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Zinc and the Cytoskeleton in the Neuronal Modulation of Transcription Factor NFAT

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Transcription factor NFAT is crucial in the development of the nervous system due to its role in neuronal plasticity and survival. In this study we characterized the role of zinc and the cytoskeleton in the modulation of NFAT in neuronal cells. The incubation of cells in zinc deficient media led to NFAT activation that was inhibited by the calcium chelator BAPTA and the antioxidants (±)-α-lipoic acid and *N*-acetyl cysteine, suggesting the involvement of calcium and oxidants in the initial steps of NFAT activation associated with zinc deficiency. At a second step of regulation, a decrease in cellular zinc led to an impaired transport of the active NFAT from the cytosol into the nucleus due to alterations in tubulin polymerization secondary to a decrease in neuronal zinc. Furthermore, disruption of the cytoskeleton structure by cold and chemical agents (colchicine (Col), vinblastine (VB), cytochalasin D (Cyt)) also inhibited NFAT transport into the nucleus. The altered nuclear transport caused a decrease in NFAT-dependent gene expression. This study demonstrates for the first time that zinc can modulate transcription factor NFAT in neuronal cells, and that microtubules are involved in NFAT nuclear translocation, crucial event in the regulation of NFAT transcriptional activity. J. Cell. Physiol. 210: 246–256, 2007. © 2006 Wiley-Liss, Inc.

The nuclear factor of activated T cells (NFAT) was first described in activated T cells (Durand et al., 1988; Shaw et al., 1988; Serfling et al., 1989; Rincon and Flavell, 1997), but it is now known to be also present in heart valves (Graef et al., 2001c) and in the central nervous system (Graef et al., 2001c). Current evidence indicates that NFAT plays important functions in nerve and brain development being involved in synaptic plasticity and memory function (Timmerman et al., 1996; Graef et al., 1999, 2001a,c, 2003; Groth and Mermelstein, 2003; Meffert et al., 2003) and in the regulation of cell survival (Benedito et al., 2005; Jayanthi et al., 2005).

At this time, five members of the NFAT family of proteins have been described: NFAT1 (also called NFATc2 or NFATp), NFAT2 (NFATc1 or NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx), and NFAT5 (TonEBP) (reviewed in Macian et al., 2001). In rest cells, NFAT is normally found in the cytoplasm. NFAT activation is dependent on calcineurin, a calcium/calmodulin-dependent phosphatase (Dolmetsch et al., 1997). The elevation of intracellular calcium leads to the binding of calcineurin to NFATc1-c4, which dephosphorylates serines within the SP repeats and serine-rich motifs in the N-terminus region of NFATc proteins. NFAT dephosphorylation unmasks the nuclear localization sequence (NLS) in the proteins allowing the translocation of NFAT into the nucleus (Beals et al., 1997). At the nucleus, NFAT binds to consensus sequences in the promoter or enhancer regions of target genes promoting transcription.

Zinc can play a central role in neuronal proliferation, differentiation, and migration, and also regulating apoptotic neuronal death (Dvergsten et al., 1983, 1984a,b; Verstraeten et al., 2004). A decrease in neuronal zinc leads to an increase in cellular oxidants. In IMR-32 cells, high levels of cell oxidants and an increased release of H_2O_2 to the media were found in cells incubated in zinc deficient media (Zago et al., 2005; Mackenzie et al., 2006b). The observed increase in cell oxidants was associated with the triggering of transcription factors NF-κB (Mackenzie et al., 2006b) and AP-1 activation (Zago et al., 2005). Although limited, there is evidence suggesting that oxidants could be also involved in NFAT activation (Huang et al., 2001a,b), in part mediated by H_2O_2 (Huang et al., 2001b). Thus, a possible oxidant-driven activation of NFAT in zinc deficient neurons will be investigated.

Downstream, zinc deficiency could affect the retrograde and nuclear transport of the active NFAT. We recently reported that a decrease in cellular zinc inhibits NF-κB-dependent gene expression. NF-κB nuclear translocation was impaired in association with a low rate of microtubule assembly in the zinc deficient cells (Mackenzie et al., 2002, 2006a). The activation of both, NF-κB and NFAT, involves the exposure of a NLS that

Abbreviations: BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetracetic acid tetrakis(acetoxymethyl ester); Col, colchicine; Cyt, cytochalasin D; FBS, fetal bovine serum; Fura 2-AM, Fura-2 pentakis(acetoxymethyl) ester; hnRNP, heterogeneous nuclear ribonucleoprotein; IP_3, Inositol 1,4,5-triphosphate; NFAT, nuclear factor of activated T cells; NLS, nuclear localization sequence; TPEN, *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylendiamine; Tx, taxol; VB, vinblastine.

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targets the transcription factor to the nuclei. The NLS determines the recognition and binding of the transcription factor to karyopherin α, and its subsequent transport and translocation through the nuclear pore (Goldfarb et al., 2004). It has been recently proposed that in neurons, NLS-bearing cargos form a complex with karyopherin α and β, and via the interaction with dynein, the retrograde transport occurs along microtubules (Hanz and Fainzilber, 2004). We hypothesize that since a functional microtubule network would be required for the axonal retrograde transport and nuclear import of NLS-bearing proteins, the alteration in tubulin polymerization that occurs when cellular zinc decreases would lead to an impaired translocation of NFAT from the cytosol to the nucleus and subsequently of a decreased NFAT-dependent gene transcription.

Due to the importance of NFAT-regulated gene expression in early stages of neuronal development, and its role in synapses to nucleus gene expression, the objective of this study was to investigate how variations in cellular zinc could affect NFAT modulation in neuronal cells. The obtained results also expand the understanding of the role of the cytoskeleton in the neuronal modulation of NFAT.

MATERIALS AND METHODS

Materials

IMR-32 and PC-12 cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media and reagents, and LipofectAMINE™ 2000 were obtained from Invitrogen Life Technologies (Carlbad, CA). The oligonucleotide containing the consensus sequence for NFAT (5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′), and β-tubulin, heterogeneous nuclear ribonucleoprotein (hnRNP) and NFATc4 antibodies were obtained form Santa Cruz Biotechnology (Santa Cruz, CA). The oligonucleotide containing the consensus sequence for OCT-1 and SP-1, the reagents for the EMSA assay, the CellTiter-Glo® Luminescent Cell Viability 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). The PathDetect NFAT cis reporting system was obtained from Stratagene (La Jolla, CA). PVDF membranes were obtained from BIO-RAD (Hercules, CA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA). The Cell Death Detection ELISA^{PLUS} was obtained from Roche Diagnostics (Indianapolis, IN). The ECL plus western blotting system was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). 1,2-Bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetracetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), (±)-α-lipoic acid, *N*-acetyl cysteine, *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylendiamine (TPEN), Fura-2 pentakis(acetoxymethyl) ester (Fura 2-AM), nerve growth factor-7S, cyclosporin A, 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).

Methods

Cell culture. IMR-32 cells were cultured at 37°C, 5% CO_2 in complex medium (55% (v/v) DMEM high glucose, 30% (v/v) Ham F-12, 5% (v/v) α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics-antimicotic (50 U/ml penicillin, 50 μg/ml streptomycin, and 0.125 μg/ml amphotericin B). Cells were differentiated as previously described (Neill et al., 1994; Erlejman and Oteiza, 2002). Briefly, cell cultures were incubated with 1 mM dibutyryl cAMP and 4 μM 5 bromodeoxyuridine for 12 days and the differentiating medium was changed every 3 days.

Rat adrenal pheochromocytoma cells (PC-12) were grown at 37°C, 5% CO_2 in high glucose DMEM medium containing 5% (v/v) FBS, 10% (v/v) horse serum, and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) on poly-L-lysine-coated cell culture dishes. For the induction of neuronal differentiation, 50 ng/ml nerve growth factor-7S were added 4 h after seeding, and the media was replaced every second day (Schimmelpfeng et al., 2004). After 5 days, the differentiating medium was replaced with media containing nerve growth factor-7S and different concentrations of zinc.

Cerebellum granule cells were obtained as described by 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 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 μM cytosine-β-D-arabinoside to inhibit non-neuronal cell proliferation, and incubated for 6 h without or with VB, Col, or Cyt. 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.

Zinc deficient serum was prepared by chelation with diethylenetriamine pentaacetic acid as previously described (Oteiza et al., 2000). The chelated serum was subsequently diluted with complex or DMEM medium to a final concentration of 3 mg protein/ml to match the protein concentration of the control non-chelated media (10% (v/v) FBS or 5% (v/v) horse serum + 10% (v/v) FBS for IMR-32 or PC-12, respectively). 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, whereas the zinc concentration of the control non-chelated complex media was 6.2 ± 0.3 μM.

Undifferentiated IMR-32 cells (90% confluence) or differentiated PC-12 (5 days in differentiating medium) and IMR-32 cells (12 days in differentiating medium) were cultured in control medium. The medium was removed and replaced with control medium or chelated media containing 1.5, 5, or 15 μM zinc for different time periods depending on the experiments. To investigate the effects of cytoskeleton disruptors, differentiated IMR-32 cells were incubated in control medium without or with the addition of 0.5 μM VB, 0.5 μM Col, or 0.5 μM Cyt and cells were harvested after 24 h in culture.

Evaluation of apoptosis. To evaluate the effects of cyclosporin A on cell death by apoptosis we measured DNA fragmentation. The cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were quantitated using the Cell Death Detection ELISA^{PLUS} kit, following the manufacturer's protocol. The amount of DNA fragments (measured from the absorbance at 405 nm) was referred to cell viability determined simultaneously for each group. Cell viability was measured with the CellTiter-GLO luminescent assay following the manufacturer's protocol. The assay measures cell ATP levels and is based on the mono-oxygenation of luciferin catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen.

Determination of cellular calcium levels. Intracellular calcium was measured with the probe Fura 2-AM at two excitation wavelength: λexc: 340 and 380 nm (λem: 510 nm). Cells were cultured in 12-well plates and after the corresponding treatments, cells were be incubated for 45 min with the probe (5 μM), washed, disrupted in PBS-Igepal 0.1% (v/v), containing the zinc chelator TPEN (100 μM), and the fluorescence was measured. Maximum and minimum fluorescence were determined in cell lysates added with 1 mM calcium or 10 mM EGTA, respectively.

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., 2006b).

For the EMSA, the oligonucleotides containing the consensus sequence of NFAT, CT-1, or SP-1 were end labeled with [γ-32P] ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1× binding buffer [5× binding buffer: 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)]. For the supershift assays, prior to the addition of the labeled nucleotide, samples were incubated in the presence of the antibody for NFATc4. The products were separated by electrophoresis in a 4%–6% (w/v) non-denaturing polyacrilamide gel using 0.5× TBE (Tris/borate 45 mM, EDTA 1 mM) as the running buffer. The gels were dried and the radioactivity quantitated in a Phosphoimager 840 (Amersham Pharmacia Biotech., Inc.).

Western blot analysis. For the preparation of total cell extracts, cells (20×10^6 cells) were rinsed with PBS, scrapped, and centrifuged. The pellet was rinsed with PBS, and resuspended in 200 μl of 50 mmol/L HEPES (pH 7.4), 125 mM KCl which contained protease inhibitors, and 2% (v/v) Igepal. The final concentration of the inhibitors was 0.5 mmol/L PMSF, 1 mg/L leupeptin, 1 mg/L pepstatin, 1.5 mg/L aprotinin, 2 mg/L bestatin, and 0.4 mM sodium pervanadate. Samples were exposed to one cycle of freezing and thawing, incubated at 4°C for 30 min and centrifuged at 15,000*g* for 30 min. The supernate was decanted and protein concentration was measured (Bradford, 1976).

Aliquots of total, nuclear or cytosolic fractions containing 25–50 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored molecular weight standards (Amersham, Piscataway, NJ) were ran simultaneously. Membranes were blotted overnight in 5% (w/v) non-fat milk, incubated in the presence of corresponding antibodies for NFATc4 (1:500), or β-tubulin (1:1,000 dilution) for 90 min at 37°C. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution), the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840.

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 pNFAT-Luc plasmid (1 μg DNA). After 24 h of initiated the transfection, cells were treated with media containing different zinc concentrations or with the control 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 cultured in coverslips were incubated for 24 h in media containing different zinc concentrations or in media containing the cytoskeleton inhibitors. After washing the cells, they were fixed with 4% (w/v) paraformaldehide, 0.12 M sucrose in PBS for 1 h at room temperature, and used for the immunocytochemical detection. For the detection of NFAT proteins, fixed cells were permeabilized by incubation with 0.01% (v/v) Triton X-100 in PBS for 5 min. Afterwards, samples were blocked with 1% (w/v) BSA in PBS for 2 h and incubated overnight with the primary antibody against NFATc4. The coverslips were rinsed twice with PBS and incubated with the appropriate Texas Red-conjugated secondary antibody. After staining, the preparations were mounted and analyzed by epifluorescence using an Olympus BX50 microscope. The intensity and distribution of the fluorescence was observed using an Image Pro Plus v.4.5 software.

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). A P-value <0.05 was considered statistically significant. Values are given as mean ± SEM.

RESULTS

Calcineurin/NFAT pathway in human IMR-32 neuroblastoma cells

The presence of the calcineurin/NFAT signaling pathway and its relevance for neuroblastoma IMR-32 cell survival was initially investigated. Cyclosporin A, an inhibitor of calcineurin, affected cell morphology (Fig. 1A) and caused a dose-dependent decrease in cell viability measured after 24 h of treatment (Fig. 1B). The

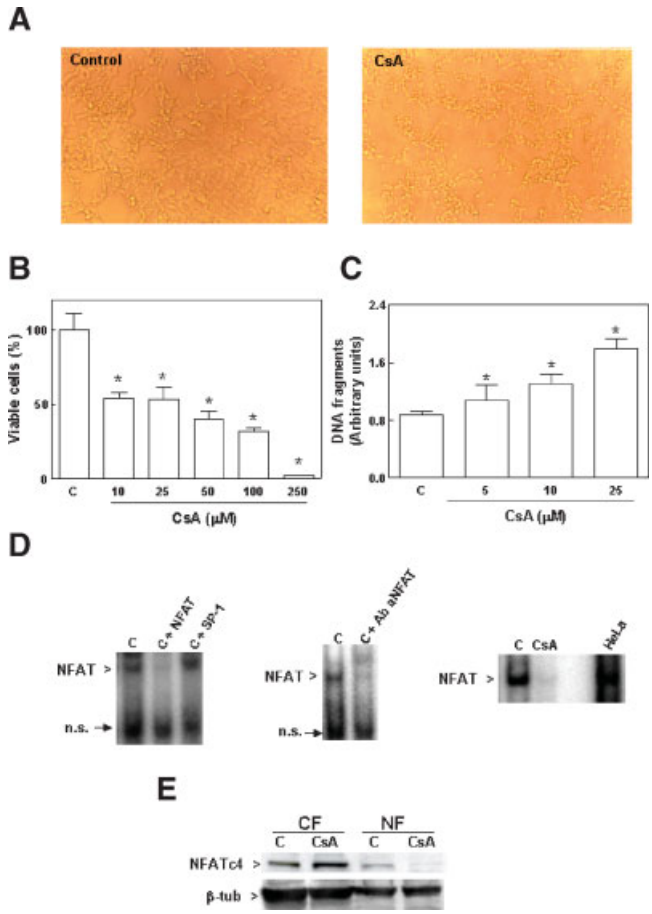

Fig. 1. Characterization of the calcineurin-NFAT signaling pathway in IMR-32 cells. Cells were incubated for 24 h, in the absence (C) or the presence of 5–250 μM cyclosporin A (CsA). **A**: Phase-contrast microscopy of cells incubated for 24 h in the absence (C) or the presence of 25 μM CsA (CsA). **B**: Cell viability values are presented as percentage of control values, **C**: DNA fragment content was evaluated measuring the amount of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) as described in Materials and Methods, and referred to cell viability. For B and C, results are shown as mean ± SEM of four independent experiments. *Significantly different compared with C cells ($P < 0.05$, one-way ANOVA test). **D**: EMSA assays showing the presence of specific bands corresponding to the interaction NFAT-DNA (consensus oligonucleotide) in nuclear fractions from IMR-32 cells. **Left part**: Nuclear fractions from control cells were incubated in the absence (C) or the presence of a 100-fold excess of cold oligonucleotide for NFAT (C + NFAT) or SP-1 (C + SP-1), n.s.: non-specific. **Central part**: Nuclear fractions incubated in the absence (C) or the presence of an antibody against NFATc4 (C + Ab aNFAT). **Right part**: Nuclear fractions isolated from cells incubated for 24 h in the absence (C) or presence of 25 μM CsA (CsA). A stimulated HeLa cell nuclear fraction (HeLa) was run simultaneously as a positive control. **E**: The presence and distribution of NFATc4 protein was evaluated by Western blot in nuclear (NF) and cytosolic fractions (CF) isolated from IMR-32 cells incubated for 24 h in the absence (C) or the presence of 25 μM CsA (CsA). β-tubulin is shown as a control of protein loading. One representative Western blot out of three independent experiments is shown.

decrease in cell viability was partially due to the triggering of apoptotic cell death, as evidenced by the cyclosporin A-induced DNA fragmentation, measured as mono and oligonucleosomes (Fig. 1C)

The specific bands for NFAT in the EMSA assays were identified by competition with a 100-fold excess of specific or non-specific oligonucleotides (Fig. 1D) or by incubation with a specific antibody against NFATc4 (Fig. 1D). As further evidence of a calcineurin-activated NFAT signal in IMR-32 cells, nuclear NFAT-DNA binding activity was decreased (Fig. 1D) and NFATc4 protein, measured by Western blot, was almost absent in nuclear fractions from IMR-32 cells treated with 25 μM cyclosporin A (Fig. 1E). Taken together, these results show that transcription factor NFAT is present in IMR-32 neuroblastoma cells, and it is regulated by calcineurin.

Low extracellular zinc concentrations induces the activation of NFAT, by increasing intracellular calcium and global oxidant levels

The effects of low extracellular zinc concentrations (1.5 and 5 μM) on total NFAT activation in human neuroblastoma IMR-32 cells was investigated. Incubation of IMR-32 cells under the current experimental conditions was shown to lead to a rapid (3–48 h) decrease in labile zinc pools (Mackenzie et al., 2002) and in total zinc content (Zago et al., 2005). NFAT-DNA binding activity was evaluated by EMSA in total fractions from cells incubated for 3–24 h in control non-chelated media or in chelated media containing 1.5–5 μM zinc (zinc deficient) or 15 μM zinc (zinc-supplemented media). The decrease in cellular zinc was

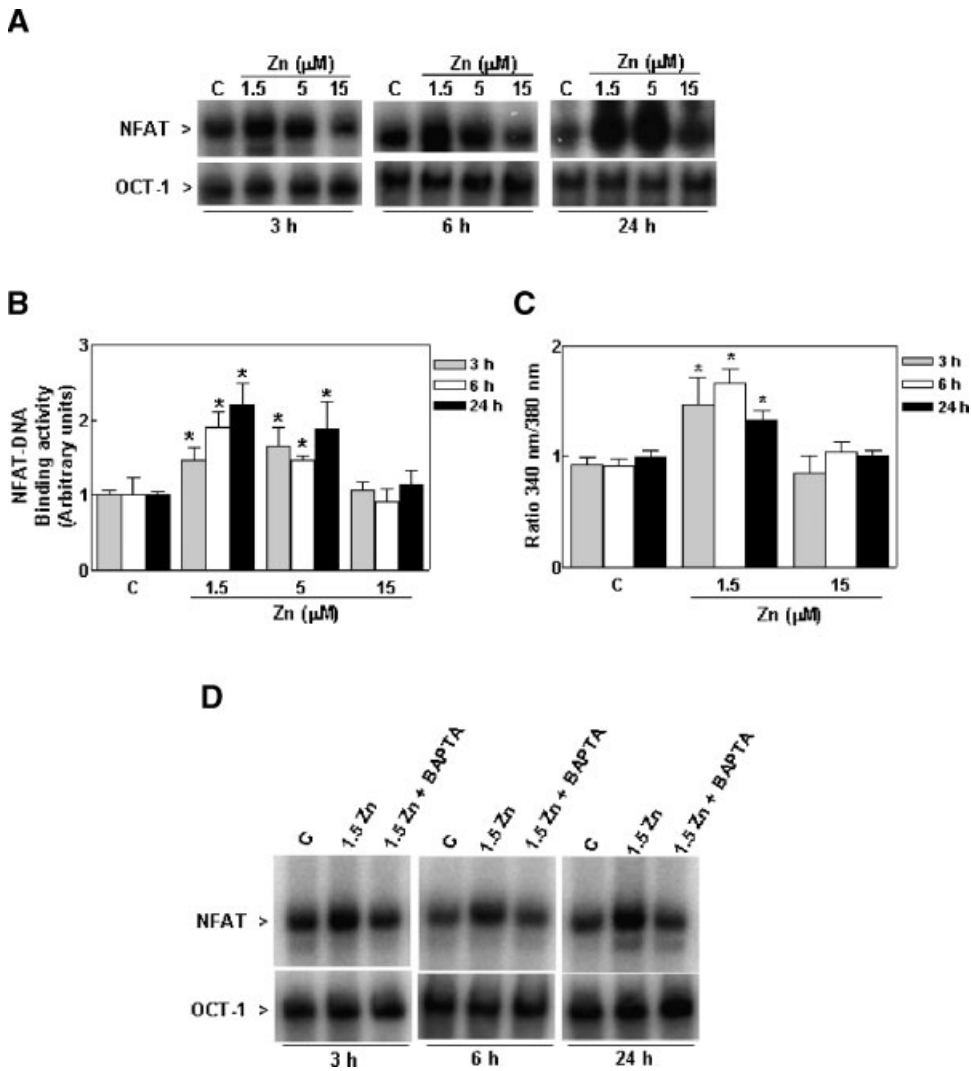

Fig. 2. Low extracellular zinc leads to NFAT activation and to an increase in cellular calcium. **A** and **B**: Total cell fractions were isolated after 3–24 h of incubating IMR-32 cells in control non-chelated media (C) or in chelated media containing 1.5, 5, or 15 μM zinc. **A**: EMSA for NFAT and OCT-1 in total fractions isolated from cells incubated for 3–24 h in the corresponding media. **B**: After the EMSA assays, NFAT bands were quantitated. Results are shown as mean ± SEM of five independent experiments. *Significantly different compared to C and 15 μM zinc groups ($P < 0.01$, one way ANOVA test). **C**: IMR-32 cells were incubated for 3–24 h in control non-chelated media (C) or in chelated media containing 1.5, 5, or 15 μM zinc. Cellular calcium levels were determined with the probe Fura 2-AM as described in the Materials and Methods. Results are shown as mean ± SEM of four independent experiments. *Significantly different compared to C and 15 μM zinc groups ($P < 0.05$, one way ANOVA test). **D**: EMSA assays of total cell fractions from cells incubated for 3–24 h in non-chelated media (C) or chelated media containing 1.5 (1.5 Zn) μM zinc in the absence or the presence of 10 μM BAPTA-AM.

associated with NFAT activation throughout the studied period. After 24 h of incubation, the DNA binding activity of NFAT in total cell fractions was higher (1.6- to 2.9-fold) in the 1.5 and 5 μM zinc cells compared to control and 15 μM zinc cells (Fig. 2A,B).

We next investigated if NFAT activation could be triggered by an increase in cellular calcium. Cellular calcium, as measured with the probe Fura 2-AM, was significantly higher in the cells incubated in media containing low zinc concentrations (1.5 and 5 μM zinc) (Fig. 2C). The rise in cellular calcium concentrations was already observed after 3 h of incubation in the zinc deficient media and remained elevated for the subsequent 24 h. The involvement of calcium in NFAT activation was investigated by incubating cells for 3–24 h in the corresponding media, in the absence or presence of the cell permeable calcium chelator BATPA-AM (Fig. 2D). Treatment with 10 μM BAPTA-AM prevented zinc deficiency-induced NFAT activation indicating the involvement of calcium in the triggering of NFAT when cellular zinc decreases.

Based on the recent finding that zinc deficiency increases cell oxidant levels in IMR-32 cells (Mackenzie et al., 2006b), that can be prevented by incubating cells in the presence of the antioxidant substances (±)-α-lipoic acid and *N*-acetyl cysteine (Mackenzie et al., 2006b), the capacity of these compounds to prevent zinc deficiency-induced NFAT activation was investigated. Both (±)-α-lipoic acid (0.5 mM) and *N*-acetyl cysteine (1 mM), added at the time of exposing cells to the different media, prevented the increase in NFAT-DNA binding activity in total cell extracts from zinc deficient cells (Fig. 3A,B). These results suggest that oxidants can also participate in the activation of NFAT when cellular zinc decreases.

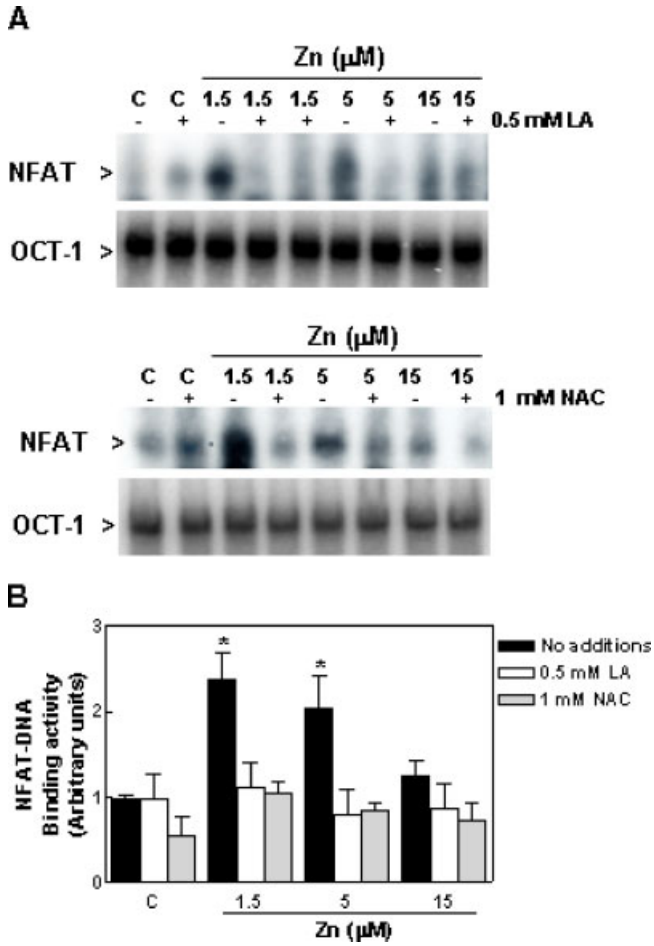

Fig. 3. The antioxidants (±)-α-lipoic acid and *N*-acetyl cysteine prevents zinc deficiency-induced NFAT activation. Total cell fractions were isolated after 24 h of incubating IMR-32 cells in control non-chelated media (C) or in chelated media containing 1.5, 5, or 15 μM zinc in the absence or presence of 0.5 mM (±)-α-lipoic acid (LA) or 1 mM *N*-acetyl cysteine (NAC). **A**: EMSA for NFAT and OCT-1 in total fractions isolated from cells incubated for 24 h in the corresponding conditions; n.s.: non-specific. **B**: After the EMSA assays, bands corresponding to total extracts isolated from cells incubated for 24 h in the corresponding media and in the absence (full bars) or presence of 0.5 mM LA (empty bars) or 1 mM NAC (gray bars) were quantitated. Results are shown as mean ± SEM of five independent experiments. *Significantly different compared to the other groups and treatments ($P < 0.05$, one way ANOVA test).

Low extracellular zinc concentrations impair the nuclear translocation of NFAT

The nuclear translocation of the active NFAT was investigated by measuring the NFAT-DNA binding activity and the distribution of NFAT proteins by Western blot in nuclear and cytosolic fractions. EMSA assays showed a higher content of active NFAT in cytosolic fractions isolated from IMR-32 cells incubated in zinc deficient media (1.5 and 5 μM zinc) (Fig. 4A, left part). The ratio nuclear/cytosolic NFAT-DNA binding activity was significantly lower in the cells incubated in the 1.5 and 5 μM zinc media compared to those incubated in control and 15 μM zinc media (Fig. 4B). Furthermore, the zinc deficient (1.5 and 5 μM zinc) IMR-32 cells showed a reduced NFAT transactivating activity compared to control and zinc supplemented (15 μM zinc) cells (Fig. 4C). While IMR-32 cells are a model of cortical neurons, differentiated PC-12 cells are a model of sympathetic neurons. Incubation for 24 h in low zinc media also impaired NFAT nuclear transport in differentiated PC-12 cells (Fig. 4A, right part).

The nuclear translocation of NFAT was also evaluated by measuring the distribution of NFAT proteins by Western blot and immunocytochemistry. NFATc4 levels, as evidenced by Western blot of nuclear and cytosolic fractions (Fig. 5A) and subsequent quantitation (Fig. 5B), were lower in the nuclear fractions and higher in the cytosolic fractions from cells incubated for 24 h in the zinc deficient media compared to control and zinc supplemented media.

The distribution of NFATc4 studied by immunocytochemistry showed that NFATc4 is evenly distributed in non-stimulated IMR-32 cells incubated in control media and in the cells incubated in chelated media supplemented with 15 μM zinc (Fig. 5C). In the zinc deficient cells (1.5 μM zinc) NFATc4 had mainly a cytosolic distribution with the appearance of fluorescence aggregates in the cytosol (Fig. 5C).

Cytoskeleton disruption impairs NFAT nuclear translocation

A decrease in cellular zinc is associated with alterations in the in vitro tubulin polymerization in IMR-32 cells and with an impaired transport of other NLS-bearing transcription factor, NF-κB (Mackenzie et al., 2002). To investigate the possible requirement of an organized microtubule network in the nuclear transport of NFAT that would explain the observed alterations in NFAT nuclear translocation in zinc deficient cells, the effects of cytoskeleton disruptors were investigated. Cells were incubated in control or zinc deficient media for 24 h in the absence or presence of VB and Col, tubulin

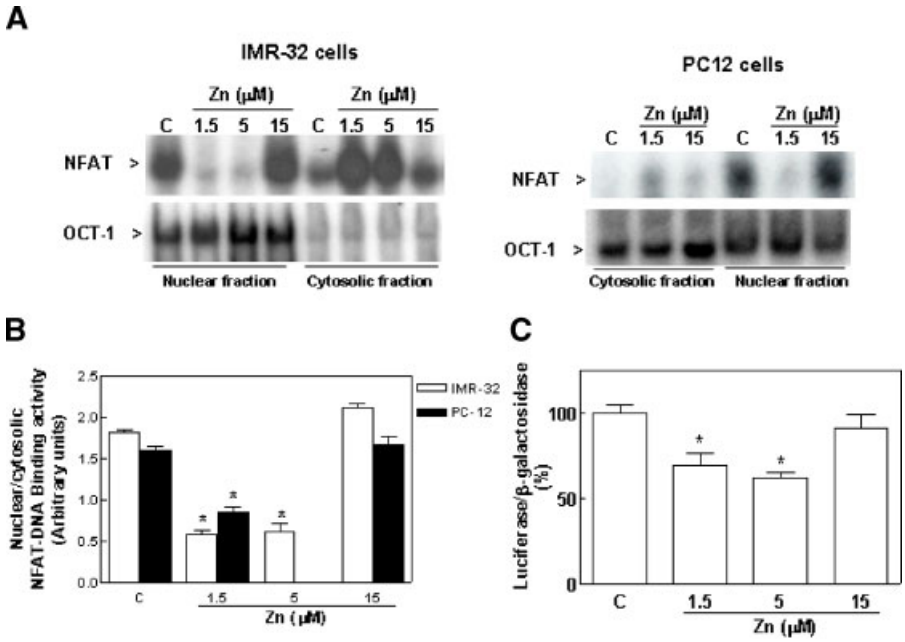

Fig. 4. Zinc deficiency leads to a decreased nuclear NFAT-DNA binding activity and decreased NFAT-dependent transactivating activity. Nuclear and cytosolic fractions were isolated after 24 h of exposure to control non-chelated media (C) or to chelated media containing 1.5, 5, or 15 μM zinc. **A**: EMSA for NFAT and OCT-1 in nuclear and cytosolic fractions isolated from IMR-32 cells (**left part**) or PC-12 cells (**right part**) incubated for 24 h in the corresponding conditions. **B**: After the EMSA assays, the bands for NFAT in IMR-32 (empty bars) or PC-12 (full bars) cell fractions were quantitated and the values expressed as the ratio nuclear/cytosolic binding activity. Results are shown as mean ± SEM of six independent experiments. *Significantly different compared with control and 15 μM zinc groups ($P < 0.01$, one-way ANOVA test). **C**: Transactivation of pNFAT-Luc plasmid. Luciferase and β-galactosidase activities were evaluated after 24 h incubation in the corresponding media. Data are expressed as the ratio luciferase/β-galactosidase activity. Results are shown as mean ± SEM of three independent experiments. *Significantly different compared to C and 15 μM zinc groups ($P < 0.05$, one way ANOVA test).

polymerization inhibitors, or Cyt, an inhibitor of actin polymerization. After 24 h of incubation, NFAT-DNA binding activity in total cell fractions was similar among the different treatment groups (data not shown). However, and similar to that observed in the zinc deficient cells, treating control cells with VB, Col, and Cyt led to the accumulation of NFAT in the cytosol and to a decreased NFAT transport into the nucleus as evaluated by EMSA assay (Fig. 6A,B). In agreement with the above, VB, Col, and Cyt caused a decreased transactivation of an NFAT-driven reporter gene (pNFAT-Luc). Cells were co-transfected with a vector expressing β-galactosidase (as a control of the transfection efficiency) and a pNAFT-Luc plasmid. After 24 h of incubation in the presence of the three cytoskeleton disruptors, the relationship luciferase/ β-galactosidase activity was approximately 55% lower than in control cells (Fig. 6C). VB, Col, and Cyt treatment did not cause an additional effect on NFAT nuclear translocation in zinc deficient cells (Fig. 6A,B).

We next investigated the effects of cytoskeleton disrupting drugs on NFAT nuclear translocation in primary cultures of cerebellum granule cells. Consistent with the results from IMR-32 cells, we observed a low NFAT-DNA nuclear binding activity in cells incubated for 6 h in the presence of 0.5 μM VB or 0.5 μM Col, compared to untreated cells (Fig. 6D). To further characterize the role of the cytoskeleton on NFAT nuclear translocation, microtubule depolymerization was induced by exposing cells to cold. In agreement with the results obtained for cells treated with VB, Col, and Cyt, NFAT-DNA binding activity was high in the cytosolic fraction, and low in the nuclear fraction, when microtubules were depolymerized by cold (1 h at 4°C). The altered distribution of NFAT was reversed by subsequent incubation of cells at 37°C for 30 min (Fig. 6E). OCT-1-DNA binding activity in nuclear fractions was not affected by cold (Fig. 6E).

We next evaluated if the stabilization of microtubules with Tx could prevent zinc deficiency-induced impairment of NFAT nuclear translocation. Cells were pre-incubated for 1 h with 1 μM Tx, followed by 24 h incubation in zinc deficient media. Pretreatment of IMR-32 cells with Tx prevented the altered NFAT nuclear translocation associated with Zn deficiency (Fig. 6F).

Cytoskeleton disruptors also affected the distribution of the NFATc4 protein. As evidenced by Western blot, after 24 h of incubation, VB, Col, and Cyt led to the accumulation of NFATc4 in cytosolic fractions and to a low NFATc4 content in nuclear fractions (Fig. 7A,B). The characterization of NFATc4 distribution by fluorescence microscopy showed that, similar to that observed in zinc deficient cells, the treatment with VB, Col, and Cyt led to the formation of fluorescence aggregates while an homogeneous fluorescence distribution was observed in control untreated cells (Fig. 7C).

DISCUSSION

This study demonstrates for the first time that zinc can modulate transcription factor NFAT in neuronal cells. A decrease in extracellular zinc leads to a decrease in cellular zinc, an increase in cellular calcium and a concomitant increase in cell oxidants. Both, calcium and oxidants trigger the activation of NFAT in the cytosol. However, the active NFAT is not efficiently translocated into the nucleus. The alterations in tubulin polymerization associated with a decrease in neuronal zinc could be

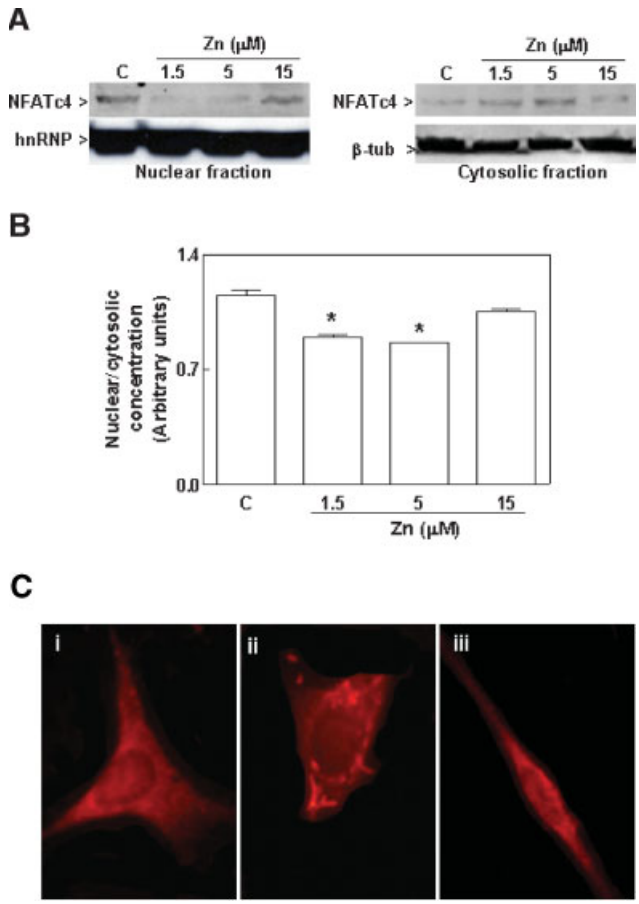

Fig. 5. Zinc deficiency affects the cellular distribution of NFATc4. The cellular distribution of NFATc4 was characterized by (**A** and **B**) Western blot and (**C**) immunocytochemistry. A: Western blot for NFATc4 in nuclear and cytosolic fractions isolated after incubating IMR-32 cells for 24 h in control (C) or chelated media containing 1.5, 5, or 15 μM zinc. hnRNP and β-tubulin were measured as loading controls in nuclear and cytosolic fractions, respectively. B: After quantitation of Western blots, results are expressed as the ratio nuclear/cytosolic content. Results are shown as mean ± SEM of four independent experiments. *Significantly lower compared to C and 15 μM zinc groups ($P < 0.05$, one way ANOVA test). C: Immunocytochemistry of NFATc4. Immunocytochemistry for NFATc4 in differentiated IMR-32 cells incubated for 24 h in (**i**) control, (**ii**) 1.5 μM zinc, or (**iii**) 15 μM zinc media. Cells cultured in coverslips were first exposed to a specific anti-NFATc4 antibody, followed by a Texas Red-labeled secondary antibody.

involved in the impaired NFAT translocation. Furthermore, disruption of the cytoskeleton structure by cold and chemical agents led to the inhibition of NFAT transport into the nucleus. Thus, we present evidence demonstrating that zinc modulates NFAT and that microtubules are involved in NFAT nuclear translocation in neuronal cells, crucial event in the regulation of NFAT transcriptional activity.

NFATc1-c4 are activated by calcineurin, a calcium/calmodulin-dependent phosphatase (type 2B serine/threonine phosphatase) (Dolmetsch et al., 1997). Calcineurin is a heterodimer formed by a catalytic subunit (subunit A) that binds calmodulin and a regulatory subunit (subunit B) that binds calcium. When cellular calcium increases, calcineurin is activated, binds to NFAT leading to its dephosphorylation and subsequent activation. Calcineurin is particularly abundant in the brain (Steiner et al., 1992; Dawson et al., 1994), with a high activity in neurons. IMR-32 neuroblastoma cells possess the calcineurin-NFAT activation pathway since cyclosporin A inhibited NFAT activation and nuclear translocation. Furthermore, and as previously demonstrated in neurons (Benedito et al., 2005), the finding that cyclosporin A-induced apoptotic cell death indicates that NFAT is involved in IMR-32 cell survival. We observed that cellular calcium increases in the zinc deficient neuroblastoma cells. The role of calcium in the initial NFAT activation when cellular zinc decreases was demonstrated by the finding that the cellular calcium chelator BAPTA-AM inhibited NFAT-DNA binding activity.

The cytosolic activation of NFAT could be also triggered by the increase in cell oxidants associated with a decrease in cellular zinc. Zinc deficiency increases cellular oxidants (Ho and Ames, 2002; Zago et al., 2005; Mackenzie et al., 2006b). We previously showed that the incubation of IMR-32 cells for 6–24 h in zinc deficient media led to an increase in cell oxidants and of H_2O_2 release to the media. This was prevented by the simultaneous incubation of cells with the antioxidants (±)-α-lipoic acid and *N*-acetyl cysteine (Mackenzie et al., 2006b) and by catalase (Zago et al., 2005). Supporting the involvement of oxidants in NFAT activation when neuronal zinc decreases, (±)-α-lipoic acid and *N*-acetyl cysteine prevented the initial activation of NFAT in the zinc deficient cells. The current evidence of a role of oxidants in NFAT activation is limited and contradictory. In PW fibroblast cells, Ni_3S_2 or $NiCl_2$ increased the production of oxidant species (Huang et al., 2001b) and activated the transcriptional activity of NFAT, which was enhanced by compounds that increased intracellular calcium levels, inhibited by calcium blockers or chelators, and by calcineurin inhibitors. A similar mechanism, NFAT activation through oxidants-calcineurin- and calcium-dependent signal transduction pathways, was proposed for vanadium (Huang et al., 2001a) and UVc irradiation (Huang et al., 2000). On the contrary, there is evidence supporting a lack of effect or the inhibition of NFAT by oxidants (Furuke et al., 1999; Devadas et al., 2002). These contradictory findings could depend on the cell type and/or the magnitude, and duration of the stimuli and/or the involvement of different regulatory processes. Our findings that the antioxidants (±)-α-lipoic acid and *N*-acetyl cysteine prevent the increase in cell oxidants (Mackenzie et al., 2006b), particularly H_2O_2, associated with a decrease in neuronal zinc, and also prevent the initial activation of NFAT indicate, that in neurons, oxidants can trigger NFAT activation. Furthermore, current evidence indicates a link between oxidants and calcium influx. In lipopolysaccharide-stimulated microglial cells, H_2O_2 promotes calcium influx through TRPM2 cation channels (Kraft et al., 2004). In rat striatal neurons, H_2O_2 leads to an increase in cellular calcium through a channel that is independent of synaptic activity or voltage-gated calcium influx (Smith et al., 2003). Zinc is a well-characterized inhibitor of the NMDA receptor (Herin and Aizenman, 2004), suggesting that the increased cellular calcium could also be secondary to the activation of NMDA receptors at low extracellular zinc concentration. Thus, in zinc deficiency, a rise in cellular calcium and oxidants is involved in the triggering of NFAT activation.

Although the levels of NFAT-DNA binding activity were high in total cell extracts from zinc deficient cells, the ratio nuclear/cytosolic NFAT-DNA binding was low, suggesting an impaired nuclear transport of the active NFAT. This was supported by a decreased transactivation of the NFAT regulated reporter gene pNFAT-Luc. The

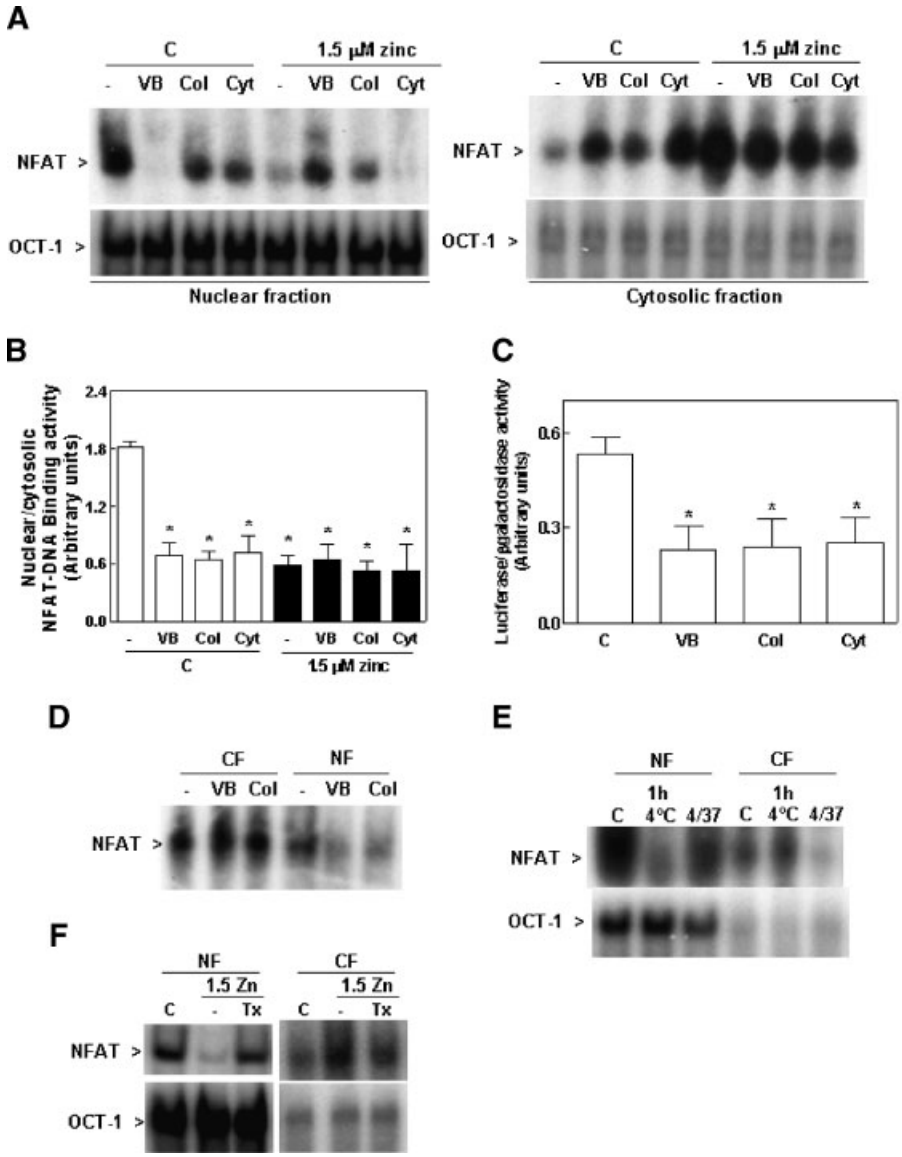

Fig. 6. Effect of cytoskeleton disruption on NFAT-DNA binding activity. Nuclear and cytosolic fractions were isolated after incubating IMR-32 cells for 24 h in control (C) or zinc deficient (1.5 μM zinc) media incubated without (—) or with 0.5 μM VB, 0.5 μM (Col), or 0.5 μM cytochalasin D (Cyt). **A**: EMSA for NFAT and OCT-1 in nuclear and cytosolic fractions. **B**: After the EMSA assays, bands were quantitated and values expressed as the ratio nuclear/cytosolic binding activity. Results are shown as mean ± SEM of four independent experiments. *Significantly different compared to the C group ($P < 0.05$, one way ANOVA test). **C**: Transactivation of a pNFAT-Luc plasmid. Luciferase and β-galactosidase activities were evaluated after 24 h incubation in control media with or without the addition of cytoskeleton inhibitors. Data are expressed as the ratio luciferase/β-galactosidase activity. Results are shown as mean ± SEM of three independent experiments. *Significantly different compared to the C group ($P < 0.01$, one way ANOVA test). **D**: NFAT-DNA binding activity in nuclear (NF) and cytosolic (CF) fractions isolated from cerebellum granule cells after incubating for 6 h without (—) or with 0.5 μM VB (VB) or 0.5 μM Col (Col). A representative EMSA out of three independent experiments is shown. **E**: NFAT- and OCT-1-DNA binding activity in nuclear and cytosolic fractions after microtubule depolymerization by cold. Nuclear and cytosolic fractions were isolated after incubating cells in control media at 37°C (37°C), for 1 h at 4°C (4°C) to allow the depolymerization of microtubules, or 1 h at 4°C and subsequent 30 min at 37°C (4/37), to allow the repolymerization of the microtubules. One representative EMSA assay out of three independent experiments is shown. **F**: EMSA for NFAT and CT-1 in nuclear (NF) and cytosolic (CF) fractions isolated from IMR-32 cells incubated for 24 h in control (C) medium or pretreated for 1 h with 1 μM taxol (Tx) and subsequently incubated in 1.5 μM zinc medium (1.5 Zn) for 24 h. One representative EMSA out of three independent experiments is shown.

alteration of NFAT nuclear transport in the zinc deficient cells was further characterized by measuring the cellular distribution of NAFTc4 by Western blot and immunocytochemistry. In agreement with the EMSA observations, Western blot analysis showed the accumulation of NFATc4 in cytosolic fractions and a decrease of nuclear NFATc4 in the zinc deficient cells. By immunocytochemistry we observed that NFATc4 fluorescence in the zinc deficient cells showed characteristic cytosolic aggregates that were not observed in control cells and that were prevented by supplementing with zinc deficient media.

We previously described that, in human neuroblastoma IMR-32 cells, a decrease in cellular zinc was associated with an impaired translocation of the active NF-κB from the cytosol into the nucleus (Mackenzie et al., 2002, 2006a). We found that the impaired NF-κB translocation was associated with a low rate of microtubule assembly in the zinc deficient cells. Both NFAT

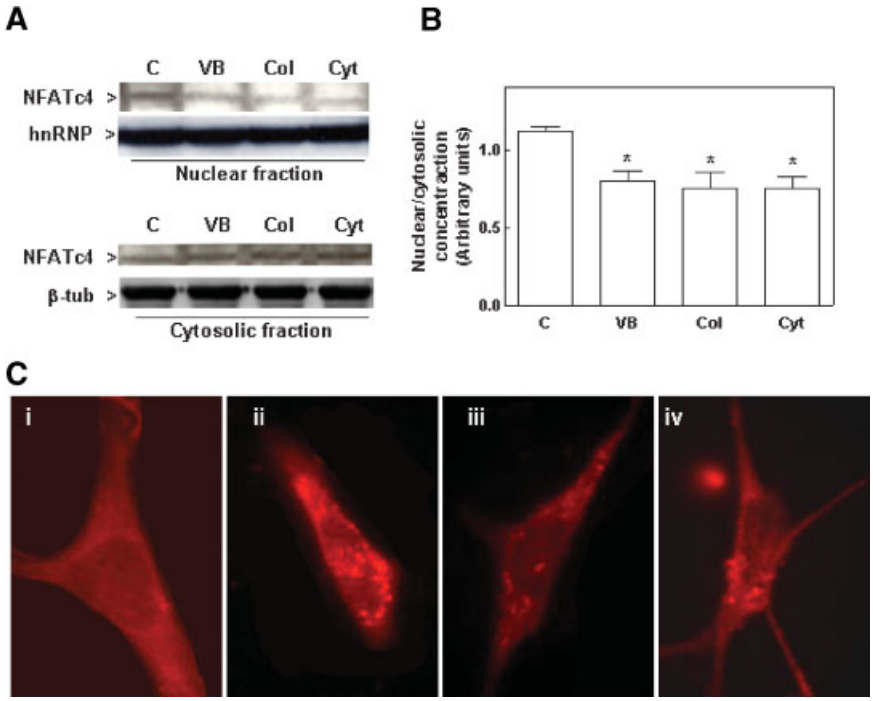

Fig. 7. Cystoskeleton disruption affects the cellular distribution of NFATc4. The cellular distribution of NFATc4 was characterized by (**A** and **B**) Western blot and (**C**) immunocytochemistry. **A**: Western blot for NFATc4 in nuclear and cytosolic fractions isolated after incubating IMR-32 cells 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 Cyt. hnRNP and β-tubulin were measured as loading controls in nuclear and cytosolic fractions, respectively. **B**: After quantitation of Western blots, results are expressed as the ratio nuclear/cytosolic content. Results are shown as mean ± SEM of four independent experiments. *Significantly lower compared to the C group ($P < 0.05$, one way ANOVA test). **C**: Immunocytochemistry of NFATc4. Immunocytochemistry for NFATc4 in differentiated IMR-32 cells incubated for 24 h in control media (**i**) without or with (**ii**) 0.5 μM VB or (**iii**) 0.5 μM Col (**iv**) 0.5 μM Cyt.

and NF-κB are NLS-bearing transcription factors. They both reside in the cytoplasm in an inactive form and, after activation, the NLS sequence is exposed which targets the transcription factor for its nuclear translocation through the nuclear pore, subsequent to the NLS recognition, and binding to karyopherin α. It has been recently proposed that a complex microtubules/dynein/karyopherin α and β/NLS-cargo is involved in the retrograde axonal transport and nuclear transport of NLS-bearing proteins (Hanz et al., 2003). In support of the involvement of microtubules in the nuclear translocation of NLS-containing proteins, p53 was shown to interact with microtubules that participate in p53 nuclear transport (Giannakakou et al., 2000). Based on a—the microtubule alterations previously found in fetal and adult rat brain (Hesketh, 1981; Oteiza et al., 1988, 1990a,b), and in IMR-32 cells (Mackenzie et al., 2002, 2006a) secondary to a condition of zinc deficiency and b—the similar altered nuclear translocation of NF-κB previously described in IMR-32 cells (Mackenzie et al., 2002), we hypothesized that functional microtubules are required for NFAT nuclear translocation. In support of this, the inhibition of tubulin polymerization with the chemical agents Col and VB and of actin polymerization with Cyt inhibited the nuclear translocation of the active NFAT as evidenced by EMSA and by the nuclear/cytosolic distribution of NFATc4 by Western blot. The depolymerization of microtubules by cold also led to the accumulation of NFAT in the cytosol. Results showing that the repolymerization of the microtubules by warming cells at 37°C restored the translocation of NFAT to the nuclei, emphasizes the concept that the cytoskeleton plays a relevant role in NFAT nuclear translocation. Moreover, the observation that the stabilization of microtubules with Tx prevented zinc deficiency-induced alterations in NFAT nuclear translocation further supports a crucial role of microtubules in the transport and nuclear translocation of NFAT in neuronal cells. The immunocytochemical characterization of NFATc4 cellular distribution showed that tubulin and actin polymerization inhibitors led to the formation of NFATc4 aggregates similar to those observed in the zinc deficient cells. These results indicate that the microtubule and actin cytoskeleton are required for the transport of NFAT into the nucleus, indicating that the altered microtubule dynamics secondary to a decrease in cellular zinc can affect NFAT transcriptional activity.

Recent experimental evidence supports a role of NFAT in the development of the nervous system. Calcineurin is abundant in the brain and has relevant roles in neuronal function and in neuronal plasticity of the mature nervous system, such as in the transition from short term to long term memory (Mansuy et al., 1998), and in the modulation of long term potentiation (Winder et al., 1998). Calcineurin regulates several genes in cerebellum granule cells including the Inositol 1,4,5-triphosphate (IP_3) receptor (Kramer et al., 2003). In rat hippocampal neurons, the transcription of IP_3 receptor is controlled by the calcium-calcineurin-NFATc pathway (Graef et al., 1999). In these cells, the entry of calcium through L-type voltage-gated channels in response to electrical activity or potassium depolarization leads to calcineurin-mediated activation, to the nuclear translocation of NFATc4 and to an enhanced NFAT-dependent transcriptional activity (Graef et al., 1999). It was recently reported that NFATc4 protects cerebellum granule cells from apoptotic death (Benedito et al., 2005), a critical process in the normal development of the nervous system. Furthermore, mice-bearing

mutations in NFATc2, c3, and c4, although not presenting alterations in neuronal differentiation and survival, showed a remarkable impairment in neurotrophins- or netrins-induced axonal out growth (Graef et al., 2003). The above evidence supports a role for the NFAT signaling cascade in neuronal survival and in neuronal plasticity during development and also in the mature brain (Graef et al., 2001b). We propose that an altered expression of NFAT-dependent genes secondary to zinc deficiency could contribute to the associated neuronal apoptosis (Verstraeten et al., 2004), affect the normal process of synaptogenesis and subsequently impair the normal development of the nervous system.

In summary, the present study shows that a decrease in neuronal zinc modulates transcription factor NFAT at different levels. Initially, through an increase in cellular calcium and oxidants species, zinc deficiency leads to the cytosolic activation of NFAT. Subsequently, alterations in the microtubule network inhibit the nuclear transport of the active NFAT into the nucleus and subsequent transactivation of NFAT-regulated genes. We present novel evidence that the neuronal nuclear translocation of NFAT requires a functional cytoskeleton.

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