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Microtubules are required for NF-κB nuclear translocation in neuroblastoma IMR-32 cells: modulation by zinc

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Abstract

The relevance of a functional cytoskeleton for Nuclear Factor-κB (NF-κB) nuclear translocation was investigated in neuronal cells, using conditions that led to a disruption of the cytoskeleton [inhibition of tubulin (vinblastine, colchicine), or actin (cytochalasin D) polymerization and zinc deficiency]. We present evidence that an impairment in tubulin polymerization can inhibit the formation of the complex tubulin-dynein-karyopherin α-p50 that is required for neuronal retrograde and nuclear NF-κB transport. Cells treated with vinblastine, colchicine or cytochalasin D, and zinc deficient cells, all showed a low nuclear NF-κB binding activity, and low nuclear concentrations of RelA and p50. The altered nuclear translocation was reflected by a decreased transactivation of NF-κB-driven genes. The immunocytochemical characterization of cellular RelA showed that cytoskeleton disruption can lead to an altered distribution of RelA resulting in the formation of peripheral accumuli. These results support the concept that cytoskeleton integrity is necessary for the transport and translocation of NF-κB required for synapse to nuclei communication. We suggest that during development, as well as in the adult brain, conditions such as zinc deficiency, that affect the normal structure and function of the cytoskeleton can affect neuronal proliferation, differentiation, and survival by altering NF-κB nuclear translocation and subsequent impairment of NF-κB-dependent gene regulation.

Keywords: karyopherin α, microtubules, nervous system, NF-κB, nuclear transport, zinc deficiency
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Synaptic activity can regulate neuronal gene expression which is essential for neuronal survival, differentiation and plasticity (West *et al*. 2002). The capacity of the central nervous system to communicate transient stimuli from the synapse to the nuclei, and thus regulate gene expression requires the relocalization of transcription factors, such as Nuclear Factor-κB (NF-κB), which after activation can migrate from the axonal cytoplasm into the nuclei (Meffert *et al*. 2003). Rel/NF-κB transcription factors are activated by multiple signals, and regulate the expression of numerous genes. In B cells, where it was first described (Sen and Baltimore 1986), NF-κB is involved in the regulation of the immune and stress response, in cell-cycle progression, in the decision of cells to undergo apoptosis, and in the maintenance of cell structure and the nearby environment (Pahl 1999). While NF-κB target genes are not fully characterized in the nervous system (Kaltschmidt *et al*. 1993; Meberg *et al*. 1996), current evidence indicates that NF-κB is involved in the regulation of neuronal plasticity (Albensi and Mattson 2000) and cell survival (Mattson *et al*. 2000). Known members of the Rel/NF-κB family of proteins include c-Rel, RelB, RelA (p65), p50 and p52. The activity of the Rel/NF-κB homo, and heterodimers, is regulated by their interaction with inhibitory IκB proteins, which maintains the transcription factor inactive in the cytosol by masking its nuclear localization signal (NLS) (Baeuerle and Baltimore 1988). In general, activation is mediated by the phosphorylation of two conserved serines in IκB by specific IκB kinases, which target IκBα for ubiquitination and

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Abbreviations used: Col, colchicine; Cyt, cytochalasin D; EMSA, electrophoretic mobility shift assay; FBS, Fetal Bovine Serum; hRNP, heterogeneous nuclear ribonucleoprotein; Jas, jasplakinolide; NLS, nuclear localization signal; NF-κB, Nuclear Factor-κB; Tx, taxol; VB, vinblastine.

degradation by the proteasome (Karin 1999). Once IκB is degraded, the NLS sequence in NF-κB is unmasked and recognized by the NLS-receptor protein karyopherin α (Cunningham *et al*. 2003). This leads to the recognition by karyopherin β, which directs the proteins to the nuclear pore complex where translocation takes place (Gorlich and Laskey 1995; Gorlich and Mattaj 1996; Jans *et al*. 2000).

The cytoskeleton of neurons is an organized structure formed by three classes of proteins: microtubules (tubulins), microfilaments (actins) and intermediate filaments. These three cytoskeletal elements interact with each other to form dynamic structures (Bourgarel-Rey *et al*. 2001). Critical functions of microtubules in neuronal cells include their ability to facilitate intracellular transport, and to regulate cell morphology. Actin microfilaments are involved in cell contractility and in the local trafficking of cytoskeletal and membrane components. The cell cytoskeleton can also participate in the modulation of cell signaling through multiple mechanisms (Ben-Ze'ev 1991). Previous studies suggest that alterations in the cytoskeleton can affect NF-κB-dependent transcriptional activity (Rosette and Karin 1995; Mackenzie *et al*. 2002; Guzik and Goldstein 2004). In HeLa S3 cells, tubulin depolymerization, induced by inhibitors such as nocodazole or by cold, activates NF-κB leading to IκBα degradation (Rosette and Karin 1995). The state of tubulin polymerization may also be crucial in the translocation of the active NF-κB from the cytosol to the nuclei. As part of our work on the characterization of the mechanisms underlying the teratogenicity of zinc deficiency, we observed that, while a decrease in intracellular zinc is associated with the activation of the cytosolic events of NF-κB activation, a decreased transcription of NF-κB-dependent genes is observed in IMR-32 cells (Mackenzie *et al*. 2002). Significantly, the zinc deficient cells also showed a low rate of *in vitro* microtubule assembly (Mackenzie *et al*. 2002). The above observation led us to question if a functional tubulin network is required for NF-κB transport and nuclear translocation. Actin microfilaments are also needed for the regulation of gene expression. In glioma cells, the induction of matrix metalloproteinase-9, a NF-κB-driven gene, requires a polymerized actin because actin depolymerization by cytochalasin inhibits the induction of the matrix metalloproteinase-9 (Chintala *et al*. 1998). In lymphocytes, an increase in gene expression regulated by Nuclear factor of Activated T cells (NFAT) (a NLS-bearing transcription factor) was observed in association with an increase in actin polymerization induced by Rho G (Vigorito *et al*. 2003).

Based on the participation of NF-κB in the transduction of the synaptic stimuli into changes in gene expression (Meberg *et al*. 1996; Routtenberg and Meberg 1998; Meffert *et al*. 2003), the present work investigated the requirement of a functional cytoskeleton in NF-κB transport and nuclear translocation in IMR-32 and in primary cultures of cerebellum granule cells and cortical neurons. In this study we show that a functional cytoskeleton is required for the nuclear transport of transcription factor NF-κB in neuronal cells. Several diverse conditions [vinblastine (VB), colchicine (Col), cytochalasin D (Cyt), and zinc deficiency] that can inhibit tubulin and actin polymerization decreased the translocation of active NF-κB into the nucleus, resulting in a lower than normal transactivation of NF-κB-driven genes. In addition, we show that the impairment in tubulin polymerization can in turn affect the formation of the complex tubulin-dynein-karyopherin α1-p50 required for the neuronal retrograde and nuclear NF-κB transport.

Materials and Methods

Materials

IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MA, USA). Cell culture media and reagents and LipofectAMINE$^{TM.}$ 2000 were obtained from Invitrogen Life Technologies (Carlbad, CA, USA). The oligonucleotides containing the consensus sequence for NF-κB (5′-AGTTGAGGGGA-CTTTCCCAGGC-3′) and OCT-1, the reagents for the EMSA assay, the enzyme assay systems for the determination of luciferase and β-galactosidase activities and the pSV- β-galactosidase control vector were obtained from Promega (Madison, WI, USA). The PathDetect NF-κB *cis* reporting system was obtained from Stratagene (La Jolla, CA, USA). Antibodies for RelA, p50, bcl-2, dynein, karyopherin α, IκBα, heterogeneous nuclear ribonucleoprotein (hRNP) and β-tubulin, and the protein A/G-Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p-IKKβ, IKKβ and p-IκBα were obtained from Cell Signaling Technology (Danvers, MA, USA). PVDF membranes were obtained from BIO-RAD (Hercules, CA, USA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA, USA). The ECL Western blotting system was from GE Healthcare (Piscataway, NJ, USA). Jasplakinolide (Jas) was obtained from EMD (San Diego, CA, USA). Vinblastine (VB), colchicine (Col), cytochalasin D (Cyt), taxol (Tx) and all other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO, USA).

Cell culture and incubation

IMR-32 cells were cultured at 37 ^{o}C in complex medium (55% DMEM high glucose, 30% Ham F-12, 5% α-MEM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics-antimicotic (50 U/mL penicillin, 50 μg/mL streptomycin and 0.125 μg/mL amphotericin B).

Zinc deficient FBS was prepared by chelation with diethylenetriamine pentaacetic acid as previously described (Oteiza et al. 2000). The chelated FBS was subsequently diluted with complex medium to a final concentration of 3 mg protein/mL to match the protein concentration of the control non-chelated media (10% FBS). The zinc concentration of the zinc deficient medium was 1.5 μM, and portions of this media were supplemented with $ZnCl_2$ to reach concentrations of 5 and 15 μM.

Cells were grown in control medium (complex medium containing 10% non-chelated FBS) until 90% confluence, after which the media was removed and replaced with control medium or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc

incubated simultaneously without, or with, 0.5 μM VB, 0.5 μM Col or 0.5 μM Cyt. Cells were harvested after 24 h in culture. To characterize the role of microtubule and microfilament stabilization, cells were pretreated with 1 μM Tx or 100 nM Jas for 1 h followed by 24 h incubation in zinc deficient media (1.5 μM zinc).

IMR-32 cells incubated for 24 h in low zinc media (1.5 and 5 μM zinc) had low ($p < 0.05$, one way ANOVA) total zinc concentrations (μmol zinc/mg protein) (3.92 ± 0.37 and 3.98 ± 0.48 for 1.5 and 5 μM zinc cells, respectively) compared to cells incubated in the control or 15 μM zinc media (5.36 ± 0.13 and 5.61 ± 0.14, respectively).

For the immunocytochemistry, cells were previously differentiated for 12 days, using dibutyryl cyclic AMP (cAMP) (1 mM) and 5 bromo-deoxiuridine (4 μM), changing the differentiating media every 3 d (Neill et al. 1994; Erlejman and Oteiza 2002). The morphological and biochemical differentiation of IMR-32 cells was followed, as previously described (Erlejman and Oteiza 2002).

Cerebellum granule cells were obtained as described by Gallo *et al.* (Gallo *et al.* 1987) with modifications (Borodinsky and Fiszman 1998). Briefly, 6–8 days old Wistar rats were decapitated and cerebella dissected in Krebs-Ringer (Sigma, St Louis, MO, USA) solution supplemented with 6 g/L glucose. The meninges were eliminated and the tissue was cut into 1 mm pieces and incubated in saline containing 0.025% (w/v) trypsin for 5 min at 37°C with continuous shaking. The enzymatic digestion was stopped with trypsin inhibitor from soybean and the tissue was mechanically dissociated in Krebs-Ringer solution using Pasteur pipettes of different diameters (15 strokes) in saline containing 0.03% (w/v) trypsin inhibitor and 0.004% (w/v) DNAse. The resulting cell suspension was sedimented at 150 × g for 10 min and after resuspending the pellet in culture medium (NeurobasalTM supplemented with the serum-free additive, B27), and cell counting, cells were plated in 100 cm^2 dishes at a concentration of 3×10^5 cells per cm^2. After 24 h, cell cultures were supplemented with 10 mM cytosine-β-D-arabinoside to inhibit non-neuronal cell proliferation, and incubated for 6 h without or with VB, Col or Cyt.

Primary cortical cultures were prepared from E19 embryos according to Goslin *et al.* (Goslin *et al.* 1998). Briefly, embryos were rapidly removed using sterile techniques, and after removal of the skull and meninges, the cerebral hemispheres were dissected out and placed in Hanks' balanced salt solution prewarmed to 37°C. Cortical tissue was dissociated by enzymatic digestion (0.025% (w/v) trypsin). Trypsinization was stopped by the addition of Hanks' solution containing 20% (v/v) FBS and the tissue was mechanically dissociated in Hanks' solution containing 0.004% (w/v) DNAse using Pasteur pipettes of different diameters. The resulting cell suspension was centrifuged at 200 × *g* for 5 min. The cells were resuspended in neurobasal medium supplemented with 2% (v/v) serum-free additive, B27 and plated on poly-L-lysine-coated dishes. Cell cultures were incubated at 37°C in an atmosphere of 95% air 5% CO2 with 90–95% humidity. After 24 h in culture, cells were treated with VB, Col and Cyt for 6 h and nuclear and cytosolic fractions were immediately prepared.

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytosolic fractions were isolated as previously described (Dignam *et al.* 1983; Osborn *et al.* 1989), with minor modifications (Mackenzie *et al.* 2002). Total cell fractions were prepared as previously described (Muller *et al.* 1997), with slight modifications (Mackenzie *et al.* 2006).

For the EMSA, the oligonucleotides containing the consensus sequences for NF-κB or OCT-1 were end labeled with [γ^{-32}P] ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the corresponding labeled oligonucleotide (20,000–30 000 cpm) for 20 min at room temperature (25°C) in 1X binding buffer [5X: 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl and 0.25 mg/mL poly(dI-dC)]. The products were separated by electrophoresis in a 4% (w/v) non-denaturing polyacrylamide gel using 0.5 X TBE (45 mM Tris/borate, 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity quantitated in a Phosphoimager 840 (GE Healthcare).

Western blot analysis

Total, nuclear and cytosolic fractions were prepared as described above. Aliquots of nuclear or cytosolic fractions containing 25–50 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Membranes were blocked overnight in 5% (w/v) non-fat milk, incubated in the presence of the corresponding antibodies for RelA, p50, bcl-2 or β-tubulin (1 : 1000 dilution), dynein or karyopherin α (1 : 500 dilution) for 90 min at 37°C. After incubation for 90 min at room temperature (25°C), in the presence of the secondary antibody (HRP-conjugated) 1 : 10000 dilution, the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840.

To evaluate the phosphorylation of IκBα and IKKβ, cell lysates were prepared in SDS-sample buffer [62.5 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) sodium docedyl sulfate (SDS), 10% (v/v) glycerol, 50 mM DTT and 0.01% (w/v) bromophenol blue], sonicated for 10 s to shear DNA and reduce sample viscosity, and then heated at 95°C for 5 min 25 μL of the lysate were loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE and Western blots were carried out as described above except that membranes were immunoblotted with the primary antibodies (1 : 1000 dilution) overnight at 4°C, and the following day for 60 min at room temperature (25°C), in the presence of the secondary antibody (HRP-conjugated) 1: 2000 dilution. The membranes were normalized by reblotting with the antibody for the corresponding non-phosphorylated form of each protein.

Equal sample loading was controlled by measuring β-tubulin content in total and cytosolic fractions and hRNP content in nuclear fractions.

Transfections

IMR-32 cells (2.5×10^6 cells) were transfected with LipofectAMINE™ 2000 according to the manufacturer's protocols. As an internal control for transfection efficiency, a vector expressing β-galactosidase (2 μg DNA) was co-transfected with the pNF-κB-Luc plasmid (1 μg DNA). Twenty four hours after the transfection was initiated, cells were incubated in control or 1.5 μM Zn media containing VB, Col or Cyt. Cells were harvested 24 h later and after

lysis, β-galactosidase and luciferase activities were determined following the manufacturer's protocols.

Immunocytochemistry

Differentiated IMR-32 cells were cultured in coverslips and incubated for 24 h in control media without or with VB or Col. After washing, cells were fixed with 4% (w/v) paraformaldehyde, containing 0.12 M sucrose, in PBS for 1 h at room temperature (25°C). For the detection of β-tubulin, actin or RelA, fixed cells were permeabilized by incubation with 0.01% (v/v) Triton X-100 in PBS for 5 min at room temperature (25°C). Samples were then blocked with 1% (w/v) bovine serum albumin in PBS for 2 h and incubated overnight with the different primary antibody. The coverslips were rinsed twice with PBS and incubated with the appropriate secondary antibody conjugated to Texas-Red (for RelA) or to fluorescein-5-isothiocyanate (FITC) (for β-tubulin). For the detection of actin, cells were incubated with FITC-conjugated phalloidin. After staining, the preparations were mounted and analyzed by epifluorescence using an Olympus BX50 microscope (Tokyo, Japan). The intensity and distribution of the fluorescence was observed using an Image Pro Plus v.4.5 software (Media Cybernetics, Sylver Spring, MD, USA).

Polymerized/soluble tubulin distribution

Cells were cultured to full confluence and, after the different treatments polymerized, soluble and total tubulin extracts were prepared as previously described (Dennerll *et al*. 1988). To isolate the soluble and polymeric microtubule fractions, cells grown in 100 mm dishes were washed gently in microtubule stabilization buffer containing 0.1 M Pipes, pH 6.75, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*-*N*′*N*-tetraacetic acid (EGTA), 1 mM $MgSO_4$, 2 M glycerol, and Mini ethylenediamine tetraacetic acid (EDTA)-free protease inhibitor cocktail. Cells were incubated at 37°C for 10 min with 0.5 mL microtubule stabilization buffer (MTSB) containing 0.1% (v/v) Triton X-100. The supernate (soluble tubulin fraction) was removed and centrifuged at $800 \times g$ for 10 min. The remaining Triton-extracted cytoskeletal ghosts were subsequently solubilized by adding 0.5 mL lysis buffer [25 mM Tris-HCl, pH 7.4, 0.4 M NaCl, and 0.5% (w/v) SDS] constituting the polymerized tubulin fraction. Total tubulin was extracted by addition of lysis buffer directly to intact cells cultured in six well plates. All extraction steps were performed at 37°C. Polymerized and total tubulin fractions were analyzed by Western blot as described above.

Immunoprecitation

Total cell lysates were prepared as described above. 500 μg of total cell proteins were pre-cleared for 30 min using 0.25 μg of the appropriate control IgG from the host specie together with 20 μL of Protein A/G-Agarose. The pre-cleared-supernatant fractions were immunoprecipitated with an anti β-tubulin antibody (1 μg), or an anti p50 antibody (2 μg) together with 25 μL Protein A/G-Agarose overnight at 4°C. After centrifugation, the beads were washed twice with PBS, and the final pellets were resuspended in 30 μL of 1X sample buffer. Samples were boiled for 5 min, 15 μL of each sample were loaded for the electrophoresis, and Western blots were immediately carried out as described above. To evaluate the specificity of the immunoprecipitation, a control total cell fraction was immunoprecipitated with control IgG together with Protein A/G-Agarose, instead of the corresponding β-tubulin or p50 antibodies. There was no evidence of non-specific immunoprecipitation when control samples were immunoprecipitated with control IgG, instead of the corresponding primary b-tubulin or p50 antibodies.

Statistical analysis

One way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe, were performed using Statview 5.0.1 (Brainpower Inc., Calabazas CA, USA). A p-value < 0.05 was considered statistically significant. Values are given as means ± SEM.

Results

Cytoskeleton disruption induces a rapid NF-κB activation in human neuroblastoma IMR-32 cells

We initially tested the NF-κB protein components in IMR-32 cells and the specificity of the EMSA. The presence of p50 and RelA as protein components of the active Rel/NF-κB in IMR-32 cells was observed by EMSA supershift assays (Fig. 1a). The specificity of the NF-κB-DNA complex in the EMSA assays was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB or OCT-1 (Fig. 1a).

One possible signal that can lead to the activation of NF-κB in certain cells is the depolymerization of the cytoskeleton network (Das *et al*. 1995; Hwang and Ding 1995; Rosette and Karin 1995). To elucidate if alterations of the cytoskeleton could activate NF-κB in IMR-32 cells, the effects of tubulin (VB and Col) and actin (Cyt), polymerization inhibitors on NF-κB-DNA binding activity, were measured in total cell fractions isolated after 1, 3, 6, 12 or 24 h of incubation in the different experimental conditions. VB (0.5 μM), Col (0.5 μM) and Cyt (0.5 μM) caused a rapid (between 1 and 3 h) NF-κB activation as observed by EMSA assay (Fig. 1b). To further study the effects of cytoskeleton disrupting drugs on NF-κB activation, we measured earlier events in the signaling cascade, IKKβ and IκBα phosphorylation. Treatment with VB, Col and Cyt increased IKKβ phosphorylation and IκBα phosphorylation after 30 min and 1 h of incubation, respectively, reaching basal levels after 2 h of incubation (Fig. 1d). In contrast, neither cells incubated in control or in zinc deficient (1.5 μM zinc) media showed significant changes in total cell NF-κB-DNA binding activity, when incubated with VB, Col or Cyt for 24 h (Fig. 1c).

Cytoskeleton alterations are associated with an impaired translocation of NF-κB into the nucleus

We previously reported that a decrease in cellular zinc concentrations results in a reduction in NF-κB-dependent gene expression. We suggest that the above reduction was due to an impaired NF-κB nuclear translocation, secondary

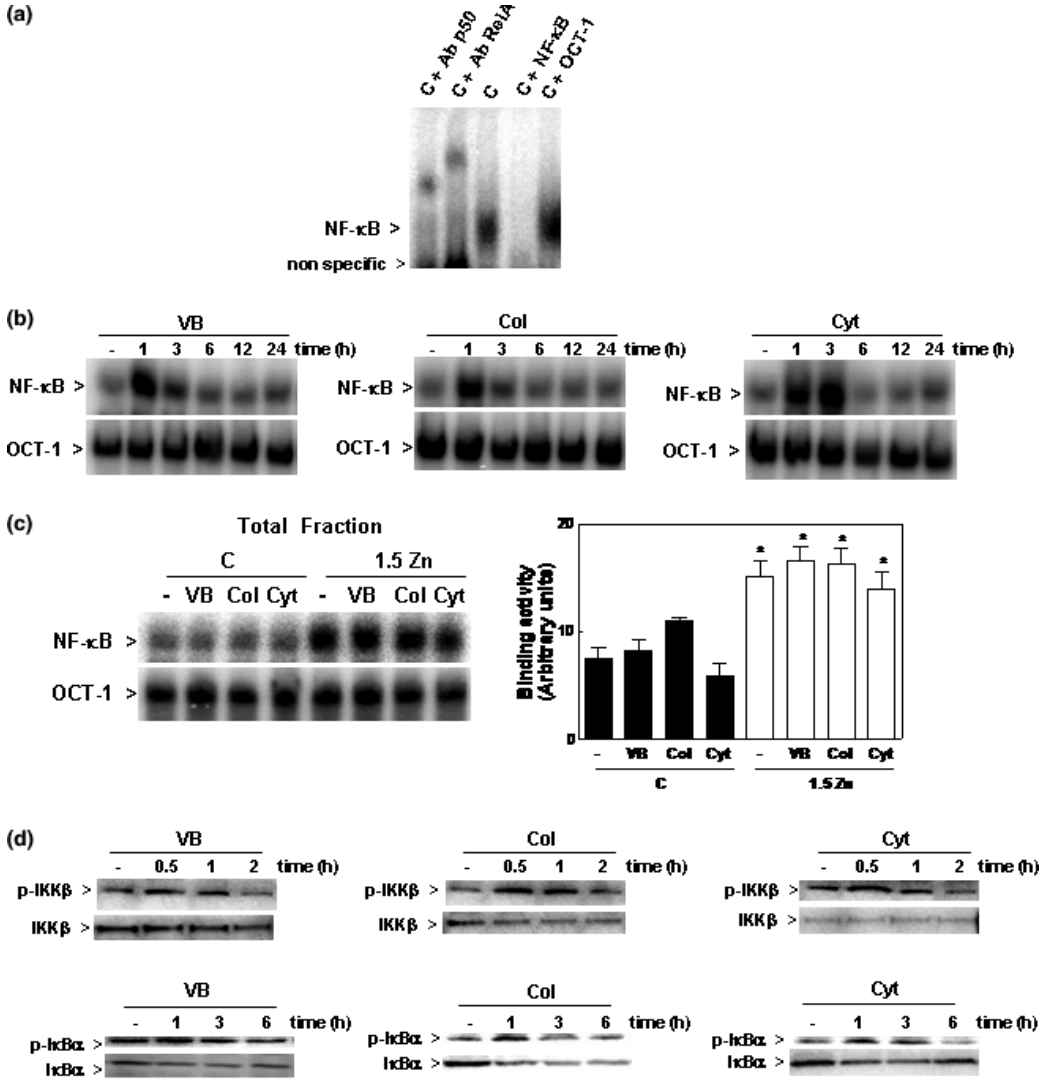

Fig. 1 Effect of cytoskeleton disruption on NF-κB activation. (a) To determine the protein components of the active NF-κB, a nuclear fraction isolated from cells incubated in control media was incubated in the absence (C) or presence of antibodies against p50 or RelA (C + Ab p50 and C + Ab RelA, respectively) for 30 min prior to the binding assay. To determine the specificity of the NF-κB-DNA complex, the control nuclear fraction C was incubated in the presence of 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB (C + NF-κB) or OCT-1 (C + OCT-1) before the binding assay. (b) EMSA assay for NF-κB or OCT-1 in total cell fractions isolated from cells incubated with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col) or 0.5 μM cytochalasin D (Cyt) for 1, 3, 6, 12 or 24 h. Representative EMSA assays out of three independent experiments are shown. (c) EMSA for NF-κB and OCT-1 in total cell fractions after 24 h of incubation. After the EMSA assays, bands were quantitated and results are shown as means ± SEM of four independent experiments. *Significantly different compared to the C group ($p < 0.05$, one way ANOVA test). OCT-1 was assessed as a loading control. (d) Western blot for IKKβ phosphorylation and IκBα phopsphorylation in total cell fractions isolated from cells incubated with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col) or 0.5 μM cytochalasin D (Cyt) for 0.5, 1, 2, 3 or 6 h. Representative Western blots out of three independent experiments are shown.

to a zinc-deficiency-induced disruption of tubulin polymerization (Mackenzie *et al*. 2002). In the present study, the effects of compounds that affect tubulin and actin polymerization on NF-κB nuclear translocation in cells incubated in control or zinc deficient media (1.5 μM zinc) were initially investigated. After 24 h of incubation in control medium, a significant reduction ($p < 0.01$) of NF-κB-DNA, but not OCT-1-DNA, nuclear binding activity was observed in the cells incubated with VB, Col and Cyt (Fig. 2a). Consistent with the above, NF-κB binding activity in the cytosolic fraction was significantly higher ($p < 0.01$) in the control cells incubated with VB, Col and Cyt (Fig. 2a) than in untreated control cells. The ratio of nuclear/cytosolic NF-κB binding activity was on average 45, 69 and 68% lower in

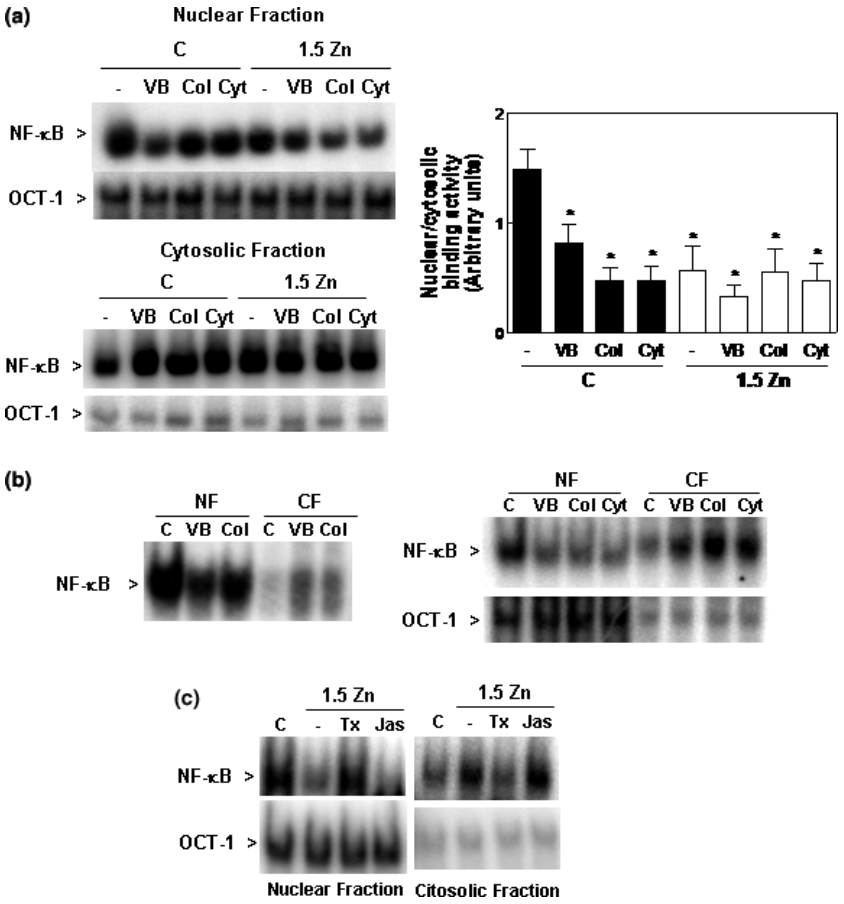

Fig. 2 Effect of cytoskeleton disruption on NF-κB nuclear translocation. Nuclear and cytosolic fractions were isolated after incubating IMR-32 cells for 24 h in control (C) or zinc deficient (1.5 Zn) media incubated without (–) or with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col) or 0.5 μM cytochalasin D (Cyt). (a) EMSA for NF-κB or OCT-1 in nuclear and cytosolic fractions. After the EMSA assays, bands were quantitated and values expressed as the ratio nuclear/cytosolic binding activity. Results are shown as means ± SEM of six independent experiments. *Significantly different compared to the C group ($p < 0.01$, one way ANOVA test). (b) NF-κB-DNA binding activity in nuclear (NF) and cytosolic (CF) fractions isolated from cerebellum granule cells (left panel) or cortical neurons (right panel) after incubating for 6 h without (C) or with 0.5 μM VB (VB), 0.5 μM Col (Col) or 0.5 μM Cyt (Cyt). Representative EMSA assays out of three independent experiments are shown. (c) EMSA for NF-κB and OCT-1 in nuclear and cytosolic fractions isolated from IMR-32 cells incubated for 24 h in control (C) or 1.5 μM zinc media (1.5 Zn) that were pre-treated for 1 h with 1 μM taxol (Tx) or 100 nM jasplakinolide (Jas). Representative EMSA assays out of three independent experiments are shown.

control cells incubated with VB, Col and Cyt, respectively, than in the cells incubated in the absence of these compounds. As previously shown (Mackenzie *et al*. 2002), NF-κB nuclear binding activity was lower in the zinc deficient cells (1.5 μM zinc) than in the control cells(Fig. 2a). Treatment of zinc deficient cells with VB, Col or Cyt was not associated with any additional impairment of NF-κB nuclear translocation (Fig. 2a). We next investigated the effects of cytoskeleton disrupting drugs on NF-κB nuclear translocation in primary cultures of cerebellum granule cells and cortical neurons. Consistent with the results from IMR-32 cells, in both primary cell cultures, we observed a low NF-κB-DNA nuclear binding activity in cells incubated for 6 h in the presence of 0.5 μM VB, 0.5 μM Col or 0.5 μM Cyt, compared to untreated cells (Fig. 2b).

We next evaluated if the stabilization of microtubules with Tx or the stabilization of microfilaments with Jas, would prevent zinc deficiency-induced impairment of NF-κB nuclear translocation. Cells were pre-incubated for 1 h with 1 μM Tx or 100 nM Jas, followed by 24 h incubation in zinc deficient media. While, pre-treatment with Tx prevented Zn deficiency-induced alterations in NF-κB nuclear transport, the pre-treatment of cells with Jas had no effect on the impaired NF-κB nuclear transport (Fig. 2c).

Low NF-κB-DNA nuclear binding activity is associated with a reduced transactivation of NF-κB-driven genes

The influence of altering the cytoskeleton by VB, Col or Cyt on NF-κB-driven transactivating activity was evaluated next using a reporter gene assay. Cells were co-transfected

with a vector expressing β-galactosidase (as a control of the transfection efficiency) and a pNF-κB-Luc plasmid. After 24 h of incubating control cells in the presence of VB, Col, or Cyt, luciferase activity, corrected for β-galactosidase activity, was approximately 65% lower in the VB, Col and Cyt cells than in control cells (Fig. 3a). As previously shown (Mackenzie *et al.* 2002), the zinc deficient (1.5 and 5 μM zinc) cells showed a reduced NF-κB transactivating activity compared to control and zinc supplemented (15 μM zinc) cells (Fig. 3a). Treatment of zinc deficient cells with VB, Col or Cyt did not cause an additional reduction in the NF-κB transactivating capacity (data not shown). The expression of the endogenous genes, p105 and bcl-2, were evaluated by measuring the concentrations of p105 and bcl-2 proteins by Western blot (Fig. 3b). The bcl-2 gene is controlled by NF-κB (Lee *et al.* 1999), and it is widely expressed in the central nervous system (Mattson *et al.* 2000). p105, the precursor for p50, is also dependent on NF-κB (Ten *et al.* 1992). After 24 h of incubation under the different experimental conditions, both p105/β-tubulin and bcl-2/β-tubulin ratios were on average 43, 46 and 38% lower in control cells incubated with VB, Col and Cyt, respectively, compared to untreated control cells (Fig. 3c). After 24 h of incubation, p105/β-tubulin and bcl-2/β-tubulin ratios were lower in the zinc deficient (1.5 and 5 μM zinc) cells than in control and zinc supplemented (15 μM zinc) cells (Fig. 3b). At 24 h, the decreased expression of p105 did not affect p50 cellular content.

Alterations in the nuclear translocation of NF-κB are also observed after measuring the nuclear and cytosolic concentration of RelA and p50

To further characterize the effects of cytoskeleton disruption on the nuclear translocation of the active NF-κB, the concentration of RelA and p50 proteins was measured in nuclear and cytosolic fractions from cells incubated in control medium without or with the addition of VB, Col or Cyt for 24 h. The content of RelA and p50 after the different treatments was determined by Western blot (Fig. 4a and c). Low nuclear/cytosolic ratios for both RelA (64, 47 and 49%) and p50 (62, 73 and 66%), were observed in cells treated with VB, Col and Cyt, respectively, compared to those observed in control cells (Fig. 4b). Similar to our previous findings (Mackenzie *et al.* 2002), after 24 h of incubation, low RelA and p50 concentrations were observed in nuclear fractions isolated from zinc deficient cells compared to control and zinc supplemented cells (Figs 4b, c).

Immunocytochemical evaluation of the cellular distribution of RelA, β-tubulin and actin

The cellular distribution of RelA was further investigated by immunocytochemistry. While RelA was uniformly

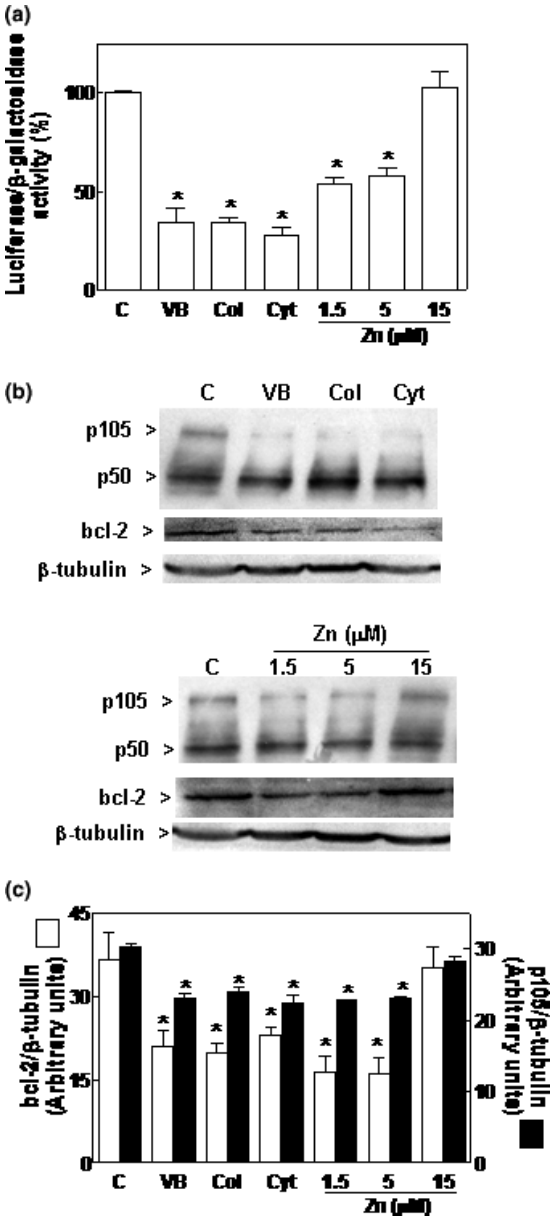

Fig. 3 Cytoskeleton disruption inhibits NF-κB-driven transactivating activity. (a) Cells were co-transfected with a pNF-κB-Luc plasmid and a β-galactosidase plasmid. 24 h after the initiation of the transfection, cells were incubated for 24 h in control media without (C) or with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col) or 0.5 μM cytochalasin D (Cyt), or in chelated media containing 1.5, 5 or 15 μM zinc. Values are expressed as the ratio luciferase/β-galactosidase activity. (b) Western blot for p105 and p50, bcl-2 and β-tubulin in total cell fractions isolated from IMR-32 cells incubated for 24 h in the different experimental conditions. (c) After quantitation of Western blots, results for p105 and bcl-2 are expressed as the ratios p105/β-tubulin (full bars) or bcl-2/β-tubulin (empty bars) content. Results are shown as means ± SEM of three independent experiments. *Significantly lower compared to C and 15 μM zinc groups ($p < 0.03$, one way ANOVA test).

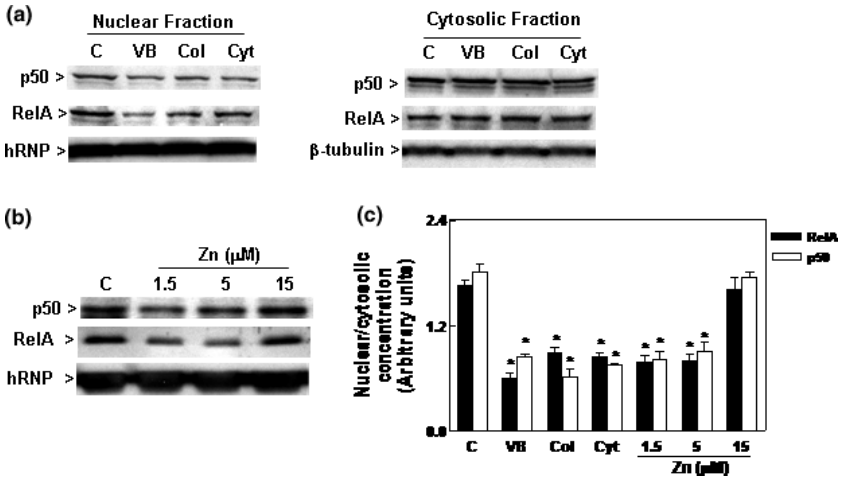

Fig. 4 RelA and p50 protein concentrations in nuclear and cytosolic fractions after microtubule depolymerization. (a) Western blot for RelA and p50 in nuclear and cytosolic fractions isolated after incubating IMR-32 cells for 24 h in control media incubated without (C) or with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col) or 0.5 μM cytochalasin D (Cyt). (b) Western blot for RelA and p50 in nuclear fractions isolated from IMR-32 cells incubated for 24 h in control (C) or chelated media containing 1.5, 5 or 15 μM zinc. C- After quantitation of Western blots, results for RelA (full bars) and p50 (empty bars) are expressed as the ratio nuclear/cytosolic content. hRNP and β-tubulin were measured as loading controls in nuclear and cytosolic fractions, respectively. Results are shown as means ± SEM of four independent experiments. *Significantly lower compared to C and 15 μM zinc groups ($p < 0.01$, one way ANOVA test).

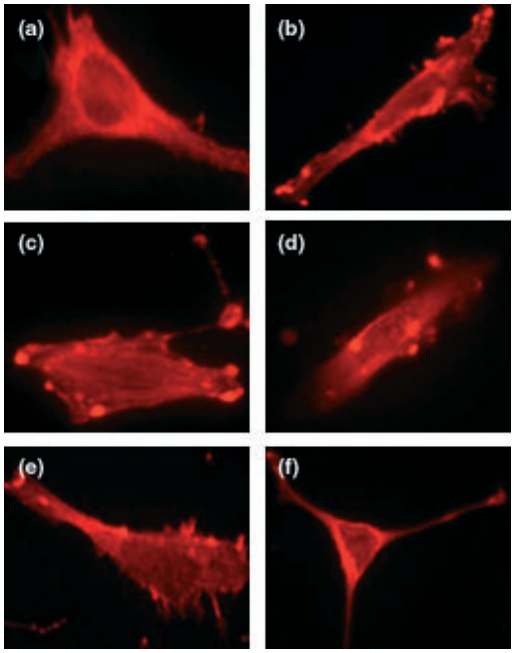

Fig. 5 Immunocytochemical evaluation of RelA distribution after microtubule or microfilament depolymerization. Differentiated IMR-32 cells were cultured on coverslips in control media incubated without or with 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col), 0.5 μM cytochalasin D (Cyt) or chelated media containing 1.5 or 15 μM zinc for 24 h. RelA was immunodetected by using a specific anti-RelA antibody, followed by a Texas-Red-labeled secondary antibody. Cells incubated in (a) control media, (b) control media with VB, (c) control media with Col, (d) control media with Cyt, (e) 1.5 μM zinc media, and (f) 15 μM zinc media, are shown.

distributed in control cells (Fig. 5a), accumules of RelA were observed in the cells incubated for 24 h with VB (Fig. 5b), Col (Fig. 5c) or Cyt (Fig. 5d). A similar RelA distribution pattern to that found for cells incubated with the cytoskeleton disrupting drugs was observed in zinc deficient (1.5 μM zinc) cells (Fig. 5e). Zinc supplementation of the zinc deficient media led to a RelA distribution pattern similar to that observed in control cells (Fig. 5f).

The organization of the microtubule and actin networks after treating cells with VB, Col and Cyt was evaluated by immunocytochemistry. Using a FITC-labeled antibody against β-tubulin, altered β-tubulin distribution patterns were observed in cells treated with VB or Col, compared to untreated cells (Fig. 6a). The observation of accumules of β-tubulin in the cells treated with VB is due to the mechanism of action of VB, which induces auto β-tubulin aggregates or accumules. We previously reported that zinc deficiency results in an impaired tubulin polymerization kinetics in IMR-32 cells (Mackenzie *et al*. 2002). To study possible structural alterations in tubulin polymerization associated with zinc deficiency, β-tubulin distribution was evaluated, by immunocytochemistry, in zinc deficient (1.5 μM zinc) and zinc supplemented (15 μM zinc) cells. Zinc deficient cells showed an altered pattern of microtubule distribution, similar to that observed in cells treated with Col (Fig. 6a). β-tubulin distribution in the 15 μM zinc cells was similar to that observed in the control cells (Fig. 6a). The relative content of polymerized tubulin was measured in cells incubated in media with different zinc concentrations, and in control cells treated with Col

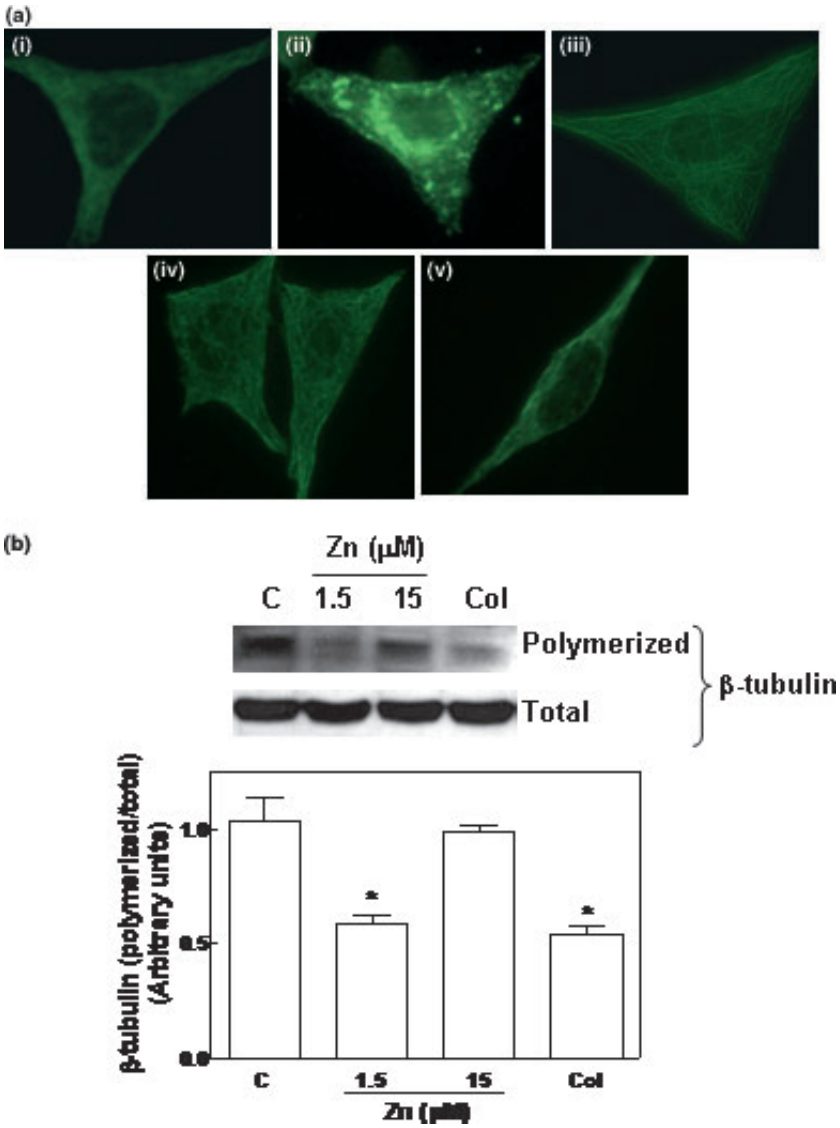

Fig. 6 Zinc deficiency affects microtubule structure and polymerization. (a) Differentiated IMR-32 cells were cultured on coverslips in control media incubated without, or with, 0.5 μM vinblastine (VB), 0.5 μM colchicine (Col), or chelated media containing 1.5 or 15 μM zinc for 24 h β-tubulin was immunodetected by using a specific antiβ-tubulin antibody, followed by a FITC-labeled secondary antibody. Cells from i- control media, ii- control media with VB, iii- control media with Col, iv- 1.5 μM zinc media, and v- 15 μM zinc media, are shown. (b) Western blots for β-tubulin in polymerized and total fractions. Bands were quantitated and values are expressed as the ratio polymerized/total β-tubulin. Results are shown as means ± SEM of four independent experiments. *Significantly lower compared to C and 15 μM zinc groups ($p < 0.05$, one way ANOVA test).

(Fig. 6b). Both, zinc deficiency and Col decreased the relative content of polymerized tubulin, suggesting that zinc deficiency mimics the effects of Col on tubulin polymerization. Zinc supplemented cells (15 μM zinc) had a polymerized tubulin content similar to that of control cells (Fig. 6b).

The organization of the microfilaments was evaluated by measuring actin distribution by immunocytochemistry using FITC-labeled phalloidin. After 24 h of incubation, actin distribution was altered in the cells incubated with Cyt, and in zinc deficient cells compared to control cells (Fig. 7). Cells incubated in zinc supplemented (15 μM zinc) media showed a pattern similar to that observed in control cells (Fig. 7).

Cytoskeleton alterations impair dynein- and karyopherin α1–β–tubulin interactions

Karyopherins are proposed to be associated with microtubules in the cytoplasm, an interaction that would allow the orientation of NLS-containing proteins for their transport into the nuclei. (Smith and Raikhel 1998; Lam *et al.* 2002; Mavlyutov *et al.* 2002). Karyopherin α participates in the

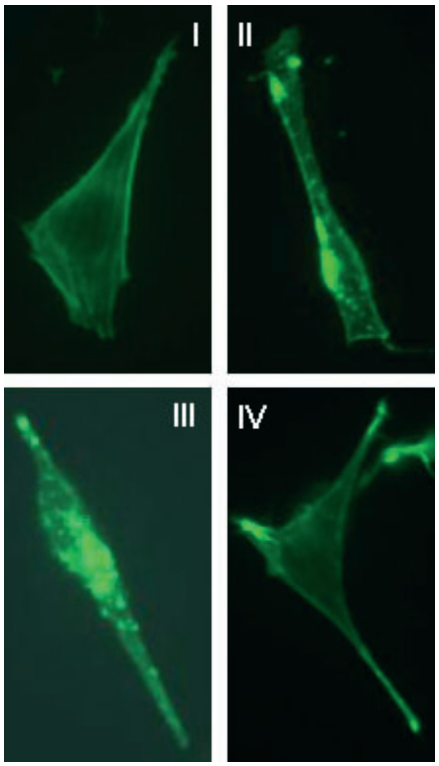

Fig. 7 Immunocytochemical evaluation of actin after microfilament depolymerization. Differentiated IMR-32 cells were cultured on coverslips in control media incubated without, or with, 0.5 μM Cyt (Cyt) or chelated media containing 1.5 or 15 μM zinc for 24 h. Cells from (i) control media (ii) control media with Cyt, and (iii) 1.5 μM zinc media, and (iv) 15 μM zinc media are shown.

axonal and nuclear transport of NLS-bearing cargos through the formation of a complex with the tubulin-associated motor protein dynein (Marelli *et al*. 2001; Hanz *et al*. 2003). Based on the above, the possible consequences of cytoskeleton alterations on the interactions among β-tubulin and karyopherin α1, dynein and p50 were investigated. After 24 h of incubating cells with cytoskeleton disruptors or in media containing different concentrations of zinc, β-tubulin was immunoprecipitated from total cell extracts. Western blots of the pellets were performed for dynein, karyopherin α1 and p50. Low amounts of karyopherin α1 were found to coprecipitate with β-tubulin in the cells incubated with VB or Col and in the zinc deficient (1.5 and 5 μM zinc) cells, compared to controls (Fig. 8a). The amount of β-tubulin precipitated was similar among the groups. Low amounts of dynein coprecipitated with β-tubulin in the cells incubated with VB or Col and in the zinc deficient cells, compared to controls (Fig. 8a). Dynein concentrations were similar among the groups (Fig. 8c). The altered interaction between dynein-β-tubulin and karyopherin α1-β-tubulin was associated with a lower level of coprecipitation of p50 and β-tubulin in the cells incubated with VB, Col and Cyt and in the zinc deficient cells (Fig. 8a). p50 content was similar among the groups (Fig. 8c). To further confirm this hypothesis, total cell fractions were immunoprecipitated with p50 followed by Western blots against dynein and karyopherin α1. After 24 h, low amounts of karyopherin α1 and of dynein were found to coprecipitate with p50 in the cells incubated with VB or Col and in the zinc deficient (1.5 and 5 μM zinc) cells, compared to controls (Fig. 8b). The amount of p50 that was precipitated was similar among the groups. These results indicate that a functional cytoskeleton is required for the correct interaction between β-tubulin-dynein-karyopherin α and subsequently for the transport of NF-κB proteins.

Discussion

In this study we show that a functional cytoskeleton is required for the nuclear transport of transcription factor NF-κB in neuronal cells. Several conditions (VB, Col, Cyt, low cellular zinc) that can inhibit tubulin and actin polymerization decreased the translocation of active NF-κB into the nucleus, resulting in a lower than normal transactivation of NF-κB-driven genes. We show that the impairment in tubulin polymerization can in turn affect the formation of the tubulin-dynein-karyopherin α1-p50 complex required for neuronal retrograde and nuclear NF-κB transport.

Neurons have a neurotransmitter-driven adaptation that allows them to modify their protein content depending on their activity (O'Neill and Kaltschmidt 1997; Gisiger 1998; Meffert *et al*. 2003). This is important both, during early stages of brain development, when cells have to decide whether to grow, migrate, differentiate or die, and in the adult brain for synaptic plasticity. NF-κB has been proposed to participate in the trans-synaptic activity that involves: NF-κB activation at the synapse, retrograde transport and translocation into the nucleus, and the subsequent regulation of gene expression (Wellmann *et al*. 2001). RelA-p50 dimers are present in synaptosomes (Kaltschmidt *et al*. 1993; Guerrini *et al*. 1995), and RelA moves by retrograde transport after stimulation with glutamate, NMDA or synaptic stimulation in hippocampal neurons (Meffert *et al*. 2003).

Current knowledge indicates that NF-κB is involved in the development of the nervous system, regulating neuronal proliferation, migration, differentiation, survival and cell interactions. In the hippocampus, NF-κB (Albensi and Mattson 2000) and Notch (Wang *et al*. 2004) seem to be critical in the regulation of synaptic plasticity. Recently, Meffert *et al*. (2003) showed that the NF-κB signaling cascade plays a major role in neuronal survival and plasticity, and that the deletion of p65 produces learning deficits (Meffert *et al*. 2003). In the cerebellum, NF-κB levels reach maximum values early during postnatal development, a period of active synaptogenesis (Kaltschmidt *et al*. 1995). NF-κB also has a central role in the regulation of neuronal survival. The activation of NF-κB by different agents and conditions protects neurons from different pro-apoptotic

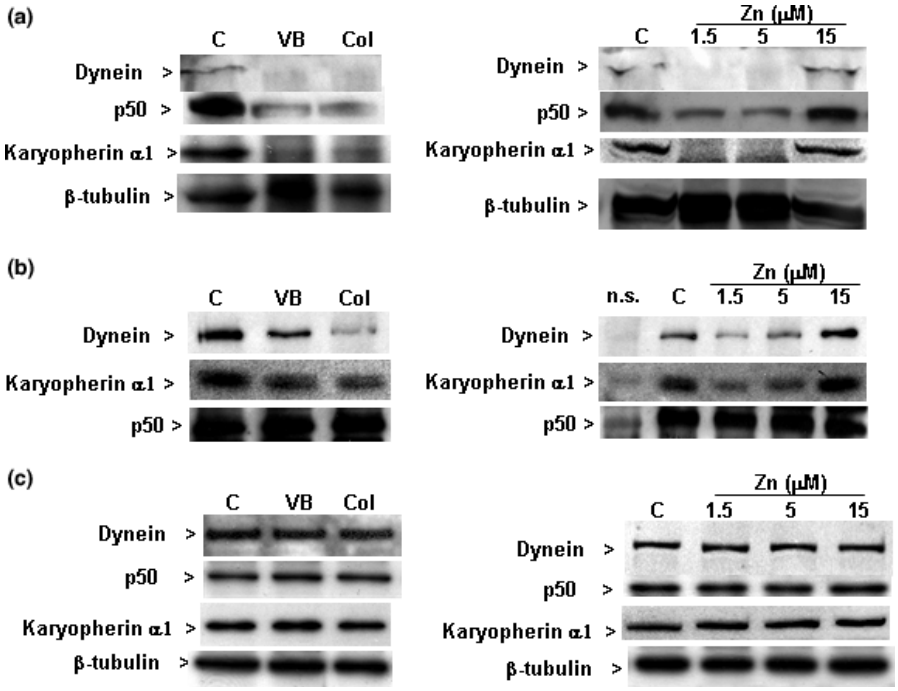

Fig. 8 Alteration of cytoskeleton network affects p50, karyopherin α1 and dynein–β–tubulin interactions. Immunoprecipitation was performed in whole cell lysates isolated after incubating cells for 24 h in control media incubated without (C), or with, 0.5 μM vinblastine (VB), or 0.5 μM colchicine (Col) or in chelated media containing 1.5, 5 or 15 μM zinc. (a) After immunoprecipitation with β-tubulin antibody adsorbed to agarose pellet beads, Western blots for dynein, p50, karyopherin α1 and β-tubulin were performed. One representative Western blot out of three independent experiments is shown. (b) After immunoprecipitating with p50 antibody adsorbed to agarose pellet beads, Western blots for dynein, karyopherin α1 and p50 were performed. One representative Western blot out of three independent experiments is shown. n.s. non-specific represents a control sample that was immunoprecipitated using IgG instead of the corresponding p50 antibody. (c) Western blots for dynein, p50, karyopherin α1 and β-tubulin in total cell fractions after incubating cells for 24 h in the different treatments. One Western blot assay out of three independent experiments is shown.

stimuli [reviewed in (Mattson *et al*. 2000)]. Hippocampal neurons isolated from p50(–/–) transgenic mice were more vulnerable to excitotoxicity than neurons from p50(+/+) and p50(+/–) mice, suggesting that NF-κB can protect neurons against excitotoxic cell death (Yu *et al*. 1999). In agreement with the above, NF-κB inactivation by proteasome inhibitors can trigger apoptosis in different areas of the central nervous system (Taglialatela *et al*. 1998) as well as in cultures of IMR-32 cells (Mackenzie *et al*. 2002). In cerebellum granule cells, survival is dependent on continuous NF-κB activation. Both serum and potassium deprivation leads to the inactivation of NF-κB (Piccioli *et al*. 2001; Lilienbaum and Israel 2003), to a decreased expression of NF-κB-regulated antiapoptotic proteins (Kovacs *et al*. 2004), and to cerebellum granule cells death by apoptosis. In addition, the presence of NF-κB during development and in the mature mouse brain in areas of active neurogenesis supports the participation of NF-κB in neuronal proliferation and migration (Denis-Donini *et al*. 2005). Collectively, the above evidence indicates a key role for NF-κB in neuronal gene expression and in the synapse-nuclei communication.

Based on the involvement of microtubules in the neuronal transport of vesicles and other molecules, we propose that the state of tubulin polymerization is crucial for normal NF-κB transport and nuclear translocation. This hypothesis was originated from our previous findings that a decrease in cellular zinc affects the nuclear translocation of NF-κB (Mackenzie *et al*. 2002), and that zinc deficiency affects *in vitro* tubulin polymerization in cell and animal models of zinc deficiency (Oteiza *et al*. 1988; Oteiza *et al*. 1990; Oteiza *et al*. 1990; Mackenzie *et al*. 2002). In IMR-32 cells, an abnormal β-tubulin distribution was observed in the zinc deficient cells with a pattern similar to that observed in the cells treated with the tubulin polymerization inhibitor Col. Furthermore, both zinc deficiency and Col decreased the amount of polymerized microtubules. Thus, in the current work, we used different experimental models to further define the role of the cytoskeleton in NF-κB nuclear translocation: cytoskeleton disrupting drugs with well described mechanisms of action, and a decrease in cellular zinc, a physiological condition that affects tubulin polymerization, were investigated.

The effect of the different cytoskeleton disrupting conditions on the activation of NF-κB (measured as the NF-κB-DNA binding activity in total cell extracts) was initially evaluated. In IMR-32 cells, cystoskeleton disruption led to a rapid and transient NF-κB activation. As recently described, zinc deficiency activates the initial NF-κB activation events through an increase in cell oxidants (Mackenzie *et al*. 2006). The requirement of the cytoskeleton for the NF-κB nuclear translocation in neuronal cells was next investigated. By EMSA we observed an impaired nuclear transport of NF-κB in cells exposed to cytoskeleton disrupting agents and to a zinc deficient media. The described alterations in NF-κB translocation were supported by our observed patterns of RelA and p50 distribution in the treated cells. An increased number of accumules of RelA was observed in the cytoplasm of cells incubated with VB and Col, and in the zinc deficient cells, compared to control and zinc supplemented cells. The observation that the supplementation of the zinc deficient cell culture media with zinc prevented the tubulin polymerization alterations observed in the cells cultured in the zinc deficient media strongly support the concept that the disruption in tubulin polymerization is a direct effect of the zinc deficiency. Moreover, while stabilizing microfilaments with Jas had no effect, the stabilization of microtubules with Tx prevented zinc deficiency-induced alterations in NF-κB nuclear translocation. This finding additionally supports a crucial role of microtubules in the transport and nuclear translocation of NF-κB. The lack of effect of Jas can be due to the fact that in zinc deficiency, alterations in the actin network structure occur secondary to the initial disruption of the microtubule network. The above finding underscores the potential physiological relevance of our findings, as zinc deficiency is a common occurrence in human populations.

The actin cytoskeleton can also regulate gene expression. IMR-32 cells incubated with Cyt showed an impaired NF-κB nuclear translocation with a subsequent inhibition in the expression of NF-κB-regulated genes. An altered distribution pattern of RelA was observed by immunocytochemistry in the cells incubated with Cyt compared to control untreated cells. The requirement of functional microfilaments for NF-κB transport in neurons can be explained by the close interrelationship between microtubules and microfilaments in sustaining the normal structure and function of the cell cytoskeleton.

We next evaluated a possible mechanism underlying the altered nuclear transport associated with microtubule alterations. NF-κB contains the NLS within its peptide sequence, which is hidden by the interaction with IκB, when NF-κB is anchored in the cytosol. Secondary to different stimuli, IκB is phosphorylated, released and degraded by the proteasome and the NLS sequence is exposed. The nuclear transport of proteins containing the NLS involves the recognition and binding of the NLS-protein with karyopherin α, the subsequent interaction with karyopherin β, the binding of the complex to the nuclear pore and the translocation of the NLS-protein through the pore in an energy-dependent process mediated by the Ran guanosine 5′-triphosphate (GTP)-binding proteins. Karyopherins are found in the neuronal axoplasm and they are involved in the retrograde transport of NLS-containing proteins (Hanz *et al*. 2003). In the microtubule-dependent retrograde axonal transport, the motor protein dynein plays a central role as the motor force for the mobilization of cargos through microtubules (Guzik and Goldstein 2004). Evidence suggests that an association between karyopherin α, dynein and microtubules is necessary for the nuclear translocation of NLS-containing proteins (Hanz *et al*. 2003). Given the above, we investigated if the altered NF-κB nuclear translocation associated with microtubule depolymerization could affect the formation of a tubulin-dynein-karyopherin α-p50 complex. In IMR-32 cells, immunoblotting assays showed that dynein, karyopherin α and p50 coprecipitated with β-tubulin. Cells incubated with VB or Col, or in zinc deficient media showed a markedly reduced association between β-tubulin and dynein, karyopherin α1 and p50. Of particular relevance is the finding that a physiological decrease in neuronal zinc, which affects microtubule polymerization and structure, causes a similar alteration in the formation of the β-tubulin-dynein-karyopherin α1-p50 complex to that induced by cytoskeleton disrupting drugs. Taken together, these results indicate that a functional microtubule network is required for the formation of the complex and subsequent neuronal transport and nuclear translocation of NF-κB. In support of this, *in vitro* studies have previously shown an association between karyopherin α and the cytoskeleton in tobacco protoplasts (Smith and Raikhel 1998). It was proposed that this association is necessary for the transport of NLS-containing-proteins from the cytosol into the nuclei (Smith and Raikhel 1998). Accordingly, the nuclear translocation of parathyroid hormone is inhibited by nocodazole-induced tubulin depolymerization (Lam *et al*. 2002).

In summary, results obtained from the described studies show that alterations in neuronal cytoskeleton assembly can affect the transport of the active NF-κB into the nucleus and the transactivation of NF-κB-driven genes. We suggest that during development, as well as in the adult brain, conditions such as zinc deficiency, which affect the normal structure and function of the cytoskeleton, can affect neuronal proliferation, differentiation, synaptic plasticity and neuronal survival secondary to impaired NF-κB nuclear translocation and subsequent impairment of NF-κB-dependent gene regulation.

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