4 Results

4.1 Essential Mitotic functions of DNA topoisomerase IIα are not adopted by topoisomerase IIβ in human H69 cells

In the first section of the results I will demonstrate that the mitotic functions of DNA topoisomerase IIα are not adopted by DNA topoisomerase IIβ. It is not clear why two isoforms of DNA-topoisomerase II have appeared in higher eukaryotes in contrast to other organisms which only possess one topoisomerase II form. The α and β isoforms are both able to decatenate and relax DNA with equal effeciency (Austin et al, 1995), but the isoforms serve different functions during the cell cycle, particularly in mitosis. Despite several attempts, the functions of the isoforms have not been found, and, furthermore, it is unknown whether these functions are unique. The most straightforward approach would be to block expression of one iso-enzyme but DNA topoisomerase IIα seems to be essential for cellular survival and mouse embryo cell lines cannot be generated when isoform IIα is completely deleted (Adachi et al, 1997). In cell lines DNA topoisomerase IIβ is dispensable for cells viability (Dereuddre et al, 1997). However, mice carrying a homozygous deletion in the gene die at birth, indicating that the isoform IIβ is required for mammalian development (J.C.Wang, personal communication). For this study a human cell line H69-VP which carries a homozygeous mutation of the nuclear localization sequence (NLS) of DNA topoisomerase IIα has been chosen. The subclone H69-VP expresses the IIα isoform outside the nucleus. It is assumed that this mutation causes this altered behaviour of DNA topoisomerase II. Here, the subclone provides a suitable model for understanding the functions of the two isoforms in the mitotic cells.

4.2 Extracellular expression of active topoisomerase IIα

4.2.1 The subclone H69/VP with a deletion in the NLS sequence

The establishment of the etoposide resistant small cell lung cancer cell lines H69-VP has been described in Jensen et al, 1993. Briefly, NCI-H69 cells were treated with increasing concentrations of etoposide (VP-16), starting at passage 70 and ending at passage 125. Cells became resistant to etoposide due to the extra-nuclear DNA topoisomerase IIα. The subclone has a homozygous deletion of 9 nucleotides in the gene of human topoisomerase IIα encoding amino acid residue 1490-1492 (KSK), which is part of a cluster of nuclear localization sequences in the carboxyl terminus of the enzyme. In this study the mutant subclone H69-VP was from passage 130 and maintained in a medium without etoposide. The parental cell line H69-WT was from passage 97. Both cell lines were viable.

4.2.2 Enzyme activity in whole cell lysate

To measure the activity of topoisomerase II in the subclone H69-VP a highly specific catalytic assay for the enzyme was used. Serial dilutions of whole cell lysate were incubated with the catenated DNA (k-DNA) from kinetoplast of *Chrithida fasciculata*. The kinetoplast, a mitochondrial DNA, which can be obtained commercially, is a catenated network of DNA rings. Topoisomerase II, but not topoisomerase I, is able to entangle k-DNA and generate DNA monomer in the presence of ATP (Marini et al, 1980). The network of the k-DNA, in relation to the monomer is too large to enter an agarose gel, but the various topoisomers formed by the enzyme can be visualized as a DNA ladder on the gel. As shown in figure 2 (a) the subclone H69-VP shows similar DNA topoisomerase activity as the parental H69-WT.

4.2.3 Enzyme levels in whole cell lysate

Furthermore, immunoblot of whole cell lysate probing with isoenzyme-specific antibodies (Fig. 2b, lanes 1 and 2) in Western blot showed that the two cell lines expressed similar amounts of topoisomerase IIα and β– antigens. Both human topoisomerase IIα and β antibodies are specific and reveal only one band at 170 kDa (IIα) and one band at 180 kDa (IIβ) seen on a SDS-polyacrylamide gel. These observations (Fig. 2a and b) confirm that H69-VP has an active full length DNA topoisomerase IIα as well as DNA topoisomerase IIβ.

4.2.4 Enzyme localization in cytosolic and nuclear fraction

Major differences between the two cell clones were observed when the subcellular organization of topoisomerase II isoforms was studied. Combined hypotonic treatment and mechanical shearing disrupted the cells and the cytosol and nuclei were separated by centrifugation. The cytosolic fractions and the nuclei were analysed for the amounts of DNA topoisomerase IIα and IIβ by immunoblotting. The results are demonstrated in figure 2 (b) (lanes 3-6). As expected, the parental H69-WT cells expressed both isoforms, mostly in the nucleus (Fig. 2b, lane 5), whereas only minor fractions (20%) of both isoenzymes were detectable in the cytosol (Fig. 2b, lane 3). In contrast, H69-VP expressed the α-isoenzyme mainly (>95%) in the cytosol (Fig. 2b, lane 4). Only a minor fraction (less than 5 %) was detectable in the nuclei (Fig. 2b, lane 6). These data confirm that the enzyme is extra-nuclear in H69-VP and can not gain access to the nuclei. However, the IIα-form present in the cytoplasma could represent a carryover from the nucleus fraction during preparation.

4.2.5 Immunoband depletion

The approach to prove that the mutant IIα-isoform in H69-VP is extra-nuclear, is to treat cells with a topoisomerase directed drug, which stabilizes a covalent catalytic intermediate of topoisomerase II and DNA. The active fraction of the DNA topoisomerase II will be trapped in covalent DNA-complexes which are far too large to migrate into a SDS-polyacrylamide gel. The enzyme catalytically interacting with the genome will be depleted from the immunoblot, hence the name immuno-band-depletion assay, and can therefore not be detected in immunostaining (method described in Boege, 1996).

In this study a topoisomerase II poison called teniposide (VM-26) was used and the representative result is demonstrated in figure 2 (c). In the parental cell line VM-26 caused an almost complete immuno-band-depletion of both topoisomerase II isoforms in a dose-dependent manner (Fig. 2c, lanes 5-8), indicating that the full cellular complement of both isoforms is engaged in catalytic DNA-turnover. In contrast, in the mutant cell line (Fig. 2c, lanes 1-4) only the β-isoform became depleted, whereas the α-isoform was not notably targeted by VM-26, even at concentrations as high as 200 μM.

These results could indicate that the major part of the mutant α-isoenzyme does not interact with the genome of the cell. It should be noted that a similar lack in band depletion of the mutant α-isoform has previously been observed using the catalytic topoisomerase II inhibitor ICRF-187 (Wessel et al, 1997). Here, a similar result would also be found if the mutant enzyme had access to the DNA but was inactive. However, as seen in figure 2 (a) the ability of the topoisomerase to decatenate kinetoplast DNA was equal in the two cell lines, indicating an active enzyme. Another explanation could be that the mutant enzyme was resistant to the drug. A high number of mutations in topoisomerase IIα

are reported to be associated with drug resistance, and it should be noted that an over-expression of the mdr1 and MRP genes are detected in the H69-VP (Brock et al, 1995). The mdr1 and MRP genes express two drug efflux pumps Pgp and MRP, which are able to decrease the accumulation of drugs in the nucleus via a pump mechanism. This could be a possible explanation for the altered sensitivity for VM26. However, the IIβ-isoform for the mutant subclone is depleted, suggesting that the drug concentration has been sufficient to interact with both isoforms.

The activity of topoisomerase IIα in the cytosolic fraction of H69-VP cells and its susceptibility to VM-26 treatment was tested. The data shown in figure 2 (d) confirms that cytosolic topoisomerase IIα of H69-VP cells was catalytically active (Fig. 2d, lane 5) and could be blocked by increasing concentrations of VM-26 (Fig. 2d, lanes 6 and 7). Thus, the lack of drug-induced band depletion of the mutant topoisomerase IIα inside the cells (Fig. 2c) must be due to the fact that the major part of the mutant enzyme is unable to enter the nucleus.

The data in figure 2 show that H69-VP cells proliferate under conditions where the β-isoenzyme of topoisomerase II is predominantly present in the nucleus and engaged in DNA processing, whereas the mutant α-isoform, although catalytically active, appears to be mostly (>95%) excluded from the nucleus. The mutant cell line did not exhibit increased levels of topoisomerase IIα or -β, nor an increase in overall cellular topoisomerase II activity, as compared to the parental cells (Fig. 2a and b), indicating that the lack of nuclear topoisomerase IIα did not stimulate an increase in regulation of either topoisomerase II isoforms. These observations seemed to suggest that isoform IIα is dispensable for cell proliferation, or can at least be complemented by the β-isoform to such an extent that an up-regulation of the enzyme is not required for maintaining unattenuated cell proliferation. From previous localization studies it has

been shown that only the α-form binds to the chromosomal scaffold during mitosis, whereas the β-isoenzyme becomes excluded from the condensed chromatin (Meyer et al, 1996). If these functions were adopted by DNA topoisomerase IIβ, it would be likely that the β-isoenzyme localizes to the chromosomal scaffold of the mutant cell line in a similar fashion as normally seen with the α-isoenzyme.

4.3 Chromosome condensation and disjunction

To test this assumption, localizations of topoisomerases IIα and -β in whole cells (Fig. 3) and chromosomal metaphase-spreads (Fig. 4) were studied. Fluorescence images of H69-WT and H69-VP in logarithmic growth stained with antibodies specific for topoisomerase IIα and -IIβ could clearly be distinguished. The specificity of the antibodies has been tested by pre-incubation with a heat inactivated recombinant topoisomerase IIα or β which blocked the signal completely. In addition, DAPI staining of DNA was used to locate the nucleus.

4.3.1 Cellular localization of topoisomerases in H69-WT

The representative images of H69-WT for the whole cell population are shown in figure 3 (a), resembling a pattern previously observed in human A431 cells (Meyer et al, 1996). The staining is for topoisomerase IIα (left), DNA (middle), and topoisomerase IIβ (right).

During interphase, the α-isoform had a mostly homogeneous distribution in the nucleoplasm, whereas the β-isoform had a highly heterogeneous reticular distribution in the nucleus. In prometaphase most of the β-isoenzyme diffused into the cytosol, and it remained there until anaphase, whereas the majority of the α-isoenzyme bound to the condensing chromatin. To get a closer picture of the distribution of the topoisomerase IIα at the chromatin in mitosis, the cells were blocked by demecolcine

swollen with KCl and after sedimentation onto the slides stained with topoisomerase antibodies. The chromosomal metaphase-spreads visualized at high resolution are shown in figure 4 (b) (top), and the staining is for topoisomerase IIα (left), DNA (middle), and topoisomerase IIβ (right). The chromosomes formed a ring with the putative centromeric regions orientated towards the centre and the arms radiating into the periphery. According to our recent studies (Meyer et al, 1996) and to other investigations (Rattner et al, 1996; Summer, 1996) topoisomerase IIα (Fig. 4b, left) was aligned in dots along the axis of the chromosome arms. Furthermore, a bright spot in the centre was also stained with the antibody. However, it was not observed in DAPI staining. Presumably this DNA-free structure is a centrosome, and it seems that our monoclonal antibody Ki-S1 cross-reacts with the centrosomes. Similar staining patterns of the centrosomes has been observed in A431 cells (Meyer et al, 1996). As expected, immunostaining of metaphase cells was negative for topoisomerase IIβ (Fig. 4b, right) because the enzyme was mainly localized in the cytosol at this stage of mitosis and probably washed away from the chromosomal spreads (compare figure 3a, right).

4.3.2 Cellular localization of topoisomerases in H69-VP

The representative images of the mutant subclone H69-VP for the whole cell population are shown in figure 3 (b). The pattern for the isoenzyme IIα in H69-VP is clearly distinct from the staining of the enzyme found in the parental cell line. In the interphase the majority of topoisomerase IIα was localized in the cytosol and not in the nucleus, whereas the β-isoenzyme exhibited a nuclear pattern similar to interphases of the parental cell line.

An unexpected result was obtained with mitotic H69-VP cells: The β-isoenzyme did not bind to the chromosomal scaffold, but rather diffused

into the cytosol and remained excluded from the chromatin throughout mitosis, just like in the parental cells. This observation indicated that topoisomerase IIβ did not substitute the lack of α-isoenzyme. An explanation could be that none of the isoforms were required for mitosis. However, the staining patterns of topoisomerase IIα in such cells showed two distinct types of mitotic events. These could be discriminated into metaphase type II and I. The high-resolution image in figure 4 (a) for topoisomerase IIα (left), DNA (middle), and topoisomerase IIβ (right), demonstrates one of the mitotic events found in the mutant cell line.

Metaphase Type I (DNA-ball)

In about 20% of mitotic H69-VP cells, an abortive type of mitosis was observed (Fig. 3b, Meta Type I). Neither topoisomerase IIα nor -β were bound to the chromatin, which appeared highly condensed and unstructured. The high-resolution image in figure 4 (a) shows such a metaphase from two views. The DNA appeared to be contracted into the shape of a flattened melon. Both topoisomerase II isoforms were excluded from this DNA-spheroid with the exception of two spots seen for topoisomerase IIα, which probably represent cross-reactions of the antibody with centrosomes.

What would happen to the survival of the mutant subclone if the metaphase type I was predominately present in the cell-culture? To test this, the mutant cells were first brought into accelerated growth by increased serum supplementation. Subsequently blocked in metaphase with demecolcine, this abortive type of metaphase occurred with high frequency (80% of the cells). After removal of the block these cells did not continue to grow but died after a few days, indicating that the ball-shaped chromatin condensed in the absence of topoisomerase II could not be further processed.

Another mitotic event was found in the mutant cell under normal growth.

This condition was designated metaphase type II.

Metaphase type II (chromosomes)

About 80% of mitotic H69-VP cells were as shown in figure 3 (b) (Meta Type II). These cells contained chromosome-like DNA-structures, which were apparently devoid of topoisomerase IIβ, but clearly stained for topoisomerase IIα. The chromatid appeared to be much less ordered compared with the chromatid in the parental cells (Fig. 4, top). Immunostaining for topoisomerase IIβ (Fig. 4b, right) was negative in such type II metaphases like in the parental cells. Topoisomerase IIα (Fig. 4b, left) appeared to be bound to the chromosomes of type II metaphases similar to the parental H69-cells, although the immunostaining was weaker and more accentuated at putative centromeric regions.

To summarize, the images in figure 3 (b) and 4 (b) suggest that topoisomerase IIβ did not (as initially assumed) substitute the mutant α-isoenzyme lacking in the nuclei of H69-VP cells. Mitosis appeared to be entirely sustained by cytosolic topoisomerase IIα, which in these cells probably entered the chromatin after breakdown of the nuclear envelope. Apparently under normal growth conditions chromatin condensation and -disjunction were possible in most cases (80%), because sufficient amounts of topoisomerase IIα gained access and bound to the DNA (Fig. 3b, Meta Type II and Fig. 4b, left). However, when topoisomerase IIα was not bound to the chromatin in detectable amounts, cells ended up with a DNA-spheroid instead of chromosomes (Fig. 3b, Meta Type I and Fig. 4a). Time seemed to be a crucial factor for binding of cytosolic topoisomerase II α to the mitotic chromatin because formation of topoisomerase IIα associated chromosomes (Meta Type II) was favoured by normal growth conditions, whereas formation of DNA-spheroids

devoid of topoisomerase IIα (Meta Type I) was favoured by accelerated cell growth.

4.3.3 Binding affinity of topoisomerases to chromatin

A fraction of topoisomerase IIα binds to chromosomes with a higher affinity than to interphase chromatin (observed in Meyer et al, 1996). The DNA-bound enzyme is extractable with an increased amount of salt concentration. To find out whether cytosolic topoisomerase IIα could also form such high-affinity chromatin bonds in type II metaphases of H69-VP cells (compare figure 4b, left), the salt stability of the chromatin binding of topoisomerase IIα and -β in comparison to the parental cell line was investigated.

The experiment was only obtained with cells under normal growth conditions. Similar experiments could not be carried out with accelerated cells because these cells were too fragile to undergo sequential salt extractions. The nuclei from cell lysis were washed and the chromatin-bound proteins were serially extracted with increasing concentrations of KCl. These fractions and the salt insoluble remnant were analysed for topoisomerases IIα and IIβ by immunoblotting and densitometry. In the four top panels in figure 5, the result for topoisomerase IIα is shown. In log-cultures of H69-WT cells (WT, I) only a minor fraction of topoisomerase IIα (30%) was not bound to the chromatin (lanes 1 and 2). The majority of the α-isoenzyme (70%) was extractable by 400 mM KCl (lane 3), but only traces (< 1%) resisted extraction by 400 mM KCl and were subsequently released from a tighter chromatin binding by 600 mM KCl or more (lanes 4 and 6). In parental cells blocked by demecolcine (WT, M) the topoisomerase IIα (about 85%) resisted salt extraction by KCl concentrations less than 600 mM (lanes 4 and 5). This indicates a recruitment of the enzyme to a high-affinity chromatin-bound state. A

markedly different pattern was observed in the mutant H69-VP cells: In log-culture (VP, I) the majority of topoisomerase IIα appeared not to be bound to the chromatin (lanes 1 and 2). When the cells were blocked by demecolcine (VP, M) and consequently formed type II metaphases, such as shown in figure 4 (b) (bottom), a small fraction of topoisomerase IIα (5%) was shifted to the 400 mM KCl extract (Fig. 5, lane 3). However, the enzyme was neither detectable in fractions subsequently extracted with higher salt concentrations (lanes 4 and 5) nor in the salt-insoluble remnant (lane 6). The four bottom panels of figure 5 show chromatin-binding of topoisomerase IIβ in interphase (I) and release of the enzyme from the chromatin at mitosis (M). Similar data were obtained in H69-WT and H69-VP cells.

These data in figure 5 indicate that in type II metaphases of H69-VP cells (compare. Fig. 3 and 4b) topoisomerase IIα did not undergo a high-affinity chromatin-bond in the same way as in metaphases of parental H69-WT (Fig. 5, WT, M) although a fraction (5%) of the enzyme was bound to the metaphase chromatin with low affinity. Furthermore, topoisomerase IIβ does not get recruited to the mitotic chromatin instead of topoisomerase IIα when the latter is lacking in the nucleus at the onset of mitosis.

4.3.4 DNA mass distribution

Finally, the DNA-mass content and distribution of the two cell lines under normal growth conditions were measured by flow cytometry (Fig. 6). The parental cell line H69-WT (Fig. 6 left panel) had a normal diploid genotype with a cell cycle distribution, not unusual for a tumour cell line (G_1= 41 ± 4 %, S = 46 ± 7 %, G_2/M = 13 ± 2 %). A more complex distribution of DNA-mass was observed for H69-VP (Fig. 6, right panel), which had an increased peak at the 2 n position and an additional broad

peak at a position of 4 n or larger. In the mutant cell line blocked in S-phase with aphidicolin or blocked in G_2/M-phase with demecolcine two peaks were observed in contrast to the one peak found in the parental cell line. It implied that more than 90% of the H69-VP cells were tetraploid or had genomes of higher ploidity, whereas the parental cells contained less than 5% aneuploid cells. The results in figure 6 together with fluorescence images showed that the nuclei of H69-VP cells were disfigured and of much larger average size than those of parental H69-cells suggesting a high frequency of non-disjunction in these cells.

4.4 DNA-topoisomerase IIα like protein is associated with centrosomes

It has puzzled our group that a high concentration of the monoclonal topoisomerase IIα antibody (named Ki-S1) was found in small globular DNA-free structures at the poles of the mitotic spindle. This was observed in two distinct cell types, a lung cancer cell line H69-WT (Fig. 4, top) and a human epidermoid carcinoma A431 (Meyer et al, 1996). These structures are suggested to be centrosomes. However, *in vivo* no findings of DNA topoisomerase II associated with the centrosomes have been reported. Here, in the second part of my thesis, I have examined if the centrosomal protein is identical to DNA topoisomerase IIα.

4.4.1 Several domains of topoisomerase IIα co-localize with γ-tubulin

Localization of topoisomerase IIα and the centrosomal protein γ-tubulin has been studied. The fluorescence images of A431 cells and human peripheral lymphocytes stained with antibodies highly specific for topoisomerase IIα and γ-tubulin are shown in figure 7. In addition, DAPI staining of DNA was used to locate the nucleus.

4.4.2 Topoisomerase IIα co-localize with γ-tubulin in A431

The representative images of the whole cell population in human A431 cells in logarithmic growth are shown in figure 7 (a). Using the monoclonal antibody Ki-S1, the enzyme appeared to be concentrated in numerous spots located in the nucleoplasm in the interphase of A431 cells as described recently in Meyer et al, 1996. Furthermore, small extra nuclear globular structures were also stained. However, these did not show up in DAPI staining. In mitotic A431 cells DNA topoisomerase IIα is labelled along the central axis of the chromosome arms, and staining of the small DNA-free globular structures located at the spindle poles of the mitotic cells was also noted with the Ki-S1. These globular structures are supposed to be centrosomes. To confirm this assumption, A431 cells were stained with γ-tubulin, which is one of several proteins found specifically in the centrosomes. This highly specific antibody for centrosomes stained the same globular structures as seen for Ki-S1, as shown in the middle panel of figure 7 (a), indicating a co-localization of topoisomerase IIα with γ-tubulin. It should be noted that both Ki-S1 and γ-tubulin stained the multiple set of centrosomes seen in a polyploid cell (Doxsey et al, 1994). Apparently, the Ki-S1 stained the centrosomes not only in the interphase but also during mitosis. Moreover, the staining of centrosomes for the Ki-S1 seems to be specific. It could be completely blocked by pre-incubating the antibodies (Ki-S1 or γ-tubulin) with heat-inactivated recombinant topoisomerase IIα, whereas the signal from the γ-tubulin antibody was not diminished (Fig. 7b).

4.4.3 Topoisomerase IIα co-localize with γ-tubulin in lymphocytes

The staining of topoisomerase at the centrosomes seen in A431 cells is not unique for human tumour cells since similar staining pattern has been seen in quiescent lymphocytes. The peripheral human blood lymphocytes

has been isolated, according to the literature (Bornens et al, 1987; Komesli et al, 1989), and cultured for 24 h. Subsequently, cells were cytospinned onto the slides, and triple stained for Ki-S1 (top), γ-tubulin (middle), and DAPI (bottom), as seen in figure 7 (c). As expected, cells which do not express normal topoisomerase IIα, the nuclei of lymphocytes were barely stained with Ki-S1. Surprisingly, a strong signal of Ki-S1 was obtained in the centrosomes, which co-localized with the γ-tubulin staining (middle in Fig. 7c). To summarize, a staining of Ki-S1 at the centrosomes in two distinct cell types A431 cells and lymphocytes was observed. To exclude the possibility that the Ki-S1 antibody cross-reacts with other proteins in the centrosome, which are not related to topoisomerase IIα, other peptide antibodies against the unique amino-terminal (Fig. 7e) or the carboxy-terminal topoisomerase IIα were used (Fig. 7d). It turned out that both antibodies stained centrosomes in A431 cells as well as the lymphocytes in the same way as for Ki-S1.

To sum up, the staining-pattern from the results in figure 7 indicates that topoisomerase IIα is a component in centrosomes. The question is whether this centrosomal protein is identical to the nuclear DNA topoisomerase IIα. However, a difference is found because the expression of this centrosomal protein is not cell cycle dependent as seen for normal topoisomerase IIα.

4.4.4 Topoisomerase IIα- like protein of 205 kDa in the cytosolic fraction

To confirm that DNA-topoisomerase IIα might be localized in the centrosomes, the cytosolic fraction obtained from proliferating A431 cells was probed in Western blot, with the use of peptide antibodies for topoisomerase IIα in figure 8 (a). Unexpectedly, a protein with an apparent size of 205 kDa was observed, using a highly specific

topoisomerase IIα antibody (lane 2). However, antibodies for topoisomerase IIβ could not recognize this protein (data not shown). In the nuclear extract the IIα-form was at the usual position, equivalent to a detected molecular size of 170 kDa (lane 3). To exclude the observed band at 205 kDa as an electrophoretic artefact, lysate of whole cells A431 was subjected to a SDS-polyacrylamide gel. The results showed that within the same lane not only the expected 170 kDa band but also the 205 kDa band was detected by topoisomerase IIα antibody (lane 1). Furthermore, by using quiescent lymphocytes, which do not express topoisomerase IIα, a similar 205 kDa protein was labelled with the topoisomerase IIα antibody. As seen for A431 cell, the protein was only detected in the cytosolic fraction (lane 5). Is the topoisomerase IIα-like protein at 205 kDa found in Western blot associated with the staining of centrosomes with various topoisomerase IIα antibodies seen in immunofluorescence?

4.4.5 The topoisomerase IIα-like protein is present in isolated centrosomes

To answer the above question, centrosomes from A431 cells were isolated by sedimented the cytosolic fraction through a sucrose density gradient and probed Western blot of the gradient fractions with antibodies against γ-tubulin (middle panel) and topoisomerase IIα (top panel) in figure 8 (b). Most of the centrosomes present in the loaded material entered the gradient and formed a strong peak at a sucrose concentration of 38-48% (fraction 2-4), which could be identified by a specific γ-tubulin antibody. The presence of tubulin in these fractions is supported by findings in the literature (Komesli et al, 1989; Hsu & White, 1998). Interestingly, the topoisomerase IIα-like protein of 205 kDa has been co-sedimented with the γ-tubulin antibody and is therefore present in the

same fractions. This implies that the protein, which differs in size from nuclear topoisomerase IIα, is associated with centrosomes and is responsible for the labelling of these organelles by topoisomerase IIα antibodies. It should be noted that detection of this centrosomal protein required alterations of the Western blotting procedure. The next step is to investigate why the centrosomal protein differs in molecular weight from the normal topoisomerase IIα.

4.4.6 The centrosomal protein is not a splice variant of normal topoisomerase IIα

The protein, which has a higher molecular weight compared to normal topoisomerase IIα, might be a splice variant of the normal enzyme. To find possible variants, PCR was performed on cDNA that had been reversely transcribed from mRNA isolated from proliferating A431 cells which contain both the centrosomal protein of 205 kDa and the normal topoisomerase IIα. Primers were selected such that the four sets of PCR-primers covered all the splice sites of the enzyme based on recent data available on the intron/exon-structure of the human DNA topoisomerase IIα-gene (Fig. 9). These amplified products were subjected to submarine electrophoresis, and, after staining with ethidiumbromide, the PCR product could be visualized on the agarose gel. The PCR products obtained were derived from normal spliced topoisomerase IIα transcript in A431 cells, as seen in figure 10 (b) (lanes 1-4), whereas no additional products of increased length, which could be due to splice variants of normal topoisomerase IIα, were observed.

The centrosomal protein of 250 kDa might not be identical with DNA topoisomerase II except in the extreme N- and C-terminal domains, which are recognized by the highly specific topoisomerase IIα antibodies. To screen for such novel gene products, a new set of primers was designed, corresponding to the peptide epitopes of rabbit antibodies

directed against the N-terminus (amino acid residues 1-12) and the C-terminus (amino acid residues 1513-1531) of human topoisomerase IIα. Using these primers, PCR was performed on cDNA from proliferating A431 cells or quiescent human blood lymphocytes, which contain the 205 kDa protein (Fig. 10a). A single PCR-product corresponding to normal topoisomerase IIα on 4.5 kb was amplified in A431 cells by combination of a forward primer of the N-terminal epitope and a reversed primer of the C-terminal epitope (lane 1). As expected no transcript of normal topoisomerase IIα was generated in the lymphocytes, and as seen for A431 cells no further PCR products were found (lane 1), indicating that the centrosomal protein is not due to a splice variant of normal DNA topoisomerase IIα. Moreover, to avoid that the centrosomal protein of 205 kDa might be derived from an unrelated gene product containing these epitopes in any other conjunction, a combination of a reverse primer of the N-terminal epitope with a forward primer of the C-terminal epitope was done (Fig. 10b). However, no PCR products in either of the cells were obtained (lanes 2 and 6). It is most likely that the centrosomal protein is a post-translationally modified variant of topoisomerase IIα.

4.4.7 The centrosomal protein is not a hyperphosphorlated form of normal topoisomerase IIα

The abnormal position of the 205 kDa band seen in Western blot probing with topoisomerase IIα could be explained by a post-translational modification as phosphorylation. Topoisomerase IIα is *in vivo* a phospho-protein and becomes hyperphosphorylated during mitosis. Our group has recently in A431 cells observed that the electrophoretic position of the endogenous topoisomerase IIα is shifted upward by phosphorylation (Meyer et al, 1996). Furthermore, other groups have also observed an electrophoretic mobility shift caused by phosphorylation of DNA

topoisomerase (Burden & Sullivan, 1994; Kimura et al, 1994). However, it has been assumed that a hyperphosphorylated population of topoisomerase IIα might exist in the cytosol. To obtain evidence that the 205 kDa protein in the cytosolic fraction from the lymphocytes could be a hyperphosphorylated form of topoisomerase IIα, the protein was treated with various phosphatases (Fig. 11). Topoisomerase IIα from nuclear extract of A431 cells was added to the cytosolic fraction to serve as an endogenous control. The protein of 205 kDa was not dephosphorylated even by extensive treatment with alkaline or acid phosphatase, whereas an effect on the normal nuclear topoisomerase IIα was observed in a similar fashion as described in Meyer et al, 1996, indicating that the enzymatic de-phosphorylation was effective (lanes 1-5). Based on these results, the centrosomal 205 kDa protein is not derived from normal topoisomerase IIα by hyperphosphorylation.

4.5 The centrosomal protein may not exhibit a type II activity

To measure the activity the centrosome extract (see Methods) made from lymphocytes was incubated with the catenated DNA from kinetoplast of *Chrithida fasciculata* (kDNA). Undiluted extract failed to detect any DNA-decatenation activity. However, recombinant topoisomerase IIα admixed with the centrosome extract was also not capable of decatenating the kDNA. Thus, it is not clear whether the centrosomal protein does not have any DNA topoisomerase II activity or whether it is inactive due to the presence of inhibitors of the enzyme activity in the centrosome extract.