Interaction of casein kinase 1 delta (CK1δ) with post-Golgi structures, microtubules and the spindle apparatus

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Received August 21, 1999
Received in revised version January 11, 2000
Accepted January 11, 2000

Casein kinase 1 delta – trans Golgi network – mitosis – microtubules – phosphorylation

Members of the casein kinase 1 family of serine/threonine kinases are highly conserved from yeast to mammals and seem to play an important role in vesicular trafficking, DNA repair, cell cycle progression and cytokinesis. We here report that in interphase cells of various mammalian species casein kinase 1 delta (CK1δ) specifically interacts with the trans Golgi network and cytoplasmic, granular particles that associate with microtubules. Furthermore, at mitosis CK1δ is recruited to the spindle apparatus and the centrosomes in cells, which have been exposed to DNA-damaging agents like etoposide or γ-irradiation. In addition, determination of the affinity of CK1δ to different tubulin isoforms in immunoprecipitation-Western analysis revealed a dramatically enhanced complex formation between CK1δ and tubulins from mitotic extracts after introducing DNA damage. The high affinity of CK1δ to the spindle apparatus in DNA-damaged cells and its ability to phosphorylate several microtubule-associated proteins points to a regulatory role of CK1δ at mitosis.

Introduction

The casein kinase 1 (CK1) family of protein kinases is common to all eukaryotic cells. At least fifteen sequence-distinct mammalian isoforms have been characterised: isoforms α, β, γ1–3, δ and ε (Fish et al., 1995; Graves et al., 1993; Gross and Anderson, 1998; Rowles et al., 1991; Zhai et al., 1995) and their various splice variants (Zhai et al., 1995). Although the isoforms differ in tissue distribution and subcellular localisation, CK1 protein kinases share several characteristics. All CK1 family members exhibit Ser/Thr-specific protein kinase activity, and some isoforms from yeast can also phosphorylate tyrosine (Hoekstra et al., 1994). They exclusively use ATP as a phosphate donor, are generally cofactor-independent, and depend on N-terminal acidic and/or phosphorylated amino acids for substrate recognition (Roach, 1990; Tuazon and Traugh, 1991). CK1 protein kinases also contain a putative nuclear localisation signal (Rowles et al., 1991; Tuazon and Traugh, 1991; Zhai et al., 1995) and possess a kinesin homology domain (Roof et al., 1992; Xu et al., 1995), which in kinesins is necessary for interactions with microtubules. All CK1 isoforms exhibit a high degree of sequence conservation within their kinase domains, but differ significantly in length and primary structure of their C-terminal non-catalytic domains. These variable C-terminal domains are responsible for substrate specificity of the different isoforms (Cegielska et al., 1998; Graves and Roach, 1995) and are involved in the regulation of interaction with other proteins, and/or subcellular structures. Phosphorylation (Cegielska et al., 1998), and perhaps dimerization (in the case of CK1δ) (Longenecker et al., 1998), are additional mechanisms that regulate CK1 activity, specificity, and subcellular localisation.

The wide spectrum of substrates suggests that the CK1 kinase family might be involved in many different cellular processes. However, so far only a few examples link CK1 activity to specific functions in vivo (Gross and Anderson, 1998; Peters et al., 1999; Sakanaka et al., 1999).

The function of CK1-related kinases has been best analysed in yeast; four different CK1 homologues have been identified (Christenson et al., 1997). Some of these CK1 homologues participate in the regulation of membrane transport processes (Murakami et al., 1999; Panek et al., 1997), in cell morphogenesis (Robinson et al., 1993) and in DNA repair pathways (Dhillon and Hoekstra, 1994). For example, in *S. cerevisiae* the *hrr25* gene encodes a CK1-related kinase, which is an essential

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0171-9335/00/79/04-240 $12.00/0

component of DNA repair processes. Interestingly, *hrr25* mutants can be complemented by the mammalian CK1δ and ε isoforms, but not by the α isoform (Hoekstra et al., 1994), suggesting that those two isoforms might have similar functions in mammals. CK1 δ/ε have also been implicated in the regulation of the tumour suppressor p53 (Knippschild et al., 1997). The induction of CK1δ by p53 in stress situations and its ability to phosphorylate p53, suggest that it may play a central role in mediating the downstream effects of p53 on cell growth and genome integrity (Knippschild et al., 1996; Knippschild et al., 1997).

Here we report that in interphase cells CK1δ is found in association with the trans Golgi network (TGN), and in addition with cytoplasmic granular particles, which decorate microtubules. During mitosis CK1δ is evenly distributed throughout the cytoplasm. However, upon genotoxic stress induced by etoposide or γ-irradiation, CK1δ specifically interacts with the mitotic spindle apparatus. Immunoprecipitation-Western blot analyses showed that CK1δ binds to all tubulin isoforms and that its affinity to tubulin isoforms changes during the cell cycle and upon DNA damage. Our results therefore suggest that CK1δ participates in regulatory processes at the onset of mitosis.

Materials and methods

Cells

Primary mouse embryo fibroblast (MEF) cultures were prepared from mechanically dispersed 17-day-old Balb/c mouse embryos by digestion with 0.2% collagenase in phosphate-buffered saline (PBS). Primary MEF cultures, C57MG cells (Vaidya et al., 1978), Balb/c 3T3 cells (Aaronson and Todaro, 1968), and F111 cells (Freeman et al., 1975) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS at 37°C in a humidified 5% CO_2 atmosphere.

Cell cycle enrichment

F111 cells, p53 -/- fibroblasts or MEFs were seeded on 10-cm plates. For arrest in G1/S phase, cells were treated with aphidicolin (1 μg/ml) and thymidine (2.5 mM) for 17 hours, then released for 8 hours and once again treated with aphidicolin and thymidine. For the enrichment of cells in G2/M phase, cells were harvested 8 to 12 hours after the second aphidicolin/thymidine block. In some cases, cells were γ-irradiated (1 to 5 gray) or treated with etoposide (0.2 to 1 μM) 30 minutes after release from G1/S arrest and then harvested at G2/M. The mitotic cells were either used for immunofluorescence microscopy, immunoprecipitation, or Western blotting.

Antibodies and production of antibodies

The peptide CGDMASLRLHAARQGARC (rat CK1δ-specific C-terminal sequence) was coupled to KLH for the first, to BSA for the second, and to ovalbumin for the third immunisation. For the immunisation of rabbits, the coupled peptide emulsified in an equal volume of complete (first immunisation) or incomplete (subsequent immunisations) Freund's adjuvant (Gibco Laboratories) was used for subsequent subcutaneous injections in rabbits. Test bleed of each animal was taken 10 days after each boost, and after three to four boosts the animals were bleeded out.

This work was initiated by using the CK1δ-specific monoclonal antibody 128A, which was kindly provided by ICOS Corporation (Washington, USA). Additionally, polyclonal antibodies against α/β-tubulin (kindly provided by Gabriel Rutter, Hamburg, Germany), γ-tubulin (Sigma, Germany) and a monoclonal anti-β-tubulin antibody (Boehringer, Germany) were used. As markers for membrane organelles we used antibodies against TGN38 (a gift from George Banting, Bristol, GB), cathepsin D (kindly provided by Annette Hille-Rehfeld, Göttingen, Germany), K58 (Sigma), rab5, rab9, α-adaptin, γ-adaptin, and dynamin II (Santa Cruz, California, USA), respectively.

Immunofluorescence microscopy

Cells used for the immunofluorescence microscopy were grown on coverslips or, in the case of mitotic shake off, cells were prepared by cytospin in a cytospin 3 centrifuge (Shandon) at 1000 rpm for 10 minutes. Cells were washed twice in PEM (0.1 M PIPES, pH 6.9; 5 mM $MgCl_2$; 1 mM EGTA) and fixed in 3% paraformaldehyde in PEM for 10 minutes at 37°C. In some cases, cells were treated with the microtubules-effecting drugs nocodazole (5 μM) or vinblastine (2 μM) in medium and fixed as above. Fixed cells were permeabilised in PBS containing 0.3% Triton X-100 at 37°C for 3 minutes. Where indicated, cells were preextracted up to 20 minutes either with KM buffer (10 mM 2-[N-morpholino] ethane sulphonic acid [MES], pH 6.8, 10 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM dithiothreithol (DTT), 1 mM EGTA, 50 μM leupeptin, 30 μg/ml aprotinin) supplemented with 1% NP40 or up to 10 minutes in PEM supplemented with 3% Triton X-100. Where indicated, cells were treated with the membrane transport-affecting drug brefeldin A (BFA, 5 μg/ml in medium) and fixed as described above. Fixed cells were washed in PBS at room temperature and blocked in PBS containing 0.2% gelatine for 1 hour followed by an incubation with primary antibodies. For multiple staining, primary antibodies were incubated sequentially for 45 minutes each. Secondary antibodies were bound for 30 minutes using Cy2-, Cy3-, FITC- or Texas-Red-labelled IgG (DIANOVA, Germany), respectively. The above incubation steps were each finished by washing in PBS (4 – 8 ×). DNA was visualised by DAPI staining (15 minutes; 0.1 μg/ml). Epifluorescence microscopy was performed using a Leica 63 ×/1.4 or 40 ×/1.0 oil immersion objective on a Leica DM-IRS microscope equipped with a confocal imaging system (TCS NT-Version) or with a Leica DM-R microscope using a high-resolution digital camera system (Diagnostic Instruments, Intas, Germany).

Preparation of cytoskeletons and immunoelectron microscopic detection of CK1δ

For electron microscopy, glass coverslip cultures of MEFs were washed and stabilised in PEM supplemented with 10% DMSO for 5 minutes at room temperature. Cells were then extracted at room temperature in the same buffer supplemented with 0.5% Triton X-100 and 1% aprotinin (Bayer Leverkusen, Germany). The cytoskeleton was fixed in PEM buffer containing 0.5% glutaraldehyde and 4% paraformaldehyde, washed and then blocked with 1% bovine serum albumin for 20 minutes. CK1δ was detected by incubation with a 1:200 dilution of the polyclonal rabbit antiserum NC10 for 30 minutes at room temperature. Subcellular localisation of CK1δ detected by the NC10 serum was visualised by an additional incubation with 9 nm gold particles conjugated to protein A for 30 minutes at room temperature. After repeated washing, the extracted cells were postfixed with 2.5% glutaraldehyde in buffer for 10 minutes and further stained with 2% aqueous solution of uranyl acetate for 30 minutes at 4°C. The extracted cells were dehydrated through a graded serious of cold ethanol and dried from tetramethylsilane. The dried cytoskeletons were shadowed with carbon at a 90° angle in vacuum (10^{-7} mbar), floated on hydrofluoric acid, washed in distilled water, and mounted on copper grids without a supporting film. Micrographs were taken in a Philips CM 120 transmission microscope.

Lysis, immunoprecipitation, SDS-PAGE and Western immunoblotting

Cells were washed in ice-cold PBS and extracted for 30 minutes with lysis buffer (TK-buffer) containing 50 mM Tris-HCl (pH 9.0), 25 mM KCl, 10% glycerol, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 μM leupeptin, 30 μg/ml aprotinin, and 1% empigen. Extracts were cleared by centrifugation for 45 minutes at 12000*g* at 4°C, diluted 1: 4 with a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% NP40, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 μM leupeptin, and 30 μg/ml aprotinin, then subjected to immunoprecipita-

tion using antibodies specific for either tubulins or CK1δ and incubated with 50 µl of settled protein A-Sepharose beads on a rotating shaker for 2 hours at 4 °C. The immunoprecipitates were washed five times with PBS containing 1% NP40, three times with PBS, and once with 0.1 × PBS. The immunocomplexes were denatured in sodium dodecyl sulfate (SDS) sample buffer, separated on SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane (Hybond C super, Amersham). The membranes were probed with CK1δ or tubulin specific antibodies. Immunocomplexes were detected using either horse radish peroxidase-conjugated anti-rabbit IgG (SAPU, Scotland, GB) or anti-mouse peroxidase-conjugated IgG, followed by chemiluminescence detection (ECL, Amersham, Germany and Pierce, USA).

Expression and purification of glutathione S-transferase fusion proteins

In this study two glutathione S-transferase (GST) fusion proteins were used: FP449 contains the full sequence of rat CK1δ fused to the C-terminus of GST in the vector pGEX-2T (Pharmacia, Sweden), whereas FP455 comprises the full sequence of human CK1ε (both plasmids were kindly provided by David Meek, Scotland, UK). The construction, expression and purification of these proteins are described in detail elsewhere (Knippschild et al., 1997).

In vitro kinase assays

In vitro kinase assays were carried out as previously described (Knippschild et al., 1996) using either bovine tubulin (ICN, California, USA), the GST-stathmin fusion protein (kindly provided by Irene Dornreiter and Silke Dehde, Hamburg, Germany), tau or the $MAP4^{640–1125}$ fragment (both proteins were a gift from Eva-Maria Mandelkow, Hamburg, Germany) as substrate. In each case, the protein was resuspended in a total volume of 20 µl containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA and 100 µM [γ-^{32}P]ATP (specific activity 5 Ci/mmol). The kinase reactions were started by adding the C-terminal truncated CK1δ (Calbiochem, Germany) or the full length GST-CK1δ fusion protein FP449 and incubated for 30 minutes at 30 °C. The reactions were stopped by adding 5 µl of concentrated SDS sample buffer as described elsewhere (Knippschild et al., 1997). The phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

Phosphopeptide analysis

In vitro phosphorylated tubulin, stathmin, tau and $MAP4^{640–1125}$ were analysed by two dimensional phosphopeptide analysis using a standard protocol as described earlier (Knippschild et al., 1995). In vitro phosphorylated proteins were separated by SDS-PAGE on 10% polyacrylamide gels, blotted onto a nitrocellulose membrane (Hybond) and isolated. Membrane fragments containing phosphate-labelled proteins were incubated in 100 mM acetic acid with 5% polyvinylpyrrolidone (PVP-360, Sigma, Germany) at 37 °C for 45 minutes, extensively washed with water and 50 mM ammonium bicarbonate buffer, digested with trypsin and oxidized with performic acid for 1 h. Phosphopeptides of either tubulin, stathmin or $MAP4^{640–1125}$ were analysed on cellulose thin layer plates by electrophoresis at pH 1.9 followed by ascending chromatography in a buffer containing either n-butanol/pyridine/acetic acid/H_2O, 15:10:3:12 (vol/vol) (Knippschild et al., 1997), or isobutyric acid/pyrimidine/acetic acid/n-butanol/H_2O, 65:5:3:2:29.

Results

Subcellular localisation of CK1δ in interphase cells

We first examined the subcellular localisation of CK1δ in Balb/c 3T3 and F111 cells using CK1δ specific antibodies for the detection of CK1δ by indirect immunofluorescence microscopy. Staining of CK1δ in Balb/c 3T3 cells with the monoclonal antibody 128A (Fig. 1a) or staining of F111 cells with the polyclonal serum NC10 (Fig. 1b) revealed a pronounced cytoplasmic distribution of CK1δ, with a predominant perinuclear localisation (Western blot analysis revealed that NC10 specifically recognises CK1δ and its hyperphosphorylated form, see Fig. 1d). Additionally, we found a weaker staining of CK1δ in the peripheral cytoplasm, which seems to be organised in linear arrays (Fig. 1a, b). Similar results were obtained in all cell lines analysed (C57MG, MCF7, swiss 3T3, data not shown).

Enlarged laser scan images of MEFs showed that the perinuclear structure stained by CK1δ-specific antibodies appeared as a tubular and cisternal network (Fig. 1c), which is sensitive to detergent treatment (data not shown).

CK1δ localises to the TGN and clathrin-coated membranes of the TGN region

CK1δ seems to be associated with a membrane network. Since the fungal secondary metabolite brefeldin A (BFA) induces fundamental changes in morphology and dynamics of several membranous organelles of the secretory pathway (Lippincott-

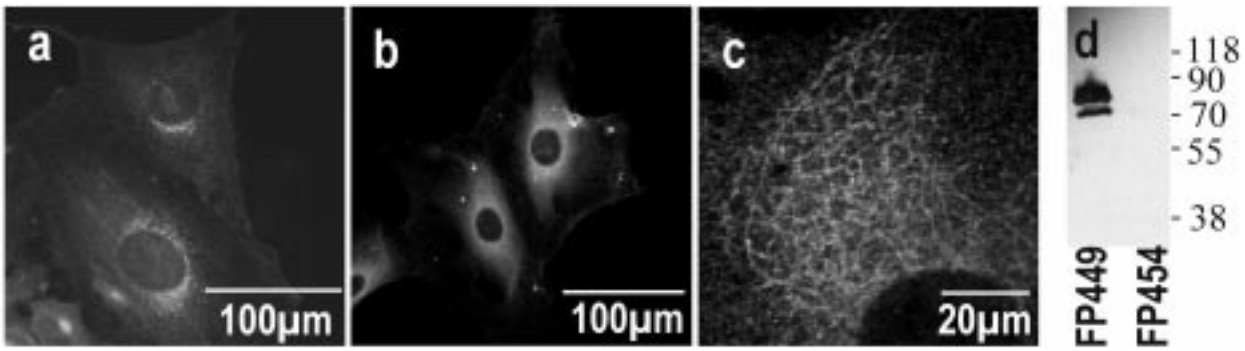

Fig. 1. Subcellular localisation of CK1δ in interphase cells. To determine the subcellular localisation of CK1δ in interphase cells, Balb/c 3T3, F111 cells and MEFs, respectively were grown on coverslips, then fixed and stained as described in Material and methods. Balb/c 3T3 cells were stained with the monoclonal anti-CK1δ specific antibody 128A (*lane a*) (Icos Corporation, Washington, USA), while F111 cells were stained with the CK1δ-specific polyclonal serum NC10 (**b**) followed by labelling with a Texas Red-conjugated IgG each. An enlarged laser scan image of the perinuclear, reticular CK1δ-positive structures in MEFs is shown in **c.** Bars as indicated. Western Blot analyses were performed to confirm the specificity of the CK1δ-specific polyclonal serum NC10 (**d**). The GST-CK1δ fusion protein FP449 was detected from bacterial extracts, whereas the GST-CK1ε fusion protein FP455 was not recognised by NC10 (Fig. 1d) (both plasmids were kindly provided by David Meek, Scotland, UK).

Schwartz et al., 1991) we analysed the effects of BFA on the morphology of CK1δ-positive, perinuclear structures. F111 cells were treated with 5 μg/ml BFA, fixed at different time points (30 minutes, 60 minutes, and 5 hours after addition of BFA) and stained for CK1δ (Fig. 2b–d). Compared to untreated cells (Fig. 2a), the distribution of CK1δ changed from the typical perinuclear, reticular-like staining to a more vesicular-like staining of CK1δ up to 60 minutes after treatment with BFA, which extended more to the periphery of the cytoplasm (Fig. 2b,c). However, after long-term treatment (5 hours) the CK1δ-positive structures nearly completely condensed into a stable structure in the perinuclear region (Fig. 2d). The observed BFA effects were reversible, as the original perinuclear reticular-like staining of CK1δ was largely re-established already 30 minutes after BFA release (data not shown). The pronounced effect of BFA on the perinuclear reticular structures stained by CK1δ-specific antibodies suggests an association of CK1δ with membrane structures of the secretory pathway and is similar to the behaviour of post-Golgi organelles (Ladinsky and Howell, 1992; Lippincott-Schwartz et al., 1991; Strous et al., 1993). We therefore attempted to co-localize CK1δ with proteins residing in distinct organelles of the secretory pathway. However, double staining of CK1δ with the Golgi-specific protein K58 did not result in co-localisation of both proteins in the laser scan microscopy (data not shown). Furthermore, we analysed the distribution of TGN38, an integral membrane protein of the TGN (Fig. 2e), and CK1δ (Fig. 2f) in F111 cells by laser scan microscopy. The merged image of CK1δ and TGN38 clearly demonstrates a co-localisation of both proteins in the perinuclear region (Fig. 2g).

To elucidate the identity of the less detergent sensitive staining of CK1δ in the periphery of the cytoplasm, we examined the co-localisation of CK1δ with vesicular and endocytotic marker proteins. Double staining of CK1δ either with early (rab5) or late endosomes (rab9), lysosomes (cathepsin D) (data not shown), or clathrin-coated vesicular structures (γ-adaptin and α-adaptin) (Fig. 2h–j, data not shown) were performed and analysed by laser scan microscopy. Furthermore, we proved whether CK1δ co-localises with dynamin II known to be involved in endocytotic processes and

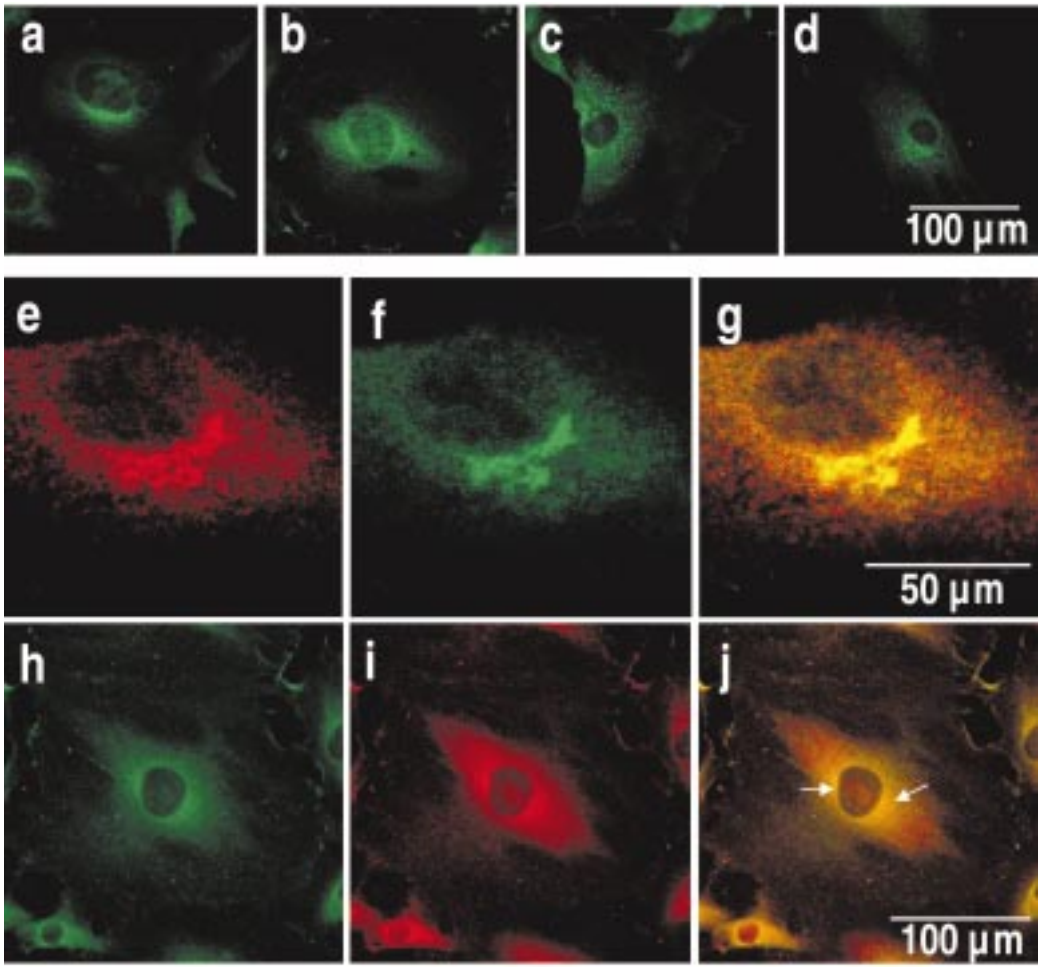

Fig. 2. CK1δ co-localises with the trans Golgi network (TGN) and AP1-positive membrane regions. To analyse alterations in the subcellular localisation of CK1δ upon BFA treatment F111 cells were incubated with BFA (5 μg/ml) either for 30 minutes (**b**), 60 minutes (**c**), or 5 hours (**d**) at 37 °C. Untreated (**a**) and treated cells (**b–d**) were fixed, permeabilised and then incubated with antibodies specific for CK1δ followed by FITC anti-rabbit IgG. Bar, 100 μm. Laser scan microscopy revealed a co-localisation of CK1δ with the trans Golgi network (TGN) (Fig. 2e–g). F111 cells were fixed, permeabilised and then incubated with antibodies to TGN38 (**e**) followed by labelling with secondary Cy3-conjugated antibodies and to CK1δ (**f**) followed by incubation with FITC-labelled antibodies. Overlays are shown in panel **g**. Bar, 50 μm. Double staining of F111 cells for CK1δ and γ-adaptin are presented in panels h–j. **h**: γ-adaptin, **i**: CK1δ, **j**: merged image of CK1δ and γ-adaptin. Regions of co-localisation are indicated by *arrows*. Bars as indicated.

to interact with clathrin-coated vesicles and microtubules (data not shown). However, only in the case of γ-adaptin (Fig. 2h), a clathrin-binding protein that resides to post-Golgi structures (Wong and Brodsky, 1992), we did observe a partial co-staining with CK1δ (Fig. 2i) in the perinuclear Golgi region of F111 cells by epifluorescence microscopy (see arrows). These results indicate that CK1δ localises at the TGN and clathrin-coated areas of the TGN region.

CK1δ-positive granular particles are associated with microtubules

We did not observe a co-localisation of CK1δ-positive granular particles with endosomal/lysosomal or vesicular marker proteins in the cell periphery. Since those particles were aligned in linear arrays we assume an association with filamentous structures (Fig. 1a). To clarify this observation, MEFs were double stained with CK1δ-specific antibodies and either antibodies against vimentin (intermediate filaments) or actin (actin filaments). However, no association between CK1δ with any of these two filament systems could be observed (data not shown). Therefore, we asked whether the CK1δ-stained particles were associated with the microtubule network. For this purpose, MEFs were double stained for α/β-tubulin (Fig. 3a, d) and CK1δ (Fig. 3b, e) and analysed by laser scan immunofluorescence microscopy. In such regions, where single microtubules could be distinguished, an association of the small, CK1δ-positive granular particles with microtubules was observed (Fig. 3c, f). The merged images of α/β-tubulin and CK1δ staining (Fig. 3c) clearly revealed that CK1δ-positive granular particles decorate the microtubules as spots. Interestingly, after detergent treatment, (Fig. 3d–f) CK1δ remained bound to microtubules indicating that it is tightly bound, directly or mediated by an unknown linker, to the microtubule network.

To support a close association of CK1δ with microtubules we performed immunoelectron microscopy with detergent-extracted cytoskeletons of MEFs (see Materials and methods). Therefore, F111 cells were extracted and labelled with the polyclonal CK1δ-specific antiserum NC10 and protein A-gold. The gold particles labelled clusters that are attached to

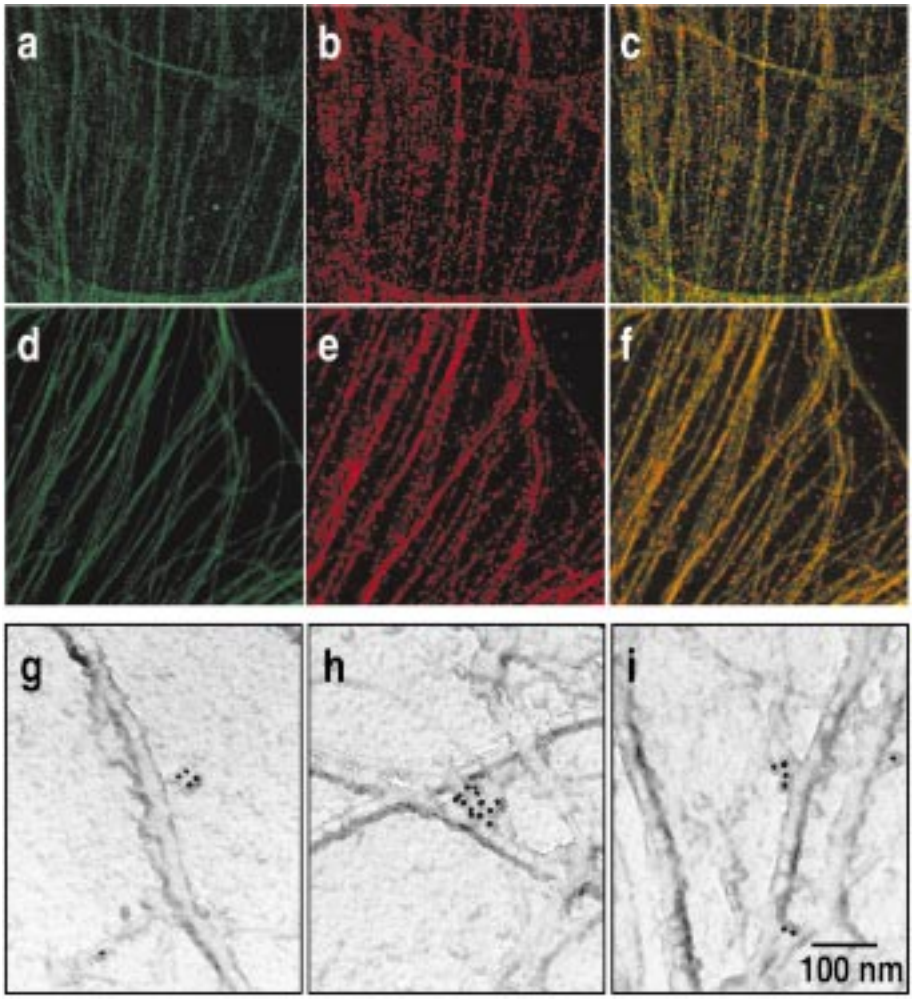

Fig. 3. CK1δ-positive granular particles are tightly associated with microtubules in interphase cells. Untreated MEFs (**a**–**c**) or MEFs treated with detergent for 20 minutes (**d**–**f**) were fixed, permeabilised and incubated with antibodies specific to α/β-tubulin (**a**, **d**) and to CK1δ (**b**, **e**), followed by incubation with Texas Red- and FITC-labelled secondary antibodies. The merged laser scan images are shown in **c** and **f**. Immunoelectron microscopic analysis of cytoskeleton preparations of MEFs using the CK1δ-specific polyclonal serum NC10 and secondary immunogold-labelled antibodies are shown in **g**, **h**, **i**. Micrographs taken in a Philips CM 120 transmission microscope underline that the 9-nm immunogold particles trace CK1δ-containing clusters attaching microtubules. Magnification 110000×. Bars as indicated.

filamentous structures. These filaments with a diameter of 22–24 nm were identified as microtubules (Fig. 3g,h,i).

Subcellular localisation of CK1δ in mitotic cells

In interphase cells, microtubules play an important role in vesicle transport, cell migration and in maintaining cellular architecture. During mitosis, tubulin builds up the mitotic spindle that is involved in chromosome segregation (Drubin and Nelson, 1996). The assembly of the bipolar mitotic spindles involves a complex rebuilding of microtubules (Zhai et al., 1996). As CK1δ is associated with the microtubule network in interphase cells, we asked whether the association of CK1δ with tubulin is maintained during mitosis. F111 cells enriched in mitotic stages (see Material and methods) were subjected to cytospin and analysed for the association of CK1δ with the spindle apparatus by immunofluorescence (Fig. 4a–d). Chro-

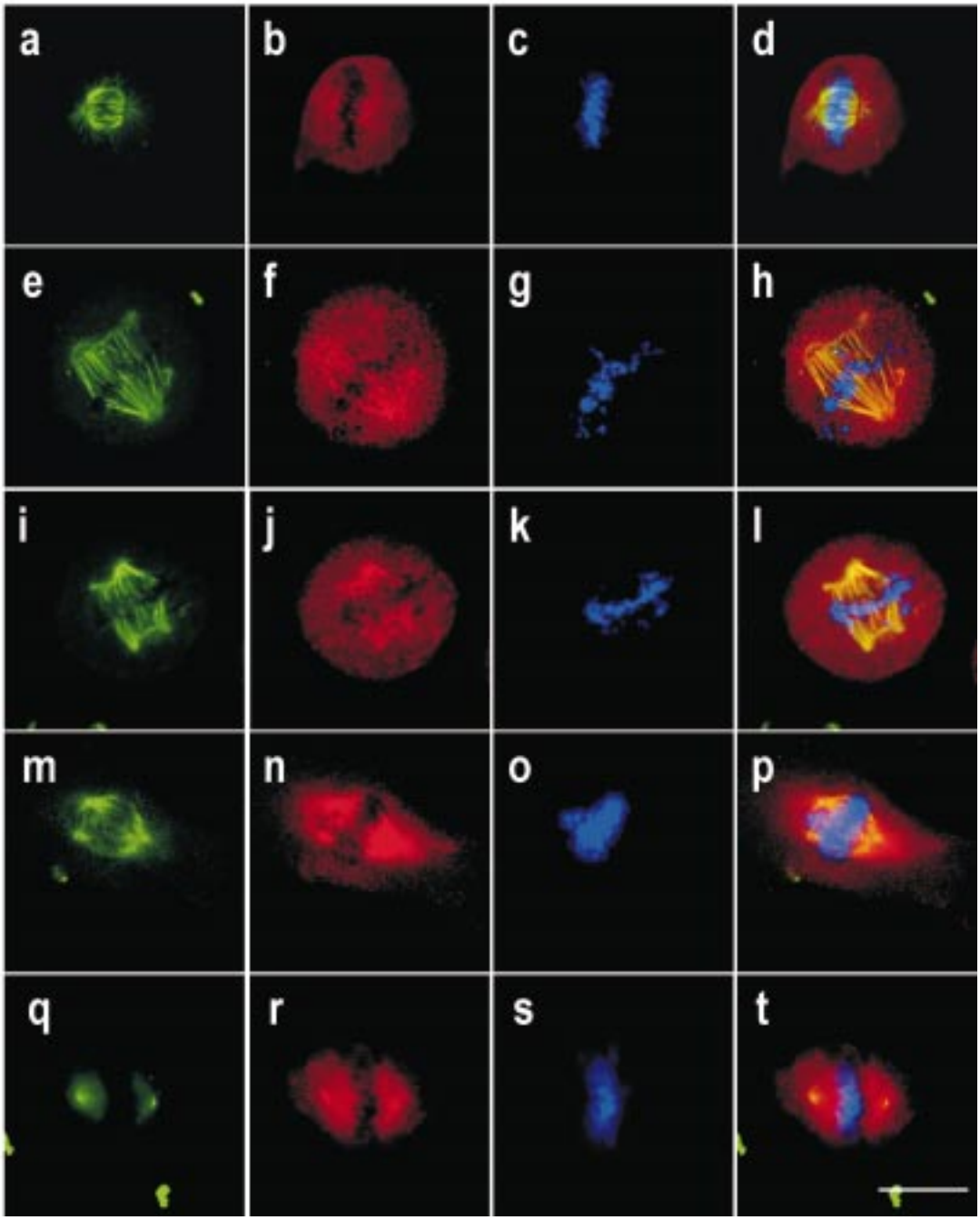

Fig. 4. CK1δ binds to the spindle apparatus if segregation failures occur. F111 cells (**a**–**d**) were released from G1/S-phase block as described in Materials and methods. Alternatively, F111 cells (**e**–**l**, **q**–**t**) were treated with etoposide (**e**–**h**) or γ-irradiated (**i**–**l**, **q**–**t**) as described in Material and methods. Images of an untreated MEF cell with an apparent centrosome defect are presented in **m**–**p**. Cells were fixed, permeabilised and incubated with anti-α/β-tubulin (**a**, **e**, **i**, **m**) or γ-tubulin antibodies (**q**) and with antibodies against CK1δ (**b**, **f**, **j**, **n**, **r**) followed by incubation with secondary Cy3- and FITC-labelled antibodies. Chromosomes were visualised by DAPI staining (**c**, **g**, **k**, **o**, **s**). Merged images of all three stainings are presented in **d**, **h**, **l**, **p**, and **t**. Bar, 10 μm.

mosomes were concomitantly detected by staining with DAPI. CK1δ showed an even distribution throughout the whole cell (Fig. 4b), and was slightly increased around the chromosomes. Although the merged image of tubulin and CK1δ staining does not point to a co-localisation of CK1δ with the mitotic spindle, we cannot exclude a weak association of CK1δ which is undetectable by immunofluorescence techniques.

There is evidence that CK1δ is activated upon genotoxic stress in a p53-dependent manner (Knippschild et al., 1997), thereby mediating signal integration onto p53. This leads to a modified p53 response and activation of cell cycle checkpoints (Meek, 1998; Schwartz et al., 1997; Schwartz and Rotter, 1998). To get hints for a possible involvement of CK1δ in mitotic checkpoint control, G1/S-arrested F111 cells, either γ-irradiated or treated with etoposide shortly after their release from the arrest, were analysed during mitosis to detect changes in the affinity of CK1δ to the mitotic spindle. Interestingly, in both, etoposide-treated (Fig. 4e–h) and γ-irradiated cells (Fig. 4i–l) we found a large amount of CK1δ co-localised with the mitotic spindle (Fig. 4h,l). Thereby, CK1δ was not evenly distributed but particularly aligned to the spindle. The spindle staining of CK1δ in γ-irradiated cells was mostly accompanied by a microscopically detectable defect in the mitotic spindle and frequent abnormalities in the chromatin structure characterised by a low condensation status (Fig. 4k). However, in etoposide-treated cells we always found spindle failure, the formation of multiple centrosomes and damage of the chromatin structure (Fig. 4g).

Without inducing DNA damage we observed a strong co-staining of CK1δ with the mitotic spindle only in the case presented in Fig. 4m–p. In these MEFs an apparent defect in centrosome division is indicated by the presence of three instead of two spindle poles. Such centrosome division failure increases the incidence of multipolar mitosis, which leads to chromosome segregation abnormalities (Lingle et al., 1998). This could also be seen in the DAPI staining of the untreated MEF where multipolarity led to an inadequate alignment of the metaphase chromosomes (Fig. 4o). The dramatic effect of such centrosome division defects underlines the important role of the centrosomes as part of the chromosome segregation machinery. To obtain information whether CK1δ also interacts with the centrosomes in addition to the spindle, γ-irradiated cells were co-stained for CK1δ and γ-tubulin, an important component of the centrosomes (Moritz et al., 1995) (Fig. 4q–t). CK1δ was found to be located throughout the whole cell with a pronounced staining at the centrosome region (Fig. 4r). The merged image of γ-tubulin and CK1δ (Fig. 4t) demonstrates that a fraction of CK1δ is indeed associated with the centrosomes during mitosis after induction by DNA damage.

The affinity of CK1δ to tubulin changes during the cell cycle and upon genotoxic stress

Our immunofluorescence data show that CK1δ is associated with microtubules in interphase cells, and with the spindle apparatus and the centrosomes in mitotic cells after DNA damage. Cellular stress situations lead to alterations in the activity of CK1δ (Knippschild et al., 1996; Knippschild et al., 1997). These alterations might influence its affinity to tubulin. Therefore we asked, whether CK1δ co-immunoprecipitates with different tubulin isoforms during the cell cycle and after genotoxic stress.

Indeed, we found CK1δ in complex with α/β- and γ-tubulin in asynchronously growing and in mitotic F111 cells in immunoprecipitation-Western analysis (Fig. 5A). The amounts of

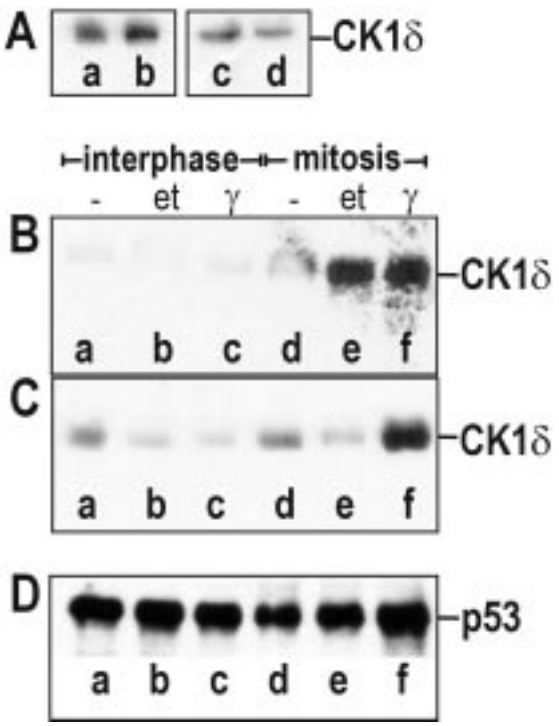

Fig. 5. Detection of complexes between CK1δ and tubulin during the cell cycle and upon genotoxic stress. **A**: α/β-tubulin (*a*, *b*), or γ-tubulin (*c*, *d*) were immunoprecipitated from lysates of asynchronously growing (*a*, *c*) or mitotic F111 cells (*b*, *d*). The immunoprecipitates were separated by SDS-PAGE, blotted onto nitrocellulose and probed for CK1δ (see Material and methods). **B**, **C**, **D**: Immunoprecipitation-Western analysis of α/β-tubulin and CK1δ are shown in **B**, those of γ-tubulin and CK1δ in **C**, whereas immunoprecipitation-Western analysis of p53 is presented in **D**. Cell lysates were obtained from cells growing under various conditions; *a:* asynchronously growing cells, *b:* asynchronously growing cells treated with 0.2 μM etoposide (et), *c:* γ-irradiated (γ) (1 gray) asynchronously growing cells, *d:* mitotic shake-off cells, *e:* mitotic shake-off cells treated with 0.2 μM etoposide (et), *f:* γ-irradiated (γ) (1 gray) mitotic shake-off cells.

CK1δ co-precipitated with α/β-tubulin slightly increased in mitotic cells (Fig. 5A, lane b) as compared to asynchronously growing cells (Fig. 5A, lane a). In contrast, the amount of CK1δ co-precipitated with γ-tubulin remained unchanged (Fig. 5A, lanes c and d) irrespectively of the synchronisation. By implementation of the reciprocal immunoprecipitation we detected all indicated tubulin isoforms in CK1δ immunoprecipitates (data not shown).

Furthermore, we determined the effect of genotoxic stress on the binding of CK1δ to α/β- or γ-tubulin. Fig. 5B shows an immunoprecipitation-Western analysis of CK1δ in asynchronously growing cells immunoprecipitated with α/β-tubulin in either untreated, etoposide-treated or γ-irradiated cells (Fig. 5B, lanes a, b, c), or in mitotic cells treated respectively (Fig. 5B, lanes d, e, f). Whereas γ-irradiation and etoposide treatment of asynchronously growing cells had only little effect on the association of CK1δ with α/β-tubulin (Fig. 5B, lanes b, c), these treatments strongly stimulated the binding of CK1δ to tubulin isolated from aphidicolin/thymidine-synchronised mitotic cells. This dramatic increase in the amount of co-precipitated CK1δ can be seen after DNA damage induced by either etoposide (Fig. 5B, lane e) or γ-irradiation (Fig. 5B, lane f) 30 minutes after release from G1/S.

Binding of CK1δ to γ-tubulin (Fig. 5C) showed some differences compared to those observed for the binding to α/β-tubulin. Firstly, a reduction in the binding of γ-tubulin was observed after etoposide treatment (Fig. 5C, lane b) and γ-irradiation in asynchronously growing cells (Fig. 5C, lane c). Secondly, etoposide treatment shortly after release from the thymidine/aphidicolin-induced G1/S block led to a decrease of the binding of CK1δ to γ-tubulin in mitotic cells (Fig. 5C, lane e). However, similar to the situation with α/β-tubulin we observed an increase in the co-precipitated amount of CK1δ (Fig. 5 C, lane f) with γ-tubulin at mitosis, if cells had been γ-irradiated in S phase. As a control we probed the same tubulin-immunoprecipitates for the tumor suppressor protein p53. In comparison to CK1δ complex formation with tubulin we observed a uniform p53 signal under various physiological conditions, indicating an unspecific binding of p53 to tubulin (Fig. 5D, lanes a – f).

Microtubule-effecting drugs influence the complex formation of CK1δ and tubulin

In a modified assay we asked whether binding of CK1δ to tubulin needs an assembled microtubule network. Therefore, we considered the role of microtubule-effecting drugs in the complex formation. Immunoprecipitation of tubulin from Balb/c 3T3 cellular extracts at 22 °C in the presence of the microtubule-destabilizing drugs nocodazole (Fig. 6A, lane c) or vinblastine (Fig. 6A, lane d) led to a significant increase in the amounts of co-immunoprecipitated CK1δ compared to that in an untreated control (Fig. 6, lane a). In addition, immunoprecipitation of tubulin at 4 °C also led to higher co-immunoprecipitation of CK1δ (Fig. 6A, lane b), indicating a strong association of CK1δ to unpolymerized tubulin in vitro.

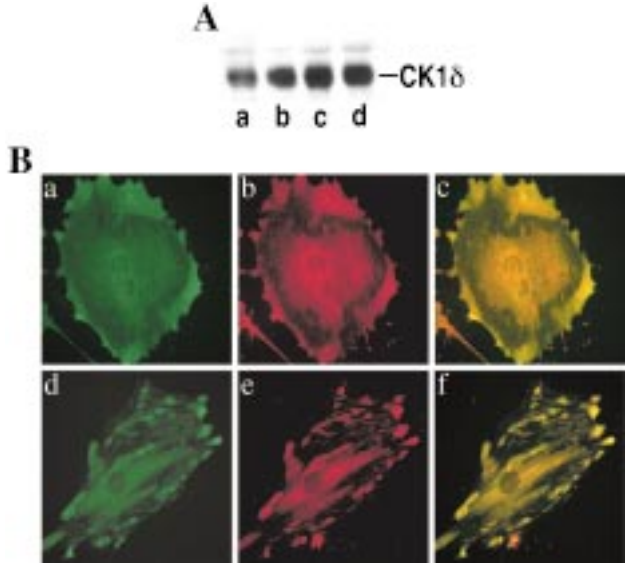

Fig. 6. The effects of the microtubule-destabilising drugs nocodazole and vinblastine on the CK1δ/tubulin complex formation and on the subcellular distribution of CK1δ. BalbC/3T3 cells were lysed and an α/β-tubulin-specific antibody was used to immunoprecipitate tubulin at 22 °C (**A**, *a*, *c*, *d*) or 4 °C (**A**, *b*) in the presence (**A**, *c*, *d*) or absence (**A**, *a*, *b*) of the microtubule-destabilising drugs. The immunoprecipitates were separated by SDS-PAGE, blotted onto nitrocellulose and probed for CK1δ. *a:* control 22 °C, *b:* cold treatment 4 °C, *c:* nocodazole (5 μM, 22 °C), *d:* vinblastine (2 μg/ml, 22 °C). MEFs were grown on coverslips and treated with 5 μM nocodazole for 17 hours. After fixation the localisation of CK1δ and tubulin was analysed by immunofluorescence (**B**, *a – c*). In addition, cells were treated with 2 μg/ml vinblastine (**B**, *d – f*), respectively. The immunostaining for CK1δ is shown in *b* and *e*, for α/β-tubulin in *a* and *d* and the superimposition of the CK1δ and tubulin staining in *c* and *f*.

To confirm that increasing complex formation between CK1δ and tubulin after disruption of microtubules by microtubule-destabilising drugs also occurs in vivo, we analysed nocodazole and vinblastine-treated MEFs by laser scan microscopy (Fig. 6B). Staining of MEFs after 2 h treatment with nocodazole resulted in a total disruption of the microtubule network and led to a partial co-localisation of CK1δ with unpolymerized tubulin (Fig. 6B, c). However, we observed an even more pronounced co-staining of CK1δ (Fig. 6B, b, e) and tubulin (Fig. 6B, a, d) after treatment of cells for 5 h with vinblastine, which leads to the building of tubulin paracrystals (Bensch and Malawista, 1968). The images of vinblastine-treated cells show a clear co-localisation of CK1δ with most tubulin paracrystals in the merged laser scan image (Fig. 6B, f).

CK1δ phosphorylates tubulin isoforms, MAP4, tau and stathmin in vitro

The association of CK1δ with interphase microtubules and the spindle fibres of DNA-damaged cells indicates a functional role for CK1δ in microtubule dynamics. Phosphorylation events play a critical role in the regulation of the turnover rate of microtubules. Especially microtubule-associated proteins (MAPs) and stathmin, a small regulatory phosphoprotein, influence the stability of microtubules (Gavet et al., 1998; Moreno and Avila, 1998). Therefore, we analysed, if MAPs, stathmin or even tubulins themselves are substrates for CK1δ.

In vitro kinase assays were performed as described in Material and methods. Purified bovine tubulin (ICN), $MAP4^{640–1125}$, tau and stathmin, respectively, were incubated in the presence of [γ-^{32}P]ATP and the C-terminal truncated CK1δ or the GST-CK1δ fusion protein FP449 (data not shown). Proteins were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 7A – D, a, b) and by autoradiography (Fig. 7, A – D, c, d). In control reactions no incorporation of phosphate into α/β-tubulin was detected (Fig. 7A – D, c). Strong signals indicated that both, the C-terminal truncated CK1δ (Fig. 7A – D, d) as well as the GST-CK1δ fusion protein FP449 (data not shown), were able to phosphorylate α-tubulin, β-tubulin, $MAP4^{640–1125}$, tau and stathmin in vitro. To obtain further information about the phosphorylation site(s), tryptic phosphopeptides of α-tubulin, β-tubulin, $MAP4^{640–1125}$, tau and stathmin were analysed by two-dimensional tryptic phosphopeptide analysis. The phosphorylated proteins were separated by SDS-PAGE, blotted, digested with trypsin, oxidized and analysed in two dimensions as described in Material and methods. In each case we observed several major and some minor phosphorylated tryptic peptides, indicating that CK1δ phosphorylates several amino acids in the proteins analysed (Fig. 7E).

Discussion

We here provide evidence that CK1δ interacts with different cellular targets. CK1δ co-localises with the TGN and interacts with microtubules in interphase cells. During mitosis CK1δ is evenly distributed throughout the cytoplasm, but upon DNA damage the association of CK1δ with the mitotic spindle is

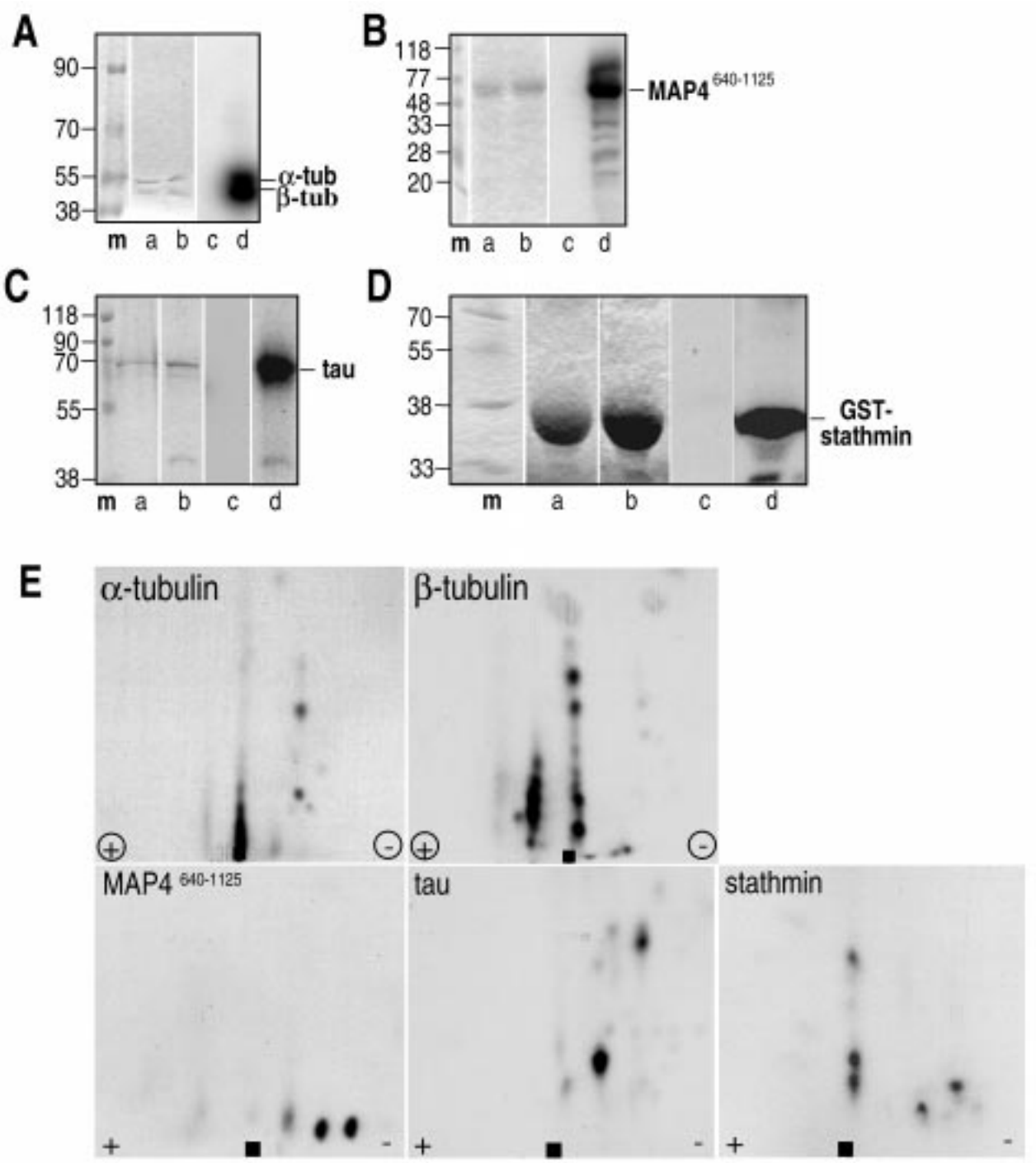

Fig. 7. α- and β-tubulin, MAP4$^{640–1125}$, tau and stathmin are phosphorylated by CK1δ in vitro. Bovine tubulin, MAP4$^{640–1125}$, tau and GST-stathmin, respectively, were used as substrates for the in vitro phosphorylation by the C-terminal truncated CK1δ (**A**–**D**, *b*, *d*) in the presence of [γ-^{32}P]ATP. Control reactions were performed in the absence of the kinase (**A**–**D**, *a*, *c*). Proteins were separated by SDS-PAGE, visualised by Coomassie staining (**A**–**D**, *lanes a, b*) and by autoradiography (**A**–**D**, *lanes c, d*). m: molecular weight marker. For two-dimensional phosphopeptide analysis proteins phosphorylated by CK1δ were separated by SDS-PAGE and blotted onto nitrocellulose. Tryptic peptides were prepared and spotted onto thin layer plates at the origin (marked with a *square*).

specifically activated, indicating that CK1δ might play a role in regulatory processes during mitosis.

Our analysis of the localisation of CK1δ in interphase cells by laser scan microscopy revealed that CK1δ antibodies stained predominantly perinuclear membrane structures which were sensitive to detergent treatment. During short-time exposure to BFA these reticular membranes showed an obvious anterograde redistribution, whereas after long-term treatment the CK1δ-stained structures collapsed to aggregates in the MTOC region. The observed alterations during BFA exposure are similar to the changes described for the TGN during BFA treatment (Ladinsky and Howell, 1992; Lippincott-Schwartz et al., 1991; Strous et al., 1993). Indeed, co-staining of CK1δ with TGN38, an integral membrane protein

of the TGN, in various cell lines of rodent origin clearly showed that CK1δ associates with the TGN. In addition, we found that CK1δ co-localises with γ-adaptin in the perinuclear region. γ-Adaptin as component of the adapter complex AP1 resides to clathrin-coated regions of the TGN that drive vesicle budding (Sosa, 1996; Wong and Brodsky, 1992). Vesicle generation in the trans Golgi network is an important site for the sorting of proteins addressed to the plasma membrane, secretory vesicles, or lysosomes (Keller and Simons, 1997). The association of CK1δ with the TGN and the partial co-staining of CK1δ with γ-adaptin suggests that CK1δ participates in regulating the budding of clathrin-coated vesicles and consequently influences sorting functions of the TGN. Kinase and phosphatase activities have been shown to regulate the interaction of adapter proteins with clathrin and thereby modulating the dynamics of vesicle formation (Chen et al., 1999; Sabatini et al., 1996; Wilde and Brodsky, 1996). An involvement of CK1 family members in TGN-specific functions has been described. For example, CK1α is associated with the TGN and participates in the regulation of vesicular trafficking (Gross and Anderson, 1998; Gross et al., 1997). Recently, it has been shown that the yeast homologue *hrr25p* of CK1δ is involved in the regulation of vesicle budding from the ER in yeast (Murakami et al., 1999). However, further experiments are necessary to prove an involvement of CK1δ in the regulation of TGN-specific processes.

Besides the perinuclear region, antibodies against CK1δ stained granular particles in the cytoplasm, which seem to be aligned in linear arrays. Extraction of soluble proteins of cells prior to fixation led to immunofluorescence images that clearly localise these granular particles to microtubules. These findings, confirmed by our immunoelectron microscopy data, point to a close association of CK1δ with tubulin. Indeed, we were able to co-immunoprecipitate CK1δ with antibodies specific for different isoforms of tubulin even in the presence of the microtuble-destabilizing drugs nocodazole and vinblastine. Therefore, we assume that CK1δ is able to interact with tubulins, either directly or mediated by an unknown linker. The association of CK1δ with microtubules and in addition with membrane structures of the TGN suggests a participation of CK1δ in microtubule-membrane interactions. This could indicate that CK1δ plays a role in microtubule-dependent transport processes. This view is also supported by the fact that CK1δ possesses a kinesin homology domain (Roof et al., 1992; Xu et al., 1995), shown to be responsible for the interaction of the motor protein kinesin with tubulin. Furthermore, it has been shown that CK1α participates in vesicle transport processes (Gross and Anderson, 1998; Gross et al., 1995).

The interaction of CK1δ with tubulin is not restricted to interphase but is also found during mitosis. While untreated mitotic cells showed no spindle association of CK1δ in the immunofluorescence microscopy, treatment with either etoposide or γ-irradiation at the onset of S-phase induces a strong particular alignment of CK1δ with the mitotic spindle and centrosomes (Fig. 4). The finding that CK1δ is recruited to the spindle in response to DNA damage is also supported by our binding affinity data. The complex formation of CK1δ with α/β-tubulin and γ-tubulin was dramatically enhanced in extracts from mitotic cells after exposure to genotoxic stress during S phase. The increased complex formation of the stress-activated CK1δ with spindle components provides evidence, that CK1δ is involved in rearrangement of the microtubule network at the onset of mitosis. Changes of microtubule dynamics are believed to be controlled by phosphorylation. In this study we present data that CK1δ is able to phosphorylate several amino acids of α- and β-tubulin. Phosphorylation of tubulins is thought to be one of the principal mechanisms adjusting the stability of microtubules (Aletta, 1996; MacRae, 1997). The CK1δ-mediated phosphorylation of α/β-tubulin might influence the turnover rate of microtubules and thereby regulating the rebuilding of microtubules during mitosis.

In addition to α/β-tubulin, CK1δ phosphorylates MAPs like MAP4 and tau (Kuret et al., 1997; Singh et al., 1995, our own data) as well as stathmin. Both, expression level and phosphorylation status of MAPs are known to be involved in the regulation of microtubule stability (Drewes et al., 1998; Moreno and Avila, 1998). Therefore, one might predict that CK1δ is involved in the control of M phase-specific rebuilding of the microtubule network. Further studies will be needed to clarify the character of the implication of CK1δ in these processes.

Other isoforms of the CK1 family have also been shown to be involved in chromosome segregation during mitosis and meiosis. For example, the α isoform is located at the spindle and the centrosomes in both mouse fibroblasts and oocytes (Brockman et al., 1992; Gross et al., 1997). So far, in all of these studies the association with the cell division machinery has been described as being constitutive. In contrast, we here demonstrate that CK1δ only associates to a significant amount with the spindle and the centrosomes when induced by genotoxic stress, thereby underlining the involvement of CK1δ in stress signalling pathways. The fact that CK1δ especially phosphorylates p53, a major stress integrator protein of the cell, at G2/M (Meek, 1998) (our own unpublished data) suggests that such a phosphorylation event could form the basis for a signal to p53 indicating the status of mitotic progression. Further studies are in process to characterise this observation.

In summary, our analyses of the cell cycle-specific localisation of CK1δ provide new clues to the function of CK1δ. The mechanism controlling the spindle association of CK1δ in response to cellular stress and the consequences for cell cycle progression remain to be elucidated.

Acknowledgements. We are grateful to our colleagues who have kindly provided us with important reagents. These include Merl Hoekstra and Toni DeMaggio (ICOS Corporation, Washington, USA) (monoclonal anti-CK1δ antibody 128A), George Banting (anti-TGN38), Annette Hille-Rehfeld (anti-cathepsin A), Eva-Maria Mandelkow (MAP4$^{640-1125}$, tau), Irene Dornreiter and Silke Dehde (GST-stathmin) and David Meek (GST-fusion proteins FP449 and FP455). We thank Tilo Patschinsky and Christine Schulze-Garg for helpful discussion and reading of the manuscript. This work was supported by a grant from the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung, to Uwe Knippschild (10 – 1285-Kn I). The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Gesundheit. This work is part of the PhD thesis of Lars Behrend, Fachbereich Biologie at the University of Hamburg.

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