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p34^{cdc2} is located in both nucleus and cytoplasm; part is centrosomally associated at G_2/M and enters vesicles at anaphase

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The *cdc2*$^+$ gene product p34^{cdc2} is located immunocytochemically in both the nucleus and cytoplasm of human cells. It is uniformly distributed throughout the cytoplasm and is irregularly distributed in the nucleus. Part of p34^{cdc2} is associated with the centrosome and centrosomal staining increases late in the cell cycle and at the onset of mitosis. This distribution is corroborated by cell fractionation which also indicates that slower migrating forms of p34^{cdc2} are found in isolated centrosomes and in Triton-insoluble fractions. We propose that one role of the p34^{cdc2} protein kinase is to modify the centrosome bringing about formation of the mitotic spindle. At anaphase p34^{cdc2} becomes associated with vesicles in the middle of the cell between the reforming nuclei. A similar location is found for p13^{suc1} and we suggest that the vesicular localization plays a role in p34^{cdc2} kinase inactivation at the end of mitosis.

Key words: cell cycle/centrosome/mitosis/p34^{cdc2}

Introduction

The centrosome acts as a microtubule organizing centre in animal cells. During the cell cycle the centrosome duplicates and the two daughter centrosomes function as mitotic poles. Isolated centrosomes are able to nucleate microtubles *in vitro* and to induce parthenogenesis in *Xenopus* eggs (Mitchison and Kirschner, 1984; Karsenti *et al.*, 1984; Bornens *et al.*, 1987), directly demonstrating the old view of the centrosome as 'the division organ' (Boveri, 1901).

Progress through the cell cycle including changes in behaviour of the centrosome is believed to be controlled by a cytoplasmic oscillator (Hara *et al.*, 1980). A crucial element of this oscillator is maturation-promoting factor (MPF) which controls entry into meiosis and mitosis in oocytes. MPF is found in many cells from yeast to humans (for a review, see Maller, 1985) and has recently been highly purified (Lohka *et al.*, 1988). Data from several laboratories have demonstrated that one component of MPF is a 34 kd protein kinase homologue of the product of the cell cycle gene *cdc2*$^+$ of fission yeast equivalent to *CDC28* in budding yeast (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbé *et al.*, 1988). The human homologue of *cdc2*$^+$ has been identified (Lee and Nurse, 1987; Draetta *et al.*, 1987), and its expression studied during the cell cycle (Lee *et al.*, 1988;. Draetta and Beach, 1988); p34^{cdc2} protein kinase activity is maximal during metaphase (Draetta and Beach, 1988; Labbé *et al.*, 1988) suggesting that protein phosphorylation plays a key role in regulating progress through the cell cycle.

Phosphoproteins are associated with the mitotic poles at the onset of mitosis (Davis *et al.*, 1983; Vandre *et al.*, 1986). An increase in centrosome-specific staining with anti-centrosome antibodies occurs during G_2 and a dramatic decrease takes place at the onset of anaphase (Gosti-Testu *et al.*, 1986). These centrosomal modifications, which are likely to correspond to a rise and fall of microtubule nucleating activity (for a review, see Bornens and Karsenti, 1984), may well involve changes in phosphorylation. In this paper we describe the cellular distribution of p34^{cdc2} in human cells and show that it is located both in the nucleus and in the cytoplasm. We establish that a fraction of the protein is associated with centrosomes suggesting that the p34^{cdc2} protein kinase may phosphorylate centrosomal proteins leading to the generation of a mitotic spindle. We also describe a shift in p34^{cdc2} distribution at anaphase leading to sequestration in vesicles, a process which could be relevant for the inactivation of the kinase at the end of mitosis. Finally the general distribution p13^{suc1}, a protein known to interact with p34^{cdc2}, was found to be strikingly similar to that of p34^{cdc2} in HeLa cells.

Results

p34^{cdc2} is found in both nucleus and cytoplasm in interphase cells and part is associated with centrosomes

Immunofluorescence of HeLa cells with retro-eluted affinity-purified antibodies against the conserved peptide EGVPST-AIREISLLKE (or 'PSTAIR') of *cdc2*-like proteins demonstrated that p34^{cdc2} was distributed in both the nucleus and the cytoplasm (Figure 1A and I). The nuclear staining was definite, irregular and apparently located in zones from which DNA was excluded (see below). Staining redistributed into fewer large zones within the nucleus in G_2 cells. The cytoplasmic staining was generally uniform, although in ~20–30% of the total population more prominent staining was observed in a small area of the cytoplasm just next to the nucleus (juxtanuclear staining; see Figure 1A).

To examine if this feature corresponded to the centrosome we used a specific marker of this organelle, the monoclonal antibody CTR453, which specifically reacts with peri-centriolar material. This antibody enabled the position of cells in the cell cycle to be approximately determined because pericentriolar staining increases as cells progress towards mitosis. Centrosomal staining is maximal in metaphase and decreases dramatically during anaphase, returning to a minimal level in telophase (unpublished result). Using this

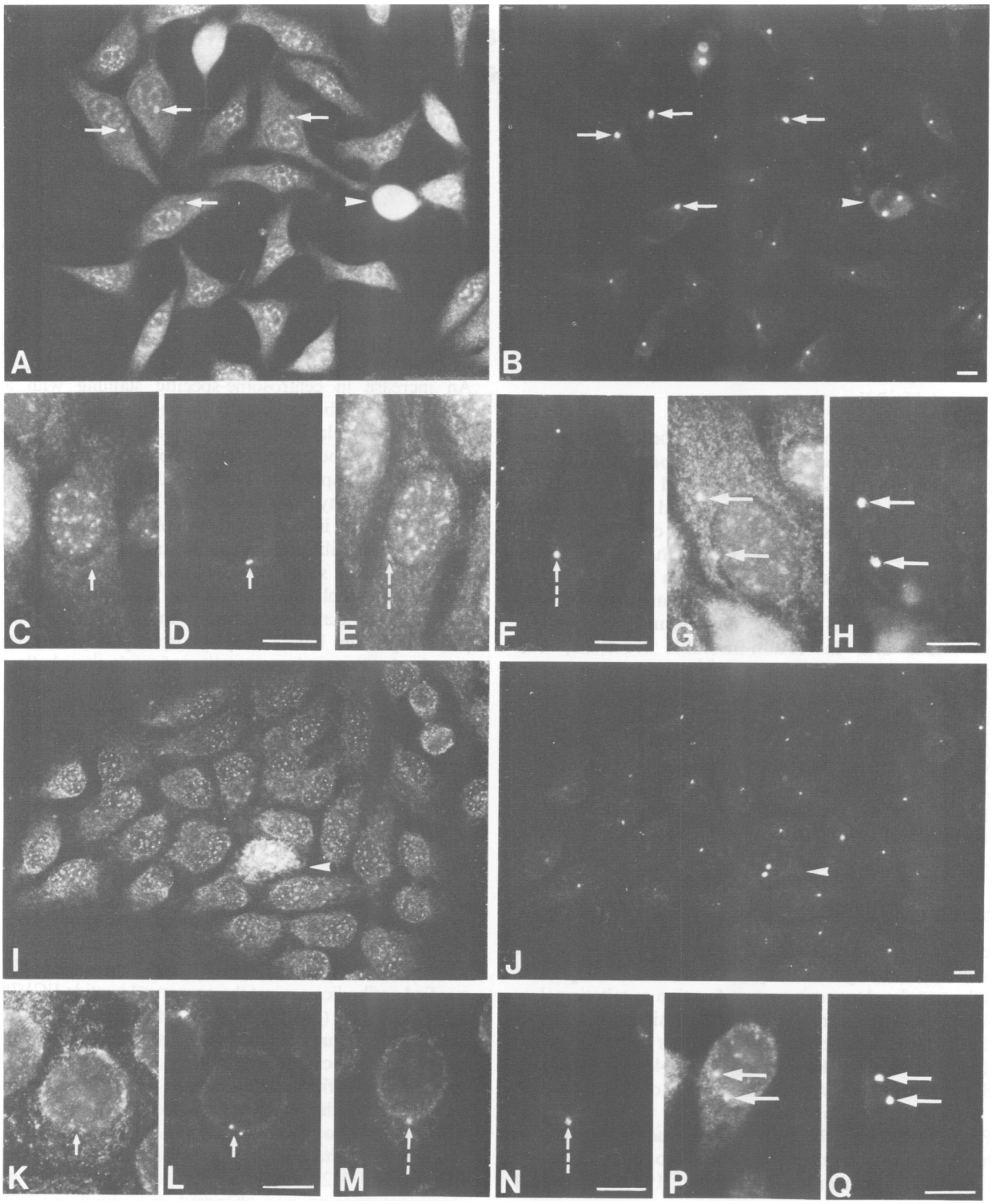

Fig. 1. Immunolocalization of p34^{cdc2} in interphase HeLa cells. Two fixation protocols have been used: methanol fixation (**A−H**) and Triton extraction before fixation (**I−Q**; see Materials and methods). Fixed cells were processed for double immunofluorescence with affinity-purified rabbit anti-p34^{cdc2} antibodies (**A,C,E,G,I,K,M,P**) and CTR453 (**B,D,F,H,J,L,N,Q**). **A,B**: low magnification of unextracted cells. p34^{cdc2} appears distributed irregularly in the nucleus and uniformly in cytoplasm (**A**). In few cells a juxtanuclear bright dot was observed (small arrows in **A**). It corresponded to centrosomes heavily stained with CTR453 (small arrows in **B**). The arrowheads in **A** and **B** indicate a cell in pre-prophase, i.e. with the duplicated centrosomes already separated. **C−H**: distribution of p34^{cdc2} according to the cell cycle. **C−D**: there is no association of p34^{cdc2} with the centrosome in cells early in the cell cycle identified by their small CTR453 centrosomal staining which figures the two centrioles (small arrows). **E,F**: a cell later in the cell cycle where p34^{cdc2} is first detected at the centrosome (dotted arrows). In the upper left corner in **F** the dots correspond to the two centrioles of a split centrosome of a cell early in the cell cycle. **G,H**: a pre-mitotic cell with the two duplicated centrosomes brightly stained with both antibodies (large arrows). **I,J**: Low magnification of Triton-extracted cells; p34^{cdc2} staining is significantly decreased in both nucleus and cytoplasm. The nuclear staining is more spotty. In a few interphase cells, identified as being in late G_2 using CTR453, p34^{cdc2} is less extracted, particularly in the nucleus (arrow heads). **K,L**: no significant accumulation of p34^{cdc2} was observed at the centrosome of G_1 cells (small arrows). Later in the cell cycle centrosome-associated p34^{cdc2} is not extracted by Triton (dotted arrows in **M,N** and large arrows in **P** and **Q**). Bars represent 5 μm.

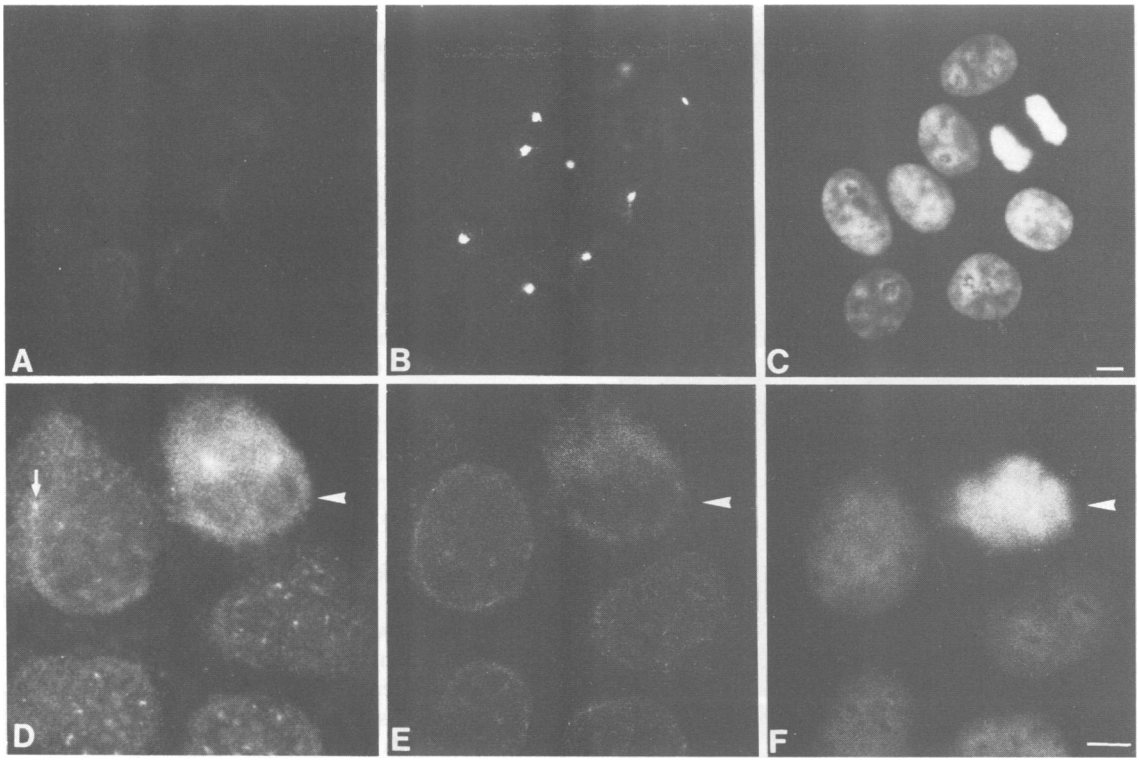

Fig. 2. Inhibition of the PSTAIR staining by the antigenic peptide and centrosomal accumulation of p34^{cdc2} in cells at the onset of mitosis. The cellular staining obtained with the affinity-purified antibody against the oligopeptide PSTAIR is abolished by pre-incubation of the antibody with the peptide (**A**) whereas the centrosomal staining with CTR453 is not (**B**). The DAPI staining is shown in **C**. Separating centrosomes at the onset of mitosis in HeLa cells are stained by P34^{cdc2} antibodies (arrowhead in **D**). Disassembly of nuclear lamina has occurred, as observed by double immunofluorescence with an antibody to lamin B (**E**) and chromosome condensation has started, as shown by DNA staining with DAPI (**F**). The small arrow in **D** points to a centrosome stained in an earlier stage. Bars represent 5 μm.

CTR453 marker and the PSTAIR antibody in double immunofluorescence we could establish that the juxtanuclear staining corresponded to the centrosome (Figure 1A and B), and that these cells were located late in the cell cycle before mitosis. In G_1 cells, which stained lightly with CTR453 as two tiny dots (Figure 1D), the PSTAIR antibody did not recognize any centrosome-associated material (Figure 1C). In contrast, it readily recognized one or two large spots (Figure 1A and G) identified as centrosomes with CTR453 in G_2 cells (Figure 1B and H). Intermediary staining was also sometimes observed (Figure 1E and F). It is noteworthy that the contour of the p34^{cdc2} centrosomal staining was not perfectly congruent with that of CTR453 staining.

To confirm that observed staining was specific for p34^{cdc2} we repeated these experiments after pre-incubating the antibody with the PSTAIR peptide and thus competing for the antigenic site on p34^{cdc2}. All the cellular staining including that associated with the centrosome was suppressed by this pre-incubation (Figure 2A–C). Further confirmation of the specificity of p34^{cdc2} was obtained by using a second affinity purified rabbit antibody raised against the peptide LDNQIKKM corresponding to the carboxy terminus of CDC2HS (Lee and Nurse, 1987). The pattern of staining with this antibody in the cytoplasm, nucleus and centrosome of the HeLa cells was similar in all respects to that obtained with the PSTAIR antibody. We conclude that p34^{cdc2} is distributed throughout the interphase cell in both the nucleus and cytoplasm, and that part of it is associated with centrosomes in G_2 cells.

Both the cytoplasmic and nuclear staining of p34^{cdc2} was significantly decreased by Triton X-100 extraction (Figure 1I and J). Compared with methanol fixation, the nuclear distribution appeared more spotty (compare Figure 1A and I). Centrosomal staining became rather more obvious, probably due to the lower cytoplasmic background (Figure 1P and Q) but was still observed only in cells located late in the cell cycle (Figure 1K and L). To check for p34^{cdc2} association with interphase pre-kinetochores we used serum of a scleroderma patient. This serum stained pre-kinetochores but in double immunofluorescence experiments with the anti-PSTAIR antibody we found no evidence for p34^{cdc2} association.

Cellular fractionation confirms localization of p34^{cdc2}

In order to confirm this cellular distribution of p34^{cdc2} we have used subcellular fractionation combined with Western blotting. For this purpose we used lymphoblastic cells (KE37 cell line) which grow in suspension and are easier to fractionate than HeLa cells, although are less suitable for immunofluorescence studies. They are particularly suitable for centrosome isolation (Gosti-Testu *et al.*, 1986; Bornens *et al.*, 1987). The cellular distribution of p34^{cdc2} in these cells as determined by immunofluorescence appeared generally similar to that of HeLa cells (Figure 3). Immunoblotting with the anti-p34^{cdc2} serum revealed that it specifically recognized a 34 kd protein in KE37 extracts (Figure 4A and B). We used different fractionation procedures to distinguish between cytoplasmic and nuclear compartments and to estimate what proportion of the protein p34^{cdc2} was readily soluble or needed detergents for

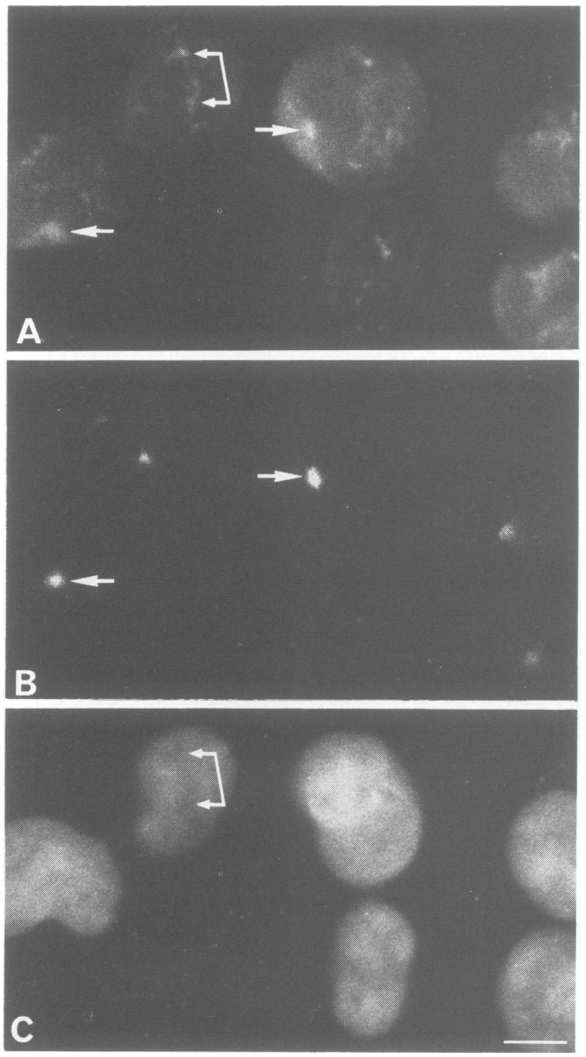

Fig. 3. Immunolocalization of p34^{cdc2} in KE37 cells. After methanol fixation cells were triple labelled with affinity-purified rabbit anti-p34^{cdc2} antibodies (**A**). CTR453 (**B**) and DAPI (**C**). The cellular distribution of p34^{cdc2} is similar to that observed in HeLa cells, including a uniform cytoplasmic distribution, centrosomal accumulation (arrows) in cells late in the cell cycle, as judged by their size, and speckled nuclear staining. Note that the nuclear staining corresponds to the DNA excluded area, as judged by DAPI staining (double arrows in **A** and **C**). Bar represents 5 μm.

solubilization (see Materials and methods). A large proprotion of the protein p34^{cdc2} was recovered in a soluble form after mechanical disruption (lanes 5 and 6, Figure 4D). Most of the insoluble fraction of p34^{cdc2} could be further solubilized by detergents (lanes 2 and 3, Figure 4D). The detergent-insoluble fraction was enriched in electrophoretically slower forms of p34^{cdc2}. These forms were apparently associated with cytoplasmic rather than nuclear structures as shown by cell fractionation using the citric acid procedure (lanes 7 and 8, Figure 4D; see Materials and methods). The latter procedure takes place at pH 3 and provides nuclei deprived of nuclear sap and nuclear envelope, corresponding roughly to a precipitate of nucleoproteins. The centrosomal fraction was enriched for p34^{cdc2} (Figure 4C) and included clearly visible slow-migrating forms (lane 4, Figure 4D). Isolated centrosomes identified with anti-tubulin antibodies were all found to contain p34^{cdc2} using double immunofluorescence (Figure 5). This contrasted with the situation *in vivo* described above when only centrosomes late in the cell cycle became stained with the antibodies. These studies confirm that p34^{cdc2} is located in both the nucleus and cytoplasm and is also associated with centrosomes.

Because slow migrating forms of p34^{cdc2} were found in centrosomes and in detergent-insoluble fractions we investigated if these forms were dependent upon the presence of microtubules. For this purpose we used HeLa cells which possess an abundant microtubular network. Cells were treated for 4 h with Nocodazole or with Taxol in order to induce respectively either complete disassembly of microtubules or complete assembly of cellular tubulin. We then extracted cells with Triton in a microtubule-stabilizing buffer; as in KE37 cells, the slow-migrating forms of p34^{cdc2} specifically resisted Triton extraction. This experiment also established that the presence of these forms was clearly independent of the presence or absence of microtubules (Figure 4E).

During mitosis p34^{cdc2} staining is lost from centrosomes and at anaphase p34^{cdc2} appears in vesicular structures

At the onset of mitosis p34^{cdc2} was distributed throughout both the nucleus and cytoplasm and the centrosomes were prominently stained. Staining for lamin B and DNA identified cells undergoing mitosis (Figure 2D–F). The cell marked with an arrowhead has undergone nuclear lamin disassembly and partial chromosome condensation, and separating centrosomes are very distinctly stained with the PSTAIR antibody. A cell earlier in the cell cycle as judged by lamin B and DNA staining has weaker staining of the centrosome using the PSTAIR antibody (marked with a small arrow in Figure 2D). It can also be seen that there is no observable p34^{cdc2} accumulation at the nuclear periphery where lamins are undergoing disassembly (Figure 2D,E).

In metaphase cells, p34^{cdc2} appeared uniformly distributed in the cytoplasm but was excluded from the chromosomes. The spindle poles were also stained (marked by arrows in Figure 6a) as confirmed using CTR453 (Figure 6b). At the onset of anaphase, a major redistribution of p34^{cdc2} was observed: it was no longer associated with the mitotic poles and appeared to concentrate in numerous spots of different sizes in the middle zone of the cell (Figure 6). These spots had precise boundaries suggestive of vesicles, which appeared to fuse with each other in an interconnected tubular system (Figure 7). These spots could be extracted by Triton X-100.

The accumulation of p34^{cdc2} in vesicle-like spots was investigated using mitotic blocks induced by the microtubule drugs Nocodazole or Taxol. More than 80% of the cells were blocked in a mitotic state after 15–20 h treatment with either drug. CTR453 staining demonstrated that the two drugs did not lead to the same centrosomal configuration. Duplicated centrosomes were separated apart in Nocodazole-treated cells with irregular pieces of pericentriolar material dissociated from them (see Figure 8B). In Taxol-treated cells, both centrosomes were usually tightly associated, suggesting that Taxol prevents their separation (see Figure 8G). In both cases, p34^{cdc2} antibody did not stain the centrosomes. In addition a major part of p34^{cdc2} appeared sequestered in Triton-extractable vesicle-like spots, the average size of which was larger in Nocodazole- than in Taxol-treated cells (Figure 8A and F).

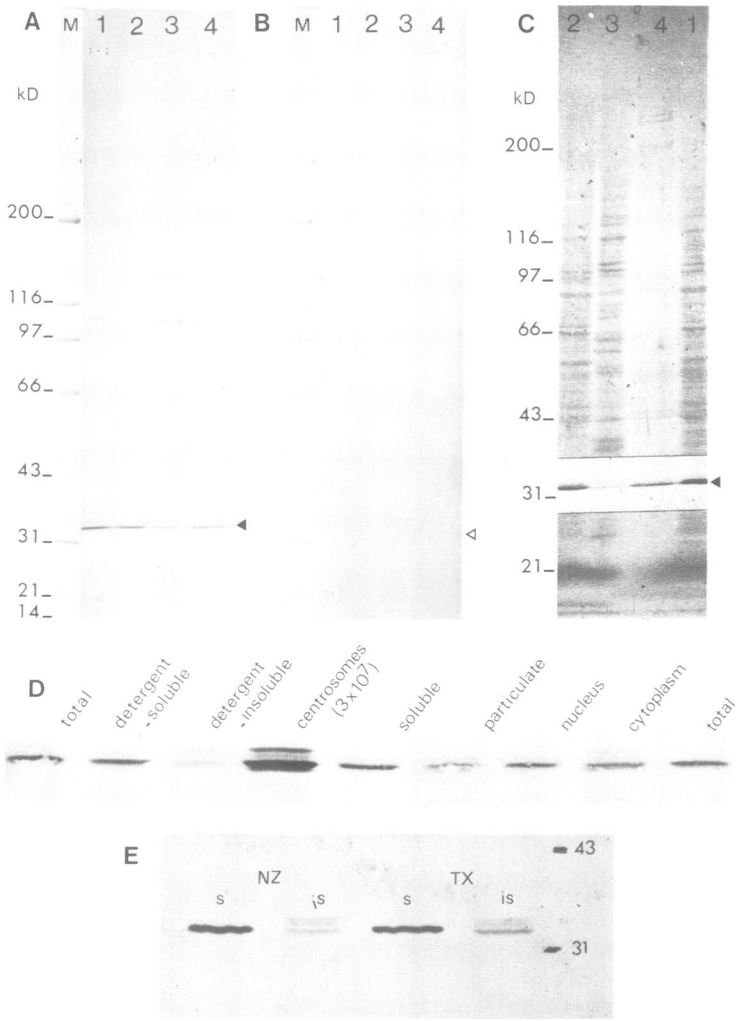

Fig. 4. Immunodetection of p34^{cdc2} in subcellular fractions. Different cellular fractions from unsynchronized KE37 (**A**–**D**) and HeLa cells (**E**) were probed for their p34^{cdc2} content by Western blot. In experiments **A,B** and **C**, total cellular proteins (1) are compared to detergent-soluble (2), detergent-insoluble (3) and centrosomal (4) proteins for their content in p34^{cdc2}. Samples were normalized at 30 μg in all cases except the centrosomes which were estimated to correspond to ~1 μg on the basis of their number (3×10^7 centrosomes; Bornens *et al.*, 1987). (**A**) The affinity-purified antibody reacts exclusively with a component of M_r 34 kd (arrowhead). The immunostaining shows an additional cross-reacting band which appeared erratically at ~100 kd in total proteins and in the detergent-insoluble fraction but which is absent in the soluble fraction and in the centrosome fraction. It is also abolished by pre-incubation of the antibody with the PSTAIR peptide. (**B**) The reaction is specific as it is suppressed by pre-incubation of the antibody with the PSTAIR peptide. (**C**) This experiment visualizes the enrichment of p34^{cdc2} in centrosomal proteins. After electrotransfer, the proteins on the nitrocellulose filter were stained with Ponceau red. Only the relevant M_r region of the filter was probed with the antibody. (**D**) In this experiment, total cellular (lane 1 and 9), detergent-soluble (lane 2), detergent-insoluble (lane 3) and centrosomal (lane 4) proteins were compared to soluble (lane 5), particulate (lane 6), nuclear (lane 7) and non-nuclear (lane 8) proteins (see Materials and methods). The protein separation was achieved on a long 6–15% gradient gel: note the presence of additional migrating forms of p34^{cdc2} in centrosomes, which could be detected also in all fractions expected to contain the centrosomes (fractions 1,3,6 and 8). (In this experiment the centrosomal proteins were obtained from a preparation which was not counted. We estimate the overload with respect to experiments **A**–**C** to be ~10-fold.) (**E**) Content of p34^{cdc2} in soluble (S) and insoluble (IS) fractions of HeLa cells treated during 4 h with Nocodazole (NZ) or Taxol (TX) and then extracted with Triton in PHEM buffer. Note that the low migrating forms specifically resist Triton extraction. Proteins were run on a 12.5% polyacrylamide gel. The migration of the mol. wt standards ovalbumin (43 kd) and carbonic anhydrase (31 kd) have been marked on the nitrocellulose filter after Ponceau red staining and before immunostaining (on the right side).

The redistribution of p34^{cdc2} into vesicle-like structures normally occurred at the onset of anaphase. However, this redistribution also occurred in cells arrested in mitosis by the drugs Nocodazole or Taxol. We conclude that impairment of spindle generation by protracted treatment with these drugs does not prevent the redistribution of the protein p34^{cdc2}. This suggests that the drugs Nococdazole and Taxol do not block strictly at metaphase, because an event such as p34^{cdc2} redistribution can still take place even though this usually occurs at anaphase.

Cellular distribution of p13^{suc1} is similar to that of p34^{cdc2}

Mitotic control in *Schizosaccharomyces pombe* involves the products of several other genes, among which p13^{suc1} has been shown to interact with p34^{cdc2} (Hayles *et al.*, 1986; Brizuela *et al.*, 1987). A 13 kd protein associated with p34^{cdc2} and cross-reacting with an antiserum raised against recombinant yeast p13^{suc1} has also been detected in HeLa cells (Draetta *et al.*, 1987). An affinity-purified antibody directed against the yeast recombinant protein, which

specifically immunoprecipitated a 13 kd protein in HeLa cells (Figure 9, inset), was found to decorate these cells in a manner strikingly similar to that observed for p34^{cdc2}: a definite and speckled nuclear staining was observed together with an apparently uniform cytoplasmic staining (Figure 9A). We could not document the co-localization of the two proteins by double immunofluorescence as the antibodies were both raised in rabbits. Despite these similarities staining differed in one important feature: the centrosomal accumulation in cells late in the cell cycle was obvious in both cases (large arrows, Figure 9A), but the centrosomal staining with the anti-p13^{suc1} antibody was observed throughout the cell cycle including in cells early in the cell cycle (small arrows, Figure 9A).

Since it has been suggested that p13^{suc1} is necessary for exit from the mitotic state (Moreno *et al.*, 1989), we investigated the cellular distribution of p13^{suc1} during mitosis. In metaphase, the whole cell was stained in a diffuse manner except in the area occupied by the chromosomes. The spindle was slightly decorated and the poles heavily so, even in the absence of any detergent extraction (Figure 9C). In ana-telophasic cells, p13^{suc1} apparently accumulated in vesicle-like dots (Figure 9E) between the segregating chromosomes (Figure 9F), a pattern which closely mimicked the decoration observed for p34^{cdc2}.

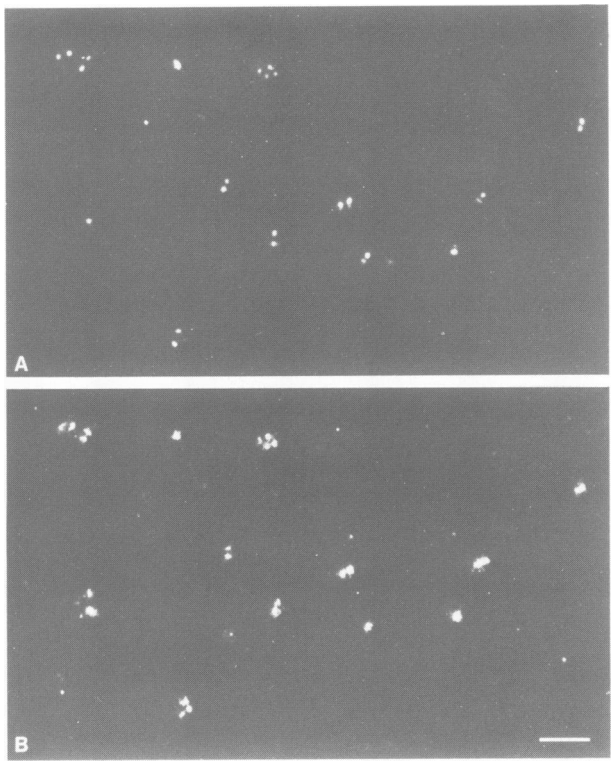

Fig. 5. PSTAIR staining of isolated centrosomes. Double immunofluorescence on isolated centrosomes with anti-tubulin (**A**) and anti-p34^{cdc2} antibodies (**B**). Bar represents 5 μm.

Discussion

Our immunocytochemical study of p34^{cdc2} in HeLa and KE37 cells was carried out with two different retro-eluted affinity-purified antibodies, one raised against the peptide EGVPSTAIREISLLKE which is conserved in all species, and the other raised against the peptide LDNQIKKM which corresponds to the carboxy terminus of the human protein. The results were identical with both antibodies. Several conclusions can be drawn. (i) In interphase cells, p34^{cdc2} is present in the cytoplasm in an apparently uniform manner, and in the nucleus in an irregular and speckled manner. Nuclear staining is located in zones from which DNA is excluded and is redistributed into a few large zones in late G_2. Staining of the centrosome is observed in cells later in the cell cycle identified as such by the increased staining obtained with an anti-pericentriolar material(PCM) monoclonal antibody. In late G_2 cells, identified by the presence of two separated centrosomes, p34^{cdc2} is still associated

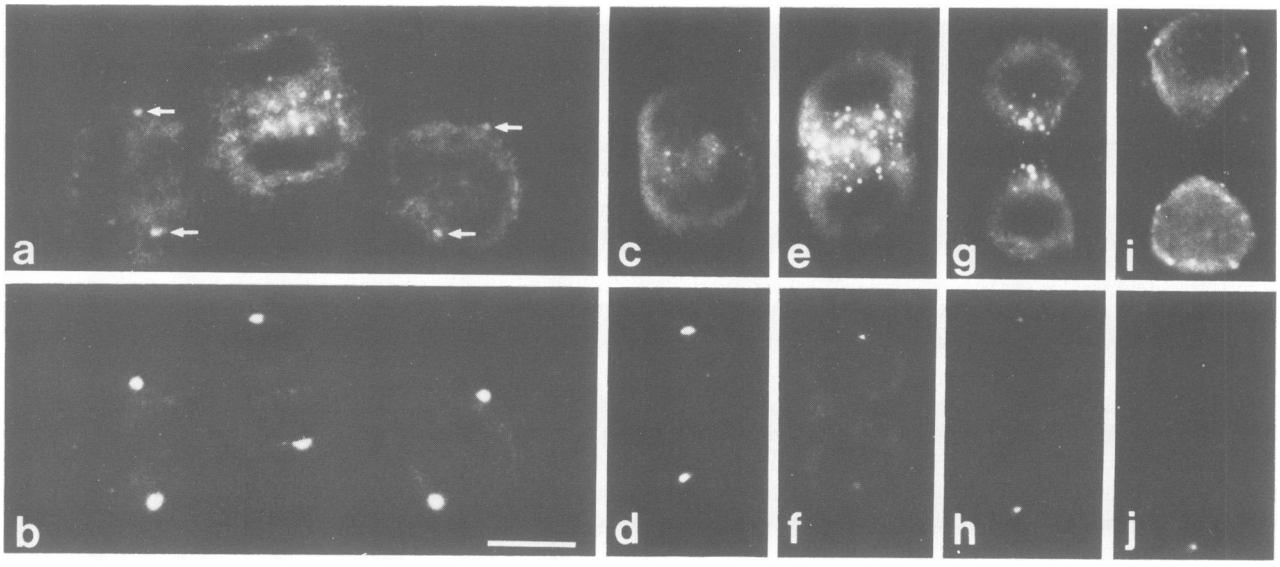

Fig. 6. Immunolocalization of p34^{cdc2} in mitotic HeLa cells. Methanol-fixed cells were double stained with PSTAIR antibody (**a,c,e,g,i**) and with CTR453 (**b,d,f,h,j**). Diffuse distribution of p34^{cdc2} was observed in metaphase cells in which discrete staining of the spindle poles could be detected (small arrows in **a**). In early anaphase (**c,d**), spindle poles were not stained and p34^{cdc2} seemed to concentrate in numerous spots of various sizes in the middle zone. This process increased during anaphase (**e**; see also the cell in the center of **a**). In telophase, the vesicle-like spots segregate in daughter cells (**g,i**). Note that centrosomal CTR453 staining dramatically decreased from metaphase to telophase. Bar represents 5 μm.

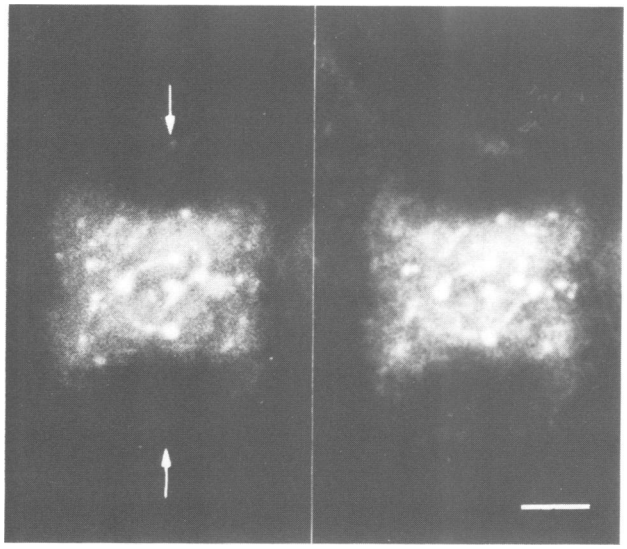

Fig. 7. Sequestration of p34^{cdc2} in anaphase. Two optical sections of the same ana-telophase HeLa cell demonstrate that p34^{cdc2} staining is concentrated in an interconnected tubular system between the two segregated sets of chromosomes. Arrows indicate the position of the spindle poles. Bar represents 2 μm.

with centrosomes. Part of the general staining can be suppressed by Triton extraction before fixation but the centrosomal protein is insoluble in these conditions and the centrosomal staining is more pronounced. (ii) In mitotic cells p34^{cdc2} becomes redistributed in the following manner. The protein is uniformly distributed through the cytoplasm of metaphase cells, with a definite accumulation at the mitotic spindle poles and with no apparent association with the chromosomes. At the onset of anaphase, the mitotic poles cease to be stained, and p34^{cdc2} becomes concentrated in small vesicle-like Triton-extractable structures which accumulate in the mid-zone between the two sets of chromosomes. The vesicles appear to interconnect in a tubular system. Most of the vesicular protein in post-metaphasic cells can be extracted by Triton, but not that which is associated with the mitotic poles in metaphase.

Our results indicate that p34^{cdc2} is distributed both in the nucleus and in the cytoplasm. The cytoplasmic location is consistent with previous reports demonstrating that enucleated oocytes induced to mature develop p34^{cdc2} kinase activity that oscillates in phase with the cell cycle (Dabauvalle *et al.*, 1988; Picard *et al.*, 1988). The fact that the cellular distribution of p34^{cdc2} is similar to that of p13^{suc1} is also expected given previous reports showing that p13^{suc1} co-immunoprecipitates with p34^{cdc2} in HeLa cells (Draetta and Beach, 1988). p13^{suc1} is probably in excess in these cells as it can be immunoprecipitated without concommitant precipitation of much p34^{cdc2} (Draetta and Beach, 1988; this work Figure 9 inset). Our data suggest that both forms of p13^{suc1} co-distribute within human cells.

The cellular distribution of p34^{cdc2} deduced from the immunofluorescence study was confirmed by biochemical data obtained using several independent fractionation procedures. p34^{cdc2} was found in both cytoplasmic and nuclear fractions. These experiments further showed in both HeLa and KE37 cells that a large amount of p34^{cdc2} was recovered in soluble form, and that higher mol. wt forms resist Triton extraction, suggesting that they are more strongly associated with insoluble structures. These structures are apparently in the cytoplasm rather than in the nucleus and do not depend upon the presence of microtubules. Although this has not been demonstrated directly, these forms of p34^{cdc2} may be hyperphosphorylated.

p34^{cdc2} is found at quite a high level in preparations of isolated centrosomes, and is associated with each individual centrosome. Higher mol. wt forms of p34^{cdc2} are also found in the centrosomal fractions. The association of p34^{cdc2} with each individual isolated centrosome is at variance with the situation *in situ*, where centrosomes are stained in G_2 and G_2/M cells only. The same discrepancy was also observed (data not shown) with another marker of G_2/M centrosomes, the monoclonal MPM2 raised against mitotic HeLa cells (Davis *et al.*, 1983). This could be explained either by p34^{cdc2} redistribution during centrosome isolation, or by the accessibility of the protein *in vivo*, which precludes its detection below a threshold concentration at the centrosome. One cannot eliminate also the possibility that the antigenic PSTAIR regions of the centrosome-associated p34^{cdc2} are masked *in vivo* through interaction with another protein which is released during the isolation procedure and during late G_2. In this respect, the cell cycle-dependent decoration observed with the PSTAIR antibody might have functional implications as microinjection of the PSTAIR peptide into starfish oocytes has recently been shown to induce meiotic maturation. This suggests that an inhibitory component in G_2-arrested oocytes interacts with the PSTAIR region of the p34^{cdc2} kinase which could block antibody staining (Labbé *et al.*, 1989). This aspect requires further work on isolated centrosomes.

The finding that p34^{cdc2} is associated with centrosomes just before the onset of mitosis has important implications for mitotic control. One of the major processes at mitosis is the formation of a mitotic spindle between the two centrosomal microtubular organizing centres. The association of p34^{cdc2} with the centrosome coincides with ultrastructural modifications of the pericentriolar material (Robbins and Gonatas, 1964. Rieder and Borisy, 1982) and with a rise in the nucleating activity of centrosomes (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981). The dissociation of p34^{cdc2} from the mitotic poles occurs when centrosomal microtubule nucleation activity is reduced in other cell systems (Snyder *et al.*, 1982). Therefore, the p34^{cdc2} protein kinase could bring about modifications of the centrosome just before mitosis by phosphorylating centrosomal proteins which lead to an increase in microtubule nucleation generating a mitotic spindle. Later on in mitosis p34^{cdc2} dissociates from the centrosomes and nucleating activity is reduced. Thus, part of the mechanism by which p34^{cdc2} initiates mitosis would be by modification of the centrosomes to generate a mitotic spindle. This could account for the release of mature starfish oocytes from interphase arrest after injection of centrosomes isolated from KE37 cells (Picard *et al.*, 1987). We also expect that p34^{cdc2} has other roles at the initiation of mitosis particularly given its rather widespread localization throughout both the cytoplasm and nucleus of the cell.

During anaphase p34^{cdc2} becomes sequestered in vesicles located at the mid-point of the dividing cell. These are particularly apparent in cells blocked in mitosis by Nocodazole or Taxol. In both cases the protein p34^{cdc2} is largely sequestrated in vesicle-like structures which are

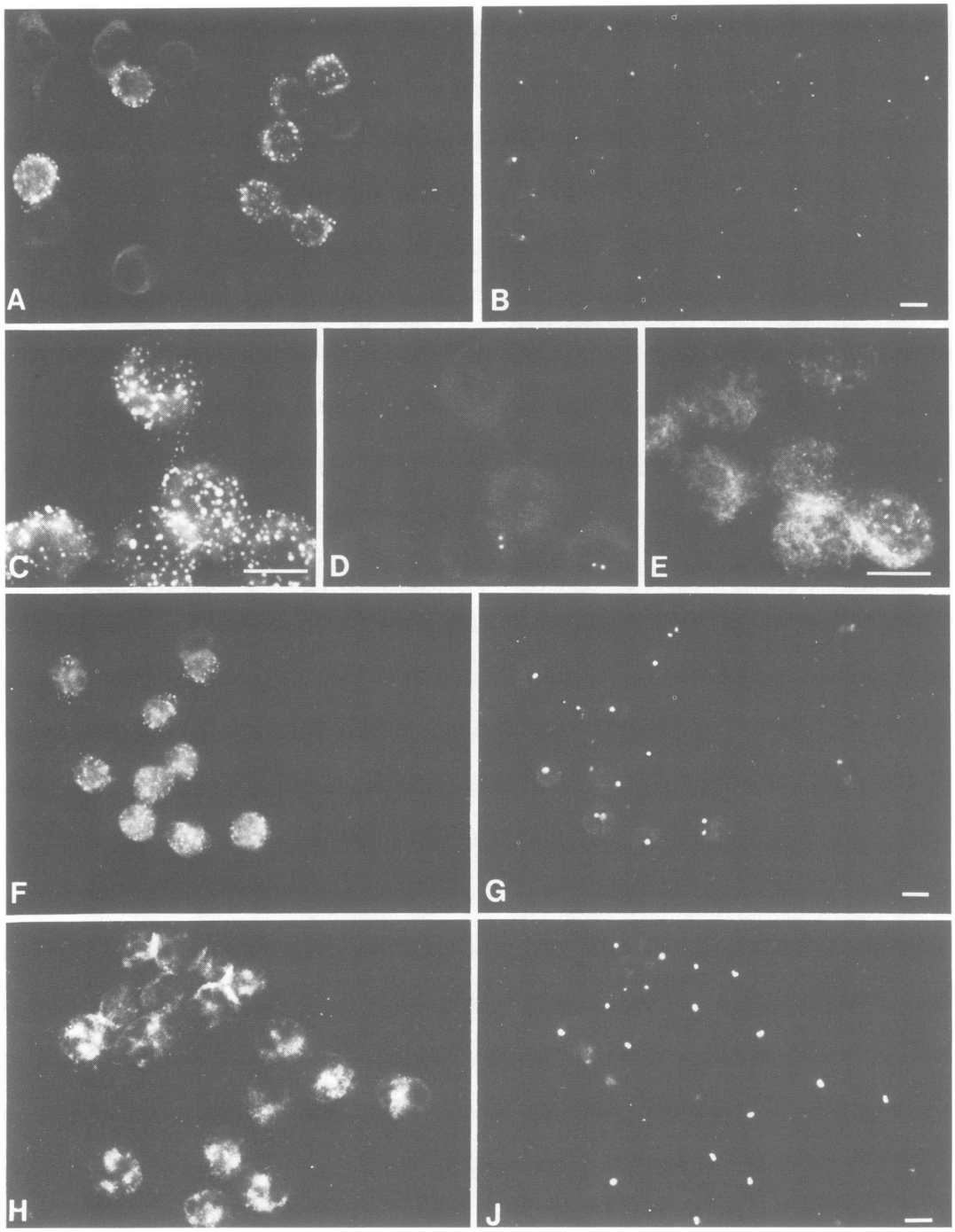

Fig. 8. Cellular localization of p34^{cdc2} in mitotic-arrested HeLa cells. Mitotic cells were blocked with Nocodazole (**A,C,E**) or Taxol (**FH**). Double staining with CTR453 (**B,D,G** and **J**) is presented in each case (except in **E**). **A−D,F,G**: methanol fixation. **E,H−J**: Triton extraction before fixation. The major part of p34^{cdc2} appeared sequestered in vesicle-like dots in mitotic blocked cells only; vesicles were larger in Nocodazole- (**A**) than in Taxol-blocked mitoses (**F**). Most of the vesicle-like dots were extracted by Triton (compare **C** to **E** and **F** to **H**). CTR453 staining demonstrated that each drug results in specific arrest of the centrosome cycle (compare **B** to **G** or **J**). Bars represent 5 μm.

Triton extractable, contrasting with the diffuse distribution observed in normal metaphase cells. This suggests that mitotic arrest induced by microtubule drugs does not prevent the redistribution of p34^{cdc2}; therefore the arrest is not strictly at metaphase because the anaphase event of p34^{cdc2} redistribution can still take place. The sequestration could ensure equal partitioning of p34^{cdc2} between sister cells, as the staining vesicles appear around the reforming nuclei in telophase. Another possibility is that sequestration may play a role in the fall of p34^{cdc2} protein kinase activity which takes place during mitosis (Draetta and Beach, 1988; Labbé *et al.*, 1988; Moreno *et al.*, 1989). In support of this possibility, we have observed that p13^{suc1} also accumulates in post-metaphasic vesicles. As the product of *suc1*$^{+}$ is required for leaving the mitotic state in *S.pombe* (Moreno *et al.*, 1989) and forms a complex with p34^{cdc2} in *S.pombe* and human cells (Brizuela *et al.*, 1987; Draetta *et al.*, 1987), it is possible that it is instrumental in the sequestration process which results ultimately in kinase inactivation. If this is the case then the sequestration process must precede kinase

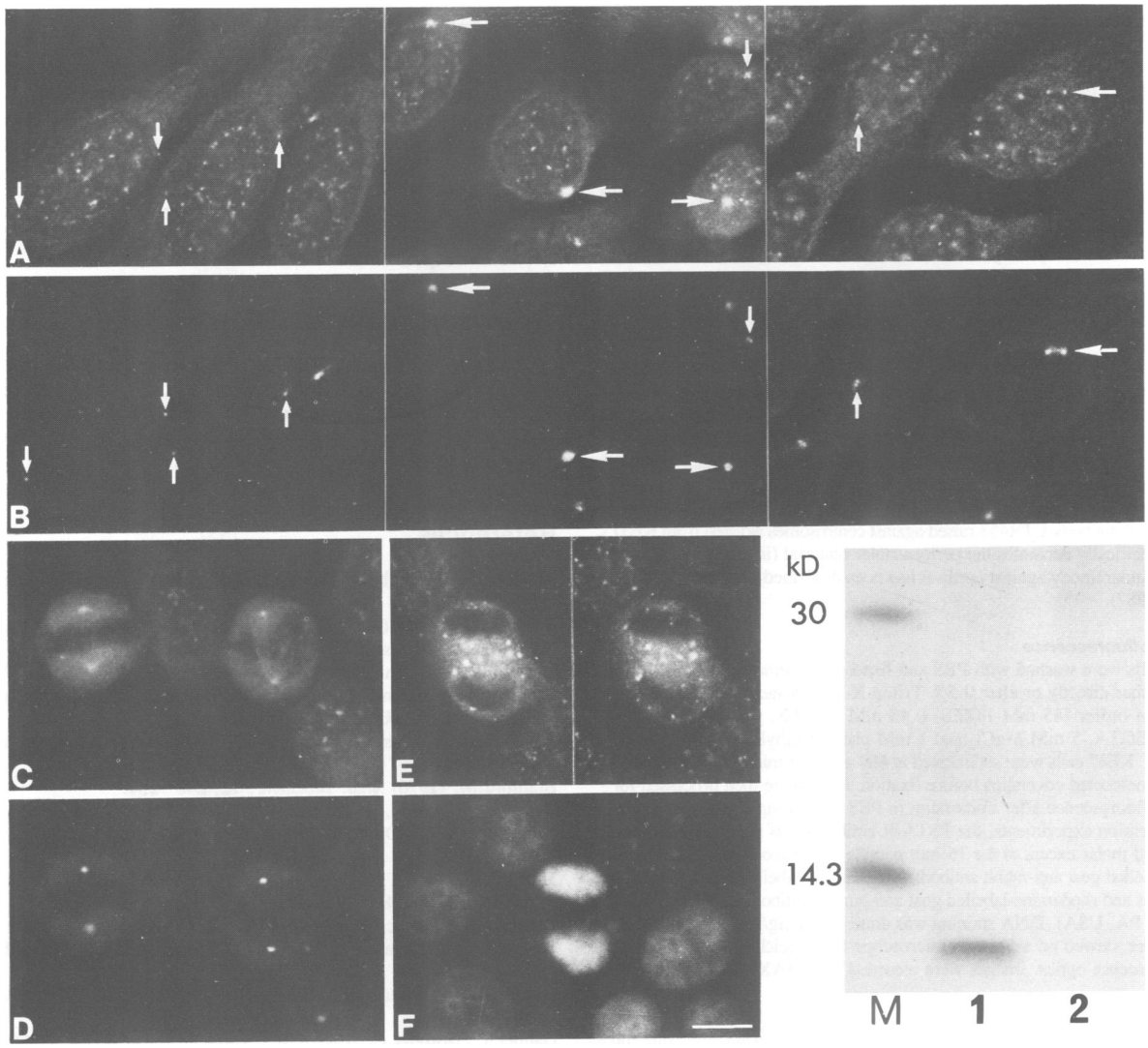

Fig. 9. Immunolocalization of p13^{suc1} in HeLa cells. After methanol fixation, HeLa cells were probed with affinity-purified antibodies against 913^{suc1} (**A,C,E**) and double stained either with CTR453 (**B,D**) or with DAPI (**F**). In interphase cells p13^{suc1} is found both in the nucleus and the cytoplasm (**A**) in a very similar manner to that observed for p34^{cdc2} (see Figure 1). However p13^{suc1} could be detected not only at centrosomes of G_2 cells (large arrows) as for p34^{cdc2} but also at centrosomes of G_1 cells (small arrows); on the left side in **A** and **B** two G_1 cells identified by the presence of two split centrioles (parallel small arrows) are shown. The fluorescence pattern observed with anti-p13^{suc1} antibodies in mitotic cells (**C** and **E**) strikingly resembles that of anti-p34^{cdc2} antibodies (compare to Figure 6). Two metaphase cells are shown in **C** which display uniform staining of the cytoplasm and conspicuous labelling of the mitotic poles. Note that the spindle also appears slightly decorated. Two focal planes of the same ana−telophasic cells are presented in **E** which shows the concentration of the staining in numerous spots between the two sets of segregating chromosomes (stained with DAPI in **F**). The inset represents an immunoprecipitation of [^{35}S]methionine-labelled mitotic HeLa cells with the same affinity-purified antibody against p13^{suc1} (see Materials and methods). M: mol. wt markers; 1: p13^{suc1} antibody; 2: control in the absence of the first antibody. Immunoprecipitates were analysed on a 15% polyacrylamide gel. Bar represents 10 μm.

inactivation because Nocodazole-treated cells block with high levels of p34^{cdc2} protein kinase activity (Draetta and Beach, 1988) as well as with high levels of vesicular located p34^{cdc2}. We are currently attempting to test this hypothesis by quantifying the amount of kinase activity associated with these vesicles.

During preparation of this work, a report has claimed that p34^{cdc2} is solely a nuclear protein based on immunocytochemical staining with one antibody (Riabowol *et al.*, 1989). Moreover p34^{cdc2} and p13^{suc1} were shown to have different cellular distributions. Our cell fractionation data, and our immunocytochemical staining obtained with affinity-purified antibodies raised against two different peptides of CDC2Hs, are in marked contrast with these conclusions. In addition we have observed a strikingly similar distribution of p34^{cdc2} and p13^{suc1} in HeLa cells, again using affinity-purified antibodies. The reasons for this discrepancy could be several. One possibility, is that the antibody used by Riabowol *et al.* (1989) reacts *in situ* with only a subset of p34^{cdc2}. This is supported by the fact that these authors observe that during 6−12 h after serum stimulation of rat 208F fibroblasts the cell population shows increasing intensity of nucelar staining. However the amount of p34^{cdc2} as quantified by immunoblotting is constant during this period. This discrepancy could be explained if only a subset of p34^{cdc2} is being detected *in situ*, and that this subset is located largely in the nucleus. Another study using rat myoblasts has also reported p34^{cdc2} in the cytoplasm with some in the nucleus, which is in broad agreement with our results for p34^{cdc2} (Akhurst *et al.*, 1989).

Materials and methods

Monolayer HeLa cells were grown in Earle modified Eagle's medium (EMEM) containing 1% non-essential amino acids and complemented with 10% fetal calf serum (FCS). The human lymphoblastic KE37 cell line was cultured in RPMI 1640 medium supplemented with 7% FCS. Both media contained glutamine, penicillin and streptomycin. 10^{-6} M Nocodazole (from Janssen, Beerse, Belgium) or 5×10^{-6} M Taxol (a generous gift from D.Guinard, Institut des Substances Naturelles du CNRS, Gif sur Yvette, France) were added for 4 or 18 h in certain experiments. Mitotic HeLa cells were obtained by a thymidine–Nocodazole double block as described in Zieve *et al.* (1980) and proteins were labelled by incubating cells with 1 mCi of [^{35}S]methionine (Amersham, France) in methionine-free medium.

Antibodies

The PSTAIR antibody was prepared as described by Lee and Nurse (1987). The 8C polyclonal antibody was raised against the LDNQIKKM peptide corresponding to the carboxyl terminus of CDCHs (Lee and Nurse, 1987). In order to eliminate any pre-immune activity of this antibody, the immunoglobulins binding to the p34^{cdc2} on immunoblots from HeLa cell proteins were affinity purified using the approach of Krohne *et al.* (1982). The anti-p13^{suc1} antibody has been described (Moreno *et al.*, 1989). The mouse monoclonal antibody CTR453 raised against centrosomes isolated from KE37 cells, specifically decorates the pericentriolar material (in preparation). The human autoantibody against lamin B has been described previously (Guilly *et al.*, 1987).

Immunofluorescence

HeLa cells were washed with PBS and fixed with methanol at −20°C for 6 min either directly or after 0.5% Triton X-100 extraction for 30–60 s, in PHEM buffer [45 mM PIPES + 45 mM HEPES, pH 6.9 containing 10 mM EGTA, 5 mM $MgCl_2$ and 1 mM phenylmethylsulphonyl fluoride (PMSF)]. KE37 cells were sedimented at 400 *g* for 10 min at 37°C on round poly(lysine)coated coverslips before fixation. Cells were then processed for immunofluorescence after immersion in PBS containing 0.1% Tween 20. In competition experiments, the PSTAIR antibody was pre-incubated with a 500-fold molar excess of the 16 mer peptide. The second antibodies were FITC-labelled goat anti-rabbit antibodies, rhodamine-labelled goat anti-mouse antibodies and rhodamine-labelled goat anti-human antibodies (Cappel, West Chester, PA, USA). DNA staining was done with 1 μg/ml DAPI (Sigma). Cells were viewed on a Polyvar microscope from Reichert equipped with epifluorescence optics. Images were recorded on TMAX 400 Kodak films.

Cell fractionation

KE37 cell extracts were prepared, after two washes in PBS, by using the following procedures. (i) A whole cell extract was prepared by resuspending the cells directly in Laemmli sample buffer (LSB); (ii) Detergent-soluble and insoluble cell fractions were obtained by a brief treatment of cells at 4°C, with 1% Nonidet P40, 0.5% sodium deoxycholate (DOC) in TNM buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) containing 1 mM PMSF, 100 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin. After sedimentation at 300 g, the pellet was washed once in TNM buffer and solubilized in LSB. The detergent-soluble protein fraction was recovered by precipitation in 90% methanol followed by centrifugation and solubilization in LSB. (iii) A cytosolic/particulate fractionation was carried out by mechanically disrupting the cells at 4°C with a Teflon/glass homogenizer in TNM buffer which contained the same cocktail of protease inhibitors as mentioned above. Both fractions were separated by a 1 h centrifugation at 150 000 *g* and processed for electrophoretic analysis as described above. (iv) Clean nuclei were prepared by the citric acid procedure known to provide demembranated nuclei devoid of any perinuclear material (Bornens, 1968). Cells were resuspended in 1% citric acid at 4°C and disrupted by 35 strokes in a tight fitting Dounce homogenizer. The nuclei were sedimented at 300 *g* and washed twice, first in 0.1% citric acid and then in TM buffer (10 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$). The soluble proteins and the structures extracted by citric acid were precipitated in 90% methanol and sedimented (non-nuclear fraction). Finally both fractions were solubilized in LSB. Centrosomes were isolated from KE37 cells as described elsewhere (Bornens *et al.*, 1987). Protein content of each fraction was assayed according to Bradford (1976).

Detergent-soluble and insoluble fractions of HeLa cells treated with 10^{-6} M Nocodazole or 5×10^{-6} M Taxol for 4 h were obtained by a brief treatment of cells at 4°C with 1% Triton X-100 in the microtubule-stabilizing PHEM buffer (see above), followed by sedimentation at 300 *g*.

Western blotting and immunoprecipitation

Gel electrophoresis analysis of each fraction was performed as described by Laemmli (1970) on 6–15% gradient SDS–PAGE. After electrotransfer to nitrocellulose filter according to Towbin *et al.* (1979), the immobilized proteins were probed with affinity-purified anti-p34^{cdc2} rabbit antibody in TBS containing 0.1% Tween 20 and 5% non fat dry milk. The immunoreactive bands were revealed by using alkaline phosphatase labelled goat anti-rabbit antibodies (Promega, Biotec) according to the manufacturer's instructions. The immunoprecipitation procedure used was as described in Draetta *et al.* (1988) except that the immunoprecipitates were collected on Protein A–Sepharose beads (Pharmacia).

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