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A 210 kDa nuclear matrix protein is a functional part of the mitotic spindle; a microinjection study using SPN monoclonal antibodies

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Six monoclonal antibodies identify a 210 kDa polypeptide which shows a cell cycle specific redistribution from the nucleus to the mitotic spindle. In interphase cells this polypeptide was localized in the nucleus and behaved during differential cell extraction as a component of the nuclear matrix. It accumulated in the centrosome region at prophase, in the pole regions of the mitotic spindle at metaphase and in crescents at the poles in anaphase, and reassociated with the nuclei as they reformed in telophase. Due to its staining pattern we call the protein the Spindle Pole-Nucleus (SPN) antigen. The localization of SPN antigen during mitosis was dependent on the integrity of the spindle since treatment of cells with nocodazole resulted in the dispersal of SPN antigen into many small foci which acted as microtubule organizing centres when the drug was removed. The SPN antigen was present in nuclei and mitotic spindles of all human and mammalian cell lines and tissues so far tested. When microinjected into the cytoplasm or nuclei of HeLa cells, one antibody caused a block in mitosis. Total cell number remained constant or decreased slightly after 24 h. At this time, about half the cells were arrested in a prometaphase-like state and revealed aberrant spindles. Many other cells were multinucleate. These results show that the SPN antigen is a protein associated with mitotic spindle microtubules which has to function correctly for the cell to complete mitosis.

Key words: microinjection/mitotic arrest/mitotic spindle proteins/monoclonal antibodies/nuclear matrix

Introduction

Relatively little is known about the proteins that nucleate microtubule formation in the living cell as opposed to the large amount of information accumulated on *in vitro* systems. Regrowth experiments after destruction of microtubules with drugs such as colcemid emphasize the importance of the centrosomal region as a microtubule organizing centre (MTOC) in interphase cells (see for instance Osborn and Weber, 1976; De Brabander *et al.*, 1986). As the cell enters prophase, microtubules of the interphase cell depolymerize and the centrosome divides to form two diplosomes, which migrate to opposite poles and then act as the nucleating centres for microtubules of the developing mitotic spindle (for review see Kirschner and Mitchison, 1986; Vandré and Borisy, 1989). The prophase to metaphase transition is paralleled by increases in electron dense material in the centrosome/pole regions (Rieder and Borisy, 1982), in protein kinase activity (Bailly *et al.*, 1989; Verde *et al.*, 1990) and in microtubule nucleating activity of these regions (Telzer and Rosenbaum, 1979; Vandré and Borisy, 1989). These results suggest that the complement of centrosomal and spindle associated proteins changes as cells progress from interphase to mitosis.

Attempts to characterize proteins associated with the centrosomes or mitotic spindle have used two approaches. In the first, which has been mainly used for centrosomes, these structures have been purified usually from cell lines. Electron micrographs show relatively pure preparations of centrioles, but gel electrophoretic patterns of such preparations still show complex patterns (Mitchison and Kirschner, 1984; Bornens *et al.*, 1987). In the second approach, human or rabbit autoantibodies, or mouse monoclonal antibodies which recognize centrosomes of interphase cells or the poles of mitotic cells or other structures associated with the mitotic spindle, have been identified in immunocytochemistry. They have then been used to define the constituent polypeptides by immunoblotting (e.g. Connolly and Kalnins, 1978; Brenner *et al.*, 1981; Calarco-Gillman *et al.*, 1983; Gosti-Testu *et al.*, 1986). Using this approach several protein components of the centrosome/pole regions e.g. 95 and 105 kDa polypeptides of the spindle (Sellitto and Kuriyama, 1988) and centromere proteins i.e. INCENPs and CENPs (Pluta *et al.*, 1990) have been recognized. Phosphorylation/dephosphorylation cycles play crucial roles in mitosis. Thus the $p34^{cdc2}$ protein kinase phosphorylates key proteins responsible for major M phase events (for reviews see Murray and Kirschner, 1989; Nurse, 1990). Certain phosphoproteins are also thought to be components of centrosomes and of the mitotic spindle (De Mey *et al.*, 1987; Kuriyama, 1989; Vandré *et al.*, 1991). Thus post-translational modifications of key proteins are also likely to be of importance for the interphase to mitosis transition. Microinjection can be a powerful way to analyse function (e.g. Kreis and Birchmeier, 1982) and in a very few instances the functional importance of some of these proteins has been assayed by microinjection of human autoantibodies or of mouse monoclonal antibodies. Thus microinjection of the CHOI antibody to the 95 and 105 kDa spindle components blocks mitosis in PtK1 cells (Nislow *et al.*, 1990) and microinjection of CENP-E antibodies delays the metaphase to anaphase progression in a human cell line (Yen *et al.*, 1991). In addition, microinjection of human autoantibodies to centromere components causes mitotic arrest either in prophase or metaphase depending on the time of injection (Bernat *et al.*, 1990) while similar antibodies interfere with chromosome movements in meiotic and mitotic mouse oocytes (Simerly *et al.*, 1990).

Our interest in proteins which translocate from the interphase nucleus to a centrosome/pole location started with the repeated isolation of monoclonal antibodies which display

a specific Spindle Pole-Nucleus (SPN) pattern in immunocytochemistry. We observed such antibodies first in 1982 when we had used insoluble cytoskeletal residues as an antigen to elicit monoclonal antibodies against intermediate filament proteins (Debus *et al.*, 1982), but at that time did not further characterize the SPN antibodies. More recently the pattern recurred repeatedly in mice immunized with a crude nuclear fraction from SW13 cells. After fusion a set of monoclonal antibodies specific for the SPN antigen was isolated. Here we show that the SPN antigen is a 210 kDa polypeptide which displays a striking and cell cycle dependent staining pattern. During interphase the SPN antigen is associated with the nuclear matrix. Starting in prophase the SPN antigen becomes associated with the centrosome/diplosome, and in metaphase and early anaphase is found at the pole regions of the mitotic spindle. The SPN antigen clearly belongs to a family of proteins which migrate from nucleus to centrosomes and poles as cells enter mitosis. Experiments in which SPN-3 antibody is microinjected into HeLa cells show that SPN is the first protein of this type for which a functional role in mitosis has been directly demonstrated. Microinjected cells are either arrested in a prometaphase-like state or become multinucleated.

Results

SPN antibody isolation

SPN hybridomas were selected by their typical Spindle Pole-Nucleus (SPN) pattern seen on either HeLa or SW13 cells in immunofluorescence microscopy. The SPN-7 clone was isolated from a fusion in which a cytoskeletal fraction of HeLa cells was used as antigen for the later isolation of hybridomas specific for human keratin 18 (Debus *et al.*, 1982). The SPN-1 to -5 clones were isolated from a fusion in which a rather crude nuclear fraction from SW13 cells was used as antigen in the hope of isolating antibodies which would recognize cell type specific nuclear proteins. SW13 cells were used because this cell line lacks intermediate filaments except for 5–10% of the cells which contain vimentin intermediate filaments (Hedberg and Chen, 1986; Osborn and Weber, 1987). Thus we expected that nuclei from SW13 cells might be less contaminated by intermediate filament proteins than are nuclei prepared from other cell types (Staufenbiel and Deppert, 1982). Even though one- and two-dimensional gel electrophoresis showed that these preparations contained a large number of different polypeptides (>100 spots on silver stained two-dimensional gels) when the sera were tested in immunofluorescence microscopy on SW13 cells the dominant pattern was to our surprise the SPN pattern.

SPN antibodies recognize a 210 kDa polypeptide

The SPN antigen is a minor cellular component. No positive reaction was seen when antibodies were used in immunoblotting on whole cell extracts of SW13 or HeLa cells. However, a 210 kDa band was detected by some but not all SPN antibodies when a nuclear extract from SW13 cells corresponding to the initial immunogen was used. All SPN antibodies reacted in the conventional indirect immunoperoxidase technique with a 210 kDa band present in the HeLa cytoskeletal fraction (Figure 1A). The band corresponding to this 210 kDa polypeptide could not be identified with certainty on the Coomassie blue stained gels, since in this region of the gel a number of minor proteins are present (see Figure 1A).

To increase the sensitivity of the immunoblotting assay, we switched to detection of the peroxidase reaction by chemiluminescence. This method was used to examine the distribution of the SPN antigen in Triton X-100 supernatant and pellet fractions of interphase and mitotic cells (Figure 1B). The 210 kDa polypeptide was detected in whole

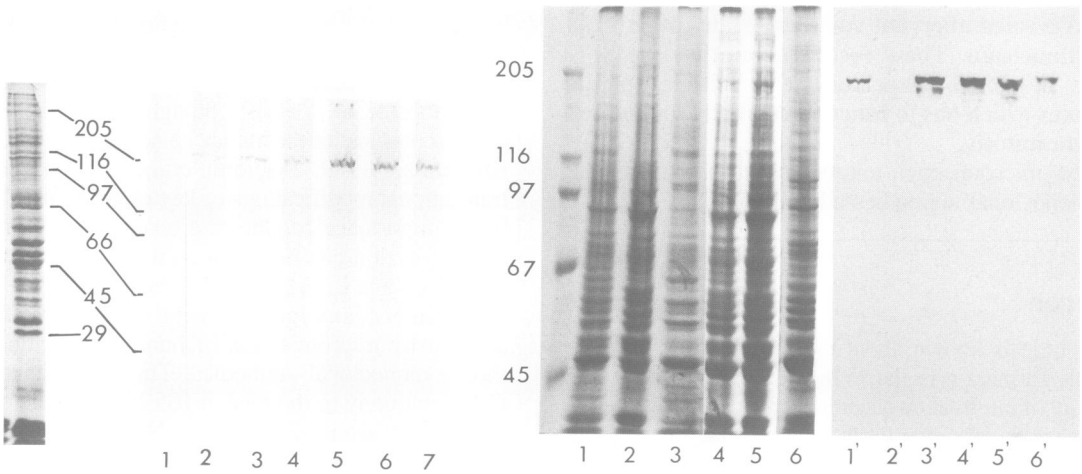

Fig. 1. (**A**) Immunoblots of cytoskeletal residues from HeLa cells with SPN antibodies. On the left is the Coomassie blue stained 10% SDS–PAGE gel. Lanes 1–7 show immunoblots after transfer from a 7.5% gel and detection with SPN antibodies followed by peroxidase conjugated rabbit anti-mouse antibody. Molecular weights of standards are given in kDa. Lane 1, negative control (no first antibody); lane 2, SPN-1 antibody; lane 3, SPN-2 antibody; lane 4, SPN-4 antibody; lane 5, SPN-3 antibody; lane 6, SPN-5 antibody; lane 7, SPN-7 antibody. (**B**) Distribution of the SPN antigen after Triton X-100 extraction of interphase (lanes 1–3) and mitotic (lanes 4–6) HeLa cells analysed by immunoblotting from a 7.5% SDS–PAGE gel. All lanes contain the equivalent fractions from 10^5 cells. Lanes 1–6 show the protein stain with Coomassie blue and lanes 1′–6′ the corresponding immunoblots with SPN-3 antibody. The peroxidase signal was detected with chemiluminescence reaction on X-ray film. In interphase cells the whole cell extract (lanes 1, 1′) shows a single 210 kDa band. The Triton X-100 supernatant of interphase cells lacks the protein (lanes 2 and 2′), which is found in the corresponding Triton X-100 pellet fraction (lanes 3 and 3′). In mitotic cells, the SPN antigen is found in the whole cell extract (lanes 4 and 4′); it is a prominent band in the blot of the Triton X-100 supernatant of mitotic cells (lanes 5 and 5′) and is present in a smaller amount in the corresponding pellet fraction (lanes 6 and 6′). Lanes 3′–6′ show some degradation of the SPN antigen possibly induced by proteolysis during sample preparation.

cell extracts of both interphase cells (Figure 1B, lane 1′) and mitotic cells (Figure 1B, lane 4′). It was not detected in Triton X-100 supernatants of interphase cells (Figure 1B, lane 2′) but was readily seen in the corresponding pellet (Figure 1B, lane 3′). Samples derived from mitotic cells showed a different distribution. The 210 kDa band was more

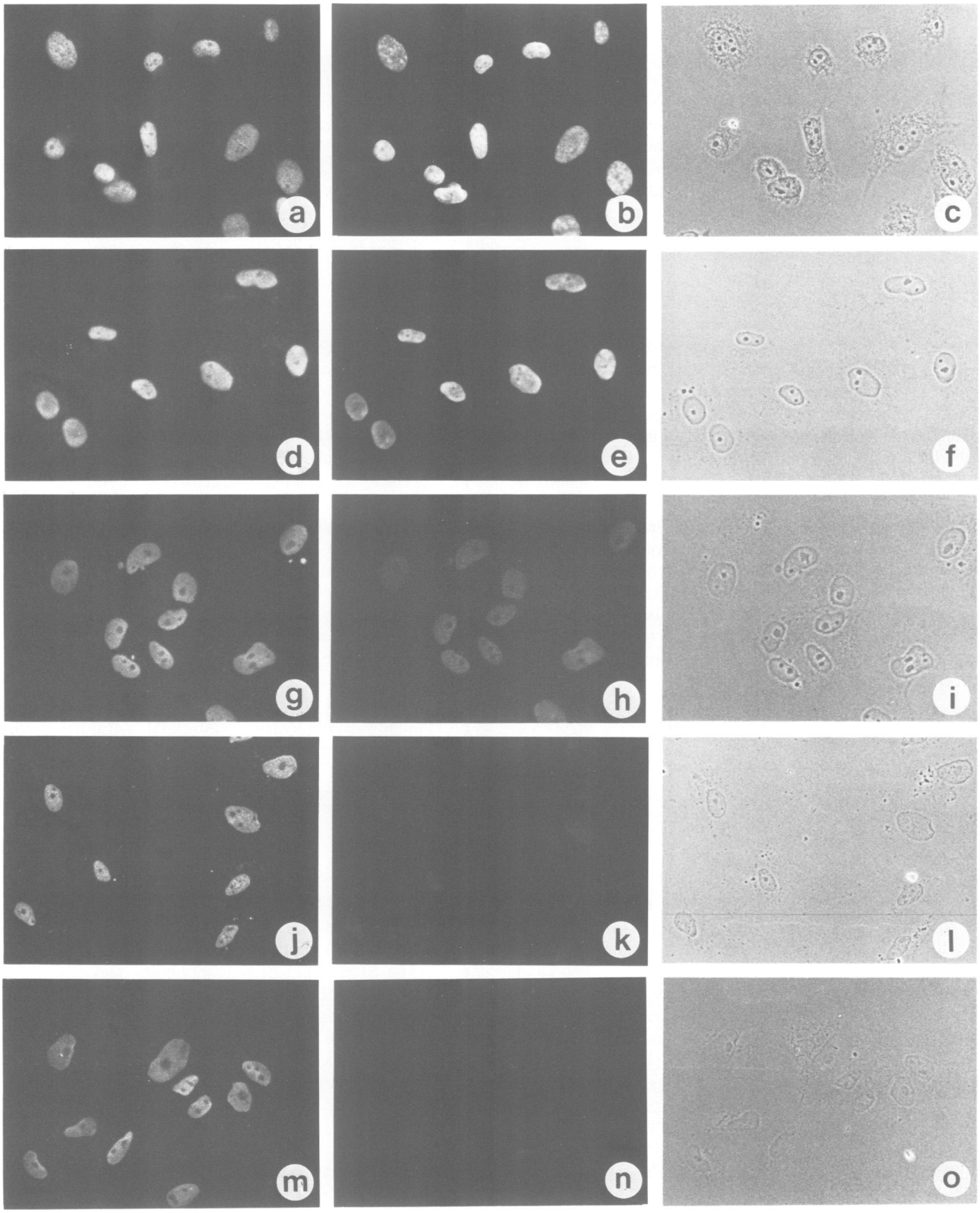

Fig. 2. Visualization of SPN antigen during sequential extraction of SW13 cells (*in situ* nuclear matrix preparation). Immunofluorescence of the SPN antigen with the SPN-3 antibody is shown in a, d, g, j and m. Panels b, e, h, k and n show the corresponding DNA staining by Hoechst 33258 and panels c, f, i, l and o are the corresponding phase contrast fields. (**a–c**) Unextracted cells. (**d–f**) NP 40-extracted cells. (**g–i**) DNase I-digested structures. (**j–l**) High salt-extracted structures. (**m–o**) DNase I and RNase A-digested structures. Note that the intensity of the SPN fluorescence remains approximately the same during the extraction procedure, even after the DNA has been removed. Magnification, ×250.

prominent in the Triton X-100 supernatant (Figure 1B, lane 5′) than in the corresponding pellet (Figure 1B, lane 6′). Thus the SPN antigen is more soluble in mitotic than in interphase cells. The additional bands just below the major band in lanes 3′−5′ of Figure 1B probably represent proteolytic breakdown products of the SPN antigen induced during sample preparation.

The SPN antigen is a component of the 'nuclear matrix' in interphase cells

In interphase cells, all SPN antibodies stained the nucleus excluding the nucleoli. Focusing through the cells showed staining throughout the nucleoplasm (Figure 2). The fate of the SPN antigen during sequential extraction of cultured cells to produce 'nuclear matrix' preparations *in situ* was monitored by immunofluorescence microscopy. Cells were first extracted with NP40, then treated with DNase I, reextracted with a high salt buffer and then further digested with DNase I and RNase A. The remaining insoluble residue contains ~10% of the total cellular protein and only traces (<0.5%) of DNA and RNA (cf. Staufenbiel and Deppert, 1984). Figure 2 shows immunofluorescence staining with SPN-3 antibody after each extraction step. The other SPN antibodies gave identical results. DNA, as judged by staining with Hoechst dye, is totally removed during the procedure. Most is extracted during the first DNase I digestion and the subsequent high salt extraction step (Figure 2h and k). While the extraction procedure results in cell residues hardly visible by phase contrast (Figure 2o), the fluorescence images observed by the SPN-3 antibody displayed approximately the same intensity of staining throughout the procedure. These results show that the SPN antigen is a component of the nuclear matrix as originally defined by Berezney and Coffey (1974) and confirm that the SPN antigen is insoluble in interphase cells.

The SPN antigen associates with the spindle in mitotic cells

The distribution of the SPN antigen was analysed by immunofluorescence microscopy of HeLa and SW13 cells. The mitotic stage was determined by staining for DNA with Hoechst dye and by phase contrast microscopy.

Prophase cells could be identified by the presence of condensed chromosomes within the intact nuclear envelope. In these cells, the SPN antigen started to accumulate at the centrosome region. Where centrosome duplication was already visible, the antigen seemed to be associated with both diplosomes (Figure 3a and double labelling with SPN-3 and tubulin antibodies in Figure 3b and c). Often in such micrographs a dark centre is seen as though the SPN antigen associates with the pericentriolar material rather than with the centrioles (Figure 3a). During metaphase the SPN antigen was very prominent in the pole regions of the mitotic spindle, forming a halo around the actual poles (Figure 3e). Although SPN staining was more concentrated towards the poles it seemed also to extend into or envelop the proximal parts of the spindle and a small amount of SPN staining was evenly distributed throughout the metaphase cell. In early anaphase a crescent-like fluorescence was observed at the spindle poles (Figure 3h). Again an unstructured fluorescence was present throughout the cell. As the chromosomes move farther apart in anaphase and the kinetochore microtubules shorten, the SPN fluorescence in the spindle pole regions gradually disappeared (Figure 3j and k). In telophase the SPN antigen associated again with the reforming nucleus and subsequently redistributed throughout the nucleus (Figure 3l and m). The midbody of telophase cells was always negative when tested with SPN antibodies (Figure 3l, m, n and o). In the few abnormal multipolar mitoses found in HeLa cell cultures, SPN antigen was always associated with the spindle pole regions (Figure 3p, q and r).

We also examined what happened to the SPN antigen when cells were treated with nocodazole to depolymerize microtubules. After a 4 h treatment of HeLa cells with nocodazole at 10 μg/ml, microtubules were depolymerized and the soluble tubulin was extracted with Triton X-100. Staining for tubulin revealed in such preparations only a perinuclear spot at the centrosome position visible in the majority of the extracted interphase cells (Figure 4b). Nocodazole treatment did not affect the nuclear SPN immunofluorescence location of interphase cells. However, nocodazole treatment and Triton X-100 extraction of mitotic cells caused the depolymerzation and complete extraction of the mitotic spindle microtubules as judged by staining with tubulin antibody. In mitotic cells the SPN reactivity was now present in multiple small dot-like structures, some of which were clearly outside the chromosomes (Figure 4a). Two hours after removal of nocodazole, microtubules were almost fully reassembled in interphase cells (Figure 4c) while SPN reactivity was still present in interphase nuclei (Figure 4d). In mitotic cells SPN fluorescence was now seen in dot-like structures and in asters extending from these structures (Figure 4d). Double staining with antibodies to tubulin showed a correspondence between the tubulin and SPN antibody staining in mitotic cells, with both antibodies staining the asters reforming in these cells. The SPN staining seemed stronger closer to the poles. Careful inspection of the coverslips showed that the multipolar spindles most often regrow from SPN positive foci located in the chromosome region (compare Figures 4e, f and g). At later times bipolar mitotic spindles are reformed.

Distribution of the SPN antigen in cell lines and in tissues

Different cell lines of human origin, as well as from other mammals, were examined by immunofluorescence microscopy with SPN antibodies (Table I). All antibodies reacted with all human cell lines tested. Staining was restricted to the nuclei of interphase cells and to the poles and mitotic spindles as described above for HeLa and SW13 cells. The SPN-3 and SPN-7 antibodies also reacted on kangaroo rat PtK2 cells and on pig kidney LLCPK1 cells but only SPN-7 reacted with rat RMCD cells. Thus Table I shows that at least three different epitopes are recognized by the SPN antibodies and that the epitope(s) recognized by antibodies SPN-3 and SPN-7 seem to be more conserved across species than the epitopes recognized by the other SPN antibodies.

Microinjected SPN-3 antibody blocks mitosis

To find out whether the SPN antigen has a critical role in mitosis, we microinjected antibodies SPN-1 to -5 into HeLa cells. While microinjection of antibodies SPN-1, -2, -4 and -5 did not affect cell proliferation or mitosis, microinjection of the SPN-3 antibody had a drastic effect. Large numbers of round cells were apparent in phase microscopy 17−24 h

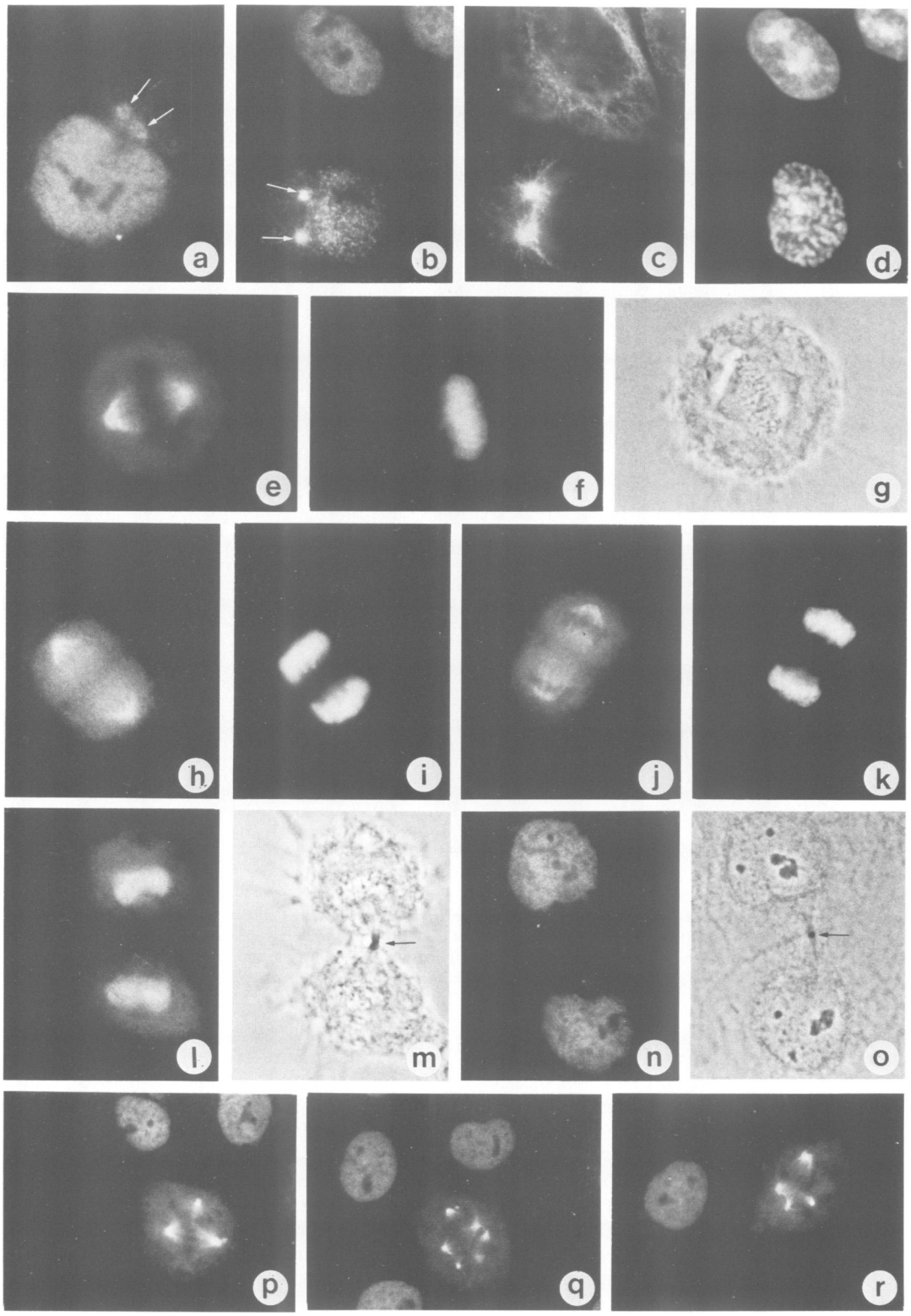

Fig. 3. Localization of SPN antigen in HeLa cells at different stages of mitosis. (**a–d**) Prophase. a, SPN-3, b–d same cell stained with SPN-3 (b), tubulin (c) and Hoechst (d). (**e–g**) Metaphase cell stained with SPN-3 (e), Hoechst (f) and in phase contrast (g). (**h–k**) Anaphase. (h and i) Early anaphase cell stained with SPN-3 (h) and Hoechst (i). (j and k) Late anaphase cell stained with SPN-3 (j) and Hoechst (k). (**l–o**) Telophase. (l and n) cells stained with SPN-3, and (m and o) same cells in phase contrast. In prophase SPN accumulates at the centrosome/diplosome regions of the mitotic spindle (arrows, a–d). In metaphase cells it is concentrated in the pole regions, but extends into the mitotic spindle and is also present diffusely in the cytoplasm (e–g). During anaphase, it is located as crescents extending from the pole regions as well as diffusely in the cytoplasm (h–k). In telophase and early G_1 it relocates to the nucleus, and is not present in the midbody (arrows) of the intracellular bridge (l–o). Micrographs **p–r** show selected examples of rare cells with multipolar spindles found in normal HeLa cultures stained with the SPN-3 antibody. Magnification a–o, ×820, p–r, ×520.

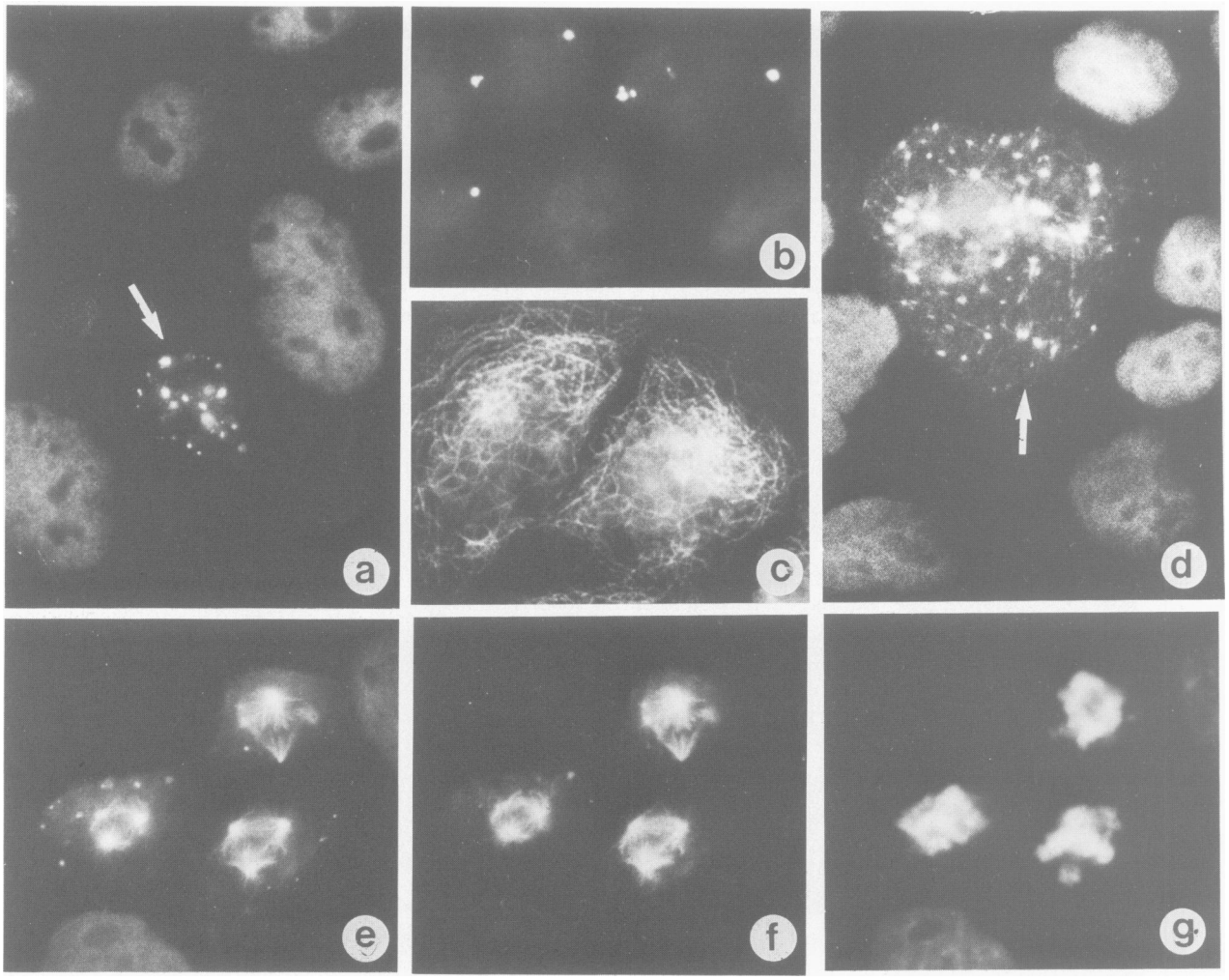

Fig. 4. Effect of nocodazole treatment on the SPN antigen arrangement in mitotic cells. HeLa cells were treated for 4 h with 10 μg/ml nocodazole and then stained either directly (a and b) or 2 (c and d) or 3 (e–g) h after removal of the drug. Cells were extracted and fixed and then stained either for SPN antigen distribution (a and d) or for tubulin (b and c). Note the foci of SPN staining apparent in the mitotic cells indicated by arrows in a and d. (e–g) show the same three cells stained for SPN (e) tubulin (f) and DNA (g). Note the similarity in the SPN and tubulin profiles. Magnification ×950.

after microinjection. Figure 5a, b and c show the same group of microinjected cells photographed 0, 17 and 24 h after microinjection. By 24 h (Figure 5c), ~50% of the cells were round and many of the non-round cells were multinucleated. A comparison of Figure 5a–c shows that the number of cells remain constant between 0 and 24 h. When the 17 h (Figure 5b) and the 24 h (Figure 5c) time points are compared, several cells that appear binucleate at 17 h appear to have become round at 24 h. Cells which are multinucleate at 17 h remain multinucleate at 24 h. Figure 5d–f show the same group of microinjected cells as in Figure 5c, after staining with a rhodamine labelled second antibody (Figure 5d) and Hoechst (Figure 5e), as well as in phase contrast (Figure 5f). Comparison of Figure 5d–f with 5c shows that many of the round cells were lost during processing. The microinjected SPN antibodies are clearly visible in Figure 5d, and the multinucleated cells in Figure 5c and f.

We also quantified the results of representative microinjection experiments by photographing the microinjected cells immediately after injection and at 4 h, 24 h and 48 h after injection using phase contrast microscopy. The total number of cells as well as the numbers of round and flat cells at each time point were counted. The results are summarized in Table II. During the first 4 h, a few microinjected cells entered mitosis (Table II, exp. 1). When microinjected colonies were followed for 24 hours, the total number of cells remained constant or decreased slightly (exp. 2–4A). After 24 h ~50% of the cells were round and arrested in mitosis (exp. 2–4A) while many non-round cells were multinucleate (Table II, exp. 2–3). Since it was sometimes hard to distinguish multinucleate from mononucleate cells in the phase contrast pictures, cells were fixed at different times after microinjection, treated with goat anti-mouse antibody and Hoechst dye, and then photographed in immunofluorescence (Figure 5d–f). Some round cells were lost in the immunofluorescence procedure. In one experiment microinjected cells were examined after 25 h (Table II, exp. 4A) and again at 48 h (Table II, exp. 4B). After 48 h many fewer round cells were present on the coverslips than at 25 h. Flat cells attached to the coverslip at 48 h were all multinucleate and at this time point dead cells were also recorded. Thus by 48 h, most cells were either lost from the coverslip or were dead.

The microinjection experiments with the SPN-3 antibody were performed initially at a concentration of 4.7 mg/ml.

Table I. IgG class and reactivity of the antibodies with cultured cells from different mammals

Antibody	Ig Class	SW13	HeLa	MCF7	Glioma	LLCPK1	PtK2	RMCD
		h	h	h	h	p	kr	r
SPN-1 (23.1)	IgG1	+	+	+	+	−	−	−
SPN-2 (83.1)	IgG2b	+	+	+	+	−	−	−
SPN-3 (347.1)	IgG2a	+	+	+	+	+	+	−
SPN-4 (344.14)	IgG1	+	+	+	+	−	−	−
SPN-5 (472.10)	IgG1	+	+	+	+	−	−	−
SPN-7	IgG1	+	+	+	+	+	+	+

h, p, r and kr indicate cells of human, pig, rat and kangaroo rat origin respectively.

We have also microinjected SPN-3 antibody into the cytoplasm at lower antibody concentrations. At 1.2 mg/ml (Table II, exp 6) and 0.3 mg/ml (Table II, exp. 7) , the same effects on mitosis were seen. However, at a concentration of 0.075 mg/ml (Table II, exp.8), no effect on mitosis was seen. Microinjection of SPN-3 antibody into the nucleus rather than the cytoplasm of HeLa cells also resulted in cells blocked in mitosis and in multinucleate cells (Table II, exp. 5). Four other SPN antibodies were also microinjected into HeLa cells. Antibodies SPN-1, SPN-2, SPN-4 and SPN-5 were microinjected at concentrations of 2.1 mg/ml, 3.5 mg/ml, 2.8 mg/ml and 5.0 mg/ml respectively. These four antibodies did not inhibit mitosis under these conditions. A representative experiment with SPN-5 is shown in experiment 9 in Table II.

The results of the experiments summarized in Table II show that microinjection of the SPN-3 antibody caused a block in mitosis.

Microinjection of SPN-3 results in aberrant spindle morphology

Several experiments were performed to try to find out how the SPN-3 antigen affects the organization of the mitotic spindle. Decoration experiments in which microinjected cells were treated only with goat anti-mouse antibodies to reveal the intracellular arrangement of the antibody decorated SPN antigen showed a high level of general fluorescence due to the excess of microinjected SPN antibody (e.g. Figure 5d). Microinjected cells were therefore extracted with microtubule stabilizing buffer containing Triton X-100 to remove the excess SPN antibody and were then stained with second antibody. After this treatment SPN staining was associated with the multiple nuclei visible in most non-round cells (compare Figure 5g with the Hoechst stain of the same cells in Figure 5h). In round cells (arrows, Figure 5g) SPN staining was associated with multiple foci although the round shape made it hard to determine the exact distribution. Staining of the same cells in Hoechst showed that such cells were arrested in a prometaphase-like state (Figure 5h).

Staining with a lamin A specific antibody (Figure 5i and corresponding Hoechst stain in Figure 5j) showed that in such cells lamin A was distributed throughout the cell (Figure 5i) consistent with its known behaviour in mitosis while in the multinucleate cells lamin A staining was associated with the nuclear lamina (Figure 5i).

Double staining with tubulin and with SPN antibodies used affinity purified rabbit tubulin antibodies. While microtubular profiles were normal in interphase cells with a nuclear SPN distribution and in mitotic cells outside the microinjected area (Figure 6a and a'), the spindle morphology was disturbed in the round, mitotically-arrested cells (Figure 6b−f). Some spindles were clearly multipolar (eg. Figure 6d and f). A comparison of the tubulin and Hoechst stain in these figures shows individual chromosomes with no hint of alignment in a metaphase plate, again suggesting that these cells were arrested in a prometaphase-like state.

Confocal microscopy was used to compare further the distribution of the microinjected SPN-3 antibody and that of tubulin in cells arrested in a prometaphase-like state 24 h after microinjection. Microinjected cells were extracted with Triton X-100 in a microtubule stabilizing buffer and then double stained. The bound microinjected SPN-3 antibody was visualized by a FITC conjugated second antibody and the tubulin distribution was monitored with a rabbit tubulin antibody and rhodamine conjugated second antibody. Some optical sections were scanned on both channels. The SPN-3 antibody binding was found in multiple dot-like structures often colocalizing with the ends of microtubule bundles in abnormal spindles (Figure 7). Thus microinjection of SPN-3 antibodies resulted in the reorganization of the SPN antigen into multiple foci.

Discussion

Properties of the SPN antigen

Whereas the SPN antigen is associated with the nuclear matrix in interphase cells, its location changes first to the centrosomal region at prophase where it seems to associate with the pericentriolar material and then to the pole regions and half spindles at metaphase and anaphase. The location of the SPN antigen in mitotic cells (Figure 3) and the changes in its distribution when mitotic cells are treated with the microtubule depolymerizing drug nocodazole (Figure 4) show that the SPN antigen is a true component of the spindle. Its distribution overlaps that of calmodulin at prophase through early anaphase (Andersen *et al.*, 1978; Welsh *et al.*, 1978) but not at other stages of the cell cycle.

All six SPN antibodies recognize the same protein as judged by immunoblotting experiments and by immunocytochemistry of cell lines, tissues and nuclear matrix preparations. A 210 kDa component is recognized both in interphase and in mitotic cells (Figure 1), making it unlikely that the SPN antibodies recognize different proteins in interphase and in mitotic cells. The data in Figure 1B indicate some proteolytic breakdown of the 210 kDa polypeptide into 180−200 kDa components. This figure also shows that the SPN antigen appears more soluble in mitotic than in interphase cells (compare the different distributions between supernatant and pellet fractions in Figure 1B), suggesting that some cell cycle dependent modification of the SPN antigen or of its binding partners occurs. While our immunoblotting results stress that the SPN

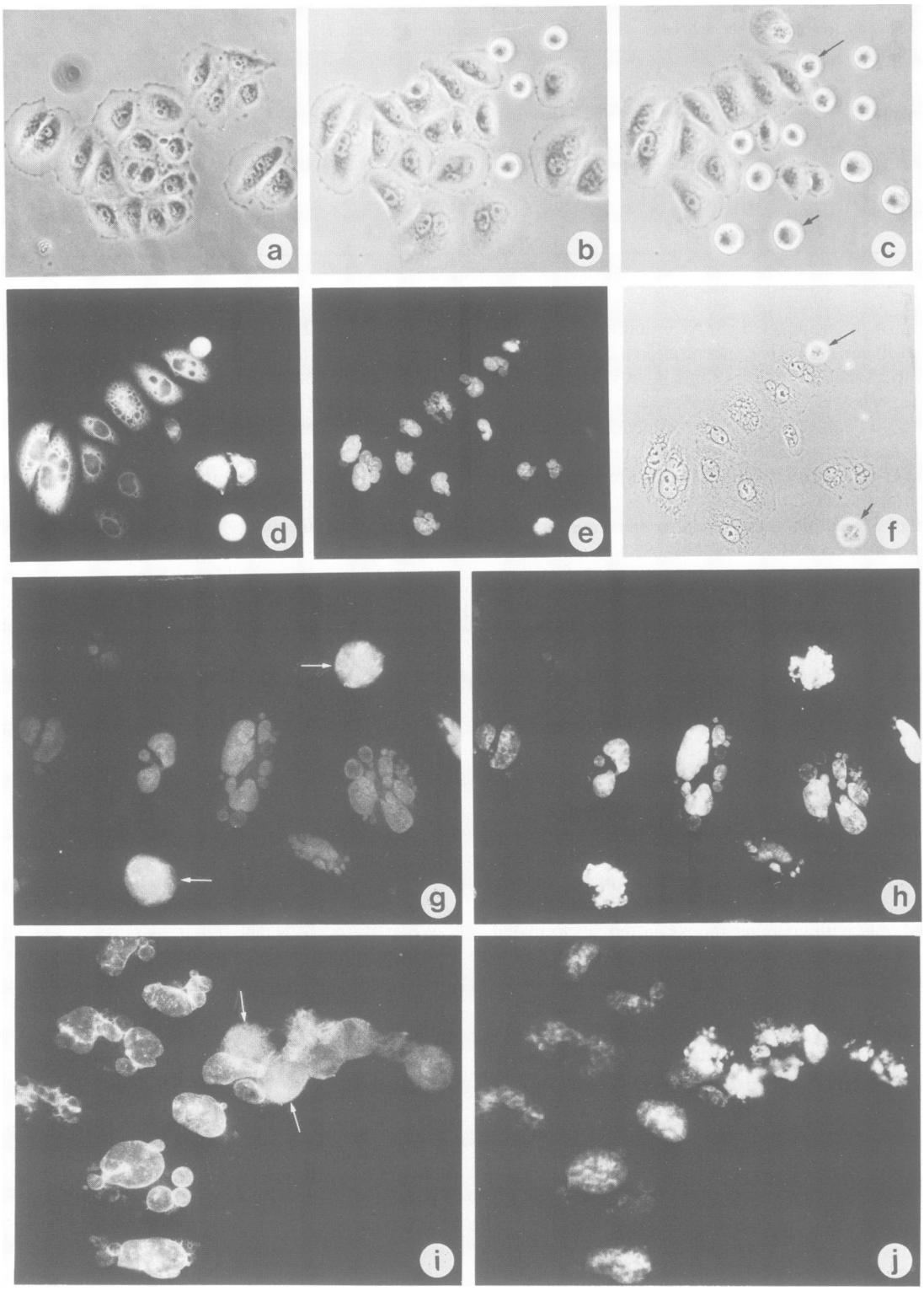

Fig. 5. Microinjection of SPN-3 antibody into HeLa cell cytoplasm blocks mitosis. (**a–f**) Cells were microinjected with SPN-3 and the same field visualized in phase contrast microscopy 0 h (a), 17 h (b) or 24 h (c) after microinjection. Note the increase in arrested mitotic cells and the increase in multinucleate cells visible in b and c. At 24 h the cells in (c) were fixed and the distribution of microinjected SPN antibody visualized with second antibody (d). Distribution of DNA by Hoechst dye in the same cells is shown in (e), the phase contrast micrograph of these cells is shown in (f). Some round cells are lost during the staining procedure (compare panels c and f; arrows indicate round cells retained on the coverslip). (**g–h**) Cells were microinjected with SPN-3 antibody, incubated for 24 h and then extracted with 0.5% Triton X-100 in microtubule stabilizing buffer to remove unbound antibody, fixed and treated with second antibody to show the SPN-3 distribution (g) and with Hoechst dye (h). Note multinucleate cells in (g). Note indication for a multifocal staining of SPN antigen in two round cells in g (arrows) which are arrested in a prometaphase-like state (h). (**i and j**) Cells microinjected with SPN-3 antibodies were stained with a lamin A specific antibody (i) or Hoechst (j). The multinucleate cells showed typical nuclear lamina (left of arrows) whereas the mitotic cells (right of arrows) show that in such cells lamin A is uniformly distributed. Magnification: a–f, ×200; g–j, ×320.

Table II. Effect of microinjection of SPN-3 antibody on Hela cells

Experiment No.	1	2	3	4A	4B	5	6	7	8	9
Antibody concentration	4.7	4.7	4.7	4.7	4.7	4.7	1.2	0.3	0.075	5.0
Injection site	cytoplasm	cytoplasm	cytoplasm	cytoplasm	cytoplasm	nucleus	cytoplasm	cytoplasm	cytoplasm	cytoplasm
No cells injected[a]	41	48	21	32	32	27	31	29	27	14
Incubation time (h)	4	23	25	25	48	21	24	24	23	24
Phase contrast										
Flat cells	38 (93%)	32 (64%)	12 (52%)	9 (36%)	8 (38%)	13 (50%)	13 (52%)	10 (37%)	44 (96%)	29 (94%)
Round cells	3 (7%)	18 (36%)	11 (48%)	16 (64%)	1 (5%)	13 (50%)	11 (44%)	15 (55%)	2 (4%)	2 (6%)
Dead cells[c]	0	0	0	0	12 (57%)[c]	0	1 (4%)	2 (7%)	0	0
Total	41	50	23	25	21	26	25	27	46	0
Immunofluorescence[b]										
Multinucleated	0	28 (56%)	12 (52%)	ND	8 (38%)	4 (15%)	9 (36%)	4 (15%)	0	0
Mitosis		abnormal	----------→						normal	normal

SPN-3 antibody was injected in experiments 1–8 and SPN-5 antibody in experiment 9. Microinjection of SPN-3 antibody affects mitosis at a concentration ≥0.3 mg/ml. Microinjection of SPN-5 at 5 mg/ml (experiment 9) and of other several SPN antibodies does not affect mitosis (see text)
[a]Cells within an individual colony were microinjected.
[b]with second antibody and with Hoechst dye to identify multinucleated cells.
[c]The number of dead cells increased at incubation times longer than 24 h.
ND = not done.

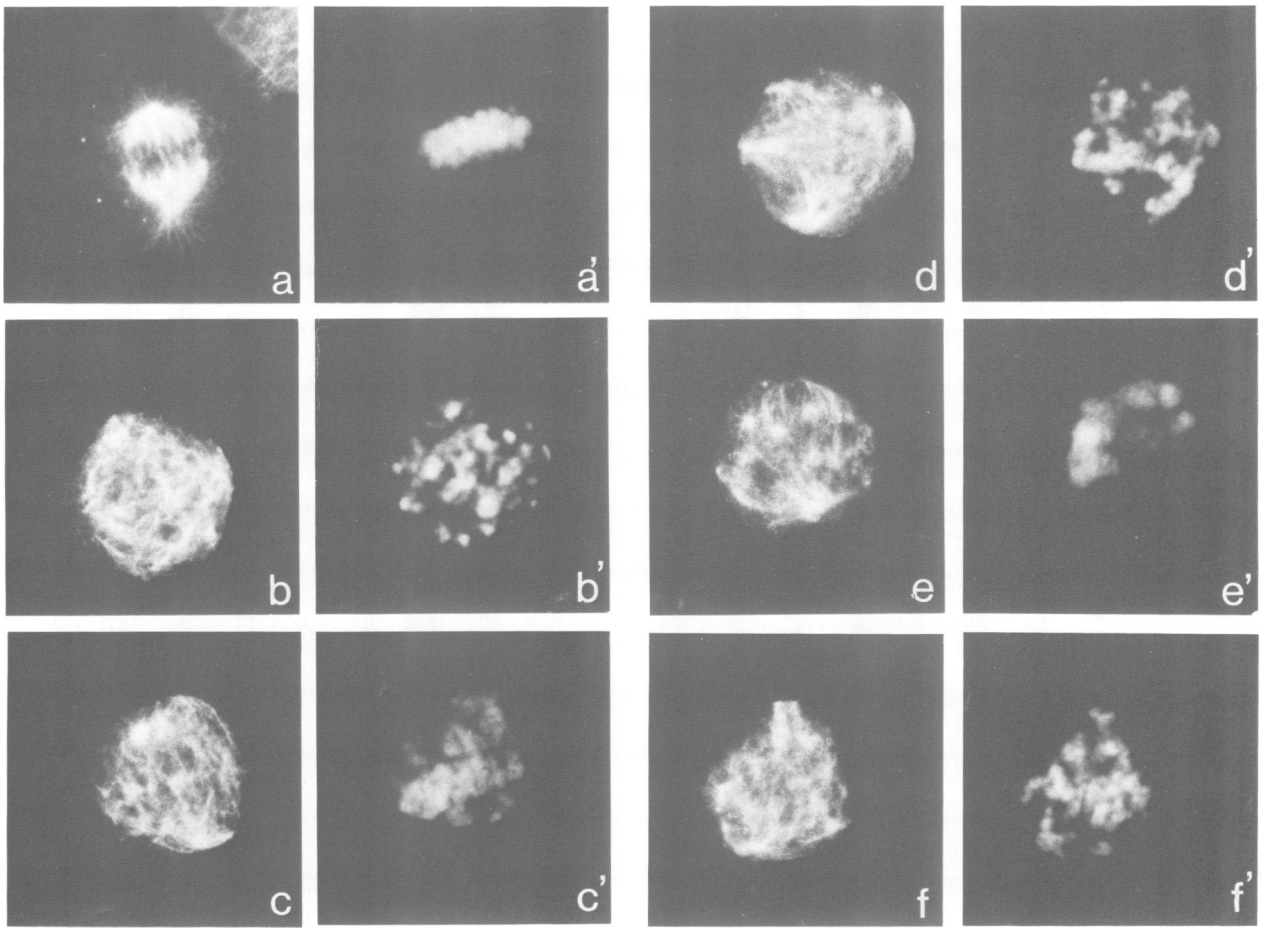

Fig. 6. Mitotic spindles of HeLa cells microinjected with the SPN-3 antibody at a concentration of 4.7 mg/ml. Cells were extracted with a 0.5% Triton X-100 in microtubule stabilizing buffer and fixed in methanol. Panels **a**–**f** show tubulin stainings and **a'**–**f'** the corresponding Hoechst stain. Panels a and a' show a normal metaphase spindle outside the microinjected area whereas panels b,b'–f,f' show a collection of microinjected, mitotically arrested cells. Note the abnormal, multipolar spindles in these cells. Magnification ×950.

antigen is a minor component both of the interphase nuclear matrix and of the mitotic spindle, it is nevertheless a very effective antigen as judged by the fact that we have observed hybridoma clones with the SPN pattern in at least ten fusions where the insoluble residue of human cell lines such as HeLa or SW13 was used as antigen.

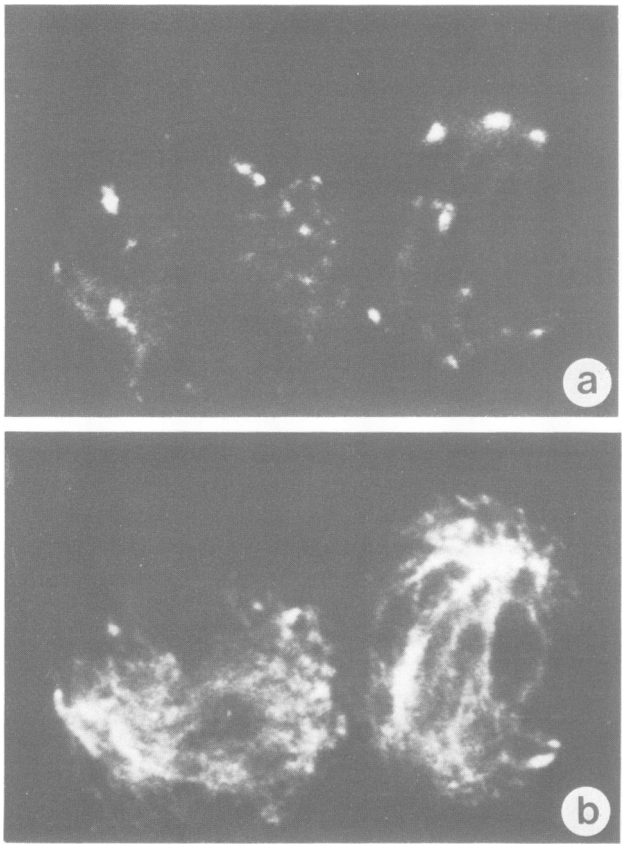

Fig. 7. Confocal microscopy of HeLa cells 24 h after microinjection with SPN-3 antibody. The cells were extracted with cytoskeletal buffer and double-labelled to reveal the SPN distribution (**a**) and the tubulin distribution (**b**). Note the localization of SPN antigen in multiple foci which are sometimes located at the end of microtubule bundles. Magnification ×1970.

The strong differences in the behaviour of the different antibodies after microinjection into HeLa cells as well as their different cross species reactivity (Table I) argue that the SPN antibodies recognize distinct epitopes on the 210 kDa polypeptide. SPN-7 has the broadest cross species reactivity but was not microinjected. SPN-3 has a broad cross species reactivity recognizing the SPN antigen in human, pig and rat kangaroo cells and drastically affects mitosis when microinjected into HeLa cells. In contrast the SPN-1, -2, -4 and -5 antibodies detect the SPN antigen only in human cells and do not affect mitosis when microinjected into HeLa cells. While the epitope of SPN-3 is available in the living cell, the lack of inhibition of the other antibodies after microinjection may be due to a too low affinity or to the fact that the epitopes are either not directly available in the living cells, or are functionally unimportant.

The microinjection experiments with the SPN-3 antibody summarized in Table II show that the SPN antigen is an important functional component of the mitotic spindle. In our experiments, microinjection of a 0.3 mg/ml solution of specific SPN-3 IgGs (2×10^{-6} M solution) is still sufficient to inhibit mitosis, whereas a 4-fold lower concentration is not. Assuming that one can replace some 5% of the cellular volume by the IgG solution this would lead to a cellular IgG concentration of $\sim10^{-7}$ M for inhibition. This low concentration again argues that the SPN antigen is only a minor cellular component, which has to remain functionally intact for the cell to progress through division.

Comparison of the SPN antigen with other proteins which relocate from the nucleus to the mitotic spindle at mitosis

The SPN antigen seems to belong to a relatively recently described group of proteins, which during interphase are predominantly nuclear but at mitosis become part of the spindle. The first member of this group is the NuMA protein discovered by Lydersen and Pettijohn in 1980. This 300 kDa polypeptide is a constituent of the nuclear matrix in interphase but is observed in metaphase in the mitotic spindle pole regions in a pattern similar to that given by the SPN antigen. The SPN antigen clearly differs from NuMA protein in molecular weight (210 kDa versus 300 kDa) and in the patterns provided by the interphase HeLa cell nucleus. While the SPN antigen gives a relatively uniform pattern the NuMA antigen is associated with 10–40 strongly staining spots per nucleus. A direct cellular function of the NuMA antigen during mitosis has not yet been demonstrated.

Properties of centrophilin, a second protein with a cell cycle dependent location, have been recently described by Tousson *et al.* (1991). Monoclonal antibodies to centrophilin were raised against a kinetochore enriched extract from HeLa cell chromosomes. Centrophilin is also a component of the interphase nucleus, and during mitosis is associated with both centromeres and centrosomes and with the midbody. Although some centrophilin staining patterns resemble those provided by the SPN antigen there are some important differences. Thus SPN reactivity is found neither on centromeres nor at the midbody of telophase cells. In addition, the SPN antigen is also diffusely distributed throughout the metaphase and anaphase cell. Disruption of the mitotic spindle with nocodazole leads to the dispersion of both centrophilin and the SPN antigen into many foci, which are insoluble in Triton X-100. The centrophilin foci are localized to the kinetochores in prometaphase and were able to nucleate microtubules. In the case of the SPN antigen, the foci acted as nucleating points for nascent microtubules after release from the nocodazole block and were often present outside the area occupied by chromosomes. In addition the centrophilin antibody recognized a 180 kDa and 210 kDa doublet in unfractionated HeLa cell extracts while the SPN antibody detects a single 210 kDa band in similar preparations (Figure 1). Although the difference in immunoblotting results of centrophilin and SPN antigens could be explained either by the recognition of phosphorylated and unphosphorylated forms of centrophilin with a different apparent molecular weight in the case of the centrophilin antibody and recognition of only the larger polypeptide by the SPN antibodies, or by a proteolytic breakdown of centrophilin resulting in an additional 180 kDa component, such speculations do not explain the differences apparent in the immunofluorescence microscopic analyses during mitosis. Thus despite the fact that the SPN antigen shows some similarity to centrophilin, the differences in the immunoblotting data and in the behaviour during mitosis suggest that they are different proteins. In addition centrophilin antibodies have not yet been shown to influence cell division after microinjection.

Recently, Compton *et al.* (1991) described the isolation of several monoclonal antibodies which decorate either kinetochores or centrosomes or both. Of these one antibody resembled the centrophilin antibody just described. A second antibody (1H1), detected a 205 kDa polypeptide and stained interphase cell nuclei in a way very similar to that observed

by the SPN antibodies; during mitosis the 1H1 antigen was localized to the pole regions at the half spindles of metaphase and anaphase cells. So far no functional assays have been reported with the 1H1 antibody.

Thus several proteins with molecular weight >180 kDa and characterized by a similar but not identical behaviour during the cell cycle have been described in different studies. The combined data favour the possibility of a group of distinct proteins with a cell cycle dependent redistribution from the nucleus to the mitotic spindle during mitosis. At least one of them, the SPN antigen, has now been shown by microinjection experiments to be functionally necessary for normal mitosis. cDNA cloning and sequencing studies are needed to resolve the possible relationship between the different proteins discussed in this section.

Functional importance of the SPN antigen for mitosis

Of the group of proteins discussed above only the SPN antigen has so far been shown to play an essential role during mitosis. The experiments summarized in Table II show that microinjection of SPN-3 antibody into the cytoplasm of HeLa cells inhibits the next division. This inhibition is dose dependent and occurs even when IgG concentrations as low as 0.3 mg/ml are introduced into the cells. Microinjected cells are blocked either in a prometaphase-like state or become multinucleate, but do not undergo further division.

The mechanism by which SPN-3 antibody blocks mitosis is not known. However, the immunofluorescence micrographs suggest that the injected antibody prevents proper spindle formation. Instead of the normal bipolar spindle, aberrant multipolar spindles are seen (Figure 6 and 7). Whether this is a direct effect, i.e. whether the SPN antigen is normally directly involved as a minus end nucleating protein of the spindle microtubules or whether it is required to bind other proteins which then act to nucleate the metaphase spindle microtubules, remains to be seen.

Materials and methods

Production of monoclonal antibodies

Balb/c mice were immunized with a urea extract of isolated nuclei (Lehner *et al.*, 1986) from a human adrenal cortex carcinoma cell line (SW13). Cells were scraped from 20 subconfluent 100 mm tissue culture dishes into 10 mM PIPES, pH 6.8 supplemented with 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100 and 1.2 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co., Munich, FRG) and centrifuged at 2000 r.p.m. for 5 min. Cell residues were washed once with the same buffer, recentrifuged and resuspended in 2 ml of the same buffer. The suspension was homogenized with a Potter–Elvehjem homogenizer (10 strokes) and centrifuged through a sucrose cushion (2.0 M sucrose in 50 mM Tris–HCl, pH 7.5, supplemented with 25 mM KCl, 5 mM $MgCl_2$ and 1.2 mM PMSF) for 30 min at 31 000 r.p.m. in a Beckman SW 60Ti rotor to pellet the nuclei. The pellet was resuspended in the same buffer without sucrose and washed a second time. It was then extracted at 4°C for 15 min with 2 ml of 5 M urea in 0.15 M Tris–HCl, pH 8.1 with 5 μM E64 and 10 μg/ml ovomucoid (Sigma Chemical Co.). The extract was centrifuged at 15 000 *g* for 10 min at 4°C. The supernatant was used for immunization. The protein concentration was estimated by the Bradford method (Bradford, 1976).

Mice were immunized by intraperitoneal injections. 50 μg protein were given at day 0 with Freund's complete adjuvant, 30 μg at days 14, 28 and 42 with Freund's incomplete adjuvant, and finally 30 μg protein at days 56 and 57 was injected without adjuvant. Fusion was on day 59 using the PAI mouse myeloma cell line. Indirect immunofluorescence microscopy with SW13 cells grown on 10-well glass slides was used to screen the hybridoma culture supernatants starting 10 days after fusion. Cloning was by limited dilution on 96-well microtitre plates. Antibodies were produced both as hybridoma supernatants and as ascitic fluid in Pristane primed mice. Immunoglobulin subclasses were determined with a mouse monoclonal antibody isotyping kit (Amersham, UK).

Gel electrophoresis and immunoblotting

Cytoskeletons. To obtain HeLa cell cytoskeletons, subconfluent cell monolayers were washed twice with PBS (137 mM NaCl, 7 mM $NaHPO_4$, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.1) and extracted for 1 min with 1% Triton X-100 in 10 mM Tris–HCl, pH 7.6, 140 mM NaCl, 5 mM EDTA and 25 μM 2-mercaptoethanol at room temperature. Extracted cells were washed once with the same buffer without Triton X-100 and scraped into the buffer. The cell suspension was centrifuged at 12 000 *g* for 15 min and the pellet was suspended in hot electrophoresis sample buffer, sonicated and boiled for 5 min.

Interphase and mitotic HeLa cell preparations. Cell numbers were determined using a haemocytometer. Whole cell preparations were made by scraping confluent cell monolayers into medium and centrifugation. Cells were washed twice with ice cold PBS. Cell pellets were suspended directly in the SDS–PAGE sample buffer at a concentration equivalent to 10^7 cells/ml. Alternatively, the cell pellets were further extracted with 0.5% Triton in PBS containing a cocktail of protease inhibitors (5 mM EDTA, 100 μg/ml ovomucoid, 2 mM PMSF and 25 μM E64) at a concentration of 2×10^7 cells/ml at 0°C. After incubation for 5 min, the suspension was centrifuged at 13 000 *g* for 5 min. The supernatant was mixed with the same volume of twice concentrated sample buffer and boiled (Triton X-100 supernatant). The pellet was suspended in sample buffer at a concentration equivalent to 10^7 cells/ml sonicated to disrupt the DNA and boiled for 5 min (Triton X-100 pellet).

To obtain populations enriched in mitotic cells, cells in exponential growth were synchronized by adding 2.5 mM thymidine to the medium for 18 h and then colcemid at 0.06 μg/ml in fresh medium for 16 h. Round, loosely attached mitotic cells were collected in medium by pipetting with a narrow bore 5 ml pipette. The proportion of mitotic cells was found by phase contrast microscopy and Hoechst 33258 staining to be >95%. The mitotic cells were then processed as described above for whole cells.

Extracts were separated in 10% polyacrylamide gels. For immunoblotting experiments 7.5% acrylamide gels containing 0.8% bisacrylamide were used. Proteins were electrophoretically transferred to nitrocellulose in a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.01% SDS and 20% methanol. Protein transfer was controlled by staining with Ponceau S. The sheets were blocked in 4% bovine serum albumin in Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.4, 0.15 mM NaCl). Undiluted hybridoma supernatants were used as primary antibodies and the second antibody was peroxidase labelled rabbit anti-mouse IgGs (Dako Immunochemicals, Klostrup, Denmark) diluted 1:200 in 1% BSA, 0.2% Tween 20 in TBS. Washing was done with 0.2% Tween 20 in TBS.

In some experiments the peroxidase signal was detected by the chemiluminescence reaction (ECL Western blotting detection system, Amersham, UK) to enhance the sensitivity. For this method, all antibodies were diluted by an additional factor of ten. The nitrocellulose membrane was washed overnight in 0.2% Tween 20 in TBS, washed for 2×10 min in 0.2% SDS, 0.5% Triton X-100 in TBS and finally in 0.2% Tween 20 in TBS. Development was by incubation in a reaction mixture made according to the manufacturer's instructions, with exposures between 5 and 60 s on X-ray film.

In situ cell fractionation. To collect more information on the biochemical properties of the SPN antigen SW13 cells grown on glass coverslips were subjected to serial extraction by non-ionic detergent, DNase, RNase and high salt (Staufenbiel and Deppert, 1984). Cells were fixed at various steps during the procedure and stained for immunofluorescence of the SPN antigen and for DNA staining with Hoechst dye. Cells were washed three times with ice cold KM buffer: 10 mM *N*-morpholinoethanesulphonic acid, pH 6.2, 10 mM NaCl, 1.5 mM $MgCl_2$, 10% (v/v) glycerol and 30 μg aprotinin (Bayer, Leverkusen, FRG).

As the first step, cells were extracted twice (3 and 27 min) with KM buffer containing 1% Nonidet P40 (NP4O), 1 mM EGTA, 5 mM DTT on ice. After three washes with KM buffer, cells were incubated for 15 min at 37°C with 50 μg/ml of DNase I (Sigma Chemical Co., Munich, FRG; No. D-4527), pretreated according to Raymond and Gagnon (1988) to eliminate possible protease contamination. Subsequent incubation was in KM buffer containing 2 M NaCl, 1 mM EGTA and 5 mM DTT on ice for 30 min. This was followed by three washes with KM buffer and treatment with 50 μg/ml of both DNase I and RNase A (Sigma Chemical Co.) in KM buffer for 30 min at 37°C. The RNase A stock solution was previously boiled for 10 min to destroy proteases. Finally, samples were washed three times with KM buffer.

Cell culture

Human cell lines used in this study were HeLa SS6 cells, the breast carcinoma cell line (MCF-7), the adrenal cortex carcinoma cell line (SW13) and the

glioblastoma cell line (U333CG/343MG glioma). The cell lines of other origin were a kidney cell line from *Potorous tridactylis* (PtK2), a pig kidney cell line (LLC PK1) and a rat mammary carcinoma cell line (RMCD). Cells were maintained in DMEM supplemented with 10% FCS and non-essential amino acids.

To test the effect of nocodazole treatment on SPN antigen distribution, HeLa cells grown on coverslips were incubated with 10 μg/ml nocodazole (10 mg/ml stock solution in DMSO) for 4 h. Cells were used either directly or after 2–3 h incubation in fresh medium without nocodazole. Cells permeabilized by buffer containing 0.5% Triton, were washed and fixed in formaldehyde as described in Tousson *et al.* (1991), and then subjected to immunofluorescence analysis.

Microinjection

For microinjection studies, IgGs were purified from ascitic fluid with a protein G column (MAb Trap, Pharmacia, Uppsala, Sweden). Pooled fractions containing electrophoretically pure IgGs were dialysed against PBS and concentrated using Centricon concentrators (Amicon Corp, Danvers, MA, USA).

Cells were grown on glass coverslips coated with 100 μg/ml poly-L-lysine. HeLa cells were plated at a low density and grown for at least two days so that they formed isolated colonies. Cells in such colonies were microinjected using a semiautomatic microinjection apparatus (Eppendorf, Hamburg, FRG) and usually all cells in a particular colony were microinjected. From 20 to 60 cells were injected on each coverslip. Colonies were photographed in phase contrast microscopy at different times after microinjection to document changes in total cell number and in the proportion of round cells. Finally, microinjected cells were subjected to immunofluorescence analysis.

Immunocytochemistry

Immunofluorescence. Cells grown on coverslips were fixed at −10°C for 10 min in methanol and air dried, and then incubated at 37°C for 45–60 min with primary antibodies. After washing three times with PBS they were incubated with fluorescein-coupled goat anti-mouse immunoglobulins (Cappel Laboratories, Cochranville, PA, USA) at 37°C for 45 min. They were then washed three times with PBS, stained for DNA with Hoechst 33258 (20 μg/ml in 25% ethanol/75% PBS) and directly mounted in Mowiol 4.88 (Hoechst AG, Frankfurt, FRG).

In microinjection experiments, rhodamine-conjugated goat anti-mouse IgG (affinity purified, Dianova, Hamburg, FRG) was used as a second antibody. In some microinjection experiments, cells were washed in microtubule stabilizing buffer: 0.1 M piperazine-*N,N'*-bis (2-ethanosulphonic acid), sodium salt, pH 7.4, 1 mM EGTA, 4% PEG 6000 and then converted to cytoskeletons with the same buffer containing 0.5% Triton X-100 for 15 s. Cells were washed in stabilizing buffer, fixed in cold methanol and air dried. Subsequent treatment with 5% normal goat serum in PBS for 15 min was used to block nonspecific staining. To visualize the injected SPN antibody and the microtubules in the same cells, coverslips were incubated for 45 min with affinity purified rabbit anti-tubulin (Osborn *et al.*, 1978) and after washing were incubated simultaneously with rhodamine-coupled goat anti-rabbit IgG [affinity purified, (Dianova)] and with fluorescein-coupled goat anti-mouse IgGs. The lamin A specific antibody was raised in a rabbit using a lamin A specific synthetic peptide. Microscopy was on a Zeiss Axiophot microscope.

Confocal microscopy

Digital images of optical sections were obtained with a Zeiss confocal laser scanning microscope (LSM) using a 63× 1.4 Planapo oil immersion lens. Fluorescein was excited at 488 nm with an external argon laser. Rhodamine was excited at 543 nm with an internal helium neon laser. Filter combinations used were BP 515–565 nm for fluorescein and LP 590 nm for rhodamine. Optical sections were made at 1 μm intervals using a minimal pinhole.

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