer (1 M Tris/HCl pH 7.4/1.5 M NaCl) and finally with 2 x SSC. After air-drying the DNA was UV crosslinked, filters were hybridized with DIG-labelled probes and colour detection was carried out as described in section 2.3.5. Positive plaques were excised, mixed with 500 µl SM-buffer/10 µl chloroform for at least 15 min at 37°C and maintained at 4°C over night, until the majority of the phages had diffused into the buffer. Screening was repeated with the excised phages, reducing the number of pfu in each round, until all phages were positive, i. e. clones were isolated (3-4 rounds).

The pBluescript plasmids containing the cDNA inserts which are part of the λ ZAP sequence were rescued by *in vivo* excision (Short et al., 1988). For this, 200 µl of an overnight culture of XL-1 blue cells resuspended in 10 mM MgSO₄ at an OD₆₀₀ of 1.0 were mixed with 250 µl phage stock and 1 µl of ExAssist helper phage (Stratagene), shaking at 37°C for 15 min. 3 ml of LB were added and the solution incubated for another 2.5 - 3 h at 37°C, shaking with 200 rpm. The tubes were heated at 65°C for 20 min and debris was sedimented by spinning at 1000g for 15 min. 10-100 µl of the supernatant were mixed with 200 µl freshly grown SOLR cells (resuspended at an OD₆₀₀ of 1.0 in 10 mM MgSO₄), incubated at 37°C for 15 min and plated on LB-amp agar plates. Colonies of SOLR cells containing the excised pBluescript-phagemids were picked and analyzed on the following day.

2.2.24 Construction of the vectors for myc/6xHis-tagging of endogenous γ -tubulin and DdCrp

The homologous recombination plasmid pUC γ myc/His-Bsr was constructed by ligating a custom-synthesized, HPLC-purified 78mer annealed oligonucleotide coding for the myc/6xHis-tag into a vector containing the γ -tubulin coding sequence. For annealing, the oligonucleotides MycHis⁺ and MycHis⁻ were mixed at a concentration of 50 pM each, heated for 3 min at 94°C and slowly cooled to room temperature. The double stranded insert was purified on an agarose gel and ligated into pB15 γ rsGFP (Ueda et al., 1997) digested with *Bam*HI and blunted with T4 DNA Polymerase.

The homologous recombination plasmid pDdCrp-myc/His was derived from pUC- γ myc/His-Bsr by excising the γ -tubulin sequence with *Eco*RI and *Bam*HI and replacing it with a DdCrp fragment generated by PCR on genomic Dd DNA using the primers CenfEco and CenrBam

(base position 74 to 497 of clone SSF324 (Tsukuba University cDNA project, Morio et al., 1998)).

2.2.25 Construction of bacterial expression vectors

The vector pQ-Cen for the expression of N'-6xHis tagged DdCrp was constructed by ligating the full-length DdCrp fragment obtained by PCR with primers CenforBam and CenrevSac using the clone SSF324 (Tsukuba University cDNA project, Morio et al., 1998) as a template into the *Bam*HI and *Sac*I sites of the pQE30 vector.

The vector pMal-Cen for the expression of an N-terminal fusion of MBP to DdCrp was constructed by ligating the full-length DdCrp fragment obtained by PCR with primers Cenfor3Eco and CenrevPst DdCenrevSac using the clone SSF324 as a template into the *Eco*RI and *Pst*I sites of the pMalC2 vector.

The vector MBP-N'DdSpc97 for the expression of an N-terminal fusion of MBP with an N-terminal fragment of DdSpc97 (base position 1-1581) was constructed by ligating the PCR generated fragment into the pMalc2 expression vector using the *Bam*HI and *Pst*I sites. The excised cDNA phagemid containing the DdSpc97 coding sequence was used as a template and the primers used to amplify the N'-DdSpc97 fragment were S97fBam and S97r3Pst.

The vector MBP-C'DdSpc97 for the expression of a C-terminal fragment of DdSpc97 (base position 1749-2975) was constructed the same way, using the primers S97f2Bam and S97r2Pst for PCR.

2.3. Biochemical and immunological methods

2.3.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

Proteins were separated on discontinuous SDS-polyacrylamide gels as described by Bollag et al. (1996). Stock solutions were filtered through a 0.4 µm polycarbonate filter to remove particles. 12.5% or 17.5% gels were always prepared freshly from the stock solutions and run in the Biorad Mini Proten III system at 15 A per gel for 20 min and then at 30 A per gel for 45 min.

Samples were either mixed with an equal volume of urea sample buffer or 1/4 volume of 5 x Laemmli sample buffer (Laemmli, 1970) and boiled for 2-5 min.

Tab. 1 Composition of SDS-polyacrylamide gels with different acrylamide concentrations.

Stock solution	3% stacking gel	12.5% separating gel	17.5% separating gel
30% acrylamide	0.68 ml	3.70 ml	5.14 ml
1% Bis -acrylamide	0.50 ml	0.93 ml	0.64 ml
Separating buffer (2M Tris/HCl, pH 8.7, 0.4% SDS)	-	2.5 ml	2.5 ml
Stacking buffer (0.25M Tris/HCl, pH 6.8, 0.4% SDS)	1.0 ml	1.8 ml	-
H ₂ O	1.78 ml	1.80 ml	0.72 ml
10% APS	35 µl	45 µl	45 µl
TEMED	7 µl	9 µl	9 µl

2.3.2 Coomassie staining

Gels were stained for 1 h in Coomassie staining solution (0.1% Coomassie Brilliant blue R250) and destained in several changes of Coomassie destaining solution (10% ethanol, 7% acetic acid) until the destaining solution remained clear.

2.3.3 Colloidal Coomassie staining

This staining method is considerably more sensitive than the conventional Coomassie R250 staining. Gels were fixed in 10% TCA for at least 1 h and washed 3 x 10 min in H₂O. The Coomassie staining stock (2 g phosphoric acid (85%), 10 g ammonium sulfate, 2 ml Coomassie G250 (5%)) was mixed with 1/4 vol methanol just before use and the gel was stained over night. Unbound color was removed by several washes in H₂O.

2.3.4 Methanol/chloroform precipitation of proteins

This method described by Wessel and Flügge (1984) is suitable for dilute protein solutions containing detergents, phospholipids or salt.

The protein solution was mixed with 5 volumes of a methanol/chloroform mixture (4:1), supplemented with 3 volumes H₂O and mixed vigorously. The upper liquid phase after centrifuging 1 min/10000g was removed and the chloroform cushion with the precipitated protein was mixed with 3 volumes of methanol and centrifuged for 2 min/10000 g. The supernatant was removed carefully and the protein pellet was air-dried and dissolved in 0.5 x urea sample solution /separating buffer.

2.3.5 Western blots and immunostaining

Polyacrylamide gels were blotted with the semidry procedure using the buffer system of Kyhse-Anderson (1984), modified by the addition of 20% methanol to the three buffers. (Buffer 1 (300 mM Tris, 0.01% SDS), buffer 2 (30 mM Tris, 0.01% SDS), buffer 3 (30 mM Tris, 40 mM *ε*-amino-*n*-capronic acid).

Blotting was carried out for 1 h at 1 mA/cm² and blots were reversibly stained with 0.25% Ponceau S (Sigma) in 40% methanol/15% acetic acid prior to immunostaining. Marker bands and prominent protein bands were labelled on the blot before blocking in TBST (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-200) containing 3% fish gelatin. Incubation with the primary antibody diluted in TBST/1% fish gelatin was carried out for 1 h at room temperature, followed by washes in TBST (3 x 5 min) and incubation with the secondary antibody (1:10000 dilution in TBST/1% fish gelatin of anti-rabbit or anti-mouse antibodies coupled to alkaline phosphatase) for 1 h at room temperature. Blots were washed 3 x 5 min with TBST, rinsed briefly in AP-reaction buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂) and colour detection was carried out by 5-60 min incubation in AP reaction buffer supplemented with 4.5 µl/ml NBT (75 mg/ml stock in 70% dimethylformamide) and 3 µl/ml BCIP (50 mg/ml in dimethylformamide).

2.3.6 Determination of protein concentration

For rough estimations of protein concentrations the OD₂₈₀ of a solution was measured, assuming about 0.7 mg/protein per 1 OD₂₈₀ (a value which is most accurate for immunoglobulins).

For accurate measurements the Bradford assay (Bradford, 1976) was carried out and a reference curve with BSA was generated for each measurement.

2.3.7 Purification of bacterially expressed proteins

2.3.7.1 Purification of bacterially expressed His-DdCrp

Purification of His-DdCrp was performed under denaturing conditions, essentially following the recommendations of the manufacturer of the Ni-NTA resin (Qiagen).

0.5 l of the transformant *E.coli* culture were grown to an OD₂₈₀ of 1.0 in LB-Amp containing 25 µg/ml kanamycin, induced with 2 mM IPTG and grown for another 2 h at 37°C. Cells were harvested (10 min, 5000 rpm, Sorvall rotor GSA, 4°C) and lysed by freeze-thawing and sonicating in 30 ml buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 8.0). The supernatant received after centrifuging for 20 min at 20000 rpm (Sorvall SS34 rotor) was

mixed with 4 ml Ni-NTA resin and was stirred for 1 h at room temperature. The resin was transferred to a column and washed with 15 ml buffer B, 15 ml buffer C (6 M urea, 0.1 M Na-phosphate, 0.01M Tris/HCl, pH 6.3) and 15 ml buffer C, pH 5.8 and finally His-DdCp was eluted with 6 ml buffer F (6 M GuHCl, 0.2 M acetic acid).

2.3.7.2 Purification of bacterially expressed, MBP-tagged proteins

0.5 l of the respective *E.coli* culture were grown to an OD₂₈₀ of 1.0 in LB-Amp containing 10 mM glucose at 37°C. After induction with 0.3 mM IPTG cells were grown for another 2-5 h at 23-37°C, depending on the construct. Cells were harvested (10 min, 5000 rpm, Sorvall rotor GSA, 4°C), resuspended in a small volume of TE buffer and frozen at -20°C. After thawing the cell suspension was sonicated and centrifuged for 20 min with 20000 rpm (Sorvall SS34 rotor). The supernatant was filtered through a 0.8 µm polycarbonate filter and adjusted to 100 mM NaCl. The solution was then applied slowly (approx. 5 sec per droplet) to an affinity column containing 1 ml amylose resin. The resin was washed extensively with column buffer (200 mM NaCl, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA) or NHS coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3) and the MBP-tagged protein was eluted with the buffer of choice (either column buffer or NHS-coupling buffer) containing 10 mM maltose. Purity of the preparation was assessed by SDS-PAGE.

2.3.7.3 Purification of myc/6xHis-tagged cytosolic γ -tubulin complexes from *D. discoideum*

Shaking cultures of approx. 5×10^9 *Dictyostelium discoideum* cells (γ myc/6xHis mutants or control AX2 cells) at a density of approx. $4-5 \times 10^6$ cells/ml were harvested and washed three times with Soerensen buffer. This and all subsequent purification steps were performed at 4°C or on ice. Cytochalasin A (Sigma, Deisenhofen, Germany) was included at a final concentration of 2 µM in the last washing step. Cell pellets were resuspended in 5 volumes of lysis buffer (50 mM Na-Hepes, pH 7.4, 100 or 400 mM NaCl, 10% sucrose, 2 mM MgCl₂, 1 mM ATP, 2 µM cytochalasin A, protease inhibitor cocktail) and cells were lysed by passage through a 5 µm mesh polycarbonate filter (Nuclepore, Costar GmbH, Bodenheim, Germany). The cytosolic supernatant after 20 min centrifugation at 10000 g was cleared with a 0.8 µm mesh polycarbonate filter and was immediately incubated with 500 µl Talon[®] affinity resin stirring on ice for 1-2 hours. The resin was washed extensively with wash buffer (50 mM Na-Hepes, pH 7.4, 100 mM or 400 mM NaCl, 2 mM MgCl₂) containing 2 mM imidazole. γ -Tubulin complexes were eluted in 1.5 ml wash buffer containing 100 mM imidazole and protease inhibitors. The eluates were incubated at 4°C overnight on a rotary shaker with 30 µl

affinity resin, containing approx. 30 μg of monoclonal anti-myc antibodies covalently linked to NHS-activated Sepharose (see section 2.3.13). Immunoprecipitations were washed 5 times with wash buffer containing 0.05% Triton-X-100 and the resin was boiled in SDS buffer (125 mM Tris/HCl, pH 6.8, 2% SDS, 5% sucrose, 0.04% bromophenole blue) without 2-mercaptoethanol to avoid dissociation of the antibody chains.

2.3.8 Preparation of whole cell extracts, nuclei and centrosomes from *D. discoideum*

Whole cell extracts for SDS-PAGE were obtained by harvesting and washing cells as described above, resuspending the cell pellet in an equal volume of 0.5 x urea sample solution/separating buffer and boiling it for 3 min.

Nuclei were isolated from 1.5 l of a *Dictyostelium* culture with a density of $3\text{-}4 \times 10^6$ cells/ml. Cells were harvested and washed three times with Soerensen buffer, including 2 μM cytochalasin A in the last washing step. The cell pellet was resuspended in 30 ml lysis buffer (10 mM Na-PIPES, pH 6.9, 2 mM MgCl_2 , 10% (w/v) sucrose, 0.25% Triton X-100, 1 x protease inhibitor cocktail, 2 μM cytochalasin A) and cells were lysed by passage through a 5 μm mesh polycarbonate filter. Nuclei were pelleted by centrifuging at 2500g for 10 min at 4°C.

Centrosomes were isolated from isolated nuclei essentially according to Gräf et al. (1998). For this, the nuclear pellet was resuspended in 30 ml pyrophosphate buffer (100 mM Na-PIPES, pH 6.9, 2 mM MgCl_2 , 30% (w/v) sucrose, 40 mM tetrasodium diphosphate, 1 mM DTT, 1% Triton X-100, 1 mM Na-ATP, 1 x protease inhibitor cocktail), vortexed for 1 min and centrifuged at 2500g for 10 min at 4°C. The supernatant was supplemented with 0.6 ml of a heparin solution (10 mg/ml) and was incubated on ice for 5 min. The solution was then filtered twice through a 5 μm polycarbonate filter and was loaded on a first sucrose step gradient (two Beckman SW40 tubes containing 0.5 ml of 80% and 1.5 ml of 50% sucrose in gradient buffer: 10 mM Na-PIPES, pH 6.9, 2 mM MgCl_2 , 0.1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mM Na-ATP, 1 x protease inhibitor cocktail). After centrifuging at 55000g for 1 h at 4°C the centrosomes were recovered from the border between the two sucrose fractions (1ml per gradient). The collected fraction was diluted with 1.5 ml gradient buffer and was sonicated in a Branson sonifier 250 (two pulses, 1 s each, lowest output). It was then loaded on a second sucrose density gradient (two Beckman SW50.1 tubes containing 0.5 ml of 80%, 0.5 ml of 70%, 1 ml of 55% and 1 ml of 50% sucrose solution). After centrifuging at 40000g for 1 h at 4°C two centrosome-containing fractions (0.6 ml and 0.8 ml) were collected from the bottom, with the second fraction containing less contaminating vesicles and DNA.

For immunofluorescence microscopy 2 μ l of centrosomes were diluted in 0.5 ml PBS and were sedimented onto round untreated coverslips by centrifugation at 2500 g for 20 min at 4°C. Centrosomes were fixed and stained as described in section 2.4.3. For SDS-PAGE nuclear and centrosome fractions were mixed with 1/4 volume of 5 x Laemmli buffer and boiled for 3 min.

2.3.9 Gel filtration

γ -Tubulin complexes were enriched by His-tag affinity purification of cytosolic extracts prepared from about 3×10^9 cells as described in section 2.3.7.3.

Eluates were concentrated with YM-30 Centricon filter devices (Millipore) and 200 μ l were loaded on a Superdex 200 gel filtration column (Amersham Pharmacia Biotech, Freiburg, Germany). Gel filtration was performed in 50 mM Hepes, pH 7.4, containing 100 or 500 mM NaCl at a flow rate of 0.5 ml/min. 1 ml fractions were collected, precipitated with the methanol/chloroform precipitation, electrophoresed and blotted. Molecular weights were estimated by comparison with globular marker proteins (Boehringer combithek, calibration kit II, cytochrome C 12.5 kDa, ovalbumin 45.0 kDa, BSA 68.0 kDa, aldolase 158.0 kDa, ferritin 450 kDa).

2.3.10 Antigen preparation and immunizations

About 3 mg of denatured, purified His-DdCrp were dialyzed against 6 M urea/ 50 mM Tris/HCl pH 7.0, concentrated in Centriprep filters and loaded on a single-well 17.5% SDS gel for further purification. The band was stained with 300 mM CuSO_4 , excised, destained in 0.3 M CuCl_2 and used for the immunization of two rabbits.

The fusion proteins of DdCrp, DdSpc97 or DdSpc97 with MBP purified on amylose columns were of sufficient purity to be used directly for the immunization of two rabbits each (using about 1mg of protein per rabbit). All immunizations were carried out by the Pineda Antikörper Service (Berlin, Germany), using a standard protocol of five immunizations over 60 days.

2.3.11 Covalent coupling of antibodies and purified proteins to NHS-activated sepharose

Monoclonal anti-myc antibodies intended for coupling were taken from mouse hybridoma cells (Evan et al., 1985) growing in serum-free medium. They were precipitated from cell culture supernatants by slowly adding an equal volume of a saturated ammonium sulfate solution, stirring on ice for at least 1 h. The pellet obtained after centrifuging for 20 min at 14000 rpm (Sorvall rotor GSA) and 4°C was resuspended in 1 ml of coupling buffer (0.2 M

NaHCO₃, 0.5 M NaCl, pH 8.3) and dialyzed overnight in 3 x 0.5 l of coupling buffer. Before coupling the sample was centrifuged for 10 min at 10000 g, the supernatant collected and concentrated to 1 ml in YM30 Centricon devices.

Purified MBP-fusion proteins intended for coupling were washed and eluted directly in coupling buffer supplemented with 100 mM NaCl and were also concentrated to a final volume of 1 ml. Purified His-DdCrp, which precipitated upon dialysis with coupling buffer, was solubilized in 0.2% SDS before coupling.

For coupling, 0.5 ml of NHS-activated sepharose (Pharmacia) were washed with ice cold 1 M HCl and were immediately mixed with 1 ml of the protein solution containing 0.5-5 mg of protein. The slurry was rotated in an Eppendorf cup for 2-4 h at room temperature or at 4°C over night. The resin was then washed several times alternating between buffers A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and B (0.1 M acetic acid, 0.5 M NaCl, pH 4.0), allowing a 20 min incubation time in buffer A at half time, for complete saturation of all unoccupied binding sites. The resin was stored in 50 mM phosphate buffer, pH 7.0 containing 0.02% NaN₃.

2.3.12 Affinity purification of antisera

Antisera were mixed with an equal volume of PBS, filtered through a 0.8 µm polycarbonate filter to remove particles and applied to sepharose columns containing the covalently coupled antigen (see section 2.3.11). The columns were washed extensively with PBS and specific antibodies were eluted with 100 mM glycine, pH 2.7 and were neutralized immediately by the addition of droplets of 1 M Tris/HCl, pH 8.7. For storage affinity purified antibodies were supplemented with 0.5% BSA and 0.02% NaN₃.

Unfortunately affinity purification did not work for any of the anti-DdCrp antibodies, so that for these only staining with the crude antisera can be shown.

2.3.13 Immunoprecipitation

For immunoprecipitations 1 ml of cytosolic extract was incubated with 30 µl sepharose beads containing immobilized antibodies (see section 2.3.11) at room temperature for 30 min. The sepharose beads were washed five times with wash buffer (50 mM Na-Hepes, pH 7.4, 100 mM or 500 mM NaCl, 2 mM MgCl₂) and resuspended in 1 x Laemmli buffer without 2-mercaptoethanol. Samples were boiled for 3 min, electrophoresed and blotted.

2.4 Cell biological methods

2.4.1 Cultivation and preservation of *D. discoideum*

Dictyostelium cells were grown shaking at 150 rpm at 21°C in AX2 medium, containing BlasticidinS or G418 in case of mutants. Under these conditions the doubling time was about 9 h. Backup cultures of adherent cells were kept in HL5 medium in small tissue culture flasks and medium was changed twice a week.

For long-term storage cells were subjected to starving conditions, inducing the formation of spores, which can easily be frozen and stored. For this, axenically growing cells were washed twice with Soerensen buffer, resuspended at a density of $2\text{-}3 \times 10^8$ cells/ml and 500 μ l of the suspension was plated out on freshly prepared phosphate agar plates. Cells formed spore-containing fruiting bodies within 2-3 days, which were washed off with sterile Soerensen buffer (about 5 ml per plate), shock-frozen in 1 ml aliquots (Nunc 2.2 ml tubes) in liquid nitrogen and stored at -80°C . For inoculation of a shaking culture, spores were thawed at room temperature, resuspended in 30 ml HL5 medium and grown shaking at 150 rpm/21°C. After 3 days the cultures usually had a density of about 5×10^6 cells/ml.

2.4.2 Cultivation and preservation of *E. coli*

E. coli cells were grown according to standard methods at 37°C on agar plates or shaking at 240 rpm in LB-medium containing the desired antibiotic (Sambrook et al. 1989). For long-term storage 500 μ l of a bacterial culture were mixed with 70 μ l sterile glycerol, frozen in liquid nitrogen and stored at -80°C .

2.4.4 Indirect immunofluorescence microscopy on whole *D. discoideum* cells and isolated centrosomes

Cells of a logarithmically growing *D. discoideum* culture were diluted to 1×10^6 cells/ml with HL5 medium and allowed to settle on a round coverslip. After about 20 min the medium was removed and cells were fixed with the respective fixation method.

Isolated centrosomes were centrifuged onto round coverslips (see section 2.3.10) and fixed like whole cells.

For this work the following fixation procedures were used:

Formaldehyde fixation: 3.7% (v/v) formaldehyde, 0.5% Triton-X-100 in 50% PHEM-buffer, 5 min fixation time.

Methanol fixation: 100% methanol (-20°C), 5-10 min fixation time.

Immunolabelling was carried out by incubating the fixed samples with one or a mixture of two different (for double staining) primary antibodies diluted in PBS, 1% BSA, 0.1% NaN₃ at room temperature for 1 h. Unbound antibodies were removed by 3 x 5 min washes with PBS and the samples were incubated for 1 h with the respective secondary antibodies (FITC, Cy3, Alexa 488 or Alexa 568 conjugates, diluted as recommended by the manufacturer) avoiding light exposure. The coverslips were finally washed three times, including DAPI (1 µg/ml) in the second washing step to visualize nuclear and mitochondrial DNA. Coverslips were embedded on a microscope slide with 5 µl anti-bleach (10% PBS, 1% phenylenediamine, 89% glycerol) and the edges were sealed with nail polish. Standard immunofluorescence microscopy preparations were viewed through a Zeiss Axiophot microscope equipped with a 100x /1.3 objective and DAPI, rhodamine and fluorescein filter sets. Images were recorded with a Hamamatsu CCD camera C5985 connected to a frame grabber and Macintosh computer.

2.4.5 Confocal microscopy

For confocal analysis preparations were viewed on an inverted microscope (Leica DM IRBE; Leica Mikroskopie und System GmbH, Wetzlar, Germany) equipped with a 100x /1.4 objective. Images were acquired using the Leica TCS NT confocal imaging system, transferred to a Macintosh computer and analyzed using NIH image 1.6.1 and Adobe Photoshop 5.5.

2.4.6 Electron microscopy

Electron microscopy images were kindly provided by Dr. Ursula Euteneuer. Paraformaldehyde and glutaraldehyde-fixation of cells and nuclei, Lowicryl post-embedding and immunostaining of cells and isolated nuclei were carried out as described by Gräf et al. (1998). Sections were viewed through a transmission electron microscope (JEOL 1200 EXII) and photographed at 80 kV.

3. RESULTS

3.1. Investigation of cytosolic γ -tubulin complexes in *Dictyostelium discoideum*

3.1.1 Cloning of DdSpc97 and the nearly complete DdSpc98 sequence

By the time this work was started very little was known about the components of γ -tubulin complexes in most species. The only γ -tubulin-interacting proteins that had been cloned were Spc97 and Spc98, which are components of the small *S. cerevisiae* Tub4p complex (Knop et al., 1997). For the α -tubulin ring complexes of higher organisms only the protein pattern on SDS-gels was known and none of the components had been cloned. However, ESTs (expressed sequence tags) for Spc97 and Spc98 homologues had been discovered in a number of species, including humans, rice and zebrafish.

Therefore it was decided to look for Spc97 and Spc98 homologues in *Dictyostelium* and to investigate their interaction with Dd- γ -tubulin in the cytosol.

Fragments of genomic sequence coding for the *Dictyostelium* homologues of Spc97 (clone JAX4a171e01 containing 935 bp of sequence coding for a protein with 39% amino acid identity to human Spc97) and Spc98 (clone JAX4a195a03 containing 1062 bp of sequence coding for a protein with 39% amino acid identity to human Spc98) were identified by a BLASTN search of the *Dictyostelium* cDNA project by homology with the human homologues (Kay and Williams, 1999; <http://genome.imb-jena.de/Dictyostelium>). These fragments were used to screen various *Dictyostelium* cDNA libraries.

Generally, both proteins were extremely poorly represented in all cDNA libraries tested. About 2×10^6 pfu of a size fractionated λ ZAPII *Dictyostelium* cDNA library with an insert size of about 2-4.5 kbp (Gräf et al., 2000) had to be screened until two clones with DdSpc97 sequence could be identified. One of them contained a truncated 3 kb fragment, but the second contained the full-length 3534 bp coding sequence (Fig. 2).

Although about 3×10^6 pfu of four different cDNA libraries (oligo-dT primed cDNA libraries, size fractionated or not, random primed cDNA libraries constructed by R. Gräf or P. Devreotes) were screened, no clone containing DdSpc98 sequence could be identified. Several other approaches aiming to clone DdSpc98 cDNA sequence, e.g. library screening by PCR according to Takumi (1997), enrichment for rare clones with the sublibrary method (Lardelli, 1997), performing of inverse PCR on circularized cDNA (Huang and Jong, 1997) and RACE (rapid amplification of cDNA ends) failed, presumably because DdSpc98 was too rare at the cDNA level.

1	ATGACAACCCCAACCACCAACATTTAATAATTTAATAAAAACCACAATCACTACCATCGACT	60
1	M T T Q P P T F N N L I K P Q S L P S T	20
61	ACAGGTATGAACGAAATAAAGAATACACCAACAAGGACAACCCTACTACATCAACACAA	120
21	T G M N E I K N T P T R T T T T S T Q	40
121	CACACTAAATATATAAAAAGAATATGTGTTAAAGAAAATAATAATAGAATTAGTAAC	180
41	H T K Y I K E D I V V K E N N N R I S N	60
181	ATGAATAATAATAATATAAGTGGAGCTAGTGGTGGCACTACATTTAATAGTTATAACTAT	240
61	M N N N N I S G A S G G T T F N S Y N Y	80
241	CTACCAAATAACTCTTGATAATTTTATAAATAATTTCTAATACAACCTACAACAACCACA	300
81	L P N N T L D N F I N N S N T T T T T	100
301	AATAATAACTAATAATAATAATAATAACAATAATAATAATAATAATAATAATAATA	360
101	N N N T N N N N N N N N N N N I I N N I	120
361	AAACAACAACGAAATAGAATAATTAACAACAAAAACATATTGAAGAGATGAAAATATTA	420
121	K Q Q H E L E L L Q Q K H I E E M K I L	140
421	CAAAATGAACAATTAATCAATTAATAAACAACAAAAGAAATTAATGAATCAGATACG	480
141	Q N E Q L N Q L K K Q Q K K L N E S D T	160
481	GATGGTAGTGACGATGATGATGACGATGATGAATATGGTGAAAATATGAATTTATCA	540
161	D G S D D D D D D D E Y G E N M N L S	180
541	CCAAGAACATTAAGAGAATTAATAAATTAAGATTAATAAATTAAGAGAAATTTAGT	600
181	P R T L R E L K N I Q R L K N L E K F S	200
601	CCACCTTATTTAATAACTAAAAAATTCACCATCAATTAACAATTACCAAATTC	660
201	P P Y L I T K K T S P S I K T L P K F P	220
661	GAATGGACAAAAGAAAGACCTTTCTTAATTTATGGTTATCTTGCAATATATCTACAAAT	720
221	E W T K E R P F L N Y G Y L A N I S T N	240
721	TATACAAGTTTAATTTACCTTCAAAATATGAACAACTAAAATTCATTAAGTTCAATA	780
241	Y T S L I L P S K Y E Q T K I Q L S S I	260
781	CCAAATTTTAGAGAACAATTTAATTTAGGATTTATTATCAACAATGATTGGAATT	840
261	P N E Q I L I E D L L S T M I G I	280
841	GAAGGAGAAATGATACATATAGGAGTTGTACAAGAGAATGAAGATGATTCATTACAGAGT	900
281	E G E C I H I G V V Q E N E D D S L Q S	300
901	AATTTAGTTAGTTTGTAGTTGAAGGAGGTTGACCCAAGTAATAATGATTTAGTTCAA	960
301	N L V S F V V E G E V D P S N N D L V Q	320
961	AGGATTTCTACCATTATGTTTCAATTTATTCATTCGTTAATGATTTTCAATTCGATAT	1020
321	R I L P L C S Y Y S F V N D F I Q I R Y	340
1021	GCCTATGAATGGGTATAATCAATCATTATTATGTTTTCATTCGATCAACTATTGAAA	1080
341	A Y E W G I I N H S L C F S I D Q L L K	360
1081	GAGTATTTAGTATTGGTATCACAAATGGAAGGTCAATAAAATCAGAGAGATTAAGTTTA	1140
361	E Y L V L V S Q L E G Q L K S E R L S L	380
1141	CAAAGAATGTGGTTCTATTACAACCAACCATCAAAACATTTGAAATACTTTATAGAGTG	1200
381	Q R M W F Y L Q P T I K T F E I L Y R V	400
1201	ACCTCGGAAATATGAGATGCAATAGTCATGGTGTTCAGTGATTAATTTACTCTTAAAG	1260
401	T S E I M R C N S H G V Q V I N L L L K	420
1261	CTACAATCGAAATATTCAGTGATAAGAAAACCGATGATCTATTTTCAATATCTCATTAAA	1320
421	L Q S K Y S S D K K T D D L F H Y L I K	440
1321	GCTACAACCTTACCTTTCTTGGAGATGTTGGACCTTTGGATTCAATCAAGGTATAATTAGA	1380
441	A T T L P F L E M L D L W I H Q G I I R	460
1381	GATACCTATTTCGAATTCATGATTGAAGAGAATACAGATTTAAAACGTGACAATATCAAT	1440
461	D T Y F E F M I E E N T D L K R D N I N	480
1441	AGGGATTTTAAATGATCTCTATTGGGAACAACATTATCAAAATTCGTCAAGATCAAATCCA	1500
481	R D F N D L Y W E Q H Y Q I R Q D Q I P	500
1501	AAATATTTACAATCACATGCTTTAAAAATTTAACAACCTGGTAAATATTTAAATGTTGTT	1560
501	K Y L Q S H A L K I L T T G K Y L N V V	520
1561	AGAGAATGTAATCAATCAATTCACAACAACAACAATAATAAATCTGACAATAATAAT	1620
521	R E C N Q S I S Q Q Q Q H N K S D N N N	540
1621	AATAATAATAATGTAATAAATAAACAGTCAACAACAATTTATTTAATAAATTAAT	1680
541	N N N N N V K L N S Q Q Q L L L L K L N	560
1681	ACAACCTCAACAACAACAACAACAACCTTTCAAGTACATCAGCATCAATGATACCAACA	1740
561	T T Q Q Q Q Q Q Q L S S T S A S M I P T	580
1741	ACAATTTATGAAATATTCAAAGAAATGAAATGTTTATTTATTTGATAAGATTTGAAAAGCATAT	1800
581	T I I E Y S K N E N V Y I D K I E K A Y	600
1801	GATTATGCAAGTGGTATATTATTTAAATTTATTAATTAATGAAAGACATTTAATCTCAAGA	1860
601	D Y A S G I L L N L L I N E R H L I S R	620
1861	TTAAAAGCTATTAACATTTCTCTTATGCAAAGGTGATTTCTTTTCTCACTTTATG	1920
621	L K A I K H Y F L L C K G D F F S H F M	640

1921	GATACCACTTATGATGAATTAAGAAACCATTGGATCAAATTAATATGGTTAAAATGAAT	1980
641	<u>D T T Y D E L K K P L D Q I N M V K M N</u>	660
1981	TCATTACTTCAACTCTCATTAAAGAACTTCTTCAATATCTGAAGAGGATGAATTTAAAGAT	2040
661	<u>S L L Q L S L R T S S I S E E D E F K D</u>	680
2041	GATTTAGAATGTGAATTCATTCCATATAAAATTTGGTTGATCAATTTAATAATATTTAAT	2100
681	<u>D L E C E F I P Y K L V D Q L L N I I N</u>	700
2101	ATTAATAATTTTAATAATCCAACAACACTACAACACTACTACAATACTTCTTCAAATAATATA	2160
701	<u>I N N F N N P T T T T T N T S S N N I</u>	720
2161	ACAGCGACTACAAATACTTCTTCAAATAATACAACACTATAAATACAACAGTTAATACAACA	2220
721	<u>T A T T N T S S N N T T I N T T V N T T</u>	740
2221	CTAATTTCAATCAACATCAGCACATGAAGTTTTAAAACTAATAAATTTATTGGTATTGAA	2280
741	<u>L I Q S T S A H E V L K T N K L F G I E</u>	760
2281	TCTTTGGCATTTAATATAATGTGTGGTTGGCCATTATCTTTAATTATTTAGTAGAAAATCT	2340
761	<u>S L A F N Y N V G W P L S L I I S R K S</u>	780
2341	TTAATAAAAATATCAAATCATATTCAGACATTTATTCCTTTGCAAACATGTTGAAAAGGTT	2400
781	<u>L I K Y Q I I F R H L F L C K H V E K V</u>	800
2401	TTGGTTGATACCTTGAGCTCAACATCAATTTAGAAGGTCATCCTATAATAAACCCTGGTCTT	2460
801	<u>L V D T W S Q H Q F R R S S Y N K P G L</u>	820
2461	TCAACTTTATTTATCCTTTACTCATTTACTTAGACATCGTATGATTCATTTCTTACAAAAT	2520
821	<u>S T L L S F T H L L R H R M I H F L Q N</u>	840
2521	TTAGAATATTTATGATGTTAGAAGTTTAGAACCAAATTTGGAATAAAATGAAAACTCA	2580
841	<u>L E Y Y M M L E V L E P N W N K M K N S</u>	860
2581	ATTAATAACTTCAAAAAGTGTGTGATGATGAATTAGGATTCATAATGATTTTTTGGAACT	2640
861	<u>I K T S K T V D D V I R I H N D F L E T</u>	880
2641	TGTTTAAACAGAATGTATGCTTACTGATACTAAATTAGTTAATATTTTAATGAAATTCATG	2700
881	<u>C L T E C M L T D T K L V N I L M K F M</u>	900
2701	TCACTTTGTATTACTTTTGCAAACCTTACAAATGAAATGATGCAGGATGAAAAGAACATT	2760
901	<u>S L C I T F A N F T N E M M Q D E K N I</u>	920
2761	GATACAGAACAATTAAGAAAGACAATTAGTAGTTCAGAAAATAAATCCATGCCATTCTT	2820
921	<u>D T E Q I K K T I S S S E N K F H A I L</u>	940
2821	AAAGTATTAATGGATACATTGAAATCATTTTCAAAGACTGAATCAAATAGACATATGATT	2880
941	<u>K V L M D T L K S F S K T E S N R H M I</u>	960
2881	CATTTAATTCATAGATTGGATTATAATGGTTATTATTCACATTATTTTGAAAAATACCA	2940
961	<u>H L I L D Y N G Y Y S H Y F E N N P</u>	980
2941	AATTTATTTAAGAATAAGACTTCTTCATCAAGTAATAAATCTAATAATGTATCACACCT	3000
981	<u>N L F K N K T S S S S N K S N N V S P P</u>	1000
3001	TTAGAACCAAGATTTTCATACCAACAACAACAGCAACAACAGCAACAACAGCAACAACAA	3060
1001	<u>L E P R F S Y Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q</u>	1020
3061	CAGCAGCAGCAGCAACAACAGCAGCAGCAACAACAACAGCAGCAACAACAACAGCAA	3120
1021	<u>Q Q</u>	1040
3121	CAGCAACAGCAGCAGCAACAACAGCAACAACAACAGCAACAACAGCAACAGAAACAA	3180
1041	<u>Q K Q</u>	1060
3181	AACCAAATACAACAACAGAAACAAAATCAAATACAGCAGCAACAACAACAAGTAGGGGT	3240
1061	<u>N Q I Q Q Q K Q N Q I Q Q Q Q Q Q S R G</u>	1080
3241	AGTACATTACCAAGTAATTTTCATTTCAAACAGCACTTCAACAACAATAAATACAACA	3300
1081	<u>S T L P S N F S F Q T A L Q Q Q I N T T</u>	1100
3301	TCAGCTACAACCACAACATCGAATCAATCACACACACATTAAATGATGATTAAAGAAA	3360
1101	<u>S A T T T T S N Q S P H T L I D D L K K</u>	1120
3361	AGAGCTTATGGAATACTTGATAGTAATAATACGAAAAAACTTTAGAAAGAACTAGCTCA	3420
1121	<u>R A Y G I L D S N N T K K T L E R T S S</u>	1140
3421	TCGCCAGCTTTAGATAACCAAAGTAATAGTTTACCAATGACTCAATCAATTTACAGTAT	3480
1141	<u>S P A L D N Q S N S L P M T Q S I S Q Y</u>	1160
3481	GCCGAAAAATTAATAAATTAAGGGCAGTTATAAATGATGAATTAATAA	3534
1161	<u>A E K L K N L R A V I K N D E L K *</u>	1178

Fig. 2 The full-length coding sequence of DdSpc97. The full-length 3534 bp DdSpc97 cDNA clone and the derived amino acid sequence are shown. The initiation methionine is underlined. The part of the sequence contained in the genomic clone JAX4a171e01 (base position 1664-2589), which had been used for screening, is shaded in grey.

One possibility to overcome the problems encountered when trying to clone a poorly expressed gene was to concentrate the search on genomic DNA. Therefore a genomic library enriched in DdSpc98 sequence was constructed. To achieve this, Southern blots of genomic DNA digested with different restriction enzymes were probed with a DdSpc98 probe in order to identify fragments of suitable length for cloning into library vectors (see section 2.2.22). Suitable fragment sizes, approx. 2 and 7 kb, for cloning into library vectors were obtained for *EcoRI* (Fig. 3), which cuts within the DdSpc98 sequence. The fragments were excised from a preparative gel and ligated into λ ZAPII-*EcoRI* arms. The titer of the 2 kb *EcoRI* fragment library was about $1,5 \times 10^5$ pfu, that of the 7 kb *EcoRI* fragment library about 10^4 pfu, due to the low *in vitro* packaging efficiency of large fragments ligated into λ ZAPII vectors.

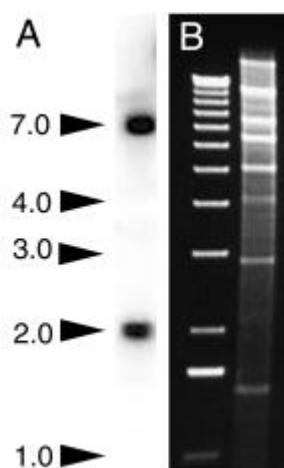


Fig. 3 Construction of a subgenomic library enriched in DdSpc98 sequence. (A) 10 μ g of genomic DNA were digested with *EcoRI* and loaded on a 0.8% agarose gel. Southern blot analysis with a 32 P-labelled DdSpc98 probe revealed the presence of two DdSpc98 fragments with sizes of 2 and 7 kb. The fragments were excised from the preparative gel shown in (B) and were ligated into λ ZAPII-*EcoRI* arms.

No positive clone could be found in the 7 kb *EcoRI* fragment library, which was not surprising considering the low titer of the library. However, screening of the 2 kb *EcoRI* fragment library was successful and one clone containing a 1.8 kb fragment overlapping at the 5'-end with the known DdSpc98 sequence was isolated. It contained a 135 bp intron at position 550, as confirmed by sequencing an RT-PCR fragment covering the region of interest. This 1867 bp fragment was obtained by priming the reverse transcription of mRNA with the DdSpc98 specific reverse primer 98-14 (base position 1852 to 1871 of the partial DdSpc98 sequence shown in Fig. 4) and performing subsequent PCR with primers 98-14 and 98-f2 (base position 3 to 24, primer sequence derived from the genomic library clone). Taking the sequence information together, 2439 bp of sequence could be cloned, including the whole 3' end with the TAA stop codon (Fig. 4).

1801	TATAAAAAGATCTTTTCATTTTATGTGGGGTATAAAAAGAGTTGAGTATTCGTTGGCTTCG	1860
601	Y K K I F H F M W G I K R V E Y S L A S	620
1861	ATTTGGCGTAAAATTAGGTCGTCAACTTCCTTATCGATTCTATCACCAATTGGTGGTGAC	1920
621	I W R K I R S S T S L S I L S P I G G D	640
1921	AATCATAAATCTCATTGATTATGAATGAAATGGTGCATTTTCATATCGAATTTTCAGTAC	1980
641	I H K S H L I M N E M V H F I S N F Q Y	660
1981	TATCTAATGTTTGGAGTTTGGAAATGTTCTTGAAGAATTTAGAGAAATTCATAGACCAA	2040
661	Y L M F E V L E C S W K N L E K F I D Q	680
2041	GAAGCTACCGATTTAGATCAACTCATGAGGCTCATCATCAATATCTTCAAGATATTGT	2100
681	E A T D L D Q L I E A H H Q Y L Q D I C	700
2101	AATAAAATGTTTATCAAATCTGATAGTTGTTATGAATGCTTTAAAAAATTATTATCA	2160
701	N K M F L S N S D S C Y E C F K K L L S	720
2161	ATTATCATCAAATTCACCTCTATTACAACTAAATTAATCAACCTTTCAATAGCTATTCAA	2220
721	I I I K F T L L Q T K L I N L S I A I Q	740
2221	AATGAAAAGAATTTCAATGAAACTCATCAAGCTCAAGTTAATAAAGAATTTAATAGTTTT	2280
741	N E K N F N E T H Q A Q V N K E F N S F	760
2281	AGAAATCATCTAAATAATCTCTATCAAGAATATACAACCTTCATTTTATAAAATTTCAATCT	2340
761	R N H L N N L Y Q E Y T T S F Y K F Q S	780
2341	GAAAATTTAAAAGTTAAAGTAAATCAAGATTTAAATCCAATTTTCATTTACAATATATGTTA	2400
781	E I L K V K V N Q D L N P I S L Q Y M L	800
2401	GATTTTAAATGAATATATGAAGAAAAAAGATAATTAA	2439
801	D F N E Y Y E E K K D N *	813

Fig. 4 The nearly complete coding sequence of DdSpc98. 2439 bp of DdSpc98 were cloned and are shown together with the derived amino acid sequence. The part of the sequence contained in the genomic clone JAX4a195a03 (base position 1376-2439), which had been used for screening, is shaded in grey. The localization of the intron that had been identified is marked with an arrowhead (▼)

Estimating from a comparison with the human Spc98 homologue (Fig. 6B) approximately 140 amino acids or 420 bp of DdSpc98 sequence are still missing at the 5' end. But since the *Dictyostelium* genome sequencing project is progressing rapidly the remaining sequence will hopefully be identified soon.

The partial sequence cloned in this work proved to be sufficient for all further experiments aiming to investigate the association of the protein with γ -tubulin.

3.1.2. Sequence analysis of DdSpc97 and DdSpc98

A comparison of amino acid identities between members of the Spc97 and Spc98 protein families (Table 2) and the phylogenetic trees derived from an alignment of the homologues (Fig. 5) show that DdSpc97 and DdSpc98 are most closely related to their vertebrate counterparts. The *Saccharomyces cerevisiae* proteins are clearly the most divergent members. Spc97 and Spc98 homologues found in all other organisms are also related to each other, which is also true for DdSpc97 and DdSpc98. They share 27.2% amino acid identity and are thus closer to each other than to their respective yeast homologues.

Tab. 2A Amino acid conservation of Spc97 homologues

	DdSpc97	hGCP2	Dgrip 84	SpSpc97	ScSpc97
DdSpc97	-	35.8	29.1	32.2	21.5
hGCP2	35.8	-	35.1	38.2	25.0
Dgrip 84	29.1	35.1	-	30.1	23.0
SpSpc97	32.2	38.2	30.1	-	24.0
ScSpc97	21.5	25.0	23.0	24.0	-

Tab. 2B Amino acid conservation of Spc98 homologues

	DdSpc98	Xgrip 109	Dgrip 91	hGCP3	ScSpc98
DdSpc98	-	33.6	30.4	33.9	22.8
Xgrip109	33.6	-	32.6	87.0	20.8
Dgrip 91	30.4	32.6	-	33.4	23.5
hGCP3	33.9	87.0	33.4	-	21.3
ScSpc98	22.8	20.8	23.5	21.3	-

Tab. 2 Pairwise comparison of amino acid identities of **(A)** all full-length homologues of Spc97 cloned so far and **(B)** comparison of all full-length Spc98 homologues, including the partial DdSpc98 sequence. Relative amino acid identities were calculated with the gap sequence alignment program (GCG package).

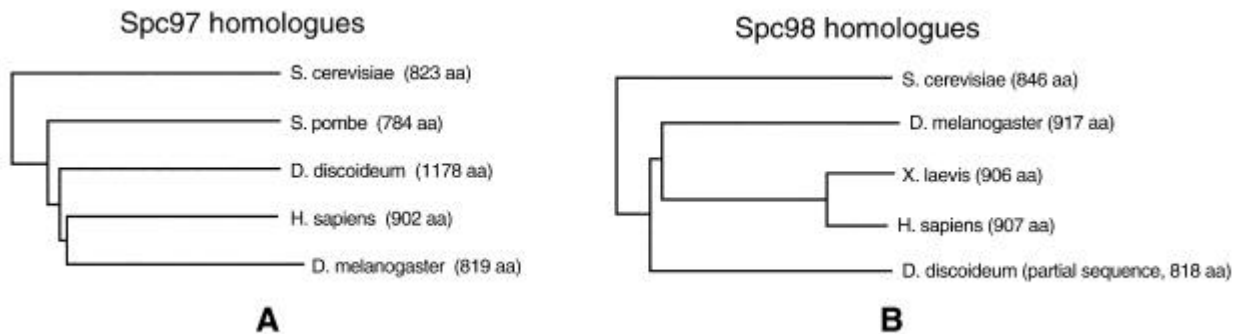


Fig. 5 Phylogenetic trees of Spc97 and Spc98 homologues. Phylogenetic trees were derived from multiple alignments of **(A)** all full-length Spc97 homologues and **(B)** all full-length Spc98 homologues including the partial DdSpc98 sequence. The alignments were made with the ClustalW programme and the trees were calculated with the PHYLIP package, version 3.5c (Felsenstein, 1993). Branch lengths represent evolutionary distances. The number of amino acids (aa) of each protein is given in brackets.

An alignment of DdSpc97 with its human counterpart hGCP2 (Fig. 6A) shows that the sequence of DdSpc97 contains some long insertions and extensions on both termini, in particular a 168 aa C-terminal extension. Some of the insertions contain stretches of highly repetitive sequence, which is quite frequently encountered in *Dictyostelium* proteins. The

functional significance of these repeats, however, has never been described. The sequence extensions make DdSpc97, which has a calculated molecular weight of 137.7 kDa, the longest member of the Spc97 family known so far.

An alignment of DdSpc98 with its human counterpart hGCP3 (Fig. 6B) also reveals a few insertions, but they are generally shorter than those found in DdSpc97 and there are hardly any stretches of repetitive sequence. The C-terminus of the protein is slightly shorter than that of hGCP3, but on the whole the DdSpc98 sequence can be aligned much better with its homologues than DdSpc97, suggesting a higher degree of conservation.

A

DdSpc97	1	MTQPPTFNNLIKQSLPSTTGMEIKNPTRTTTTSTGHTKYIK	DIVVKEN	NRIS	NMNNNIS	CSGGTTFNSYN	YLN	NNLDNF	INN	NTTTTT
hgCP2	1MS	ERI	HHDV	NLLS	LLRVHGG	CA	EVYID	LLQK	NR
DdSpc97	101	NNNNNNNNNNNNNNIN	NKQOHE	EL	QOKH	TE	EMKI	ONE	QNLK	QOKK
hgCP2	56	FSR	PE	DF	LYK	YDEL	KSK	NR	NLDP	LV
DdSpc97	198	.KFSPPY	LIT	K	TSP	S	IKT	L	K	F
hgCP2	154	L	L	K	R	K	M	L	R	D
DdSpc97	294	EDDSL	OS	N	LVS	Y	VE	GE	V	DS
hgCP2	237	PLA	GR	Q	S	R	.	I	L	P
DdSpc97	394	F	E	I	Y	R	V	T	S	E
hgCP2	335	M	I	A	S	L	A	S	L	V
DdSpc97	494	F	E	I	Y	R	V	T	S	E
hgCP2	434	F	E	I	Y	R	V	T	S	E
DdSpc97	594	D	K	E	K	A	M	D	V	A
hgCP2	486	E	Q	L	E	K	A	M	D	V
DdSpc97	694	O	L	L	N	I	N	I	N	N
hgCP2	684	O	L	L	N	I	N	I	N	N
DdSpc97	794	C	K	H	V	E	K	V	L	D
hgCP2	648	C	K	H	V	E	R	Q	L	C
DdSpc97	894	N	I	L	M	K	F	M	S	C
hgCP2	746	K	V	F	R	K	L	M	S	C
DdSpc97	953	T	E	S	N	R	H	I	L	H
hgCP2	846	S	D	C	H	G	M	A	S	V

B

DdSpc98	1N	S	T	F	V	I	P	K	L	N
hgCP3	101	S	E	D	R	R	Q	P	S	K	V
DdSpc98	60	I	T	L	Q	N	Y	Q	P	A	T
hgCP3	201	S	R	L	A	W	L	S	A	N	A
DdSpc98	159	V	M	R	C	E	G	W	L	F	K
hgCP3	291	A	V	R	S	E	H	L	N	K	H
DdSpc98	258	I	D	G	S	L	L	R	L	V	M
hgCP3	361	I	L	L	R	L	V	M	Y	P	D
DdSpc98	358	L	E	K	T	K	E	K	F	A	I
hgCP3	456	T	D	R	I	W	H	D	K	Y	L
DdSpc98	458	E	K	T	K	E	L	N	I	N	K
hgCP3	534	I	D	A	A	F	F	T	S	K	Y
DdSpc98	558	L	V	N	R	L	D	E	A	L	L
hgCP3	617	L	L	R	L	V	R	L	L	V	S
DdSpc98	657	N	F	O	Y	I	L	M	F	E	V
hgCP3	717	Q	M	O	Y	I	T	F	E	V	L
DdSpc98	748T	H	Q	A	O	N	K	F	N	S
hgCP3	816	E	Q	G	V	T	A	E	E	E	E

Fig. 6 Sequence alignment of human and *Dictyostelium* Spc97 and Spc98 homologues. (A) Pairwise alignments of DdSpc 97 with its human homologue hGCP2 and (B) of DdSpc98 with its human homologue hGCP3 using the gap alignment program (GCG package). Identical amino acids are shaded in black.

3.1.3 Southern and Northern blot analysis of DdSpc98 and DdSpc97

For all homologues of Spc97 and 98 described so far only one isoform has been reported. Bearing in mind the divergence of DdSpc97 Southern blot analysis was performed to examine whether this is also true for the two *Dictyostelium* proteins. Genomic DNA prepared from axenically growing *Dictyostelium* amoebae was digested with different restriction enzymes and probed for DdSpc98 and DdSpc97.

For DdSpc97 all enzymes yielded only one band, indicating the existence of only one gene isoform (Fig. 7A). The same result was obtained for Spc98 (Fig. 7B). The only enzyme producing two bands was *EcoRI*, which cuts internally.

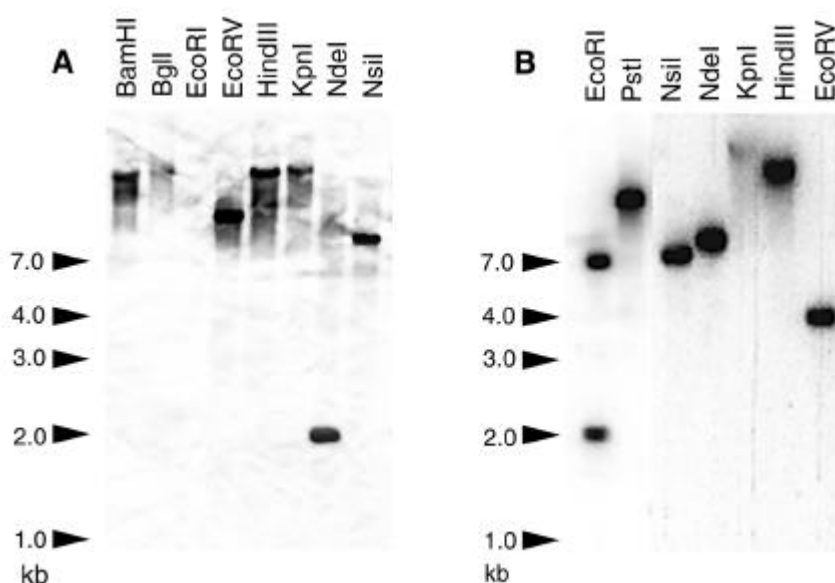


Fig. 7 Southern blot analysis of DdSpc97 and DdSpc98 indicates single gene isoforms.

Southern blots loaded with 10 μ g digested genomic DNA were probed with (A) a digoxigenin-labelled DdSpc97 probe (base position 1652-2261 of the full-length DdSpc97 sequence) and (B) a 32 P-labelled DdSpc98 probe (base position 1784-2424 of the partial DdSpc98 sequence), respectively. The hybridization conditions were 37°C in Easy Hyb solution (Boehringer Mannheim) for (A) and 37°C, 50% formamide, 2 x SSC for (B). In all lanes only one single band is detectable, except for the *EcoRI* lane in (B), which cuts within the DdSpc98 sequence. This indicates the existence of only one gene isoform for both DdSpc97 and DdSpc98.

To examine the nature of the transcripts present in vegetative cells Northern blot analysis was additionally performed on mRNA isolated from axenically growing amoebae. A single 3 kb transcript was found for DdSpc98 (Fig. 8B), but unexpectedly three different transcripts with sizes of about 2.8, 3.5 and 4.5 kb were detected for DdSpc97 under the same hybridization conditions (Fig. 8A). This suggests that different transcripts, presumably splice variants, are produced from the single DdSpc97 gene. The presence of a 2.8 kb transcript was especially surprising, because it is actually shorter than the 3534 bp coding sequence of DdSpc97.

However, anti-DdSpc antibodies recognized one single band on Western blots (see section 3.1.4.2), indicating that only one protein species is synthesized from these transcripts.

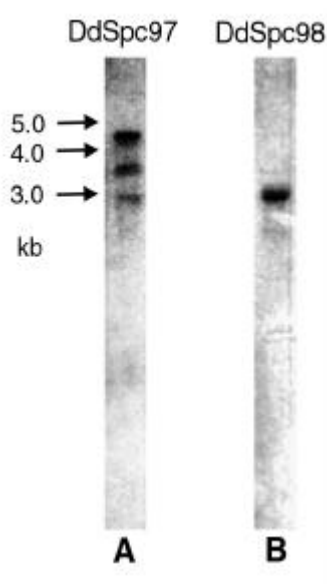


Fig. 8 Northern blot analysis of DdSpc97 and DdSpc98. Northern blots with RNA isolated from axenically growing amoebae were probed with ^{32}P -labelled probes (base positions 1652-2261 of DdSpc97 and 1428-2561 of DdSpc98). Under the same hybridization conditions (50% formamide, 2 x SSC, 37°C) three bands were detected for DdSpc97 (A) and one single band for DdSpc98 (B).

3.1.4 Investigation of the subcellular distribution of DdSpc97 and DdSpc98

3.1.4.1 Generation of polyclonal antibodies and GFP-tagged mutants

N-terminal (base position 1-1581) and C-terminal (base position 1749-2975) parts of DdSpc97 were cloned into the pMalc2 vector for bacterial expression. Highly repetitive regions of the protein were excluded, because they might interfere with antibody specificity. The fusion proteins migrated on SDS gels with the expected molecular weights of about 80 kDa for the C-terminal fragment and 90 kDa for the N-terminal fragment (Fig. 9). The fusion proteins were purified on an amylose resin and used for the immunization of two rabbits each. Antisera were affinity-purified prior to their use in immunofluorescence microscopy and on Western blots.

Tagging of DdSpc97 with GFP and the myc-epitope was attempted without success, but for DdSpc98, for which no suitable antibodies for immunofluorescence microscopy were available, a *Dictyostelium* mutant expressing base position 69-2378 of the cloned partial sequence with an N-terminal GFP-tag could be generated. With this GFP-tagged mutant the subcellular distribution of DdSpc98 could be investigated in microscopy and on Western blots.

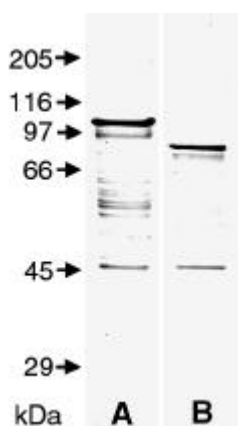


Fig. 9 MBP-fusions with N- and C-terminal fragments of DdSpc97 were expressed and purified. N-terminal (base position 1-1581) and C-terminal (base position 1749-2975) fragments of DdSpc97 were bacterially expressed as fusion proteins with maltose binding protein. The resulting fusion proteins had apparent molecular weights of about 100 kDa for MBP-N'-DdSpc97 (**A**) and about 90 kDa for MBP-C'-DdSpc97 (**B**). The proteins were used for the immunization of two rabbits for each construct.

3.1.4.2 Western blots, immunofluorescence microscopy and electron microscopy

The subcellular distribution of DdSpc97 and DdSpc98 was investigated on Western blots, in immunofluorescence microscopy and by electron microscopy. On immunoblots anti-DdSpc97 antibodies recognized a single band migrating at about 118 kDa. It was present both in cytosolic extracts and in isolated centrosome fractions, whereas the preimmune serum was negative (Fig. 10A). The protein migrated faster than expected, considering its calculated molecular weight of 137 kDa. But the migration behaviour was still in the range of variability observed for SDS-denatured proteins and, most importantly, no other protein that might correspond to DdSpc97 was detectable. Therefore, the 118 kDa band most likely represents DdSpc97.

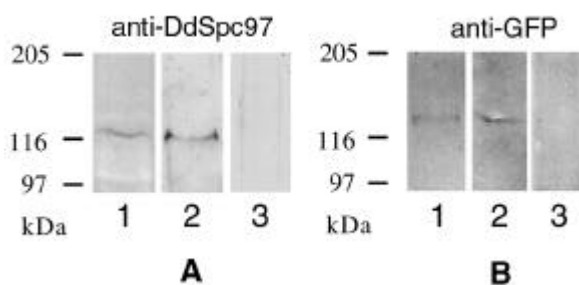


Fig. 10 Western blots show the presence of DdSpc97 and GFP-DdSpc98 in cytosolic extracts and isolated centrosome fractions. In lanes A1 and B1 cytosolic extracts prepared from about 10^7 *Dictyostelium* GFP-DdSpc98 cells were loaded per lane and stained with anti-DdSpc97 (**A**) or anti-GFP antibodies (**B**). In lanes A2 and B2 centrosome preparations from about 10^8 cells were loaded and stained with anti-DdSpc97 (**A**) and anti-GFP (**B**) antibodies. The single band recognized by the anti-DdSpc97 antibody migrates faster than expected, but can still be reconciled with the calculated molecular weight of the protein of 137 kDa. As a control for the specificity of the anti-DdSpc97 antibody the preimmune serum was tested on isolated centrosomes in lane A3. To confirm that the band recognized by the GFP antibody was specific, centrosomes isolated from AX2 cells were stained with the anti-GFP antibody in lane B3. Both controls were negative.

In immunofluorescence microscopy all anti-DdSpc97 antibodies strongly labelled isolated centrosomes, whereas the preimmune serum produced no labelling (Fig. 11A, C). In whole fixed *Dictyostelium* cells anti-DdSpc97 antibodies specifically stained the centrosome and no other cellular structure, whereas the preimmune serum produced no specific labelling (Fig 11E, G). Staining with anti-DdSpc97 antibodies and the preimmune serum is shown in red (Cy3 channel), merged with the blue DNA stain (DAPI). As a control, isolated centrosomes and whole cells were double-stained with a monoclonal antibody recognizing the centrosomal protein DdCP224, which is shown in green (FITC channel, Fig. 11B, D, F, H).

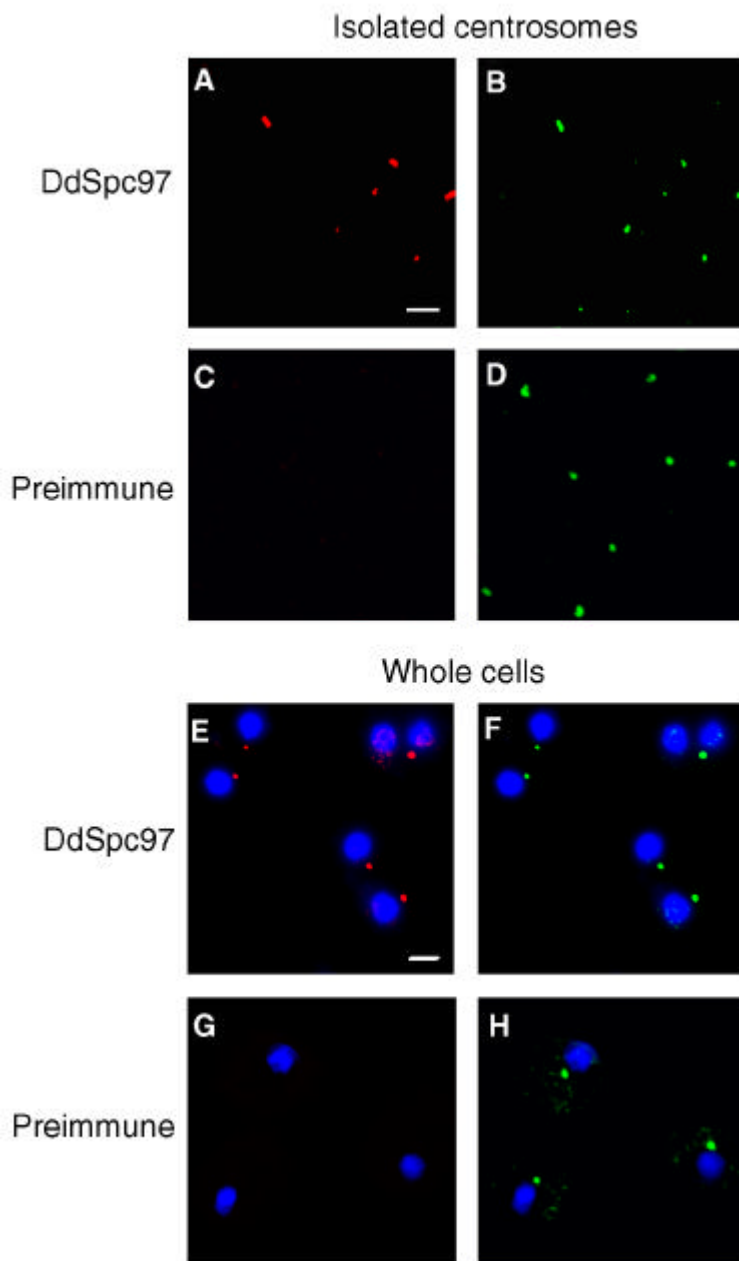


Fig. 11 Anti-DdSpc97 antibodies recognize the centrosome in immunofluorescence microscopy.

Isolated centrosomes fixed with methanol were brightly labelled with anti-DdSpc97 antibodies (A, red). The centrosomes were double stained with an anti-DdCP224 monoclonal antibody (B, green). The negative control (C, red) shows that no labelling was obtained with the DdSpc97 preimmune serum. Centrosomes were again double-stained with anti-DdCP224 antibodies (D, green).

The corresponding staining pattern observed on whole methanol fixed cells is shown in (E-H). (E, red) shows the centrosomal labelling obtained with anti-DdSpc97 antibodies, (F, green) shows the corresponding double-staining with anti-DdCP224 antibodies. (G, red) shows the labelling with the DdSpc97 preimmune serum and (H, green) the double-staining with anti-DdCP224 antibodies, which corresponds to (G). A merge of DAPI (blue) and Cy3/FITC-labelling is shown. (Bar: 2 μ m).

The labelling with anti-DdSpc97 antibodies remained associated with the centrosome throughout the cell cycle (Fig. 12A-C, red). The double staining obtained with the anti-DdCP224 antibody is shown in Fig. 12A'-C' (green).

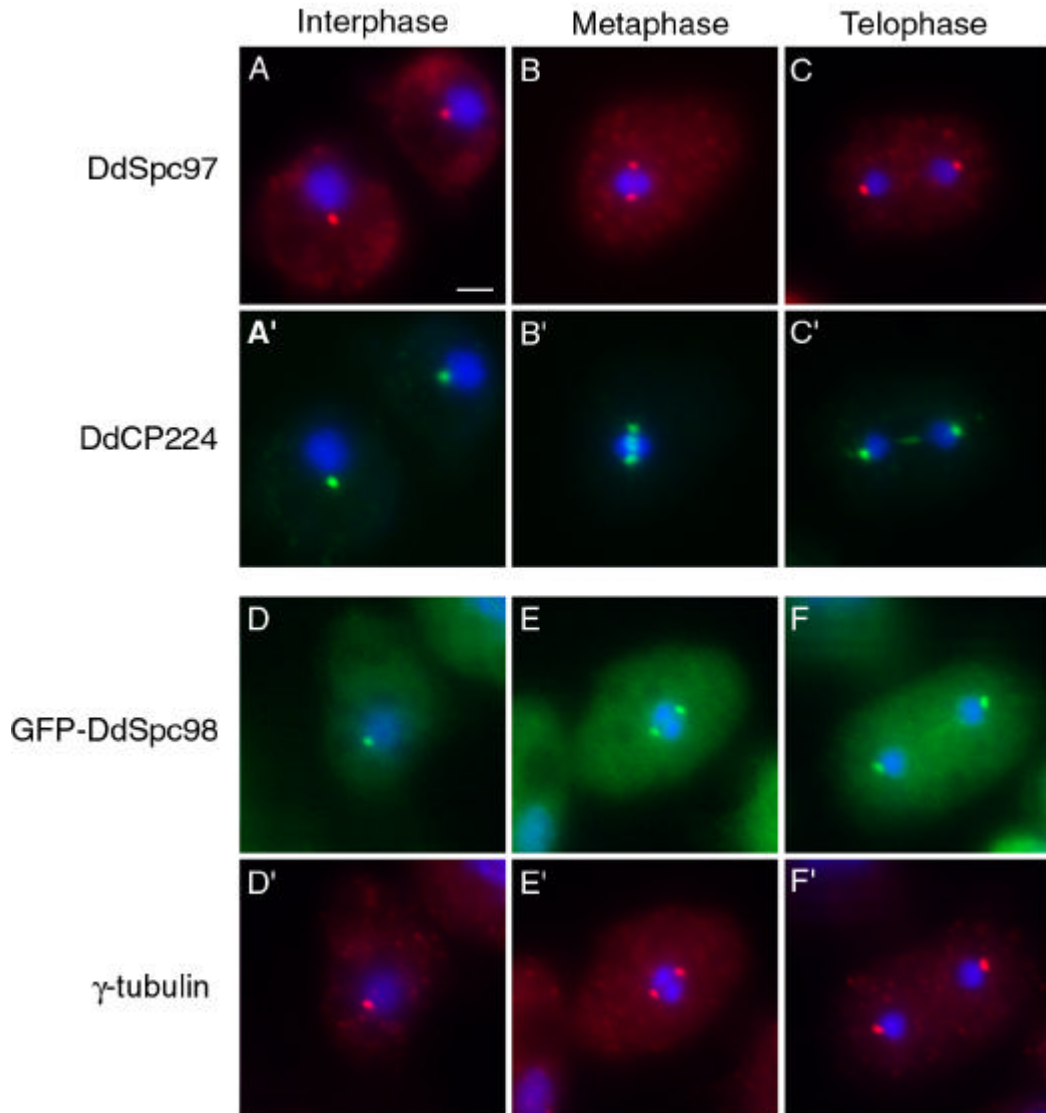


Fig. 12 DdSpc97 and DdSpc98 localize to the centrosome in interphase and mitosis.

Immunofluorescence microscopy images of interphase (A, D) and mitotic (B, C, E, F) cell cycle stages reveal that DdSpc97 and DdSpc98 remain associated with the centrosome throughout the cell cycle. The upper panel shows methanol fixed AX2 cells stained with rabbit anti-DdSpc97 antibodies (red; A, B, C) that were double-labelled with a mouse monoclonal antibody against the *Dictyostelium* centrosomal protein DdCP224 (green; A', B', C). In (D, E, F) the green fluorescence of methanol-fixed GFP-DdSpc98 cells was recorded (green) and cells were labelled with rabbit anti- γ -tubulin antibodies (red; D', E', F'). Secondary antibodies were Alexa 488 (green) and Alexa 568 (red), respectively. (Bar: 2 μ m)

The localization of the protein was also examined in developing cells (tested until aggregation stage). No change in the subcellular distribution of DdSpc97 was observed in starving cells (Fig. 13 A, B).

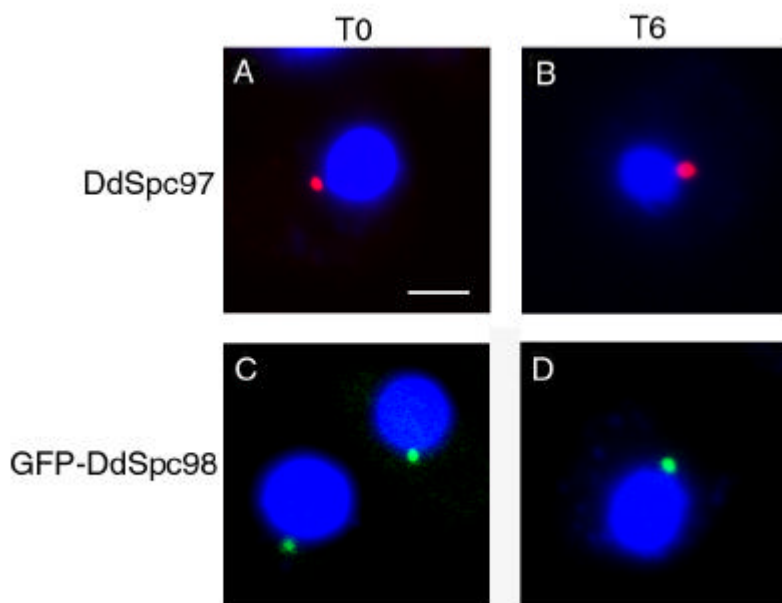


Fig. 13 DdSpc97 and DdSpc98 remain at the centrosome during starvation.

The intensity of the centrosomal anti-DdSpc97 staining (using Cy3 labelled secondary antibodies) remained unaltered after 6 hours of starving (T6, **B**) compared to normally feeding cells (T0, **A**). The same result was obtained for centrosomal GFP-DdSpc98 fluorescence (green) in feeding (T0, **C**) and starving (T6, **D**) cells. (Bar: 2 μ m)

The subcellular distribution of DdSpc98 was investigated using the GFP-tagged *Dictyostelium* mutants. The GFP-DdSpc98 fusion protein migrated with the expected molecular weight of about 120 kDa and was found in cytosolic extracts and isolated centrosomes of GFP-DdSpc98 *Dictyostelium* cells (Fig. 10B). As a control, cytosolic extracts prepared from AX2 wild-type cells were labelled with the anti-GFP antibody (Fig. 10B, lane 3). The 120 kDa band was not detectable in wild-type cells, confirming that the 120 kDa band corresponds to the recombinant GFP-tagged protein. Fluorescence microscopy revealed that GFP-DdSpc98 localizes to the centrosome and remains, like DdSpc97, associated with it during interphase and mitosis (Fig. 12). GFP-DdSpc98 fluorescence is shown in green (Fig. 12D-F), whereas the double labelling with a polyclonal anti- γ -tubulin antibody is shown in red (Fig. 12D'-F'). The distribution of GFP-DdSpc98 was also investigated in starving *Dictyostelium* amoebae, showing an unaltered centrosomal label until aggregation stage (Fig. 13 C, D).

In standard immunofluorescence microscopy the localization of anti-DdSpc97 labelling and of GFP-DdSpc98 fluorescence was indistinguishable. To investigate the colocalization of DdSpc97, DdSpc98 and γ -tubulin at a higher resolution, electron microscopy was also performed (kindly provided by U. Euteneuer). Isolated nuclei of GFP-DdSpc98 cells were fixed with paraformaldehyde/glutaraldehyde and thin sections were labelled with anti-DdSpc97, anti-GFP and anti- γ -tubulin antibodies, respectively. All three antibodies recognized the centrosome, as identified by an enrichment of gold particles, but the total number of gold particles was generally very low, especially when using anti-GFP antibodies. Therefore it was difficult to decide which centrosomal substructures they preferentially bound to. When a larger number of centrosomes was evaluated by counting the gold particles found in the core structure, the corona or both centrosomal substructures an interesting tendency was observed: For DdSpc98 and γ -tubulin almost all gold particles were confined to the corona and the core structure was hardly ever labelled (Fig. 14A, B). For DdSpc97, on the other hand, gold particles were also repeatedly found in the centrosome core (Fig 14C). However, this finding needs to be interpreted with caution, because the anti-DdSpc97 antibodies tended to give some non-specific background staining in standard immunofluorescence microscopy, whenever glutaraldehyde was present in the fixative, which was necessary to maintain sufficient structural preservation for electron microscopy.

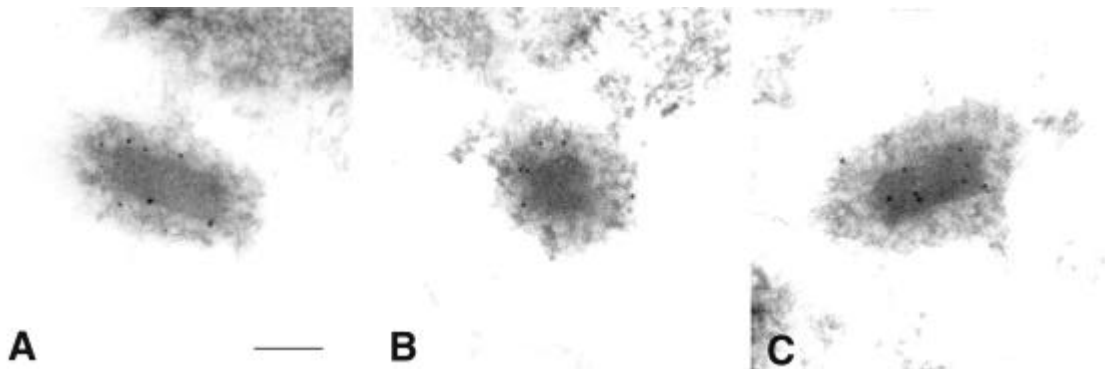


Fig. 14 Electron microscopy suggests that DdSpc97 and DdSpc98 localize to different centrosomal substructures Isolated nuclei of GFP-DdSpc98 cells were stained with anti- γ -tubulin, anti-GFP and anti-DdSpc97 antibodies. Gold particles were exclusively found in the centrosomal corona using anti- γ -tubulin antibodies (A). The same pattern was observed with anti-GFP antibodies (B), which indicate the localization of GFP-tagged DdSpc98. For DdSpc97 (C) some gold particles were also found in the electron-dense core structure in addition to the centrosomal corona. (Bar: 0.2 μ m)

3.1.4 Generation of a mutant stably expressing myc/6xHis-tagged γ -tubulin

One important approach towards the investigation of cytosolic γ -tubulin complexes was to purify endogenous γ -tubulin and proteins interacting with it from cytosolic *Dictyostelium* extracts. For this, γ -tubulin endogenously expressed by *Dictyostelium* amoebae was tagged with a combined myc/6xHis tag to make use of sequential affinity purification steps. The homologous integration plasmid pUC γ mycHisBsr used for the transformation of *Dictyostelium* cells carries a promoterless version of the γ -tubulin gene lacking the first 211 bp (Fig. 15). Transformants expressing γ -tubulin-myc/6xHis following successful homologous recombination were identified by the centrosomal localization of the c-myc tag in immunofluorescence microscopy (Fig. 16A, B). PCR on genomic DNA, Western and Southern blots confirmed that the endogenous copy of γ -tubulin was indeed disrupted and replaced by the tagged gene (Fig. 16B-D). Mutant cells had a normal phenotype, grew like wild-type cells and underwent unaltered development. Due to genomic integration of the vector, expression of γ -tubulin-myc/6xHis was stable and was not lost in the absence of the antibiotic. Both tags were accessible for purification, confirming that the tags were exposed on the outside of γ -tubulin-complexes.

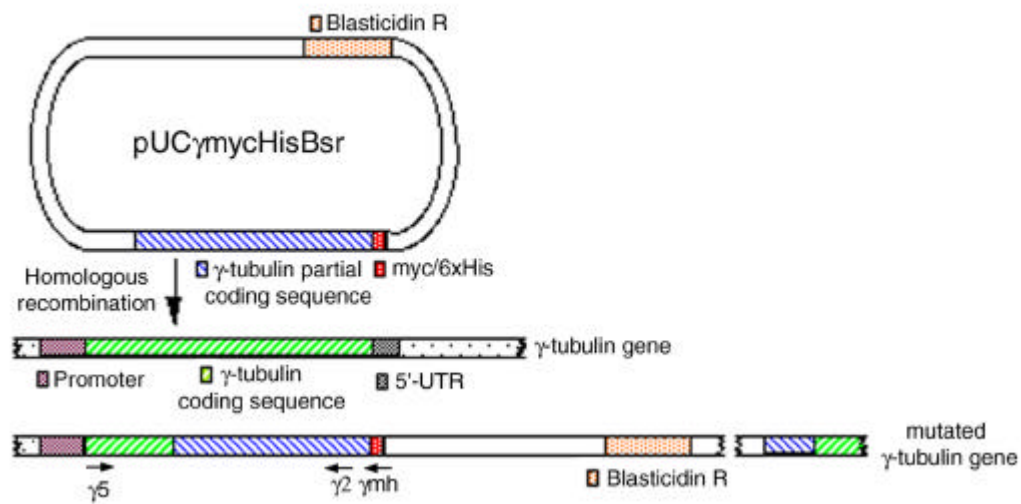


Fig. 15 The homologous integration plasmid pUC-g-myc/His-Bsr. The plasmid used for transformation of *Dictyostelium* cells carries a 5'-deleted copy of the γ -tubulin gene followed by a myc/6xHis tag and a Blastidicin resistance cassette. By homologous recombination the vector inserts into the endogenous γ -tubulin gene, leading to the expression of a full-length, tagged version of the γ -tubulin gene with a simultaneous disruption of the endogenous gene. The sites of the primers γ 5, γ 2 and γ mh, which were used for PCR to confirm the successful integration event (Fig. 16B), are indicated with arrows.

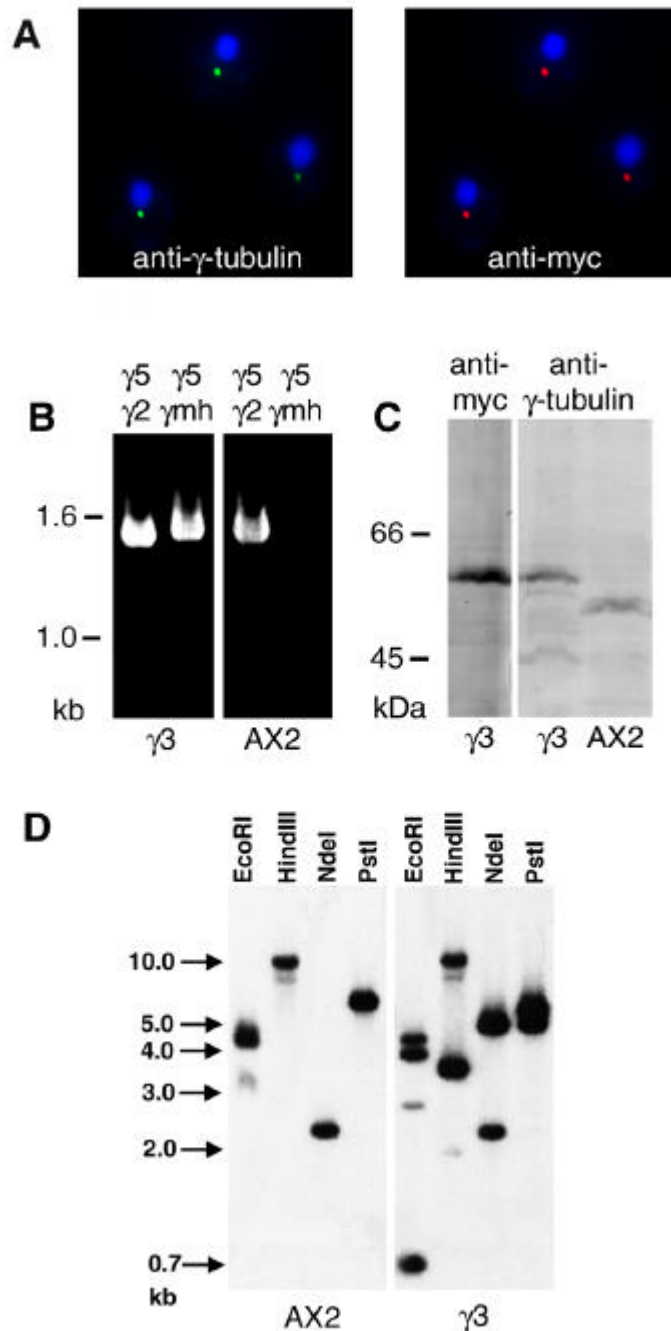


Fig. 16 Evidence for successful homologous integration of pUC-g-myc/His-Bsr. (A) Immunofluorescence analysis of γ -tubulin myc-his cells with anti- γ -tubulin or anti-myc antibodies revealed that γ -tubulin co-localizes with the myc-tag at the centrosome. (B) PCR experiments confirmed that genomic DNA of recombinant γ 3 cells contains a full-length γ -tubulin gene which contains the 5'-end missing on the plasmid p γ Myc-His-Bsr, and which carries a myc/6xHis tag. Primer sites are indicated by arrows in Fig. 15. (C) Western blots of cytosolic extracts of γ 3 and AX2 cells stained with anti- γ -tubulin and anti-myc antibodies show that in transformants only one, tagged copy of γ -tubulin is expressed which migrates slower than the wild-type protein on SDS-gels. (D) Southern blots of genomic DNA probed with a 32 P-labelled 1 kb γ -tubulin probe revealed the presence of additional bands in γ 3 cells compared to AX2 wild type cells. Restriction digests with PstI and NdeI resulted in only one additional band, whose size corresponds to that of the gene replacement vector (approximately 56 kb). Digests with EcoRI and HindIII, both of which cut twice within the vector sequence, each resulted in two additional bands which are together about 5.6 kb long. These results are consistent with the occurrence of the desired recombination event. Band intensities suggest a double insertion of the vector, which should not affect the mutants because the truncated γ -tubulin gene on the replacement vector lacks a promoter and cannot be transcribed.

3.1.4 Gel filtration of purified γ -tubulin complexes

To determine the size of *Dictyostelium* γ -tubulin complexes cytosolic extracts were purified on a His-tag affinity resin and subjected to size exclusion chromatography. Assuming a globular conformation of the complexes their size was estimated by comparing the elution volume with that of globular marker proteins of known size. Generally, heterogeneity of γ -tubulin-complex sizes and size shift under different buffer conditions were observed. A reproducible and very important finding, however, was that no peaks of γ -tubulin larger than about 500 kDa were detectable, even if the buffer conditions were identical to those used for the purification of γ TuRCs in *Drosophila* (Oegema et al., 1999). This suggests that no γ -tubulin complexes with a size comparable to γ -TuRCs are present in the *Dictyostelium* cytosol. To exclude that larger complexes were not detected due to aggregation, the first fractions, including the exclusion volume of the gel filtration column, were also collected. γ -Tubulin was consistently absent from the first fractions and predominantly appeared in fractions around a broad peak at about 450 kDa and a second, sharper peak at about 50 kDa. The 450 kDa peak represents the prevailing γ -tubulin-containing complexes, whereas the 50 kDa peak presumably consists of monomeric γ -tubulin. Interestingly, the 450 kDa peak was more pronounced in the presence of 500 mM NaCl, whereas the fraction of monomeric γ -tubulin was increased if the salt concentration was lowered to 100 mM NaCl (Fig. 17). This suggests that the prevalent 450 kDa complex in *Dictyostelium* is stabilized under high salt conditions.

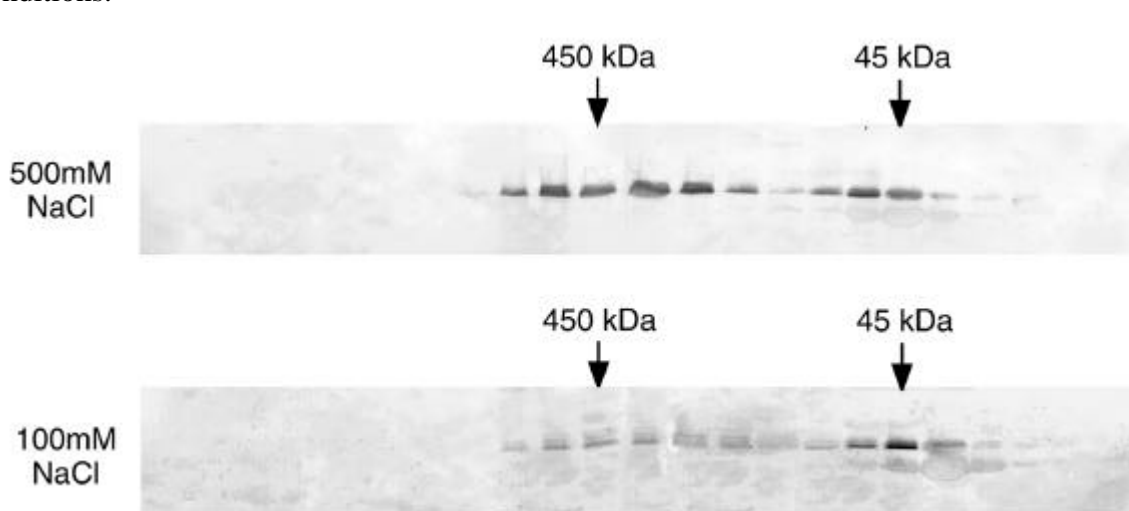


Fig. 17 The prevailing *Dictyostelium* γ -tubulin complex is stabilized under high salt conditions. Gel filtration was performed with cytosolic γ -tubulin complexes enriched in the presence of 100 mM or 500 mM NaCl. 0.75 ml fractions were collected, precipitated and loaded on SDS-gels. Western blots were stained with anti-myc antibodies. The molecular weight of selected globular marker proteins (ferritin, 450 kDa and albumin, 45 kDa) is indicated by arrows.

In contrast, the complex partially dissociated when the pH was lowered from 7.5 to 6.5 (Fig. 18A, B). The addition of 5% glycerol (Fig. 18C), which had been recommended to be included in the washing steps of the His-tag affinity resin, or the addition of 0.25% Triton-X-100 (Fig. 18D) which is often used for detergent lysis of *Dictyostelium* cells, also caused partial dissociation of the γ -tubulin complexes. Therefore these substances were omitted in all further purifications. The addition of 100 μ M GTP, which had proved to be useful to prevent aggregation of γ -TuRCs in *Drosophila* (Oegema et al, 1999), on the other hand, had no measurable effect on the size of γ -tubulin complexes (Fig. 18E).

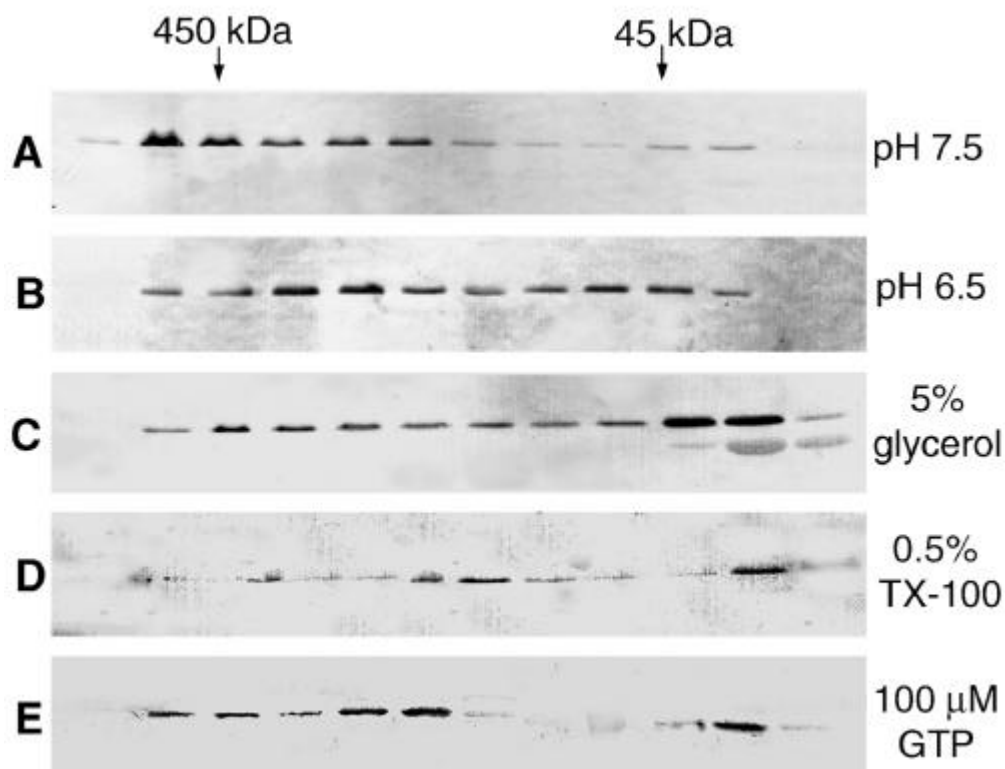


Fig. 18 Gel filtration of isolated *Dictyostelium* γ -tubulin complexes using different buffer conditions. γ -Tubulin complexes enriched under different buffer conditions were subjected to size exclusion chromatography and γ -tubulin-containing fractions were blotted and stained with anti-myc antibodies. The purification in (A) was performed at a pH of 7.5, whereas the pH was lowered to 6.5 in (B). Lowering of the pH apparently destabilized the prevailing *Dictyostelium* γ -tubulin complex, resulting in a broader distribution over many molecular weight fractions. 5% glycerol was added to the pH 7.5 buffer in (C) and 0.5% Triton-X-100 was added in (D). Both had a strong destabilizing effect with a drastic increase of monomeric γ -tubulin. The addition of 100 μ M GTP in (E) apparently did not influence the stability of the *Dictyostelium* γ -tubulin complex. The elution volumes of 45 kDa and 450 kDa marker proteins are indicated by arrows on the top of the panel.

3.1.5 Purification and identification of the components of *D. discoideum* γ -tubulin complexes.

In order to identify putative *Dictyostelium* γ -tubulin binding proteins the eluates from the His-affinity columns were further purified by immunoprecipitation with anti-myc antibodies. At first conditions similar to the purification of γ -TuRCs in other organisms, in particular low salt conditions, were used. However, under these conditions no specific interactors could be identified. A weak background of proteins was detectable in all purifications, but the protein pattern was always indistinguishable from that of AX2 mock purifications performed simultaneously under the same conditions (data not shown). Since no proteins specifically interacting with the His-affinity and anti-myc resin ought to be present in AX2 cells the observed protein pattern must be caused by non-specific binding of proteins to the affinity resins.

Because the results of the gel filtration experiments had suggested a stabilization of γ -tubulin-complexes under high-salt conditions, the purification was repeated with the addition of 500 mM NaCl to all buffers. Under these conditions a clear difference between γ -tubulin complex preparations from γ myc/His cells and AX2 mock purifications was detectable. Consistently two bands of about 55 and 100 kDa were observed in γ myc/His cells, which were not detectable in the AX2 mock purifications (Fig. 19A). Additional weak bands were visible in some preparations (e. g. around 205, 30 and 20 kDa), but they were also present in AX2 mock preparations and are therefore most likely non-specific contaminants. Western blots confirmed that the 55 kDa protein represents myc/6xHis-tagged Dd- γ -tubulin, which is strongly enriched during the purification procedure (Fig. 19B). As expected, no γ -tubulin was enriched in AX2 mock preparations (Fig. 19B), which confirmed the specificity of the purification. Furthermore, enrichment of γ -tubulin was shown to be more efficient under high salt than low salt conditions (Fig. 19C).

To determine which protein was represented by the 100 kDa band, it was excised from an SDS-polyacrylamide gel stained with colloidal Coomassie and subjected to mass spectrometry analysis (Toplab, Martinsried, Germany). The peptide masses of fragments obtained upon tryptic digest were compared to those calculated for *Dictyostelium* protein libraries and selected *Dictyostelium* centrosome components, including DdSpc97 and DdSpc98. 19 peptide masses were identical to those calculated for the partial DdSpc98 sequence, which suggests strongly that the 100 kDa band co-purifying with γ -tubulin in fact represents DdSpc98.

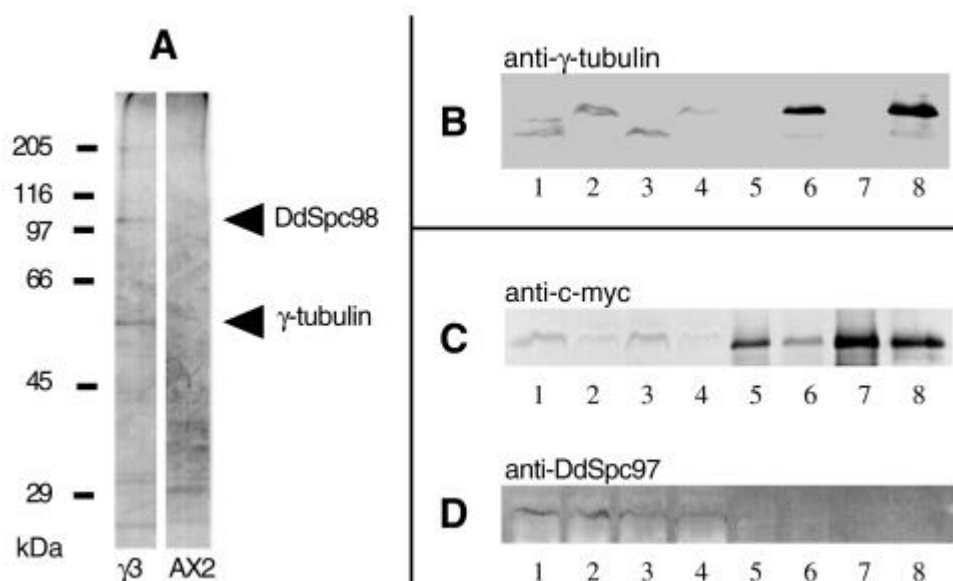


Fig. 19 Cytosolic *Dictyostelium* g-tubulin complexes consisting of g-tubulin and DdSpc98 can be purified under high salt conditions. (A) Colloidal Coomassie staining of cytosolic γ -tubulin complexes purified from $\gamma 3$ cells and mock purifications of AX2 cells. The two most prominent bands, representing γ -tubulin and DdSpc98, are indicated by arrows. (B) Western blot of samples from a γ -tubulin complex purification ($\gamma 3$) and a simultaneously performed mock purification (AX2) stained with anti- γ -tubulin antibodies, showing strong enrichment of γ -tubulin in purifications from $\gamma 3$ cells. Lanes 1-8: 1, AX2 cytosolic extract; 2, $\gamma 3$ cytosolic extract; 3, AX2 supernatant after His-affinity purification, containing cytosolic proteins that do not bind to the affinity resin (His-SN); 4, $\gamma 3$ His-SN; 5, AX2 eluate from His-affinity resin (His-E); 6, $\gamma 3$ His-E; 7, AX2 anti-myc immunoprecipitation (anti-myc IP); 8, $\gamma 3$ anti-myc IP. (C and D) Western blots of γ -tubulin complex preparations from $\gamma 3$ cells under high salt (HS) and low salt (LS) conditions stained with anti-c-myc antibodies (C) and anti-DdSpc97 antibodies (D). Lanes 1-8: 1, cytosolic extract HS; 2, cytosolic extract LS; 3, His-SN HS; 4, His-SN LS; 5, His-E HS; 6, His-E LS; 7, anti-myc IP HS; 8, anti-myc IP LS. Lanes 1-8 in (B-D) contain equivalents of 5×10^6 (1-4), 7×10^7 (5, 6) and 1.7×10^9 cells (7, 8).

Quite unexpectedly no band co-purifying with tagged γ -tubulin was detectable around 137 kDa, the calculated molecular weight of DdSpc97 (Fig. 19A). Western blots confirmed that DdSpc97 stays in the supernatant of the first affinity-binding step in γ -tubulin complex preparations under high salt and low salt conditions (Fig. 19D). Neither was DdSpc97 detectable in immunoprecipitations performed with anti- γ -tubulin antibodies in cytosolic extracts of GFP-DdSpc98 cells under conditions where DdSpc98 co-immunoprecipitated efficiently (Fig. 20). Both findings support the notion that DdSpc97 does not interact with γ -tubulin in the cytosol of *Dictyostelium* cells.

This finding is in contrast to all other systems where the interaction of γ -tubulin, Spc97 and Spc98 has been investigated so far and where the three proteins have been shown to form very stable complexes in the cytosol (Knop et al., 1997; Knop and Schiebel, 1997; Murphy et al., 1998; Oegema et al., 1999).

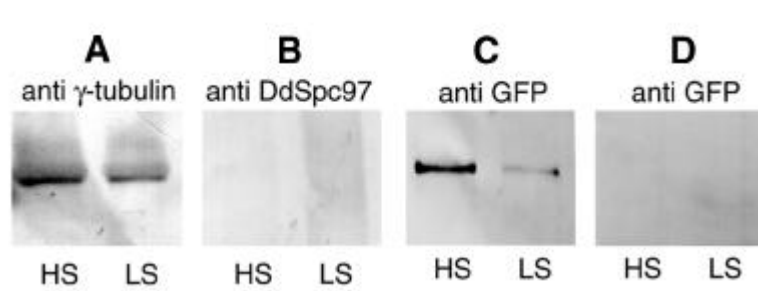


Fig. 20 γ -Tubulin coimmunoprecipitates with DdSpc98, but not with DdSpc97.

Immunoprecipitations from cytosolic extracts of GFP-DdSpc98 cells were performed with anti- γ -tubulin antibodies under high salt (*HS*) and low salt (*LS*) conditions. (**A**, **B**, **C**). Western blots were stained with anti- γ -tubulin (**A**), anti-DdSpc97 (**B**) and anti-GFP antibodies (**C**). γ -Tubulin and GFP-DdSpc98 were both precipitated efficiently under high salt conditions (*HS*), whereas DdSpc97 could not be detected. As a control for the specificity of the copurification of GFP-DdSpc98 immunoprecipitations were also performed with anti-myc-antibodies, which should not bind any protein specifically in GFP-DdSpc98 cells (**D**). As expected, no GFP-DdSpc98 could be detected, confirming that the band detected in (**C**) is not due to non-specific binding to the resin.

3.2. Investigation of the *D. discoideum* homologue of centrin

3.2.1 Cloning and sequence analysis of DdCrp

Centrins are centrosomal proteins ubiquitously found in all organisms investigated so far. Therefore, a homologous protein was expected to be present in *Dictyostelium*, too. By a BLASTN homology search of a *Dictyostelium* cDNA library sequenced in Tsukuba/ Japan a sequence with similarity to the centrin family was identified (clone SSF324, Morio et al., 1998). Clone SSF324 contains 506 bp of coding sequence, including one complete open reading frame (base position 45-500) (Fig. 21).

1	ACAATAAAAAACAACAACACCAACACCAACAACAATACAATATGAAAACATAAAACG	59
1	<u>M K T K T</u>	19
60	TGTAATAAATTTAATTTCAAACGAACAAATCCAAGAAGTACAAGAAGTTTATGATACTTTT	119
20	C K N L I S N E Q I Q E V Q E V Y D T F	39
	▼	
120	AAATGTAGAAATGGAGAAATGGATAAAATTAATATACAATATGGATTTAGAGCGTTAGGT	179
40	K C R N G E M D K I N I Q Y G F R A L G	59
180	GTTAGATTAGGAGAAGAAGAGGTTGAAAAGATATTTAAAAATCAACAATACATTAATTTT	239
60	V R L G E E E V E K I F K N Q Q Y I N F	79
	▼	
240	AATTCATTTTATAGATATTGTTACACCATTAATCTATAAAATTGATGTCTATGCATCATT	299
80	N S F L D I V T P L I Y K I D V Y A S F	99
300	GAACAGGCATTTAGTTTATTTGATAGAGATGGCTCTGGTTATATTACATTTGATGATTTA	359
100	E Q A F S L F D R D G S G Y I T F D D L	119
360	AAAACAGTTGCTATTAATTTAGGTGAAGCAAGATCAGATTCAAAATTGTACAATATGATA	419
120	K T V A I N L G E A R S D S K L Y N M I	139
	▼	
420	AAACGTGCAGATTTAAATGGGGATAAAAAAATTAGTAAAATAGAATTTATCCAATTATTA	479
140	K R A D L N G D K K I S K I E F I Q L L	159
480	TATTGAAAAAGATATATTGATTAGA	506
160	Y W K K I Y *	168

Fig. 21 The centrin-related *Dictyostelium* cDNA clone SSF324. The cDNA sequence of clone SSF324 obtained from the Tsukuba cDNA library (Morio et al, 1998) is shown. The putative coding sequence is shaded in grey and the initiation methionine is underlined. The sites of the introns identified by sequencing a PCR fragment on genomic DNA are indicated by arrowheads (▼).

The protein sequence derived from the open reading frame of clone SSF324 shows 32% amino acid identity and 40% similarity to its closest centrin relative from the amoebflagellate *Naegleria gruberi* (Levy et al., 1996). This protein most likely represents the *Dictyostelium* homologue of centrin, but due to its poor conservation and some aberrant sequence features explained in detail in section 3.2.2 it will be referred to as DdCrp (*Dictyostelium* centrin-related protein) instead of *Dictyostelium* centrin. Since the N-terminus of DdCrp was shorter than expected (see section 3.2.2) cDNA 5' ends were amplified from cDNA libraries, to confirm that the putative initiation methionine is indeed the first in-frame methionine. A PCR product of about 360 bp was obtained by PCR with the centrin-specific

reverse primer SSC444-2 (base position 324-342 of clone SSF324) and the λ -phage specific primer λ for (Fig. 22B). The sequence of the PCR product was identical to the Tsukuba cDNA clone SSF324 and thus it is very unlikely that there is another start codon upstream of the putative initiation methionine. PCR on genomic DNA using primers Cen for/Cen rev yielded a PCR product of about 800 bp instead of the expected 500 bp (Fig. 22C, D). Subsequent sequencing of the PCR product revealed that the gene contains three short introns of about 100 bp each. The localizations of the introns are indicated in Fig. 21. The genomic sequence obtained by PCR proved to be identical to overlapping genomic fragments recently identified in the course of the *Dictyostelium* genome sequencing project (clones JC1c136g07, JC1b226c07, JC1c48a01, IIAFP1D).

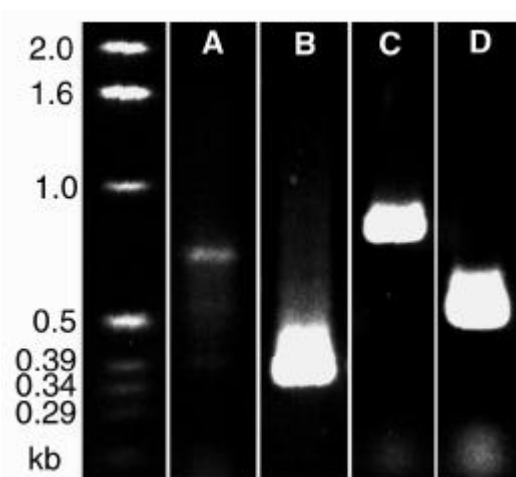


Fig. 22 PCR experiments confirmed the putative initiation methionine and revealed the presence of three introns . (A, B) PCR was performed on a cDNA library to investigate the 5' region of DdCrp. (A) shows the first round of PCR using primers λ forward /SSC444-1 (SSC444-1: base position 380-402 in Fig. 21). With nested primers λ for /SSC444-2 (SSC444-2: base position 324-342 in Fig. 21) on the first reaction a strong band of about 360 bp was obtained (B). Sequencing of the PCR product confirmed the putative initiation methionine. (C, D) PCR was performed with primers Cen for (pos. 45-62 in Fig. 21) and Cen rev (pos. 483-500 in Fig. 21) on genomic DNA (C) and on the cDNA clone SSF324 (D). The PCR product obtained in (C) is about 300 bp longer and sequencing of the PCR product revealed the presence of three introns of about 100 bp each.

3.2.2 Southern and Northern blot analysis of DdCrp

In some organisms more than one centrin gene have been identified: there are at least three different centrin isoforms in vertebrates (Errabolu et al., 1994; Lee and Huang, 1993; Middendorp et al., 1997), three closely related centrin genes in *Paramecium tetraurelia* (Madeddu et al., 1996) and a centrin and a centrin-related protein in *Arabidopsis thaliana* (EU Arabidopsis sequencing project, accession numbers O82659 and O23184). To investigate whether the *Dictyostelium* genome contains other centrin isoforms or genes closely related to DdCrp Southern blots of genomic DNA prepared from axenically growing wild-type AX2 cells digested with a number of restriction enzymes were performed. All of them, except for *Nsi*I, which cuts internally, yielded only one band, suggesting that there is only one isoform and no other closely related gene in *Dictyostelium* (Fig. 23).

In addition, Northern blots were prepared to investigate the expression of DdCrp. mRNA prepared from axenically growing amoebae was blotted and probed with a full-length DdCrp probe under high-stringency conditions. A single, strong band of about 1.2 kb appeared (Fig. 23B), indicating that the gene is expressed in amoebae in substantial amounts. The size of the transcript was larger than expected for a 150 aa protein. But since the nature of the untranslated regions was not in the focus of interest of this work it was not investigated further. However, since the 5'-end of the DdCrp cDNA has been investigated in more detail it can be stated that untranslated regions must be located at the 3'-end.

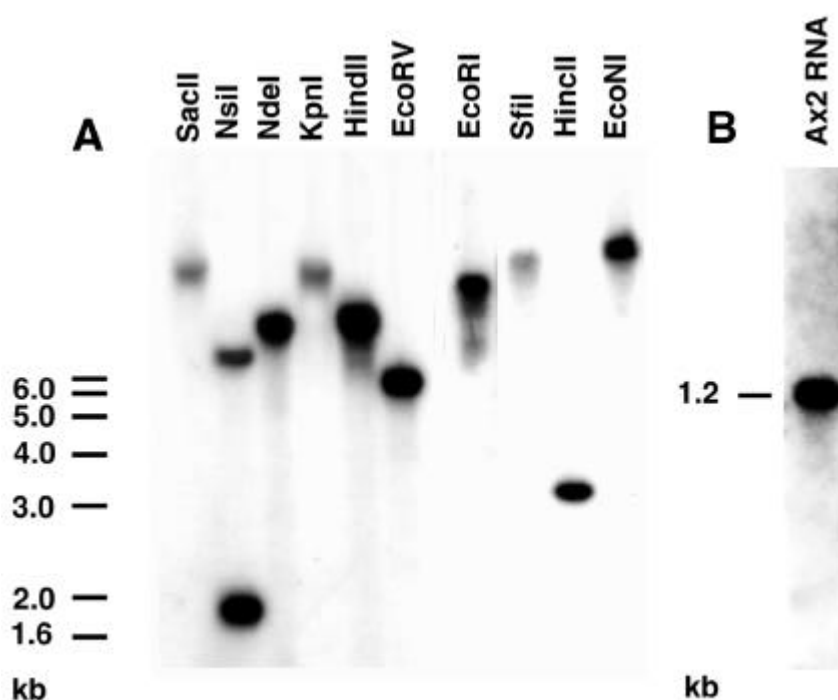


Fig. 23 Southern and Northern blot analyses of DdCrp suggest the existence of only one DdCrp isoform. (A) Southern blot of genomic *Dictyostelium* DNA which was cleaved with different restriction enzymes. The blot was probed with a full-length ^{32}P -labelled DdCrp probe (base position 44-500 in Fig. 21). The size of standard DNAs is given on the left. Single bands were obtained with all enzymes except *NsiI*, which cuts within the DdCrp sequence, indicating that there is no other closely related isoform of the gene. (B) Northern blot of mRNA prepared from axenically growing *Dictyostelium* amoebae. The blot was probed with the same probe as above. A single mRNA species of 1.2 kb is expressed in the cells.

3.2.3 Sequence analysis of DdCrp

An alignment of DdCrp with 23 other published centrin sequences (Fig. 24) and the phylogenetic tree derived from this alignment (Fig. 25) reveal that DdCrp is clearly the most divergent member of the centrin family known to date. Sequence conservation is especially

low in the N-terminal part of the molecule, which is generally the most variable region among centrins. With an extension of only 9 amino acids the DdCrp N-terminus is by far the shortest compared to extensions of 16-35 amino acids present in other centrins.

The protein derived from the cDNA sequence is 151 amino acids long and has a calculated molecular weight of 17.7 kDa, which makes it the smallest centrin known so far. With an isoelectric point of 6.71 it is also the least acidic, compared to the pK_is of 4.3-5.3 of other centrins (summary in Schiebel and Bornens, 1995). The most striking aberrance of DdCrp, though, is the lack of conservation of two of the four Ca²⁺-binding EF-hand domains which are an essential sequence feature of all other centrins. The EF-hand motif was first described by Kretsinger and Barry (1975) and consists of a twelve residue Ca²⁺-binding loop, flanked on both sides by alpha-helices, the E- and F-helix. Six residues (in positions 1, 3, 5, 7, 9 and 12) in the Ca²⁺-binding loop are involved in binding. Residue 12 is always a Glu or Asp and provides two oxygens for liganding calcium. The *Dictyostelium* homologue lacks the first two EF-hand consensus motifs, whereas the third and fourth EF-hand are present, albeit less strictly conserved than in most other centrins.

Homology searches showed that another EF-hand protein, *Dictyostelium* calmodulin (CaM) (Goldhagen and Clarke, 1986), is also closely related to DdCrp. DdCaM is therefore included in the phylogenetic tree, where it is actually closer to the other centrins than DdCrp. Because of the divergence of DdCrp and its relatedness to DdCaM one might suggest that DdCrp is not a centrin, but a calmodulin-like protein or a novel centrosomal EF-hand protein. But there are a couple of arguments against that, which will be discussed in detail in section 4.2.1.

(Next page)

Fig. 24 Multiple sequence alignment of centrin homologues

All full-length centrin sequences published by the time of this work were aligned with DdCrp and DdCaM using the ClustalW programme. Abbreviations and protein accession numbers are: AtCen-like, *Arabidopsis thaliana* caltractin-like protein (O23184); AtCen, *A. thaliana* centrin (O82659); AnCen, *Atriplex nummularia* centrin (P41210); CrCen, *Chlamydomonas reinhardtii* centrin (P05434); DdCrp, *Dictyostelium discoideum* centrin-related protein; DdCaM, *D. discoideum* calmodulin (PO2599); DsCen, *Dunaliella salina* centrin (P54213); EoCen, *Euplotes octocarinatus* centrin (Q9XZV2); GICen (a), *Giardia intestinalis* centrin (Q24956); GICen (b), *G. intestinalis* centrin (Q94836); HsCen1, *Homo sapiens* centrin1 (Q12798); HsCen 2, *H. sapiens* centrin 2 (P41208); HsCen 3, *H. sapiens* centrin 3 (O15182); MvCen, *Marsilea vestita* centrin (O4999); MmCen1, *Mus musculus* centrin 1 (P41209); MmCen2, *M. musculus* centrin 2 (Q9R1K9); MmCen3, *M. musculus* centrin 3 (O35648); NgCen, *Naegleria gruberi* centrin (P53441); PtcenICL1A, *Paramecium tetraurelia* centrin ICL1A (Q27177); PtcenICL1B, *P. tetraurelia* centrin ICL1B (Q27179); PtcenICL1C, *P. tetraurelia* centrin ICL1C (Q27178); ScCdc31, *Saccharomyces cerevisiae* Cdc31p P06704); SdCen, *Scherffelia dubia* centrin (Q06827); SpCen, *Schizosaccharomyces pombe* centrin (O74435); XICen, *Xenopus laevis* centrin (Q91643). Amino acids which are strongly conserved (identical amino acids in at least 22 of the 25 sequences) are shaded in grey. Residues where DdCrp differs in otherwise conserved positions are shaded in black. EF-hand regions are indicated with asterisks. Centrin-

specific sequence features, such as an N-terminal extension and C-terminal positively charged and aromatic amino acids, are underlined.

```

DdCen      -----MKTKT-----CKNLH SNEQIQE VQEVYDFEKKR- NGE MDK INIQYGFRALGVRLGEEVEKIFKNQ-----
DdCaM      -----ASQESL TEEQIAEFK EAFSLFDKDGDS ITTKELGTVMRSLGQNPTAEALQDMINEVDADGN
XlCen      -----MASNYKKPSLGVTTQRKKP-----VPKTEL TEEQKQEIREFDLFDTDGTGT IDVKELKVAMRALGFEPKKEEIKKMIADIDKEGT
HsCen1     -----MASGFKKPSAASTGQKRKV-----APKPEL TTDQKQEVREFDLFDVDSGSGT IDAKELKVAMRALGFEPRKEEMKKMISEVDREGT
HsCen2     -----MASNFKKANMASSQRKRM-----SPKPEL TEEQKQEIREFDLFDADGTGT IDVKELKVAMRALGFEPKKEEIKKMISEIDKEGT
HsCen3     -----MSLALRSELLVDKTKR-----KKRREL TSEEQKQEIREFDLFDTDKDEA IDYHELKVAMRALGFDVKKADV LKILKDYDREAT
MmCen1     -----MASTFRKSNVASTSYKRKV-----GPKPEL TTDQKQEVREFDLFDSGSGT IDVKELKVAMRALGFEPRKEEMKKMISEVDKEAT
MmCen2     -----MASNFKKTTMASSAQRKRM-----SPKPEL TTDQKQEIREFDLFDADGTGT IDIKELKVAMRALGFEPKKEEIKKMISEIDKEGT
MmCen3     -----MSLALRSELVVDKTKR-----KKRREL TSEEQKQEIREFDLFDTDKQA IDYHELKVAMRALGFDVKKADV LKILKDYDREAT
PtICL1A    MARRGQQPPQP--QAPPAQKNQTKGFNP AE-----FVKPGL TEEVLEIK EAFDLFDTDGTS IDPKELKAAMTSLGFEAKNQTIYQMISDLDTDGS
PtICL1B    MARRGQQPPPPQQQAPP-QKNQAGKFNPAE-----FVKPGL TEEVLEIK EAFDLFDTDGTS IDPKELKAAMTSLGFEAKNQTIYQMISDLDTDGS
PtICL1C    MARRGQQPPPPQQQAPPQTKNQAGKFNPAE-----FVKPGL TEEVLEIK EAFDLFDTDGTS IDPKELKAAMTSLGFEAKNQTIYQMISDLDTDGS
EoCen      -----MIKKPEFGMLQPPKKR-----VRQEL TSEEQKQEIREFDLFDTNKTS IDYHELKVAMRALGFDVKKPEILELMNEYDRGN
NgCen      -----MQYGSKKIGATSATSSNQ-----KVQIEL TDEQKQEIREFDLFDMGSGK IDAKELKVAMRALGFEPKKEEIKKMI SGID-NGS
GlCen      -----MNRAAIAAGKPSGSI STGKPR-----KTRAEVSEEMKHE IREFDLFDADRSGR IDFHELKVAMRALGFDVKKEEIQRIMNEYDRDQL
GlCen2     -----MA-----RKP NARST-----EPDL TEEQKHE IREFDLFDSGSGR IDVKELKVAMRALGFEPRKEELKRMIAEVDTSGS
SdCen      -----MS-YRK-AASARRDKAK-----TRSAGL TEEQKQEIREFDLFDTDGSGT IDAKELKVAMRALGFEPKKEEIKKMIADIDKDG
CrCen      -----MSYKAKTVVSARRDQKK-----GRVGL TEEQKQEIREFDLFDTDGSGT IDAKELKVAMRALGFEPKKEEIKKMIADIDKDG
DsCen      -----MSYR-KTVVSARRDQKK-----GRVGL TEEQKQEIREFDLFDTDGSGT IDAKELKVAMRALGFEPKKEEIKKMIADIDKAGS
MvCen      -----MSNFRKGVGTGRRDKNK-----GRAQGL TSEEQKQEIREFDLFDTDGSGT IDAKELKVAMRALGFEPKKEEIKKMIADIDKDG
AtCen      -----MSSIYRTVSRKEKPR-----RHHGL TTQKQEIREFELFDTDGSGT IDAKELNVAMRALGFEEMTEEQINKMIADVDKDG
AtCen-like -----MSEAAQLR-RGLKPK-----GKTYGL TNQKRRE IREFDLFDIDGSGS IDASELNVAMRSLGFEMNNQIQINELMAEVDKNQS
AnCen      -----MSSA-RTV-RKDKPR-----GRHHL TQKQEIREFELFDTDGSGT IDAKELNVAMRALGFEEMTEEQINQMIADVDKDG
ScCdc31    -----MSKNRSSLQS-----GPLNSE LLEQKQEIREFSLFDMNNDGFLDYHELKVAMRALGFELPKREILDLIDEYDSEGR
SpCen      -----MFANARAKRRSRASSPTPARLGGYAPLRV EITTEQRQD INEAFKLFDSDKDNA IDYHELRAAMRALGFNAEKSEVLKILRDFDKTGK

```

```

DdCen      -YINFNFLDIDVTPLIYKIDVYASFEQAFSLFDRDGSYITFDLKVVAI N LGEARSDS LYNMIKRADLN GDKKISKIEFIQLH YWKKIY-- 151
DdCaM      GNIDFPEFLTMARKMQDIDTEEIR EAFKVFDDKNGYISAAELRHVMTSLGKLTNEEVDEMIREADLDGQGQVNYDEFVKMIVRN---- 151
XlCen      GKIAFSDFCAMTQKMAEKDSKEEIMKAFRLFDDETGKISFKNLKRVAKELGENLTDEELQEMI DEADR DGDGEVNEEFLRIMKKTSLF-- 172
HsCen1     GKISFNDFLAVMTQKMS EKD TKEEILKAFRLFDDETGKISFKNLKRVANELGENLTDEELQEMI DEADR DGDGEVNEEFLRIMKKTSLY-- 172
HsCen2     GKMNFGDLFLVMTQKMS EKD TKEEILKAFKLFDDDETGKISFKNLKRVAKELGENLTDEELQEMI DEADR DGDGEVNEEFLRIMKKTSLY-- 172
HsCen3     GKITFDNFNEVTDWILERDPHEEILKAFKLFDDDDSGKISLRNLRRVARELGENMSDEELRAMIEEFDKDGDGEINQEEFIAIMTGD I---- 167
MmCen1     GKISFNDFLAVMTQKMAEKDSKEEILKAFRLFDDETGKISFKNLKRVANELGESLTDEELQEMI DEADR DGDGEVNEEFLKIMKKTNL Y-- 172
MmCen2     GKMNFGDLFLVMTQKMS EKD TKEEILKAFKLFDDDETGKISFKNLKRVAKELGENLTDEELQEMI DEADR DGDGEVNEEFLRIMKKTSLY-- 172
MmCen3     GKITFDNFNEVTDWILERDPHEEILKAFKLFDDDDSGKISLRNLRRVARELGENMSDEELRAMIEEFDKDGDGEINQEEFIAIMTGD I---- 167
PtICL1A    QQIDFAEFLKIMTARIS ERDSKADIQKVFNLFD SERAGVVTLDLKRKVALELGETMDDSELQEMI DRADS DGAQVTFDEFYNIMTKKTFA-- 181
PtICL1B    QQIDFAEFLKIMTARIS ERDSKADIQKVFNLFD SERAGVITLKLDRKVALELGETMDDSELQEMI DRADS DGAQVTFDEFYNIMTKKTFA-- 182
PtICL1C    QQIDFAEFLKIMTARIS ERDSKADIQKVFNLFD SERAGVITLKLDRKVALELGETMDDSELQEMI DRADS DGAQVTFDEFYNIMTKKTFA-- 183
EoCen      GYIGFDDFLDIMTEIKINRDPVEEILKAFKVFEDN SGKISLRNLKRVAKELGENLSDEELQAMI DEFDKDQDGEISEQEF LNMKQTSIY-- 168
NgCen      GKIDFNDFLQIMTAKMS EKD SHA EIMKAFRLFDEDDSGFITFANLKRVAKDLGENMTDEELREMI BEADR SNQGI SKEDFLRIMKKTNL F-- 172
GlCen      GEITFDQDFEVMIEKISNRDPTEEILKAFRLFD DDTGRI SLKLNRRVAKELSENISDEELLA MIQEFDRDGDGEIDEEDFIAILRSTSAFS- 176
GlCen2     GMIDLNDFFRIMTAKMAERDSRE EILKAFRLFDEDDTGKISFKNLKRVAKELGENLTDEELQEMI DEADR DGDGEINEEFLRIMRRTSLYQ- 162
SdCen      GTIDFEEFLQMTAKMGERDSRE EIMKAFRLFDDDETGKISFKNLKRVAKELGENMTDEELQEMI DEADR DGDGEVNEEFLRIMKKTSLF-- 168
CrCen      GTIDFEEFLTM TAKMGERDSRE EILKAFRLFDDN SGTITIKDLRRVAKELGENLTDEELQEMI AEADR NDDNEIDEDEFIRIMKKTSLF-- 169
DsCen      GTIDFEEFLQMTAKMGERDSRE EIKAFKLFDDDN TGFITLKNLKRVALELGENLTDEELQEMTDEADR NGDQIDEDEFYRIMKKTSLF-- 169
MvCen      GTIDFEDFLQMTTKMGERDSKE EIMKAFRLFDDDETGKISFKNLKRVAKELGENMTDEELQEMI DEADR DGDGEINEEFLYRIMKKTSLF-- 170
AtCen      GAIDFDFVHM TAKIGERDTKEELTKAFQIIDLKNGKISPDDIKRMAKDLGENFTDAEIREMVEEADRDRDGEVNMDFMRMMRRTAYGGN 169
AtCen-like GAIDFDFVHM TTKGERDSIDELSKAFKIDHNNNGKISPRDIKMI AKELGENFTDNDIEEMI BEADR DKDGEVNL EEFM KMKRTSYG-- 167
AnCen      GAIDFDFCHM TTAKIGERDTKEELMKAFRIDQDNNGKISPEDIQRI AKELGENFTVKDIQDMI BEADRDRDGEVNV EFLRMMKRTSYAY- 167
ScCdc31    HLMKYDDFYIVMGEKILKRDPLDEIKRAFQLFDDH TGTKISIKNLRRVAKELGETLTDEELRAMIEEFDL DGDGEINENE FIAICTDS---- 161
SpCen      GYLQMEDFVRVMT EKIVERDPLEEIKRAFELFDDDETGKISLRNLRRVAKELNENID DQLEAMI EEFDL DQDGEINEQEFIAIMMDEA--- 176

```

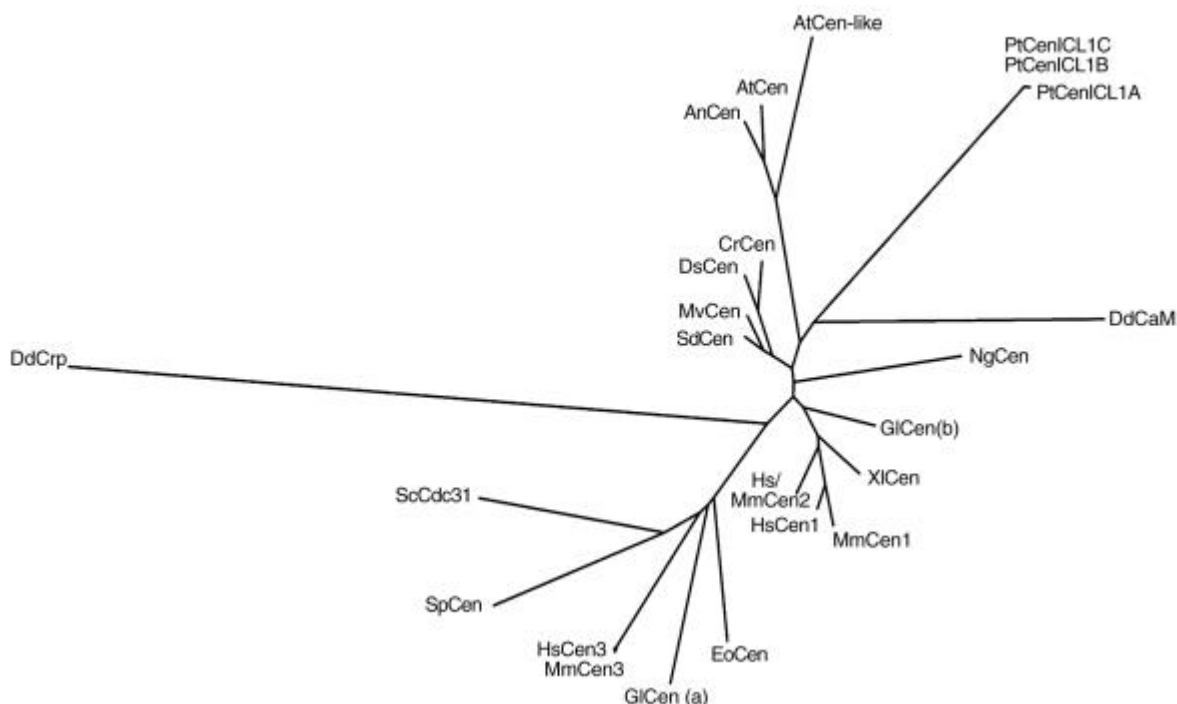


Fig. 25 Phylogenetic tree of centrin homologues. The alignment from Fig. 24 was used to calculate a phylogenetic tree with the PHYLIP package version 3.5c (Felsenstein, 1993). Branch lengths represent evolutionary distances. DdCrp belongs to none of the obvious clusters of centrin sequences and is clearly the most divergent member of the protein family.

3.2.4 Investigation of the subcellular distribution of DdCrp

3.2.4.1 Generation of polyclonal antibodies raised against DdCrp

DdCrp was fused to an N-terminal 6 x His tag and expressed in bacteria. The fusion protein precipitated under native conditions and could only be purified under denaturing conditions (Fig. 26A), therefore a second construct with an N-terminal fusion to MBP was made, which was soluble under native conditions (Fig. 26B). Both constructs, 6 x His- and MBP-tagged DdCrp, were used for the immunization of two rabbits per construct.

The four antisera gave similar results in immunoblots and showed the same patterns in immunofluorescence microscopy, except for anti-His-DdCrp1, which did not work in immunofluorescence microscopy. For simplicity, all data presented in this work were obtained with the same antibody, anti-MBP-Crp2, which generally yielded the best results.

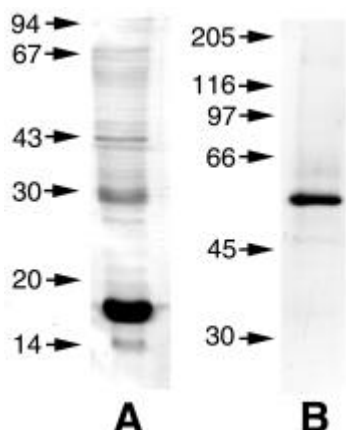


Fig. 26 His- and MBP-tagged DdCrp were expressed and purified for immunizations. DdCrp was expressed as an N-terminal fusion with a 6xHis tag and was purified on a Ni-NTA resin under denaturing conditions. The purified recombinant fusion protein migrates with an apparent molecular weight of about 18 kDa (A). The band was excised from a CuSO_4 -stained gel and was used for the immunization of two rabbits. Furthermore, DdCrp was expressed as an N-terminal fusion protein with MBP and was purified on an amylose resin. The eluate containing the 55 kDa fusion protein was used directly for the immunization of two rabbits (B).

3.2.4.1 Subcellular distribution of DdCrp on Western blots and in immunofluorescence microscopy

As expected, anti-MBP-DdCrp2 strongly reacted with both recombinant His-DdCrp and MBP-DdCrp, whereas bands were barely visible with the preimmune serum (Fig. 27).

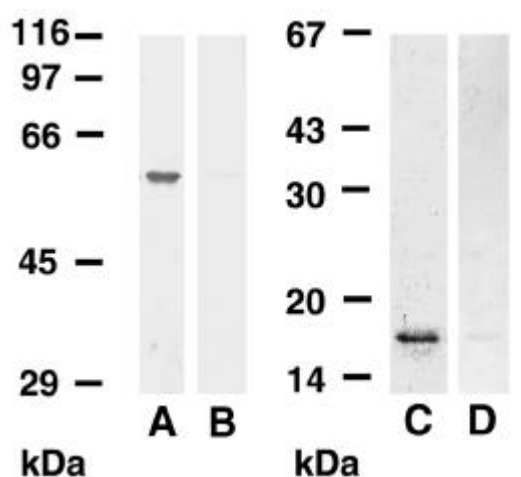


Fig. 27 Anti-MBP-DdCrp2 antibodies strongly react with recombinant His-DdCrp and MBP-DdCrp. Recombinant, purified MBP-DdCrp (A) and His-DdCrp (C) were strongly labelled with anti-MBP-DdCrp2 antibodies. The preimmune serum tested on the respective recombinant proteins in (B) and (D) was almost completely negative.

The specificity of anti-MBP-DdCrp2 was shown on immunoblots loaded with *Dictyostelium* cytosolic extracts, enriched nucleus/centrosome fractions and isolated *Dictyostelium* centrosomes. In all lanes one 19 kDa band was predominantly stained, which represents DdCrp (Fig. 28A-C). As a control isolated centrosomes were labelled with the preimmune serum (Fig. 28D), which was negative.

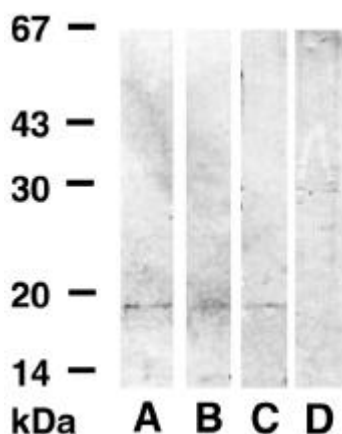


Fig. 28 Anti-MBP-DdCrp2 antibodies recognize a 19 kDa protein in *Dictyostelium* cell extracts.

To examine the specificity of the anti-MBP-DdCrp2 antiserum cytosolic extracts of approximately 10^7 *Dictyostelium* cells (A), enriched nuclei/centrosome fractions of approximately 5×10^6 cells (B) and centrosomes isolated from approx. 10^8 cells (C) were loaded on a 17.5 % polyacrylamide gel. Anti-MBP-DdCrp2 antibodies predominantly stained a band of about 19 kDa in all fractions on Western blots. The native protein stained in (E-G) migrates slower than the recombinant protein detected in (C).

Immunofluorescence microscopy with anti-DdCrp antibodies showed bright labelling of isolated centrosomes fixed to coverslips (Fig. 29A, red), whereas the preimmune serum was negative (Fig. 29C, red), which proves the centrosomal localization of DdCrp. As a control for the presence and localization of centrosomes on the coverslips double staining with anti-DdCP224 antibodies was performed for both samples (Fig. 29 B, D, green).

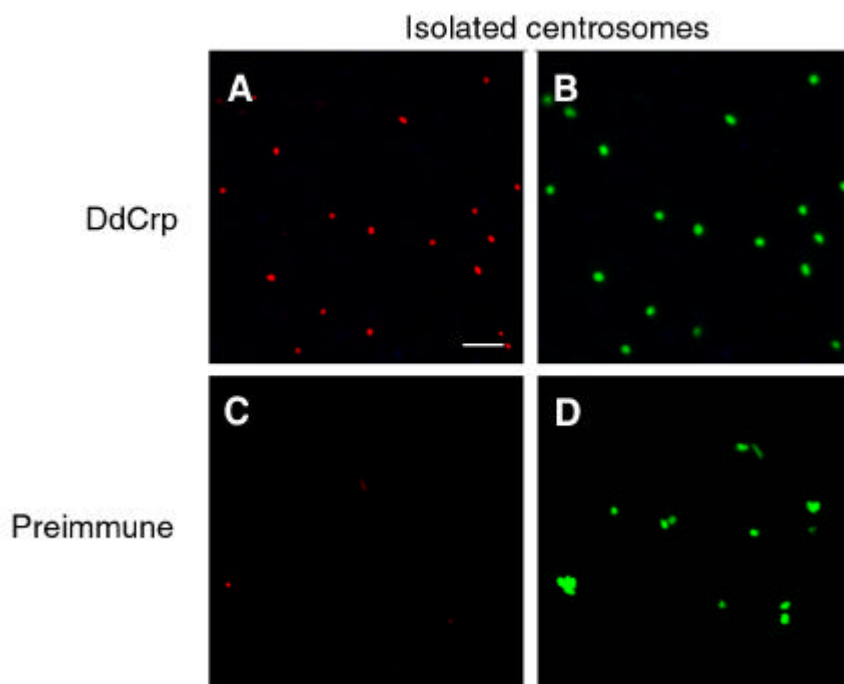


Fig. 29 Anti-DdCrp antibodies recognize the centrosome. Isolated centrosomes were fixed with methanol and stained with anti-MBP-DdCrp2 antibodies (A, red) or the respective preimmune serum (C, red). (B, green) shows the double staining of (A) with a monoclonal antibody against the centrosomal protein DdCP224 and (D, green) shows the double staining of (C) with anti-DdCP224. The centrosomes were labelled with anti-DdCrp antibodies, whereas the preimmune serum produced no labelling. Secondary antibodies were anti-rabbit Cy3 and anti-mouse FITC. (Bar: 2 μ m)

In whole interphase cells, the centrosome and a second, nuclear structure of about the same size in close proximity to the centrosome were the most intensely labelled structures (Fig. 30A, red). The latter, centrosome-associated structure was even more intensely stained than the centrosome itself. Furthermore, little dots distributed all over the nuclei were visible in addition to the centrosome and the associated structure. Again, double-labelling of the centrosomal protein DdCP224 was performed (Fig. 30B, green). Except for a weak overall background staining the preimmune serum was also negative on whole cells (Fig. 30C, red).

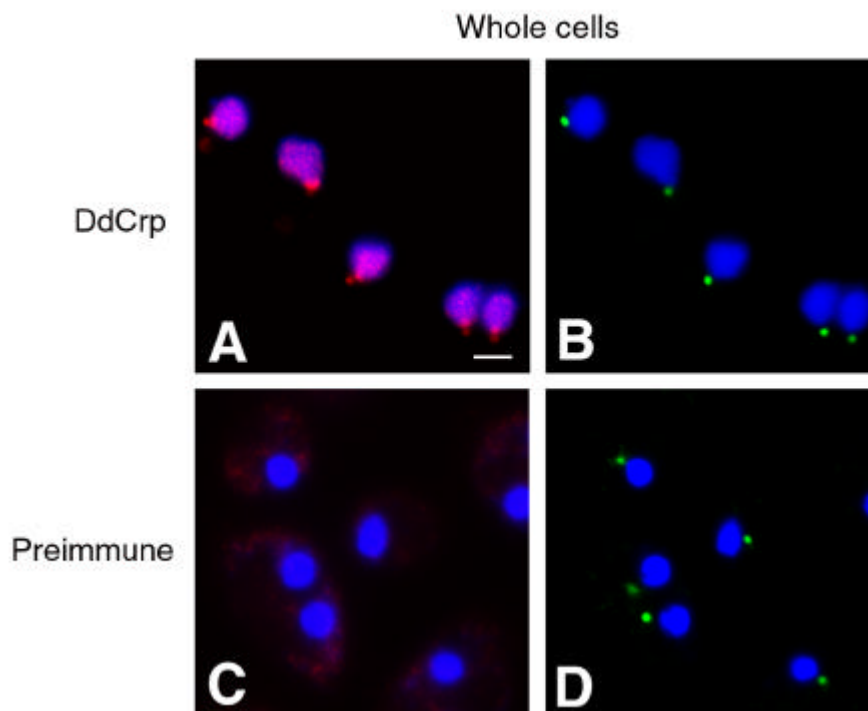


Fig. 30 Immunofluorescence of whole cells stained with anti-MBP-DdCrp2 antibodies.

Whole *Dictyostelium* amoebae were fixed with methanol and stained with anti-MBP-DdCrp2 (A, red). The antibody labels the centrosome, a second, centrosome-associated structure close to the nucleus and the nucleus itself. The respective double staining with the centrosomal marker anti-DdCP224 is shown in (B, green). The preimmune serum merely produces a weak background staining (C, red). The sample from (C) was again double-stained with anti-DdCP224 antibodies (D, green). Secondary antibodies were anti-rabbit Cy3 and anti-mouse FITC. A merge with the blue DAPI staining is shown in all samples. (Bar: 2 μ m)

A closer look at the centrosomal label in interphase cells revealed that DdCrp is localized at the centrosomal corona (Fig. 31). It appears as a doughnut-shaped structure surrounding the inner, unlabelled core structure. This staining pattern corresponds to that of antibodies against γ -tubulin or DdCP224 (Euteneuer et al., 1998; Gräf et al., 2000), which are both components of the corona of the *Dictyostelium* centrosome.

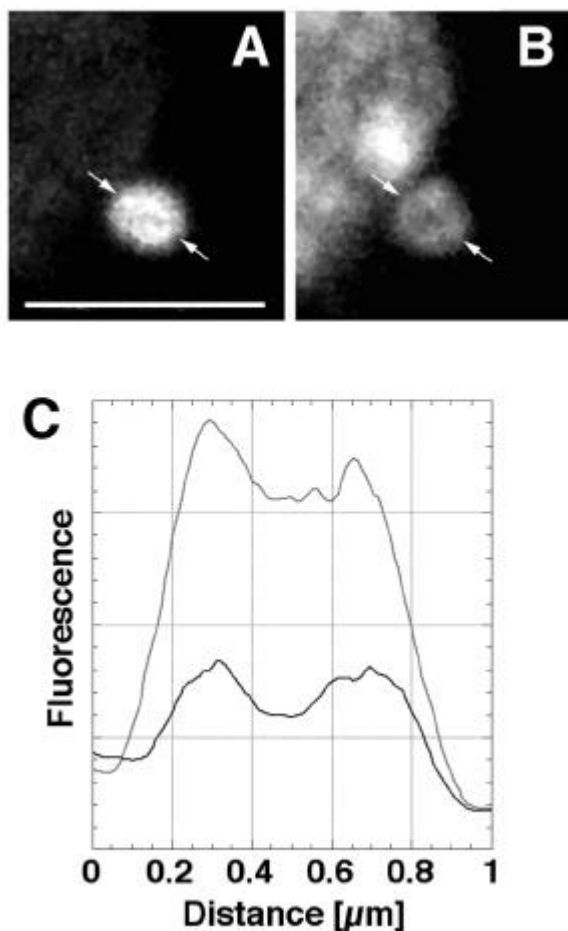


Fig. 31 DdCrp is localized at the centrosomal corona.

Intensity measurements made on confocal microscopy images revealed that DdCrp is localized at the centrosomal corona. The corona was labelled with monoclonal anti-DdCP224 as a control (A) and anti-DdCrp antibodies (B). The graph in (C) shows tracings of fluorescence intensity along a line through the center of the centrosome label in (A) and (B). The straight line selection used for measurement is indicated by two arrows. The positions of the maxima of fluorescence intensity of anti-DdCP224 labelling (upper, grey line) match that of anti-DdCrp labeling (lower, black line) which proves colocalization of both proteins at the corona. Secondary antibodies were anti-rabbit-Alexa488 and anti-mouse-Cy3. (Bar: 2 μm)

Unfortunately, attempts to visualize the labelling with anti-MBPDdCrp2 antibodies in immunogold-EM were not successful, because the antibodies did not work in fixation procedures providing sufficient structural preservation for EM (data not shown).

3.2.4.3 Generation of a *D. discoideum* mutant expressing myc/6xHis tagged DdCrp

To confirm the subcellular distribution of DdCrp obtained in immunofluorescence microscopy it was additionally attempted to study DdCrp localization by generation of stable *Dictyostelium* mutants overexpressing tagged versions of DdCrp. But, unfortunately, DdCrp was either not expressed or could not be localized within the cell, when tagged with GFP or the myc-epitope, regardless of whether the tags were fused to the N- or C-terminus. To exclude that cells overexpressing tagged DdCrp could not be isolated due to cell division defects caused by the overexpression of this centrosomal protein it was also tried to express

the protein under the control of the inducible Discoidin promoter. But again, no label was observed in cells growing under permissive conditions.

However, partial success was obtained with *Dictyostelium* mutants in which the endogenous copy of the DdCrp gene was replaced with a copy of the gene carrying a C-terminal myc/6xHis tag. For this, a similar approach was used as described in detail for γ -tubulin in section 3.1.5. The DdCrp-myc/6xHis transformants were examined for expression of the myc-tag by immunofluorescence microscopy with anti-myc antibodies. In most cells no label could be detected, but in about 1% of transformants a centrosomal label was observed. In some cells the centrosome-associated structure was also faintly visible next to the centrosome (Fig. 32A, red), which confirms the labelling obtained with anti-DdCrp antisera. Fig. 32B shows the double labelling with a polyclonal anti- γ -tubulin antibody (green) staining only the centrosome. Unfortunately it was not possible to establish clonal lines from any of the DdCrp-myc/6xHis transformants for further analyses, because DdCrp-myc/6xHis expression was not detectable any more after some passages.

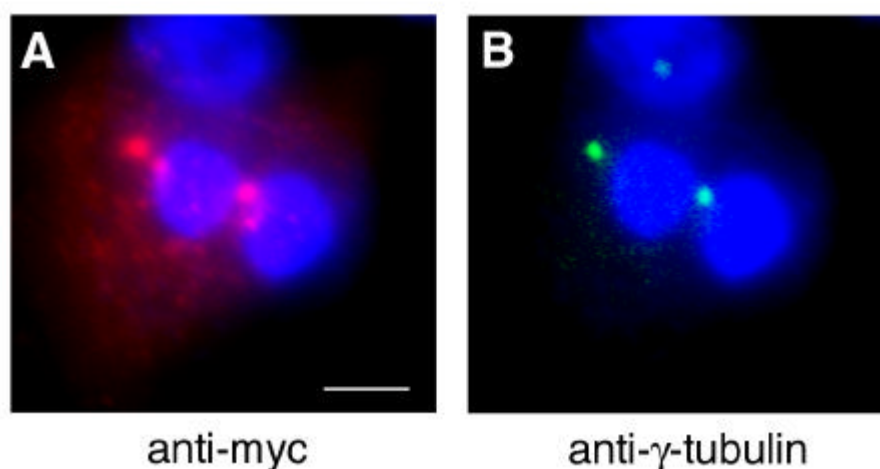


Fig. 32 The centrosome and a centrosome-associated structure are labelled with anti-myc antibodies in cells expressing myc/6xHis tagged DdCrp. Immunofluorescence using the anti-myc-antibody 9E10 (Evan et al., 1995) reveals a centrosomal and a centrosome-associated label in cells transformed with the homologous recombination plasmid pDdCrp-myc/His (**A**, red). Cells were double-stained with anti- γ -tubulin antibodies (**B**, green). Secondary antibodies were anti-mouse Cy3 and anti-rabbit FTIC. A merge with the blue DAPI staining is shown in both images. (Bar: 2 μ m)

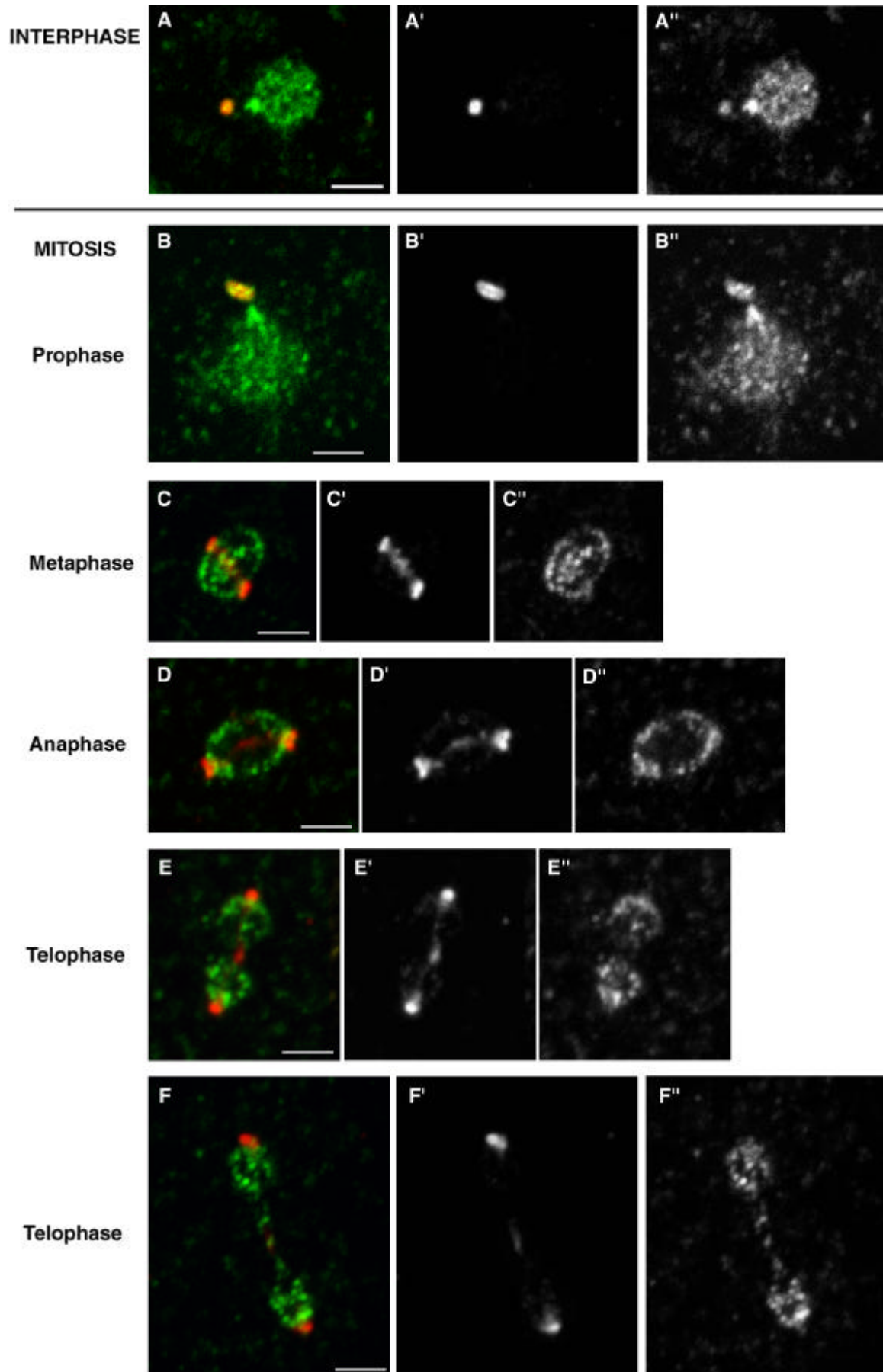
3.2.4.4 Cell cycle dependent changes in the localization of DdCrp

Using confocal microscopy the distribution of the DdCrp antigen during different stages of the cell cycle was investigated. Whereas centrins from other organisms generally are permanently associated with the MTOC, anti-DdCrp labelling was found to be subject to cell

cycle dependent changes (Fig. 33). The centrosomal and centrosome-associated label were still pronounced during prophase, when the *Dictyostelium* centrosome starts to enlarge (Fig. 33B). However, in metaphase fluorescent labelling of the two spindle poles was only barely visible, whereas labelling of the centrosome-associated structure had already disappeared completely (Fig. 33C). The patchy nuclear staining, in contrast, remained unaltered throughout the cell cycle. Especially the anaphase and telophase images show that the nuclear label was stronger on the outer rim of the nucleus, which may indicate that the antigen is localized close to the nuclear envelope, which remains intact during the closed mitosis of *Dictyostelium* amoebae (Fig. 33D, E). The label extended into the small remains of nuclear material connecting the two separating telophase nuclei.

(Next page)

Fig. 33 DdCrp dissociates from the centrosome and the centrosome-associated structure during mitosis. The distribution of DdCrp during the cell cycle was examined by confocal microscopy using anti-MBP-DdCrp2 antibodies. The cell cycle stages (indicated on the left) were identified according to Ueda et al. (1999) and Gräf et al. (2000). Single optical sections (**A-C**) and projections of the two optical sections with maximal spindle pole labelling (**D-F**) are shown. Cells were double labelled with anti-DdCrp antibodies (**A''-F''**) and the mouse-monoclonal anti-DdCP224 antibody (Gräf et al., 1999) as a reference for centrosome, spindle, kinetochore and midbody labelling (Gräf et al., 2000) (**A'-F'**). In the merged images (**A-F**), DdCrp labeling is shown in green and DdCP224 in red. Secondary antibodies were anti-rabbit-Alexa488 and anti-mouse-Cy3. (Bars: 2 μ m)



4. DISCUSSION

4.1 Investigation of cytosolic γ -tubulin complexes in *D. discoideum*

4.1.1 Tagging of endogenous γ -tubulin with a myc/6xHis tag

Like all centrosomal proteins γ -tubulin and its interacting proteins are present in very low amounts in the cell. Standard biochemical approaches are therefore of limited use in the purification of γ -tubulin complexes. Thus it was decided to tag endogenous γ -tubulin with a combined myc/6xHis tag. The C-terminal localization of the tag was chosen, because this side of the protein has been predicted to be exposed on the surface of *Xenopus* γ TuRCs (Felix et al., 1994). In fact, a C-terminal myc/6xHis tag has been used successfully before to immunoprecipitate mammalian γ TuRCs (Murphy et al., 1998). The plasmid used for the transformation of *Dictyostelium* cells carries an N-terminally truncated, promoterless version of the γ -tubulin gene (Fig. 15). Since the N-terminus of γ -tubulin has been shown to be required for correct localization (Leask and Stearns, 1998), tagged γ -tubulin will only localize to the centrosome following homologous integration.

By immunofluorescence microscopy, PCR on genomic DNA, Western and Southern blots it could be confirmed that in transformants the endogenous copy of γ -tubulin was indeed disrupted and replaced by the tagged version of the gene (Fig. 15). The tagged protein is not overexpressed, but expressed under the control of the endogenous promoter, without a background of endogenous wild-type γ -tubulin. Therefore stoichiometries of γ -tubulin and its interaction partners can be expected to be unaltered compared to wild-type γ -tubulin-complexes. Because transformants had a completely normal phenotype, fusion of the tag apparently does not interfere with protein function and interaction with other centrosomal proteins seems not to be impaired. Both tags were accessible for purification, confirming that the C-terminus of γ -tubulin is exposed on the outside of γ -tubulin-complexes.

These were important prerequisites for a successful purification of γ -tubulin-interacting proteins.

4.1.2 The size and stability of *Dictyostelium* γ -tubulin complexes

In contrast to *Xenopus* and *Drosophila* egg or early embryonic extracts (Oegema et al., 1999; Stearns and Kirschner, 1994; Zheng et al., 1995), where γ -TuRCs appear as sharp peaks in sucrose gradients, γ -tubulin complexes purified under similar conditions from *Dictyostelium* cytosolic extracts displayed considerable size heterogeneity and were restricted to fractions much smaller than γ -TuRCs. However, size heterogeneity of γ -tubulin complexes has also

been observed in mammalian somatic and brain cells (Detraves et al., 1997; Meads and Schroer, 1995; Moudjou et al., 1996), *Drosophila* somatic cells (Debec et al., 1995), *Physarum* plasmodia (Lajoie Mazenc et al., 1996) and *Aspergillus* conidia (Akashi et al., 1997). The prevalence of γ -TuRCs may therefore be a special feature of eggs and early embryonic cells, which have to store large amounts of pre-assembled centrosomal building blocks for subsequent rapid cell divisions (see INTRODUCTION).

Furthermore, the composition of purified γ -tubulin complexes has been shown to vary in different systems and under different purification conditions. For example, a large complex with a sedimentation coefficient of 38-48 S that contains the protein pericentrin has been purified in mammalian cells (Dictenberg et al., 1998). In *Saccharomyces cerevisiae* the γ -tubulin homologue Tub4p seems to associate with only two other proteins, Spc98p and Spc97p, although some Tub4p is also present in fractions with higher molecular weight (Knop et al., 1997). Similar small complexes, supposedly representing γ -TuSCs, are released from larger complexes under high salt conditions in many other systems (Akashi et al., 1997; Meads and Schroer, 1995; Oegema et al., 1999; Stearns and Kirschner, 1994; Zheng et al., 1995). Furthermore, γ -tubulin has also been found in association with chaperonin TCP1 (Melki et al., 1993; Vassilev et al., 1995), with α - and β -tubulin, Hsp70 and EF1 α (Marchesi and Ngo, 1993), with the centrosomal proteins CP60 and CP190 (Raff et al., 1993) and with centrin, Hsp70 and Hsp90 (Uzawa et al., 1995). The diversity of γ -tubulin complexes observed in different systems and the absence of γ -TuRC-like structures demonstrated in yeast, and now also in *Dictyostelium*, suggest that the frequently drawn picture of the γ -TuRC as the only microtubule nucleating unit is probably too simple.

Unfortunately, a functional characterization has not been reported for most of the γ -tubulin complexes found in various systems. The only exception is the nucleation of microtubule assembly *in vitro* demonstrated for γ TuRCs (and to a very small extent for γ TuSCs) isolated from *Xenopus* eggs (Zheng et al., 1995) or *Drosophila* embryos (Oegema et al., 1999). Yet, the physiological significance of this observation remains elusive. The ability to nucleate microtubules must be tightly suppressed in the cytoplasm, since ectopic microtubule nucleation is generally not observed *in vivo*, despite an abundance of cytoplasmic γ TuRCs. Possibly the presence of nucleation-competent complexes in the cytosol is a special feature of oocytes or early embryonic cells, since microtubule nucleation could not be demonstrated in any other system so far. The lack of synthesis of centrosomal material during the first cell divisions of fertilized *Xenopus* or *Drosophila* eggs may require the presence of preformed microtubule nucleation sites in the cytosol, which is not necessary or maybe even detrimental in somatic cells.

Another striking property of isolated *Dictyostelium* γ -tubulin complexes, besides the absence of higher molecular weight complexes under low salt conditions, was the stabilization of the prevailing small complex upon addition of salt. In contrast, a dissociation of large γ -tubulin complexes under high salt conditions has been reported for *Drosophila* (Oegema et al., 1999), human (Stearns and Kirschner, 1994), sheep (Detraves et al., 1997) and *Aspergillus* (Akashi et al., 1997). In all these systems a shift from higher molecular weight fractions towards a complex of about 8-10 S, presumably representing γ -TuSCs, has been observed. Unfortunately no data is available showing whether these small complexes, like the small complexes found in *Dictyostelium*, are stabilized upon addition of salt. But it has been demonstrated that they are very salt stable, resisting the addition of up to 1 M salt (Gunawardane et al., 2000a).

Taken together, the nature of the interaction tethering the components of the *Dictyostelium* γ -tubulin complex clearly differs from that tethering γ -TuRC components and bears more resemblance to the salt-stable interaction of γ -TuSCs or other small γ -tubulin complexes.

Furthermore, because both low salt conditions and the addition of glycerol and the detergent Triton-X-100 caused a dissociation of *Dictyostelium* γ -tubulin complexes, its components seem to be mainly tethered by hydrophobic forces.

4.1.3 The composition of the prevailing *D. discoideum* γ -tubulin complex

The most surprising finding when analyzing the composition of cytosolic *Dictyostelium* γ -tubulin complexes was that the prevailing small complex in *Dictyostelium* appeared to consist exclusively of DdSpc98 and γ -tubulin, whereas DdSpc97 did not co-purify. In other systems the interaction of the three proteins was very strong, resisting treatment of up to 1 M salt (Gunawardane et al., 2000a). It could be ruled out that the presence of the myc/6xHis tag interfered with the association of γ -tubulin and Spc97, because DdSpc97 did not co-immunoprecipitate with γ -tubulin in cytosolic extracts of AX2 or GFP-DdSpc98 cells either, whereas GFP-DdSpc98 did co-purify in GFP-DdSpc98 cells (Fig. 20). Thus it seems as if *Dictyostelium* is the first example investigated so far where the Spc97 homologue does not interact stably with γ -tubulin and the Spc98 homologue in the cytosol. One possible explanation for this is the divergence of *Dictyostelium* γ -tubulin, which shares only 60-75% amino acid identity with other γ -tubulins and is thus, besides yeast Tub4p, the most divergent member of the protein family (Euteneuer et al., 1998). Furthermore, the unusual sequence extensions of DdSpc97 might be responsible for an altered interaction with γ -tubulin and DdSpc98.

Band intensities of γ -tubulin and DdSpc98 were about equal in all γ -tubulin complex purifications, suggesting that the stoichiometry is two γ -tubulins to one DdSpc98p. Since the estimated molecular weight of the prevailing complex was about 400-500 kDa it may consist of four molecules of γ -tubulin and two molecules of DdSpc98. Other proteins besides these two can only be present in substoichiometric amounts, judging from the band intensities of γ -tubulin complex purifications.

An interesting question was whether the small *Dictyostelium* γ -tubulin complex is capable of nucleating microtubules. Unfortunately it was not possible to solve this issue, because *in vitro* microtubule nucleation assays with purified γ -tubulin complexes have so far failed in *Dictyostelium* (unpublished observations), as in yeast (E. Schiebel, pers. communication). Presumably microtubule nucleation sites have adapted to the very divergent tubulins present in these two species and are thus unable to interact with the pig brain microtubules commonly used for microtubule nucleation experiments. This was shown with isolated centrosomes and SPBs, which were only able to nucleate pig brain microtubules *in vitro* if they retained remnants of endogenous α - and β -tubulin at the nucleation sites (Gräf et al., 1998; Rout and Kilmartin, 1990). Since sufficient amounts of polymerization-competent microtubules cannot be purified from yeast and *Dictyostelium*, it remains unclear whether the small yeast Tub4p complex or the small γ -tubulin complex isolated from *Dictyostelium* are sufficient to nucleate microtubules on their own or whether additional components at the MTOC are required.

However, it seems quite unlikely that the small *Dictyostelium* γ -tubulin complex possesses a measurable microtubule nucleating activity. So far the only example for successful *in vitro* microtubule nucleation experiments using isolated γ -tubulin complexes is the γ -TuRC isolated from *Drosophila* early embryonic extracts or *Xenopus* eggs, respectively (Oegema et al., 1999, Zheng et al., 1995). As mentioned before, the presence of nucleation-competent γ -tubulin complexes may thus be a special feature of these early embryonic systems. Furthermore, the microtubule-nucleating activity of the small *Drosophila* γ -TuSC, which is more similar to the small *Dictyostelium* γ -tubulin complex concerning size and composition, was very small, just above buffer controls. Considering that the *Dictyostelium* γ -tubulin complex has a composition even simpler than γ -TuSCs it is hard to imagine that it is still capable of nucleating microtubules at a measurable rate.

4.1.4 The possible significance of γ -tubulin complexes in *D. discoideum*

Taken together, the composition and nature of interaction of γ -tubulin complex subunits seems to be quite unique in *Dictyostelium*. However, since its centrosome cycle has also been shown to be unusual (Ueda et al., 1999, see Fig. 34) differences in the assembly and

regulation of cytosolic γ -tubulin complexes are not surprising. Unlike in mammalian cells or budding yeast, where centrosome duplication is initiated in G1 (Winey and Byers, 1992), duplication and separation of the *Dictyostelium* centrosome take place during prophase/prometaphase (Ueda et al., 1999). Most importantly, in *Dictyostelium* there is no drastic increase of microtubule nucleation during mitosis as observed in mammalian cells (Kuriyama and Borisy, 1981), where the number of microtubules increases at least five-fold, or as in *S. pombe* (Hagan and Hyams, 1988), which nucleates microtubules at the SPB only at mitosis. Instead, in *Dictyostelium* the corona, which contains γ -tubulin, is lost during the transition of prophase to prometaphase, resulting in a loss of cytoplasmic microtubules. During prometaphase the two outer layers of the centrosome core separate and γ -tubulin reassembles with the inner surfaces of these layers, forming nucleation sites for spindle microtubules (see Fig. 34 for an overview of the *Dictyostelium* centrosome cycle). Apparently there is no requirement for storing pre-assembled MT-nucleating complexes in the cytosol, since there is no rapid increase of MT nucleation, but merely a redistribution of the limited number of nucleation sites present in interphase cells. Therefore in *Dictyostelium* a rudimentary complex of Dd- γ -tubulin and DdSpc98 present in the cytosol may be sufficient to activate microtubule nucleation at the centrosome. One conceivable model is that the γ -tubulin/DdSpc98 complex associates with DdSpc97 and probably other proteins directly at the centrosome to create functional microtubule nucleating units, equivalent to γ TuRCs.

As mentioned before, the data obtained from electron microscopy have to be interpreted with caution, due to problems with the anti-DdSpc97 antibodies after glutaraldehyde fixation. However, they support the observation that in *Dictyostelium*, as the only organism investigated so far, there are apparent differences between Spc97 and Spc98 concerning their subcellular distribution. The EM pictures suggest that DdSpc97 occurs both in the centrosomal corona and the core structure, whereas DdSpc98 is, like γ -tubulin, confined to the corona. This finding is consistent with the differences observed in the interaction of DdSpc97 and DdSpc98 with γ -tubulin in the cytosol. The possible significance concerning the role of DdSpc97 and DdSpc98 in microtubule nucleation can only be speculated on. One might suggest that DdSpc98 is more directly involved in microtubule nucleation, since it associates tightly with the microtubule-nucleator γ -tubulin in the cytosol and is confined to the *Dictyostelium* centrosome corona, the site of microtubule nucleation. DdSpc97, on the other hand, might fulfill some structural functions, since it is also found in the centrosome core, which is the structural backbone of the *Dictyostelium* centrosome, and possesses some unusual repetitive sequence insertions, which might be required for structural aspects. To take

the speculation even further, DdSpc97 might be required at the centrosome core to serve as an anchor for the rudimentary complex of γ -tubulin and DdSpc98.

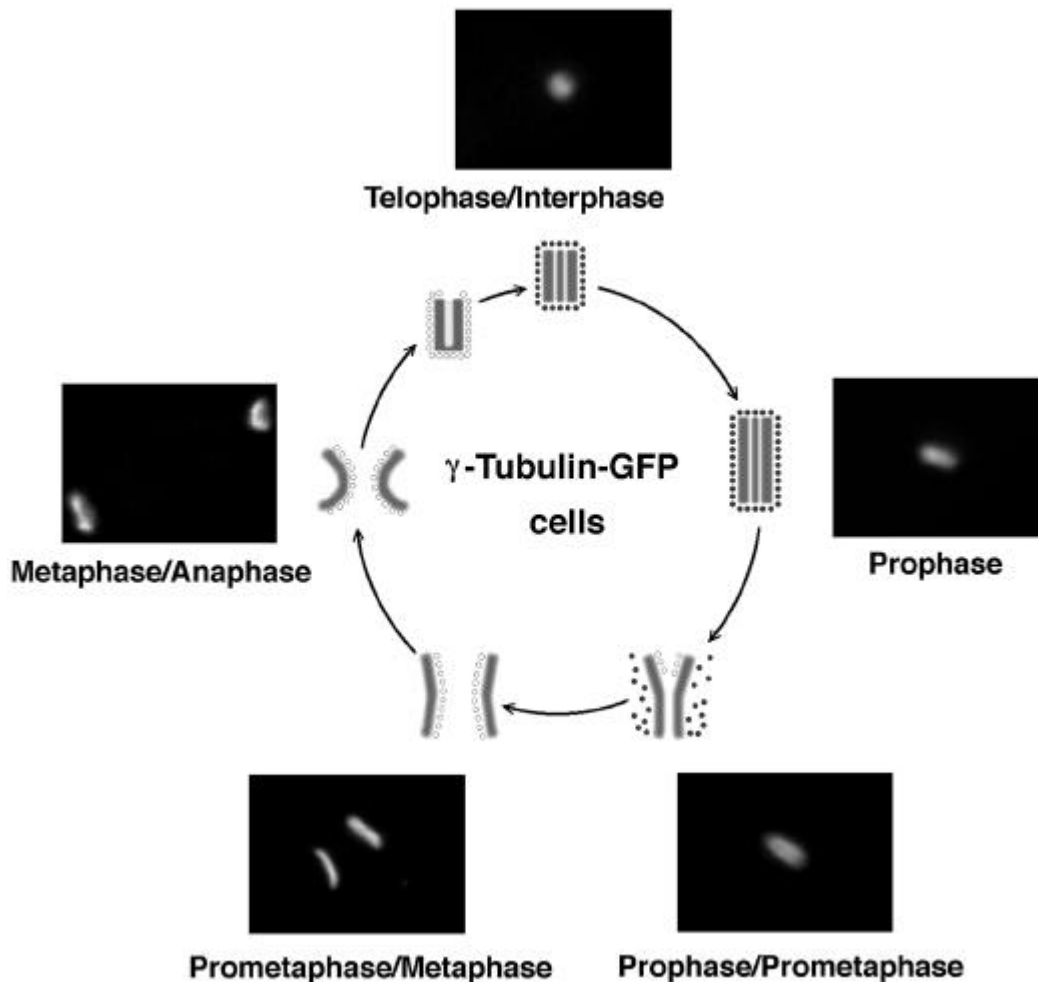


Fig. 34 The *Dictyostelium* centrosome cycle. Confocal microscopic images (kindly provided by M. Ueda) of γ -tubulin-GFP cells (Ueda et al., 1997) are shown, which document the morphological changes of the *Dictyostelium* centrosome cycle during mitosis (Ueda et al, 1999). The images are complemented by schematic diagrammes. The multi-layered core structure of an interphase centrosome (grey bars) is surrounded by an amorphous corona (filled circles). At the onset of prophase the core structure increases in size. At the transition from prophase to prometaphase the corona, in which γ -tubulin is present, dissociates from the core, resulting in a loss of cytoplasmic microtubules. The two outer layers of the core structure then separate and γ -tubulin re-associates with the layers at their inner surfaces (open circles), forming new nucleation sites for spindle microtubules. The daughter centrosomes start to curl beginning in metaphase and throughout anaphase. They finally fold up in telophase and assemble new inner layers, resulting in two complete interphase centrosomes. This centrosome cycle is remarkable in several aspects: Both structural duplication and separation take place during prophase, in contrast to G1 in mammalian cells and budding yeast. Furthermore, duplication and separation are very fast, taking only a few minutes, while mitosis occupies approximately 15 minutes.

4.2 Investigation of the *D. discoideum* homologue of centrin

4.2.1 DdCrp as the most divergent centrin homologue

The sequence alignment of DdCrp with other centrin homologues had revealed that the *Dictyostelium* homologue is the most divergent member of the protein family. It is even more aberrant than the *Saccharomyces cerevisiae* homologue Cdc31p, which is often considered to represent a separate class of centrin-related proteins. Therefore one might suggest that DdCrp is not a real centrin, but a completely different EF-hand protein with incidental similarity to centrins and that the actual *Dictyostelium* homologue of centrin still remains to be discovered. However, no other protein family could be identified that DdCrp would be more closely related to than the centrin family. Furthermore, neither Southern blot or Northern blot analyses nor extensive searches of the *Dictyostelium* genome project, which had reached a 5-fold coverage by the time of this work, gave any hint towards another *Dictyostelium* protein with similarity to centrins besides DdCrp. The only exception is the EF-hand protein DdCaM, which is actually more similar to the other centrins than DdCrp. Thus, one conceivable objection would be that the protein published as DdCaM is actually the *Dictyostelium* homologue of centrin.

But there are a number of observations strongly arguing against this. First of all, DdCaM is undoubtedly a real, highly conserved calmodulin that shares 87% amino acid identity with mammalian calmodulin and is functionally homologous to its mammalian counterpart (Marshak et al., 1984). DdCaM also shows a different localization than centrins, being greatly enriched on membranes of the contractile vacuole complex (Zhu and Clarke, 1992; Zhu et al., 1993). A second line of evidence is the presence of a number of important sequence features in DdCrp, which distinguish it from the calmodulin family and support its relatedness to centrins. Levy et al. (1996) identified 40 amino acids which are conserved in centrins, but differ in calmodulins. In 11 of these positions *Dictyostelium* carries the centrin-specific amino acid and in only two of them the calmodulin-specific. However, 27 of them are neither centrin- nor calmodulin-specific, which underlines the aberrance of DdCrp. Another observation distinguishing centrins and calmodulins is the presence of an aromatic amino acid (Y or F) at the C-terminus of all centrins (except for Cdc31p and HsCen3), which is missing in calmodulins. DdCrp possesses a C-terminal Y, which speaks for its relatedness to the centrin family. Also specific for centrins is an N-terminal extension before the first strongly conserved amino acid. This extension is very short in *Dictyostelium*, but is still longer than in all other calmodulins (except for *Naegleria gruberi* flagellar calmodulin) and contains a number of positively charged amino acids, which is another centrin-specific property. Unlike

calmodulins but like centrins DdCrp also carries two positively charged amino acids (KK) at the C-terminus. All in all there are 33 amino acids which are strictly conserved in all centrins except DdCrp, which differs in 12 out of these 33 positions (Fig. 22).

Thus, DdCaM is not a potential centrin homologue, although a first glance at the phylogenetic tree might suggest that, and DdCrp is closer to the centrin family than to the calmodulins. DdCrp is definitely the most divergent of all members of the centrin family, but it is close enough to be considered at least a centrin-related protein.

4.2.2 DdCrp lacks two of the four EF-hand consensus motifs

The most striking difference of DdCrp compared to all other centrins is the absence of the first two EF-hand consensus motifs. Apparently Ca^{2+} plays an important role in directing centrin function, however, it is not clear whether the presence of all four EF-hands i.e. the ability to bind four Ca^{2+} per centrin molecule, is essential. Although four EF-hands are present in all other centrins, some of them seem to be non-functional. In *Chlamydomonas* centrin all four EF-hands bind Ca^{2+} , two of them with high affinity and two with low affinity (Weber et al., 1994). But in human centrin2 (HsCen2), for example, only EF-hand IV is canonical, i.e. contains an oxygen-bearing side chain in the critical position for binding calcium. Under physiological conditions HsCen2 binds only one Ca^{2+} per molecule (or rather two Ca^{2+} per centrin-dimer). Binding of the peptide melittin, though, apparently activates one of the "dead" EF-hands in HsCen2, so that two Ca^{2+} per centrin molecule are bound (Durussel et al., 2000). For other centrins besides HsCen2 and *Chlamydomonas* centrin no direct Ca^{2+} binding studies exist, but sequence comparison suggests that different centrins probably bind Ca^{2+} in different stoichiometries (Durussel et al., 2000). In yeast Cdc31p only mutations in the first and fourth EF-hand result in non-functional proteins, in which the Ca^{2+} -induced conformational change is impaired. Mutants of the second and third EF-hand respond to Ca^{2+} as wild-type (Geier et al., 1996). *Naegleria gruberi* centrin has a deletion in the putative Ca^{2+} -binding loop of the first EF-hand, which might well interfere with Ca^{2+} -binding (Geier et al., 1996). Thus, there is evidence that some functional centrins actually bind less than 4 Ca^{2+} . Consequently, DdCrp may well be a functional centrin, although it possesses only two EF-hands.

4.2.3 Cell-cycle dependent changes of DdCrp localization

Using specific antibodies the localization of DdCrp in whole *Dictyostelium* cells and at isolated centrosomes was investigated. Confocal microscopy showed that DdCrp is localized to the centrosomal corona. Remarkably, in addition to its typical localization at centrosomes

and spindle poles a second, centrosome-associated structure within the nucleus was stained with anti-DdCrp antibodies. Most likely this label represents a real DdCrp localization, because the antibodies specifically stained DdCrp in Western blots and the same staining pattern was obtained with all three different antisera raised against two different recombinant DdCrp preparations. Furthermore, a similar labelling pattern was observed in some of the *Dictyostelium* mutants in which the myc/6xHis tag was fused to the DdCrp C-terminus by homologous recombination.

The unusual distribution of DdCrp might be attributed to the unique structure of the *Dictyostelium* MTOC. Unlike the yeast SPB (Francis and Davis, 2000) the *Dictyostelium* centrosome is not embedded in the nuclear membrane, but lies outside the nucleus and is anchored to the nuclear membrane by a strong, fibrous linkage (Omura and Fukui, 1985). It seems as if DdCrp is localized at both ends of this fibrous linkage, at the centrosome and at its distal, nuclear end which may be the site of kinetochore clustering in early prophase (Ueda et al., 1999). Unfortunately the subcellular localization of DdCrp could not be investigated at a higher resolution, since the antibodies did not work well in fixations suitable for electron microscopy.

An investigation of the centrosomal and centrosome-associated label during the cell cycle revealed that DdCrp starts to disappear from both localizations at the transition from prophase to metaphase. This is not surprising, because at this stage the corona, which harbors most if not all of the centrosomal DdCrp, has dissociated from the centrosomal core. The linkage structure is not required any more then, since the duplicated centrosomes have entered their fenestra within the nuclear envelope (Ueda et al., 1999).

Centrosomal centrins from all other species investigated so far seem to be permanently associated with the MTOCs throughout the cell cycle. However, a change of the intensity of the centrosomal label was monitored in PtK2 and HeLa cells (Baron et al., 1994; Errabolu et al., 1994). In these cells, the staining was strongest and most focussed during late G1-phase until metaphase. A partial redistribution of centrin into the cytoplasm was observed from the metaphase/anaphase transition until early G1-phase.

After the disappearance of centrosomal and centrosome-associated labels in mitotic *Dictyostelium* cells, only the patchy nuclear staining remained. Since this was also observed with all three different antibodies, but not with preimmune sera, it is most likely due to labelling of DdCrp and not of a cross-reacting protein. The presence of a centrin within the nucleus is not unprecedented. Paoletti et al. (1996) have detected HsCen2 in a demembrated nuclei fraction and, most interestingly, Araki et al. (2001) very recently have shown that nuclear HsCen2 is involved in nucleotide excision repair as part of the xeroderma

pigmentosum group C complex (XPC complex). The presence of DdCrp in the nucleus may be the first evidence that this function in nucleotide excision repair is evolutionarily conserved.

4.2.4 Possible implications for DdCrp

Functional analyses of DdCrp proved very difficult, because the protein could not be overexpressed and there was not enough sequence information available for knock-out experiments. Besides, it could not be isolated in a native state suitable for Ca^{2+} -binding studies. Thus, the possible functions of DdCrp can only be speculated on, judging from its sequence and subcellular localization. The presence of two EF hand consensus motifs and the conservation of many centrin specific sequence characteristics suggest that DdCrp responds to changes in Ca^{2+} levels with conformational changes, like other centrins, although its ability to bind Ca^{2+} may be lower than in some other centrins. The presence of DdCrp at the MTOC is in correspondence with the centrin functions in centrosome duplication and centrosome separation described previously for other centrin homologues (Middendorp et al., 2000; Spang et al., 1993). Since DdCrp is also present in the nucleus it may, like HsCen2, also be involved in nucleotide excision repair (Araki et al., 2001) These different functions in centrosome duplication, separation and nucleotide excision repair are apparently fulfilled by different centrin isoforms in many species. But, most likely, in *Dictyostelium* they all have to be accomplished by DdCrp, because it is apparently the only centrin isoform. This, as well as the distinct structure and duplication mode of the *Dictyostelium* centrosome, may explain the striking differences of DdCrp to all other known centrins and its unique position within the phylogenetic tree of centrin-like proteins.

5. CONCLUSION

In this work three new *Dictyostelium* centrosomal proteins, DdCrp, DdSpc97 and DdSpc98, could be identified and cloned. An analysis of the three proteins revealed striking differences to homologues in other organisms. DdCrp turned out to be the most divergent member of the centrin family: it lacks two Ca²⁺-binding EF-hand domains and shows an unusual subcellular, cell-cycle dependent localization, which is not found in any other organism.

DdSpc97 also displayed some unusual sequence features and, more importantly, an altered interaction with DdSpc98 and γ -tubulin in the cytosol. *Dictyostelium* is thus the first example investigated so far where Spc97, Spc98 and γ -tubulin do not form stable complexes in the cytosol. Despite the divergence of these important centrosomal proteins *Dictyostelium* possesses a fully functional, highly dynamic microtubule skeleton. Therefore it may provide valuable insight into the discrimination between strictly conserved, essential proteins or sequence features and species-specific, structural proteins.

The generation of mutant cell-lines expressing tagged centrosomal proteins which were successfully used for microscopy or biochemical purification underlines the excellent accessibility of *Dictyostelium* for genetic and biochemical approaches.

Taken together, the results from this work emphasize the role of the *Dictyostelium* centrosome as an invaluable model system usefully complementing research on the yeast spindle pole body and the animal centriolar centrosome.

REFERENCES:

- Adachi, H., T. Hasebe, K. Yoshinaga, T. Ohta, and K. Sutoh. 1994. Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* 205:1808-1814.
- Akashi, T., Y. Yoon, and B.R. Oakley. 1997. Characterization of γ -tubulin complexes in *Aspergillus nidulans* and detection of putative γ -tubulin interacting proteins. *Cell Motil. Cytoskeleton.* 37:149-158.
- Araki, M., C. Masutani, M. Takemura, A. Uchida, K. Sugasawa, J. Kondoh, Y. Ohkuma, and F. Hanaoka. 2001. Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. *J. Biol. Chem.* 276:18665-72.
- Baron, A.T., V.J. Suman, E. Nemeth, and J.L. Salisbury. 1994. The pericentriolar lattice of PtK2 cells exhibits temperature and calcium-modulated behavior. *J. Cell Sci.* 107:2993-3003.
- Baum, P., C. Furlong, and B. Byers. 1986. Yeast gene required for spindle pole body duplication: homology of its product with Ca^{2+} -binding proteins. *Proc. Natl. Acad. Sci. USA.* 83:5512-6.
- Bhattacharya, D., J. Steinkotter, and M. Melkonian. 1993. Molecular cloning and evolutionary analysis of the calcium-modulated contractile protein, centrin, in green algae and land plants. *Plant Mol. Biol.* 23:1243-54.
- Bollag, D.M., M.D. Rozycki, and S.J. Edelstein. 1996. Protein Methods. Wiley-Liss, Inc., New York. 415 pp. pp.
- Boveri, T. 1887. Über Differenzierung der Zellkerne während der Furchung des Eies von *Ascaris megacephala*. *Anat. Anz.* 2:688-693.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-54.
- Brugerolle, G., G. Bricheux, and G. Coffe. 2000. Centrin protein and genes in *Trichomonas vaginalis* and close relatives. *J. Eukaryot. Microbiol.* 47:129-38.
- Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. *J. Bacteriol.* 124:511-23.
- Claviez, M., K. Pagh, H. Maruta, W. Baltus, P. Fisher, and G. Gerisch. 1982. Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. *EMBO J.* 1:1017-1022.
- Dauderer, C., M. Schliwa, and R. Gräf. 1999. *Dictyostelium discoideum*: a promising centrosome model system. *Biol. Cell.* 91:313-320.
- Debec, A., C. Detraves, C. Montmory, G. Geraud, and M. Wright. 1995. Polar organization of γ -tubulin in acentriolar mitotic spindles of *Drosophila melanogaster* cells. *J. Cell Sci.* 108:2645-2653.
- DelVecchio, A.J., J.D.I. Harper, K.C. Vaughn, A.T. Baron, J.L. Salisbury, and R.L. Overall. 1997. Centrin homologues in higher plants are prominently associated with the developing cell plate. *Protoplasma.* 196:224-234.
- Detraves, C., H. Mazarguil, I. Lajoie-mazenc, M. Julian, B. Raynaud-messina, and M. Wright. 1997. Protein complexes containing γ -tubulin are present in mammalian brain microtubule protein preparations. *Cell Motil. Cytoskeleton.* 36 (2):179-189.
- Dicthenberg, J.B., W. Zimmerman, C.A. Sparks, A. Young, C. Vidair, Y.X. Zheng, W. Carrington, F.S. Fay, and S.J. Doxsey. 1998. Pericentrin and γ -tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* 141:163-174.

- Durussel, I., Y. Blouquit, S. Middendorp, C.T. Craescu, and J.A. Cox. 2000. Cation- and peptide-binding properties of human centrin 2. *FEBS Lett.* 472:208-12.
- Erickson, H.P., and D. Stoffler. 1996. Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γ -tubulin. *J. Cell Biol.* 135:5-8.
- Errabolu, R., M.A. Sanders, and J.L. Salisbury. 1994. Cloning of a cDNA encoding human centrin, an EF-hand protein of centrosomes and mitotic spindle poles. *J. Cell Sci.* 107:9-16.
- Euteneuer, U., R. Gräf, E. Kube-Grandenrath, and M. Schliwa. 1998. *Dictyostelium* γ -tubulin: molecular characterization and ultrastructural localization. *J. Cell Sci.* 111:405-412.
- Evan, G.I., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* 5:3610-3616.
- Fava, F., B. Raynaud Messina, J. Leung Tack, L. Mazzolini, M. Li, J.C. Guillemot, D. Cachot, Y. Tollon, P. Ferrara, and M. Wright. 1999. Human 76p: A new member of the γ -tubulin-associated protein family. *J. Cell Biol.* 147:857-868.
- Felix, M.A., C. Antony, M. Wright, and B. Maro. 1994. Centrosome assembly *in vitro*: role of γ -tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* 124:19-31.
- Felsenstein, J. 1993. PHYLIP - Phylogeny inference package (Version 3.2). *Cladistics.* 5:164-166.
- Francis, S.E., and T.N. Davis. 2000. The spindle pole body of *Saccharomyces cerevisiae*: architecture and assembly of the core components. *Curr. Top. Dev. Biol.* 49:105-32.
- Geier, B.M., H. Wiech, and E. Schiebel. 1996. Binding of centrins and yeast calmodulin to synthetic peptides corresponding to binding sites in the spindle pole body components Kar1p and Spc110p. *J. Biol. Chem.* 271:28366-74.
- Geissler, S., G. Pereira, A. Spang, M. Knop, S. Soues, J. Kilmartin, and E. Schiebel. 1996. The spindle pole body component Spc98p interacts with the γ -tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment. *EMBO J.* 15:3899-3911.
- Goldhagen, H., and M. Clarke. 1986. Identification of the single gene for calmodulin in *Dictyostelium discoideum*. *Mol. Cell Biol.* 6:1851-4.
- Gräf, R., C. Daumberger, and M. Schliwa. 1999. Cell cycle-dependent localization of monoclonal antibodies raised against isolated *Dictyostelium* centrosomes. *Biol. Cell.* 91:471-477.
- Gräf, R., C. Daumberger, and M. Schliwa. 2000. *Dictyostelium* DdCP224 is a microtubule-associated protein and a permanent centrosomal resident involved in centrosome duplication. *J. Cell Sci.* 113:1747-1758.
- Gräf, R., U. Euteneuer, M. Ueda, and M. Schliwa. 1998. Isolation of nucleation-competent centrosomes from *Dictyostelium discoideum*. *Eur. J. Cell Biol.* 76:167-175.
- Gunawardane, R.N., S.B. Lizarraga, C. Wiese, A. Wilde, and Y. Zheng. 2000a. γ -Tubulin complexes and their role in microtubule nucleation. *Curr. Top. Dev. Biol.* 49:55-73.
- Gunawardane, R.N., O.C. Martin, K. Cao, L. Zhang, K. Dej, A. Iwamatsu, and Y. Zheng. 2000b. Characterization and Reconstitution of *Drosophila* γ -tubulin ring complex subunits. *J. Cell Biol.* 151:1513-1524.
- Hagan, I.M., and J.S. Hyams. 1988. The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* 89:343-357.
- Hart, P.E., and S.M. Wolniak. 1999. Molecular cloning of a centrin homolog from *Marsilea vestita* and evidence for its translational control during spermiogenesis. *Biochem. Cell Biol.* 77:101-8.

- Horio, T., S. Uzawa, M.K. Jung, B.R. Oakley, K. Tanaka, and M. Yanagida. 1991. The fission yeast γ -tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99:693-700.
- Huang, B., A. Mengersen, and V.D. Lee. 1988. Molecular cloning of cDNA for caltractin, a basal body-associated Ca^{2+} -binding protein: homology in its protein sequence with calmodulin and the yeast CDC31 gene product. *J. Cell Biol.* 107:133-40.
- Huang, S.H., and A.Y. Jong. 1997. Rapid amplification of gene ends (RAGE) from gene libraries by anchored PCR. *Methods Mol Biol.* 67:295-300.
- Joshi, H.C. 1994. Microtubule organizing centers and γ -tubulin. *Curr. Op. Cell Biol.* 6:55 - 62.
- Joshi, H.C., M.J. Palacios, L. McNamara, and D.W. Cleveland. 1992. γ -tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature.* 356:80-3.
- Kay, R.R., and J.G. Williams. 1999. The *Dictyostelium* genome project: an invitation to species hopping. *Trends Genet.* 15:294-7.
- Kellogg, D.R., M. Moritz, and B.M. Alberts. 1994. The centrosome and cellular organization. *Annu. Rev. Biochem.* 63:639-674.
- Knop, M., G. Pereira, S. Geissler, K. Grein, and E. Schiebel. 1997. The spindle pole body component Spc97p interacts with the γ -tubulin of *Saccharomyces cerevisiae* and functions in microtubule organization and spindle pole body duplication. *EMBO J.* 16:1550-1564.
- Knop, M., and E. Schiebel. 1997. Spc98p and Spc97p of the yeast γ -tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. *EMBO J.* 16:6985-95.
- Knop, M., and E. Schiebel. 1998. Receptors determine the cellular localization of a γ -tubulin complex and thereby the site of microtubule formation. *EMBO J.* 17:3952-3967.
- Ko, J.H., J.Y. Kim, and S.H. Lee. 1999. Molecular cloning and characterization of a cDNA encoding caltractin from *Dunaliella salina*. *Plant Cell Physiol.* 40:457-61.
- Kretsinger, R.H., and C.D. Barry. 1975. The predicted structure of the calcium-binding component of troponin. *Biochim. Biophys. Acta.* 405:40-52.
- Kuriyama, R., and G.G. Borisy. 1981. Microtubule-nucleating activity of centrosomes in Chinese hamster ovary cells is independent of the centriole cycle but coupled to the mitotic cycle. *J. Cell Biol.* 91:822-826.
- Kyhse-Anderson, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Meth.* 10:203-209.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680-5.
- Lajoie Mazenc, I., C. Detraves, V. Rotaru, M. Gares, Y. Tollon, C. Jean, M. Julian, M. Wright, and B. Raynaud Messina. 1996. A single γ -tubulin gene and mRNA, but two γ -tubulin polypeptides differing by their binding to the spindle pole organizing centres. *J. Cell Sci.* 109:2483-2492.
- Laoukili, J., E. Perret, S. Middendorp, O. Houcine, C. Guennou, F. Marano, M. Bornens, and F. Tournier. 2000. Differential expression and cellular distribution of centrin isoforms during human ciliated cell differentiation in vitro. *J. Cell. Sci.* 113:1355-64.
- Lardelli, M. 1997. Generation and PCR screening of bacteriophage λ sublibraries enriched for rare clones (the "sublibrary method"). *Methods Mol Biol.* 67:345-55.
- Leask, A., and T. Stearns. 1998. Expression of amino- and carboxyl-terminal γ - and α -tubulin mutants in cultured epithelial cells. *J. Biol. Chem.* 273 (5):2661-2668.
- Lee, V.D., and B. Huang. 1993. Molecular cloning and centrosomal localization of human caltractin. *Proc. Natl. Acad. Sci. USA.* 90:11039-43.

- Levy, Y.Y., E.Y. Lai, S.P. Remillard, M.B. Heintzelman, and C. Fulton. 1996. Centrin is a conserved protein that forms diverse associations with centrioles and MTOCs in *Naegleria* and other organisms. *Cell Motil. Cytoskel.* 33:298-323.
- Liu, Y.C., and Y.C. Chou. 1990. Formaldehyde in formaldehyde/agarose gel may be eliminated without affecting the electrophoretic separation of RNA molecules. *Biotechniques.* 9:558-560.
- Madeddu, L., C. Klotz, J.P. Lecaer, and J. Beisson. 1996. Characterization of centrin genes in *Paramecium*. *Eur. J. Biochem.* 238:121-128.
- Maeda, Y., K. Inouye, I. Takeuchi, and (eds.). 1997. *Dictyostelium*: A model system for cell and developmental biology. Universal Academic Press, Inc., Tokyo.
- Malchow, D., B. Nägele, H. Schwarz and G. Gerisch. 1972. Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *Eur. J. Biochem.* 28: 136-142.
- Marchesi, V.T., and N. Ngo. 1993. In vitro assembly of multiprotein complexes containing α , β , and γ -tubulin, heat shock protein HSP70, and elongation factor 1α . *Proc. Natl. Acad. Sci. USA.* 90:3028-3032.
- Martin, M.A., S.A. Osmani, and B.R. Oakley. 1997. The role of γ -tubulin in mitotic spindle formation and cell cycle progression in *Aspergillus nidulans*. *J Cell Sci.* 110:623-33.
- Martin, O.C., R.N. Gunawardane, A. Iwamatsu, and Y. Zheng. 1998. Xgrip109: A γ -tubulin-associated protein with an essential role in γ -tubulin ring complex (γ TuRC) assembly and centrosome function. *J. Cell Biol.* 141:675-687.
- McFadden, G.I., D. Schulze, B. Surek, J.L. Salisbury, and M. Melkonian. 1987. Basal body reorientation mediated by a Ca^{2+} -modulated contractile protein. *J. Cell Biol.* 105:903-12.
- Meads, T., and T.A. Schroer. 1995. Polarity and nucleation of microtubules in polarized epithelial cells. *Cell Motil. Cytoskeleton.* 32:273-288.
- Melki, R., I.E. Vainberg, R.L. Chow, and N.J. Cowan. 1993. Chaperonin-mediated folding of vertebrate actin-related protein and γ -tubulin. *J. Cell Biol.* 122:1301-1310.
- Meng, T.C., S.B. Aley, S.G. Svard, M.W. Smith, B. Huang, J. Kim, and F.D. Gillin. 1996. Immunolocalization and sequence of caltractin/centrin from the early branching eukaryote *Giardia lamblia*. *Mol. Biochem. Parasitol.* 79:103-108.
- Middendorp, S., T. Kuntziger, Y. Abraham, S. Holmes, N. Bordes, M. Paintrand, A. Paoletti, and M. Bornens. 2000. A role for centrin 3 in centrosome reproduction. *J. Cell Biol.* 148:405-16.
- Middendorp, S., A. Paoletti, E. Schiebel, and M. Bornens. 1997. Identification of a new mammalian centrin gene, more closely related to *Saccharomyces cerevisiae* CDC31 gene. *Proc. Natl. Acad. Sci. USA.* 94:9141-9146.
- Moens, P.B. 1976. Spindle and kinetochore morphology of *Dictyostelium discoideum*. *J. Cell Biol.* 68:113-122.
- Moritz, M., M.B. Braunfeld, J.W. Sedat, B. Alberts, and D.A. Agard. 1995. Microtubule nucleation by γ -tubulin-containing rings in the centrosome. *Nature.* 378:638-640.
- Morio, T., H. Urushihara, T. Saito, Y. Ugawa, H. Mizuno, M. Yoshida, R. Yoshino, B. N. Mitra, M. Pi, T. Sato, K. Takemoto, H. Yasukawa, J. Williams, M. Maeda, I. Takeuchi, H. Ochiai, and Y. Tanaka. 1998. The *Dictyostelium* cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. *DNA Res.* 5: 335-340.
- Moudjou, M., N. Bordes, M. Paintrand, and M. Bornens. 1996. γ -Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.* 109:875-887.
- Murphy, S.M., L. Urbani, and T. Stearns. 1998. The mammalian γ -tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. *J. Cell Biol.* 141:663-674.

- Oakley, B.R., C.E. Oakley, Y. Yoon, and M.K. Jung. 1990. γ -tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell*. 61:1289-301.
- Oegema, K., C. Wiese, O.C. Martin, R.A. Milligan, A. Iwamatsu, T.J. Mitchison, and Y. Zheng. 1999. Characterization of two related *Drosophila* γ -tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* 144:721 - 733.
- Ogawa, K., and T. Shimizu. 1993. cDNA sequence for mouse caltractin. *Biochim. Biophys. Acta.* 1216:126-8.
- Omura, F., and Y. Fukui. 1985. *Dictyostelium* MTOC: Structure and linkage to the nucleus. *Protoplasma.* 127:212-221.
- Paoletti, A., M. Moudjou, M. Paintrand, J.L. Salisbury, and M. Bornens. 1996. Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J. Cell Sci.* 109:3089-102.
- Pereira, G., and E. Schiebel. 1997. Centrosome-microtubule nucleation. *J. Cell Sci.* 110:295-300.
- Pickett-Heaps, J.D. 1969. The evolution of the mitotic apparatus: An attempt at comparative ultrastructural cytology in dividing plant cells. *Cytobios.* 3:257-280.
- Raff, J.W., D.R. Kellogg, and B.M. Alberts. 1993. *Drosophila* γ -tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823-835.
- Raper, K. B. 1931. *Dictyostelium discoideum*, a new species of slime mold growing from decaying forest leaves, *J. Agric. Res.* 50: 133-147.
- Roos, U.P. 1975. Fine structure of an organelle associated with the nucleus and cytoplasmic microtubules in the cellular slime mould *Polysphondylium violaceum*. *J. Cell Sci.* 18:315-326.
- Rout, M.P., and J.V. Kilmartin. 1990. Components of the yeast spindle and spindle pole body. *J. Cell Biol.* 111:1913-1927.
- Salisbury, J.L. 1998. Roots. *Journal Of Eukaryotic Microbiology.* 45 (1):28-32.
- Sambrook J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, New York.
- Sanders, M.A., and J.L. Salisbury. 1989. Centrin-mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 108:1751-60.
- Sanders, M.A., and J.L. Salisbury. 1994. Centrin plays an essential role in microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 124:795-805.
- Schiebel, E., and M. Bornens. 1995. In search of a function for centrins. *Trends Cell Biol.* 5:197-201.
- Schliwa, M., J. van Blerkom, and K. B. Pryzwansky. 1982. Structural organization of the cytoplasm. In: Cold Spring Harbour Symposium on Quantitative Biology. Vol. XLVI. Cold Spring Harbour Laboratory. 51-67.
- Short, J. M., J. M. Fernandez, J. A. Sorge and W. D. Huse. 1988. λ ZAP: a bacteriophage λ expression vector with in vivo excision properties. *Nucl. Acid Res.* 16: 7583-7600.
- Sobel, S.G., and M. Snyder. 1995. A highly divergent γ -tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:1775-88.
- Spang, A., I. Courtney, U. Fackler, M. Matzner, and E. Schiebel. 1993. The calcium-binding protein cell division cycle 31 of *Saccharomyces cerevisiae* is a component of the half bridge of the spindle pole body. *J. Cell Biol.* 123:405-16.
- Stearns, T., and M. Kirschner. 1994. In vitro reconstitution of centrosome assembly and function: the central role of γ -tubulin. *Cell.* 76:623-637.

- Sunkel, C.E., R. Gomes, P. Sampaio, J. Perdigo, and C. Gonzalez. 1995. γ -Tubulin is required for the structure and function of the microtubule organizing centre in *Drosophila* neuroblasts. *Embo J.* 14:28-36.
- Taillon, B.E., S.A. Adler, J.P. Suhan, and J.W. Jarvik. 1992. Mutational analysis of centrin: an EF-hand protein associated with three distinct contractile fibers in the basal body apparatus of *Chlamydomonas*. *J Cell Biol.* 119:1613-24.
- Takumi, T. 1997. Use of PCR for cDNA library screening. *Methods Mol Biol.* 67:339-44.
- Tassin, A.M., C. Celati, M. Moudjou, and M. Bornens. 1998. Characterization of the human homologue of the yeast Spc98p and its association with γ -tubulin. *J. Cell Biol.* 141:689-701.
- Ueda, M., R. Gräf, H.K. MacWilliams, M. Schliwa, and U. Euteneuer. 1997. Centrosome positioning and directionality of cell movements. *Proc. Natl. Acad. Sci. USA.* 94:9674-9678.
- Ueda, M., M. Schliwa, and U. Euteneuer. 1999. Unusual centrosome cycle in *Dictyostelium*: correlation of dynamic behavior and structural changes. *Mol. Biol. Cell.* 10:151-160.
- Uzawa, M., J. Grams, B. Madden, D. Toft, and J.L. Salisbury. 1995. Identification of a complex between centrin and heat shock proteins in CSF-arrested *Xenopus* oocytes and dissociation of the complex following oocyte activation. *Dev. Biol.* 171:51-59.
- Vassilev, A., M. Kimble, C.D. Silflow, M. LaVoie, and R. Kuriyama. 1995. Identification of intrinsic dimer and overexpressed monomeric forms of γ -tubulin in Sf9 cells infected with baculovirus containing the *Chlamydomonas* γ -tubulin sequence. *J. Cell Sci.* 108:1083-1092.
- Vogel, J.M., T. Stearns, C.L. Rieder, and R.E. Palazzo. 1997. Centrosomes isolated from *Spisula solidissima* oocytes contain rings and an unusual stoichiometric ratio of alpha/beta tubulin. *J. Cell Biol.* 137:193-202.
- Weber, C., V.D. Lee, W.J. Chazin, and B. Huang. 1994. High level expression in *Escherichia coli* and characterization of the EF-hand calcium-binding protein caltractin. *J. Biol. Chem.* 269:15795-802.
- Wessel, D., and U.I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141-143.
- Williams, K.L., and P.C. Newell. 1976. A genetic study of aggregation in the cellular slime mould *Dictyostelium discoideum* using complementation analysis. *Genetics.* 82:287-307.
- Winey, M., and B. Byers. 1992. Spindle Pole Body of *Saccharomyces cerevisiae*: A model for genetic analysis of the centrosome cycle. In *The centrosome*. V.I. Kalnins, editor. Academic Press, Inc., San Diego. 197 - 218.
- Winsor, B., and E. Schiebel. 1997. Review: An overview of the *Saccharomyces cerevisiae* microtubule and microfilament cytoskeleton. *Yeast.* 13:399-434.
- Wright, R.L., S.A. Adler, J.G. Spanier, and J.W. Jarvik. 1989. Nucleus-basal body connector in *Chlamydomonas*: evidence for a role in basal body segregation and against essential roles in mitosis or in determining cell polarity. *Cell Motil. Cytoskeleton.* 14:516-26.
- Wright, R.L., J. Salisbury, and J.W. Jarvik. 1985. A nucleus-basal body connector in *Chlamydomonas reinhardtii* that may function in basal body localization or segregation. *J. Cell Biol.* 101:1903-12.
- Zhang, L., T.J. Keating, A. Wilde, G.G. Borisy, and Y. Zheng. 2000. The role of Xgrip210 in γ -tubulin ring complex assembly and centrosome recruitment. *J. Cell Biol.* 151:1525-1536.
- Zheng, Y.X., M.L. Wong, B. Alberts, and T. Mitchison. 1995. Nucleation of microtubule assembly by a γ -tubulin-containing ring complex. *Nature.* 378:578-583.
- Zhu, J.K., R.A. Bresson, and P.M. Hasegawa. 1992. An *Atriplex nummularia* cDNA with sequence relatedness to the algal caltractin gene. *Plant Physiol.* 99:1734-1735.

- Zhu, Q., and M. Clarke. 1992. Association of calmodulin and an unconventional myosin with the contractile vacuole complex of *Dictyostelium discoideum*. *J. Cell. Biol.* 118: 347-358.
- Zhu, Q., T. Liu, and M. Clarke. 1993. Calmodulin and the contractile vacuole complex in mitotic cells of *Dictyostelium discoideum*. *J. Cell. Sci.* 104: 1119-1127.

LEBENS LAUF

Persönliche Daten:

Name: Christine Zoglmeier, geb. Dauderer
Geburtsdatum: 16.03.1972
Geburtsort: München
Eltern: Dr. Gertrud Dauderer, geb. Biechl und Dr. Max Dauderer
Geschwister: Michael Dauderer (* 12.03.1969), Peter Dauderer (* 13.03.1971),
Johannes Dauderer (* 16.03.1972)
Familienstand: seit 15.04.2000 verheiratet mit Peter Zoglmeier
Kinder: Tobias Zoglmeier (* 22.07.2000), Leonie Zoglmeier (* 11.04.2002)

Schulbildung:

9/78-9/81: Grundschule Pullach
9/81-9/89: Gymnasium Pullach
9/89-12/89: Farlingaye High School, Woodbridge, England
1/90-6/91: Gymnasium Pullach
6/91: Allgemeine Hochschulreife (Note: „1.0“)

Hochschulbildung:

11/91-11/93: Grundstudium Biologie, Ludwig-Maximilians-Universität München
3-4/92: Praktikum der Molekularbiologie bei Prof. Dr. Hahn, Molekularbiologisches
Labor der Poliklinik I, Universität Erlangen
11/93: Vordiplom (Note „1.1“)
10/93-10/94: Studium der Biochemie, University of Kent, Canterbury, England
Certificate („with distinction“)
11/94-6/97 Hauptstudium Biologie, Ludwig-Maximilians-Universität München
10/96-6/97 Diplomarbeit bei Prof. Dr. Wiczorek, Zoologisches Institut
Thema: „Charakterisierung der V-ATPase von SF9-Zellen“ (Note: „1.0“)
6/97 Diplomprüfung (Gesamtnote: „1.0“)
9/97-6/01 Promotionsstudium am Institut für Zellbiologie, München, Forschungs-
tätigkeit zur vorliegenden Arbeit.
Leitung: Dr. Ralph Gräf, Abt. Prof. Dr. Schliwa
Betreuung: Dr. habil. Birgit Wetterauer

Stipendien:

11/91-6/97: Bayerische Hochbegabtenförderung
2/95-6/97: Studienstiftung des Deutschen Volkes

DANKSAGUNGEN

Mein ganz besonderer Dank gilt Dr. Ralph Gräf für die optimale Betreuung dieser Arbeit und seinen unermüdlichen, wertvollen und bewundernswert geduldigen Rat und Beistand bei allen nur denkbaren Problemen und Problemchen.

Ebenfalls besonders danken möchte ich Prof. Dr. Manfred Schliwa für sein reges Interesse an der Arbeit, seine Diskussionsbereitschaft und die Unterstützung bei allen notwendigen Anschaffungen.

Herzlich danken möchte ich auch Dr. habil. Birgit Wetterauer für die Vertretung dieser Arbeit vor der Fakultät auch nach Beendigung Ihrer Tätigkeit am Zoologischen Institut, für ihre wertvollen Anregungen und ihr Interesse.

Ein ganz besonders herzliches Dankeschön gilt Dr. Ralph Gräf, Andrea Hestermann, Markus Rehberg und Hieu Ho für ihre tatkräftige Unterstützung während meiner Baby-Teilzeit-Laborarbeit.

Dr. Ursula Euteneuer möchte ich herzlich für die elektronenmikroskopischen Aufnahmen sowie für ihre rege Diskussionsbereitschaft und das Interesse an der Themenstellung danken.

Bedanken möchte ich mich auch bei Nicole Brusis und Hieu Ho für die technische Unterstützung.

Der Arbeitsgruppe um Prof. Dr. Michael Schleicher, insbesondere Prof. Schleicher selbst, Klaus-Peter Janssen und Annika Gloss danke ich sehr herzlich für ihre Hilfe bei Dictyostelium-spezifischen Fragestellungen.

Andreas Schneider, Dr. Günther Woehlke, Dr. Ralph Gräf und ganz besonders Christian Horn danke ich für ihre bereitwillige Unterstützung bei Computerproblemen.

Ein herzliches Dankeschön geht auch an Andrea Hestermann, Alexandra Lepier, Ralph Gräf, Prof. Manfred Schliwa und meine Mutter für das kritische Durchlesen von Manuskripten.

Mein persönlicher, herzlicher Dank gilt allen lieben MitarbeiterInnen des Instituts für Zellbiologie für das wunderbare Arbeitsklima, die Kameradschaftlichkeit und stete Hilfsbereitschaft, die einem das Durchhalten bei so manchem Fehlschlag erleichtert haben.

Schließlich danke ich meiner Mutter für das liebevolle Babysitten, Tobi für seinen ausgedehnten Mittagsschlaf und Peter für seine Geduld mit der Doktormami, die die Fertigstellung dieser Arbeit erst ermöglicht haben.