Proteome Analysis of the Human Mitotic Spindle*\textsuperscript{f}

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The accurate distribution of sister chromatids during cell division is crucial for the generation of two cells with the same complement of genetic information. A highly dynamic microtubule-based structure, the mitotic spindle, carries out the physical separation of the chromosomes to opposite poles of the cells and, moreover, determines the cell division cleavage plane. In animal cells, the spindle comprises microtubules that radiate from the microtubule organizing centers, the centrosomes, and interact with kinetochores on the chromosomes. Malfunctioning of the spindle can lead to chromosome missegregation and hence result in aneuploidy, a hallmark of most human cancers. Despite major progress in deciphering the temporal and spatial regulation of the mitotic spindle, its composition and function are not fully understood. A more complete inventory of spindle components would therefore constitute an important advance. Here we describe the purification of human mitotic spindles and their analysis by MS/MS. We identified 151 proteins previously known to associate with the spindle apparatus, centrosomes, and/or kinetochores and 644 other proteins, including 154 uncharacterized components that did not show obvious homologies to known proteins and did not contain motifs indicative of a particular localization. Of these uncharacterized proteins, 17 were tagged and localized in transfected mitotic cells, resulting in the identification of six genuine spindle components (KIAA0008, CdcA8, KIAA1187, FLJ12649, FLJ90806, and C20Orf129). This study illustrates the strength of a proteomic approach for the analysis of isolated human spindles and identifies several novel spindle components for future functional studies. *Molecular & Cellular Proteomics* 4: 35–43, 2005.

During mitosis, the two newly forming daughter cells must receive one copy of each chromosome. To accomplish this task, the mitotic spindle pulls sister chromatids toward opposite poles of the dividing cell. This microtubule-based structure comprises dynamic polymers made of αβ-tubulin heterodimers, associated with a large variety of microtubule-associated proteins (1–5). At the transition from interphase to mitosis, the microtubule network undergoes a profound morphological change. In particular, the microtubule organizing centers of animal cells, the centrosomes, position to opposite sides of the nucleus and increase their microtubule nucleation capacity. Following nuclear envelope breakdown, microtubules emanating from the centrosomes capture each chromosome at the kinetochore, a protein complex assembled on centromeric DNA (6). Appropriate bipolar attachment of sister chromatids is monitored by a surveillance mechanism, the spindle checkpoint (7). Once all kinetochores are attached to microtubules emanating from opposite poles, the connection between sister chromatids is severed and chromatids are pulled apart. In addition to its central role in chromosome segregation, the mitotic spindle also determines the positioning and orientation of the cleavage plane. Therefore, proper positioning of the mitotic spindle is of particular importance for asymmetric cell divisions during development (8, 9).

A large number of proteins associate with the mitotic spindle and regulate its dynamic formation and function. Stabilizing and destabilizing proteins control the high turnover rate of mitotic microtubules, which have a half-life of less than 60 s (3). Furthermore, motor proteins of the kinesin and dynein families play crucial roles in the formation of a bipolar mitotic spindle (10, 11). By interacting with microtubules during early mitosis, they push the spindle poles apart, then play important roles in chromosome-capture by microtubules and power chromosome movement throughout mitosis. Finally, the spindle harbors several regulatory proteins, notably protein kinases and phosphatases (12), which coordinate spindle function in time and space.

Although our understanding of microtubule dynamics and spindle formation has greatly advanced during the last two decades, the complexity of the spindle continues to hamper its investigation. A more complete inventory of the mitotic spindle may thus contribute to a better understanding of how exactly the spindle is assembled, what role it plays in the spindle checkpoint, and how it induces cleavage furrow formation. Recent proteomic studies have begun to address the composition of the human centrosome (13), the spindle midbody in hamster cells (14), and in vitro-assembled spindle structures (15, 16). Here we describe a comprehensive proteomic study of human spindles isolated from synchronized HeLa S3 cells (17–19). MS identified a total of 795 proteins.
including 151 previously known spindle-associated components. Of 17 previously uncharacterized proteins analyzed further, six were found to be bona fide spindle components.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa S3 cells were maintained in Dulbeccos modified Eagles medium containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37 °C in a humidified incubator with a 5% CO₂ atmosphere.

**Mitotic Spindle Enrichment**—HeLa S3 cells were first synchronized at the G₂/S phase boundary by treatment for 16 h with 1.6 μg/ml aphidicolin. They were then released for 14 h into medium containing 40 ng/ml nocodazole to block them in mitosis. Mitotic cells were harvested by shake-off, followed by centrifugation at 300 × g, washed twice with PBS, and released into normal medium for 30–40 min, until most of them had reached metaphase. (This was controlled by immunofluorescence analysis of aliquots of 4,6-diamidino-2-phenylindole (DAPI)1-stained cells for metaphase plate formation). Microtubules were subsequently stabilized by addition of 5 μg/ml taxol to the medium for 3 min. Cells were then harvested, washed with PBS containing 2 μg/ml latrunculin B, 1 μm PMSF, 5 μg/ml taxol, and then incubated for 15 min at 37 °C in lysis buffer: 100 μm piperoxidine-1,4-bis(2-ethanesulfonic acid) (PIPES), pH 6.9, 1 mM MgSO₄, 2 mM EGTA, 0.5% Nonidet P-40, 5 μg/ml taxol, 2 μg/ml latrunculin B, including nucleases (200 μg/ml DNAseI, 10 μg/ml RNase A, 1 μl/micrococcal nuclease, 20 U/ml benzoate), protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μm PMSF), and 20 μm β-glycerophosphate as a phosphatase inhibitor. Lysed cells were harvested by centrifugation at 700 × g for 2 min and resuspended in the same buffer, incubated for 5 min, and harvested again by centrifugation.

Spindles were subsequently isolated by incubating the lysed cell “ghosts” in isolation buffer (1 mM PIPES, pH 6.9, 5 μg/ml taxol) for 5–10 min (until differential interference contrast (DIC) microscopy revealed spindles to be free from intermediate filaments) and collected by sedimentation at 1,500 × g for 5 min in SDS-PAGE sample buffer before loading onto NuPAGE gradient gels (Invitrogen, San Diego, CA).

**Gel Electrophoresis and Western Blotting**—For MS analysis, NuPAGE gradient gels were used in a Bis-Tris buffer system according to the manufacturer’s instructions (Invitrogen). Gels were stained with 0.5% Coomassie in 50% methanol/10% acetic acid and destained in 50% methanol/10% acetic acid. For Western blotting, proteins were separated in homemade gels and subsequently transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) using a semi-dry blotting device (Hoefer). Filters were blocked in PBS buffer containing 0.05% Tween 20 and 5% milk and subsequently probed with anti-α-tubulin (1:4,000; Sigma-Aldrich, St. Louis, MO), anti-Hec1 (1:1,000) (21), anti-Eg5 (2 μg/ml) (22), anti-Arsf1 (3 μg/ml) (23). After incubation with secondary horseradish peroxidase-coupled antibodies (Amer sham Pharmacia Biotech, Piscataway, NJ), signals were visualized by ECL.

**MS**—Protein digestion was performed essentially as reported (24), and peptides were desalted and concentrated using C18 extraction tips (25). The peptide mixture was analyzed with a CAPLC nanoHPLC system (Waters, Milford, MA) coupled to a Q-TOF Ultima, Micromass, London, United Kingdom). The column setup was essentially as described by Meiring et al. (26). Samples were dissolved in 5 μl of 0.1% formic acid and loaded by the CAPLC autosampler onto a 2.5-cm long 100-μm inner diameter precolumn packed with 5-μm AQUASIL C18 phase (NewObjective, Woburn, MA) at a flow rate of 2 μl/min. The precolumn was coupled to a 14-cm pulled fused-silica capillary packed with ReproSil-Pur C18-AQ (Dr. Maisch GmbB, Ammerbuch, Germany) 3-μm reverse-phase material. This fritless capillary column had an internal diameter of 75 μm and a tip opening of 8 μm (NewObjective) and was prepared as described by Ishihama et al. (27). Peptides were separated by a stepwise 90-min gradient of 0–100% between buffer A (2% ACN, 0.5% formic acid) and buffer B (80% ACN, 0.5% formic acid) at a flow rate of 170 nl/min. Throughout the analysis 1.5-s MS acquisitions were followed by 7-s MS/MS experiments in information-dependent acquisition mode. Combined peak lists were searched against the Mass Spectrometry Protein Sequence Database (MSDB) (csc-fserve.hh.med.ic.ac.uk/msdb.html) or the human International Protein Index (IPI) database (www.ebi.ac.uk/IPI/IPIhelp.html) using in-house Mascot (www.matrixscience.com) Version 1.7 allowing ±0.15 Da tolerance for both peptide and MS/MS fragment ion mass values. Proteins identified by two or more peptides with a combined peptide score of greater than 50 or by one single peptide with a score of higher than 60 were considered significant, whereas all lower-scoring proteins were either included or discarded after inspection of individual spectra.

**Plasmid Construction**—For cloning, the following cDNAs were obtained from the “Deutsches Ressourcenzentrum für Genomforschung” (RPZD): CGI-115 (IMAGp958L171641), Cdc8A (IMAGp958p6068), FLJ12649 (IMAGp998B0111718 and HeLa cDNA library), 38D8-2 (IMAGp958F0602), DJ383D4 (IMAGp958A231049), CGI-99 (IMAGp998K2012605), Tob3-like (IRALp962A1928), KIAA0008 (IRAKp611M1813), PM5 (DKFZp676N0525), C200r129 (IMAGp958M1212), FLJ90806 (hSPC24 (IMAGp958K072621), FLJ35834 (IRAKp611L1148), CIRHN (DKFZp676E1577); and/or from the Kazusa DNA Research Institute: KIAA0007, KIAA0663, KIAA1187, KIAA1698, KIAA1988 (CIRHN). The CIRHN gene was assembled of two cDNAs (DKFZp676E1577 and KIAA1988), and FLJ12649 was assembled from IMAGp998B0111718 and a cDNA fragment amplified from a HeLa cDNA library (Ciontect, Palo Alto, CA). All coding DNA sequences were amplified by PCR using specific primers and cloned into a pRCMV vector in-frame with a sequence encoding an aminoterminal triple myc-tag.

**Transfection and Immunofluorescence Microscopy**—HeLa S3 cells were grown on coverslips and transfected with 1.5 μg of plasmid DNA using Fugene 6 reagent (Roche, Indianapolis, IN) according to the manufacturer’s recommendations. After 36 h cells were washed with PBS and fixed for 10 min in PTEMF buffer (50 mM PIPES, pH 6.8, 10 mM EGTA, 1 mM MgCl₂, 0.2% Triton X-100, 4% formaldehyde). Coverslips were washed three times with PBS and incubated with anti-myec10-TRITC (Santa Cruz Biotechnology, Santa Cruz), anti-α-tubulin-FITC (Sigma), anti-Hec1 followed by anti-rabbit Alexa-green, and DAPI in PBS buffer containing 1% BSA. After 1-h incubation, coverslips were washed three times with PBS and mounted onto glass slides. Immunofluorescence microscopy was performed with a Zeiss Axioplan II microscope and an Apochromat 63 × 1.4 n.a. oil-immersion objective. Pictures were taken with a Micromax charged-coupled device camera (Princeton Instruments) and Metaview software (Universal Imaging Corp.), and images were processed with Photoshop (Adobe Systems).

**RESULTS AND DISCUSSION**

**Enrichment of Mitotic Spindles**—To purify mitotic spindles from human cells to high homogeneity and in sufficient
amounts for mass spectrometrical analysis, previously described methods were further optimized (28–30). The final procedure involved the stabilization of microtubules by taxol and is schematically depicted in Fig. 1. HeLa S3 cells were first synchronized at the G1/S phase transition by an aphidicolin block. Following release from this block, cells were treated with low concentrations of nocodazole (40 ng/ml), thereby affecting microtubule dynamics. Under these conditions spindle asters could still form, but cells were arrested in mitosis as a result of spindle checkpoint activation. The arrested cells were then harvested by shake off and released from the nocodazole block (to allow formation of mitotic spindles). When most cells had reached a metaphase stage, taxol was added to stabilize microtubules. To samples to be used as negative controls, a high concentration of nocodazole was added, resulting in microtubule depolymerization. Cells were then lysed and treated with DNase to remove chromosomes from the microtubule (MT) network and with latrunculin B to depolymerize the actin cytoskeleton, which would otherwise contaminate the MT fraction (31). Finally, incubation of the lysed cell “ghosts” in low-ionic-strength buffer resulted in depolymerization of the intermediate filaments, whereas the mitotic spindles remained intact. Spindles were collected by centrifugation.

Examination of the spindle isolates by DIC light microscopy proved the effectiveness of the method (Fig. 2A). Spindles, mostly from prometa- and metaphase stages, could be observed, largely free of other cellular structures. As expected, no spindle structures could be seen when the isolation was performed in the presence of nocodazole (data not shown). In addition, analysis of the spindle isolates on Coomassie blue-stained gels (Fig. 2B) showed a strong enrichment of tubulin and many other proteins, compared with the control (nocodazole) sample. The presence of spindle components was further confirmed by Western blot analysis (Fig. 2C), revealing an enrichment not only of α-tubulin, but also of the microtubule-associated motor Eg5 (22), the mitotic kinase Plk1 (32), and the kinetochore protein Hec1 (21). None of these proteins were detected in the control (nocodazole-treated) sample. Likewise, the chromatin assembly factors Asf1a and Asf1b (23) analyzed as negative controls were absent from the spindle isolates, as expected (Fig. 2C).

**Identification of Mitotic Spindle-Associated Proteins Using MS** — For the identification of proteins within the spindle isolates, proteins were separated by size on a SDS-PAGE gradient gel. After Coomassie blue staining, each lane was cut into 20 pieces, covering different protein mass ranges. The proteins were then in-gel digested with trypsin, and extracted peptide mixtures were analyzed by nanoLC-MS/MS. Analyses of several spindle preparations resulted in the identification of almost 7,000 peptides. As an example for the quality of these analyses, Fig. 3 illustrates fragmentation spectra of one peptide derived from KIAA1187 and one from FLJ12649, two newly identified spindle proteins (see below). These spectra show excellent signal-to-noise ratios and good coverage of expected fragment masses, thus enabling unambiguous protein identification. To ensure nonredundancy of the identified proteins, a sequence database was created in fasta-format, and every new entry was searched for homologues or identical sequences using a local installation of the blastp program.

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**Fig. 1. Scheme for the isolation of mitotic spindles.** Mitotic HeLa S3 cells were obtained by a nocodazole block/release protocol. Spindles were stabilized by taxol and isolated as depicted. Microtubules are shown in green, chromosomes in blue, actin fibers in pink, intermediate filaments in yellow, and centrosomes in red. Actin fibers, chromosomes, and intermediate filaments were removed as described in detail in “Experimental Procedures.” Proteins of the spindle isolate were then resolved by SDS-PAGE and digested with trypsin. The extracted peptide mixtures were analyzed by nanoLC-MS/MS.
Fig. 2. Characterization of mitotic spindles. A, DIC pictures of isolated mitotic spindles. Scale bar is 10 μm. B, Coomassie-stained gel of total lysates and mitotic spindles isolated in the presence of taxol (T) or nocodazole (N). Note that the loading of the isolated spindles corresponds to about 300 times more cells than that of the total lysate. C, Western blot analysis of purified spindles isolated in the presence of taxol (T) or nocodazole (N). Total lysates were loaded as a control. The loading of the isolated spindles corresponds to 25 times more cells than that of the total lysate.
These database searches resulted in the identification of a total of 795 different proteins (see supplemental table). Of these, 579 proteins were identified by more than three peptides. However, 87 proteins were identified by only a single peptide and 129 proteins were identified by two peptides. Stringent criteria for the evaluation of the latter MS/MS spectra (see “Materials and Methods”) were applied before the corresponding proteins were accepted.

Of the 795 proteins, 151 were previously reported to associate with mitotic spindle structures and/or microtubules and hence could be denoted as genuine spindle components (Fig. 4). Even though the previously known spindle proteins account for only 19% of all detected proteins, the majority of the 30 top-scoring proteins in the MS analysis were genuine spindle components, indicating a high enrichment of spindles in the analyzed preparations. The list of known spindle components (see supplemental table) includes not only major structural proteins (e.g. NUMA, TPX2) and kinesins (e.g. MKlp1, MKlp2, Eg5, Kid), but also regulatory proteins, including protein kinases (Aurora-A and Aurora-B, Plk1) and phosphatases (PP2A), and components of the spindle checkpoint (Bub1, Bub3, BubR1, Mad1, and Mad2). This clearly shows that the spindle preparations analyzed here comprised proteins localizing to kinetochores (36 proteins) and centrosomes
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(19 proteins), in addition to microtubule-associated proteins. However, the majority of the predicted constituents of the interphase centrosome were not identified, which indicates that some centrosome proteins had dissociated prior to spindle formation, or were lost during spindle preparation (13). Alternatively, considering that centrosomes contribute a relatively small part to the spindle, some proteins may have gone undetected due to limited sensitivity.

The remaining proteins (644) were grouped into five different categories on the basis of reported functions and/or localizations, the presence of particular domains (e.g. nucleic acid-binding motifs), and/or similarities with previously characterized proteins. These categories compromise 165 known or predicted nucleic acid binding proteins, 54 mitochondrial proteins, 50 ribosomal proteins, and a group of 221 proteins with proposed functions and/or localizations linked to diverse cellular processes (Fig. 4). The fifth category comprised 154 previously uncharacterized proteins (Fig. 4). This latter group was considered most likely to comprise novel spindle components. This does not exclude, however, that bona fide spindle components could also be present in the other categories. For example, the category “diverse cellular processes” comprises 10 nucleoporins, and it is possible that some of these might associate with kinetochores during mitosis, as suggested by recent studies on the nuclear pore subcomplex Nup107–160 (33). In addition, microtubules not only play a role in sister chromatid segregation, but also serve as major tracks for the intracellular transport of ribosomes (34, 35), mitochondria (36) (37), membrane vesicles (38), and RNA granules (39). Hence, the identification of a high number of mitochondrial and ribosomal proteins could indicate that these proteins interact with the mitotic spindle in vivo.

Novel Spindle Proteins—The above results suggested that the spindle apparatus might contain many more proteins than hitherto assumed. As a first step toward examining this possibility, several previously uncharacterized proteins were studied further. Based on the availability of cDNAs, 17 novel gene products were chosen for further characterization (Table I). These cDNAs were fused to a triple myc-tag and transiently transfected into HeLa S3 cells. The tagged proteins were then localized by indirect immunofluorescence microscopy. Of the 17 proteins analyzed, six showed clear localization to diverse spindle structures (Figs. 5 and 6). For three of these proteins, KIAA0008 (also termed HURP), CdcA8 (Borealin/Dasra), and FLJ90806 (Spc24), their localizations were independently confirmed by other groups in the course of this study. KIAA0008 (HURP) localizes to spindle microtubules and contains a GKAP homology domain (GH1) that is found in a number of guanylate kinase-associated proteins (Fig. 5A) (40). Its function is unknown, but as illustrated in Fig. 5A it displays a striking exclusion from the spindle pole region. FLJ90806 (Spc24) was shown to form a complex with the kinetochore proteins Hec1, Nuf2, and hSpc25, and, as expected, it co-localizes with Hec1 at the kinetochores (Fig. 5B) (41, 42). In addition, centrosomal staining was observed (Fig. 5B). CdcA8 (Borealin/Dasra) was independently identified as a new chromosomal passenger protein interacting with Aurora-A, INCENP, and survivin and localizes to kinetochores from pro- to metaphase and to the midzone and midbody from ana- to telophase (Fig. 5A and data not shown) (43, 44).

The three other novel spindle proteins identified here, FLJ12649, KIAA1187, and C20Orf129, have not previously been described. FLJ12649 and KIAA1187 localized to microtubules throughout mitosis (Fig. 6 and data not shown). Interestingly, both proteins contained coiled-coil (cc) motifs with sequence similarity to corresponding structures in the microtubule-associated protein 7 (MAP7), but they are clearly distinct outside of these domains. Overexpression of FLJ12649 and KIAA1187 resulted in strong microtubule bundling in in-

![FIG. 4. Classification of identified proteins. Absolute numbers and percentages (100% = 795) are indicated.](http://www.mcponline.org/Downloaded from)
terphase cells (data not shown), suggesting that these proteins could directly bind to microtubules. The third novel protein, C20Orf129, localized to spindle microtubules with a clear concentration toward the spindle poles (Fig. 6). Surprisingly, this protein contains an aminoacyl-tRNA synthetase class-1 signature and a domain with weak homology to a phospholipase D active site motif. The mRNA levels of this protein were reported to be up-regulated during G2/M (45), consistent with a mitotic function for C20Orf129.

Criteria for the Selection of Likely Spindle Proteins—As the
expression of at least some spindle proteins is known to be cell cycle regulated, the analysis of mRNA levels might constitute a helpful criterion for the selection of spindle candidates. The usefulness of this approach is illustrated by the example of C20Orf129 discussed above. Unfortunately, however, the approach could not be extended to the entire class of 154 uncharacterized proteins, because their representation in published mRNA expression datasets is scarce (45, 46). Specifically, expression patterns were available for only nine of the 17 proteins that were further studied. Of those nine proteins, six (the new spindle protein FLJ12649 and five discarded candidates) showed no cell cycle regulation of their mRNAs, whereas three proteins did (the new spindle proteins CdcA8 and C20Orf129 and the discarded candidate DJ383J4) (45, 47). Thus, with presently available datasets it is difficult to assess the validity of using cell cycle regulation of mRNA levels as a selection criterion.

We have also considered the predictive value of structural features. Analysis of the domain structures of the 17 analyzed proteins showed that eight contained cc domains whereas nine were lacking this structure. Remarkably, five of the six newly identified spindle proteins contained cc domains. When all proteins identified in our spindle inventory were analyzed for the presence of cc domains (using the algorithm colis2 at smart.embl-heidelberg.de), we found that 50% of the known spindle proteins contained one or more cc domains, whereas only 28% of the 644 other proteins showed this motif. The presence of cc structures therefore appears to constitute a valid criterion for narrowing down the number of candidate spindle proteins for future detailed analysis. Except for cc domains, however, no structural motif was found to be statistically more abundant in known spindle proteins than in other components of our preparations.

Interestingly, 83% of the known spindle proteins in the preparation, but only 72% of proteins with other reported localizations or functions, were represented by more than two peptides. Moreover, of the previously unknown proteins that were chosen for detailed localization studies, all of those validated as bona fide spindle components were identified by four or more peptides, whereas five of the proteins that did not localize to the spindle were identified by only one peptide. Thus, the number of peptides identified for a given protein may constitute a useful criterion for deciding which proteins are likely spindle components.

Concluding Remarks—As is true for all complex multiprotein machines, an inventory of the component parts of the mitotic spindle constitutes an important starting point for functional studies. Our present analysis has led to the identification of several novel spindle components and, in addition, provides an extensive list of potential candidates for future analysis. Extensions of the proteomic approach described here should also make it possible to investigate the variations in spindle composition during mitotic progression. Moreover, considering that many spindle proteins undergo cell cycle-de-
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