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Molecular and Cellular Neuroscience 24 (2003) 1038–1050
MCN Molecular and Cellular Neuroscience
www.elsevier.com/locate/ymcne

Rb binding protein Che-1 interacts with Tau in cerebellar granule neurons Modulation during neuronal apoptosis

Christian Barbato,[a,b] Nicoletta Corbi,[c] Nadia Canu,[a,b] Maurizio Fanciulli,[d] Annalucia Serafino,[a] MariaTeresa Ciotti,[a] Valentina Libri,[c] Tiziana Bruno,[d] Giuseppina Amadoro,[a,b] Roberta De Angelis,[d] Pietro Calissano,[a,b] and Claudio Passananti[c,*]

[a] *Istituto di Neurobiologia e Medicina Molecolare, CNR, Viale Marx 43, 00137 Rome, Italy*
[b] *Dipartimento di Neuroscienze, Università di Roma Tor Vergata, 00173 Rome, Italy*
[c] *Istituto di Biologia e Patologia Molecolari, CNR, Viale Marx 43, 00137 Rome, Italy*
[d] *Regina Elena Cancer Institute, Laboratory "B", Via delle Messi d'Oro 156, 00158 Rome, Italy*

Received 29 March 2003; revised 28 May 2003; accepted 13 August 2003

Abstract

Che-1 is a recently identified human Rb binding protein that inhibits the Rb growth-suppressing function and regulates cell proliferation. Che-1 contacts the Rb and competes with HDAC1 for Rb-binding site, removing HDAC1 from the Rb/E2F cell cycle-regulated promoters. We have investigated the expression of Che-1 in neuronal cells and we showed that Che-1 directly interacts with Tau. Tau is a microtubule-associated protein involved in the assembly and stabilization of neuronal microtubules network that plays a crucial role modulating neuronal morphogenesis, axonal shape, and transport. In rat cerebellar granule neurons (CGNs) Che-1 partially colocalizes with Tau in the cytoplasm. Che-1 binds the amino-terminal region of Tau protein, which is not involved in microtubule interactions. Tau and Che-1 endogenous proteins coimmunoprecipitate from CGNs cellular lysates. In addition, Che-1/Tau interaction was demonstrated both in overexpressing COS-7 cells and CGNs by FRET analysis. Finally, we observed that Tau/Che-1 interaction is modulated during neuronal apoptosis.

Introduction

Recent data suggest that pathological cell cycle reentry might be an important mechanism associated with neuronal apoptosis. In this view, it was suggested that cell cycle regulatory molecules, such as Rb and E2Fs, play a critical role in survival and apoptotic pathways in postmitotic neurons (Liu and Greene, 2001). Moreover, it was hypothesized that entry of postmitotic neurons into cell cycle, followed by an aberrant progression through the cell cycle, can be involved in the pathogenesis of neurodegenerative disease such as Alzheimer's (Vincent et al., 1997; Busser et al., 1998; Husseman et al., 2000; Raina et al., 2000; Yang et al., 2001; Herrup and Arendt, 2002). For these reasons it is crucial to depict, in neuronal cells, the functional role of genes involved in cell cycle regulation. Recently, we have isolated and characterized a novel human gene, named Che-1, that affects cell growth by interfering with the recruitment of histone-deacetylase-protein-1 (HDAC1) by retinoblastoma protein (Rb) (Fanciulli et al., 2000; Bruno et al., 2002). Che-1 protein contains a canonical leucine zipper motif and three nuclear receptor-binding *LXXLL* consensus sequences distributed throughout the protein (Heery et al., 1997; Torchia et al., 1997). The Che-1 is involved in transcription control and it is highly conserved during evolution (Thomas et al., 2000). It appears to be ubiquitous expressed, showing a dotted nuclear pattern of distribution. Che-1 contacts directly the core subunit 11 of RNA polymerase II,

* Corresponding author. IBPM-CNR, Viale Marx, 43, 00137 Rome, Italy. Fax: +0039-0686090340.
E-mail address: passananti@itbm.rm.cnr.it (C. Passananti).

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doi:10.1016/j.mcn.2003.08.002

and can form complexes with the Rb gene product using two distinct protein portions (Fanciulli et al., 2000). In addition, the rat Che-1 orthologous gene product interacts with Dlk/ZIP kinase, which is tightly associated with chromatin and phosphorylates histones (Page et al., 1999). We characterized Che-1 expression in postmitotic neuronal cells using as a model system the cerebellar granule neurons (CGNs) culture. Interestingly, in these neurons Che-1 shows its typical nuclear localization and in addition colocalizes in the cytoplasm with the neuronal microtubule-associated protein Tau.

Tau protein is a component of the neuronal cytoskeleton, and it plays a crucial role in maintaining the microtubule network (Weingarten et al., 1975; Cleveland et al., 1977; Himmler et al., 1989). Tau modulates neuronal morphogenesis, axonal shape, and transport (Goedert et al., 1991; Hirokawa, 1994; Stamer et al., 2002), and it shows a cytoplasmic localization, but it is also associated with plasma membrane and nucleus (Brandt et al., 1995; Arrasate et al., 2000; Greenwood and Johnson, 1995; Lefebvre et al., 2003). Tau undergoes a complex pattern of physiological and pathological posttranslational changes (Bueè et al., 2000). It is the major component of the intraneuronal fibrillar lesions that characterize Alzheimer's disease and the neurodegenerative tauopathies (Lee et al., 2001).

In this report, we demonstrate in vitro and in vivo that two regions of Che-1 protein are directly involved in contacting the amino-terminal portion of Tau protein in CGNs. Moreover, the Che-1/Tau interaction was studied using confocal microscopy-based fluorescence resonance energy transfer (FRET) techniques. Finally, we showed that Che-1/Tau association was modulated in cell death evoked by low K^+ in CGNs (D'Mello et al., 1993).

Results

Che-1 expression in neuronal cells

Although Che-1 shows an ubiquitary pattern of expression, we have investigated, by western blot analysis, its expression in postmitotic rat CGNs and in SY5Y human neuroblastoma cell line (Fig. 1A). As expected the human Che-1 neuronal protein profile in SY5Y cells has a higher molecular weight in comparison to the rat protein observed in CGNs. This molecular weight difference is due to an extra exon present only in the human gene (Bruno et al., 2002).

We have investigated Che-1 localization in CGNs by indirect immunofluorescence. Che-1 immunostaning (Fig. 1B), in addition to a typical nuclear dot-like compartmentalization, showed an unexpected, intense, polarized signal at the level of the neuronal cytoplasm, resembling the Golgi apparatus. This signal was further characterized by a double staining with both Che-1 and wheat germ agglutinin (WGA), used as vesicles Golgi-derived specific marker in CGNs (Galli et al., 1998). Merge analysis indicated the Che-1 colocalize with WGA marker. To confirm this peculiar Che-1 localization, neuronal cells were cultured in the presence of Brefeldin A (BFA), a fungal metabolite that disrupts the Golgi network. After BFA treatment, Che-1 and WGA indirect immunofluorescences were both dispersed into the neuronal cytoplasm (Fig. 1B).

With the intent to better characterize the above-described neuronal Che-1 localization, a series of double indirect immunofluorescence studies was performed in CGNs using different neuronal antibody markers. Interestingly, Tau, a neuronal microtubule-associated protein appeared to partially colocalize in the minute cytoplasm district of CGNs (Fig. 1C).

Che-1 interacts with Tau

We examined this emerging codistribution of Che-1 and Tau proteins by performing a series of experiments to test the possibility that the two proteins directly interact. Fig. 2 shows that Che-1 is able to directly contact Tau. In fact, coimmunoprecipitation of Tau, in rat CGNs extracts, followed by western blot analysis of the precipitants for the presence of Che-1, indicated an interaction between these two proteins (Fig. 2A). The reciprocal coimmunoprecipitation experiments, performed with Che-1 immunoprecipitation and western blot analysis of the precipitants for the presence of Tau, confirmed a specific interaction of Che-1 and Tau proteins in CGNs (Fig. 2B). In these two sets of coimmunoprecipitation experiments, nonspecific goat IgG and nonimmune rabbit IgG antibodies respectively, were used as negative controls. Moreover, pull-down experiments showed that only GST-Che-1 bacterial purified fused protein, but not GST alone, was able to bind Tau (Fig. 2C). In addition, we used a Tau affinity chromatography, in which equal amounts of purified GST protein and purified GST-Tau N-terminal fusion protein (GST-Tau 230), irreversibly coupled to CNBr-activated Sepharose 4B, were engaged in a pull-down assay with whole cell extract of rat CGNs (Fig. 2D). The recovered interacting proteins were then blotted and incubated with the Che-1 antibody, showing that GST-Tau 230 is able to bind and retain Che-1 protein. These data, taken together, demonstrate that Che-1 and Tau proteins directly interact.

Protein regions involved in Tau and Che-1 interaction

With the purpose to define the Tau protein region involved in Che-1 contact, a series of coimmunoprecipitation assays were performed. COS-7 monkey cells were cotransfected with Flag tagged Che-1 (Flag-Che-1) construct plus a series of Myc tagged Tau full-length and deleted constructs (Myc-Tau), as described in the scheme presented in Fig. 3A. The analysis of the results, shown in Fig. 3B and C, indicates that the Tau region spanning from amino acid position 44 to 230 is still sufficient to mediate protein-protein inter-

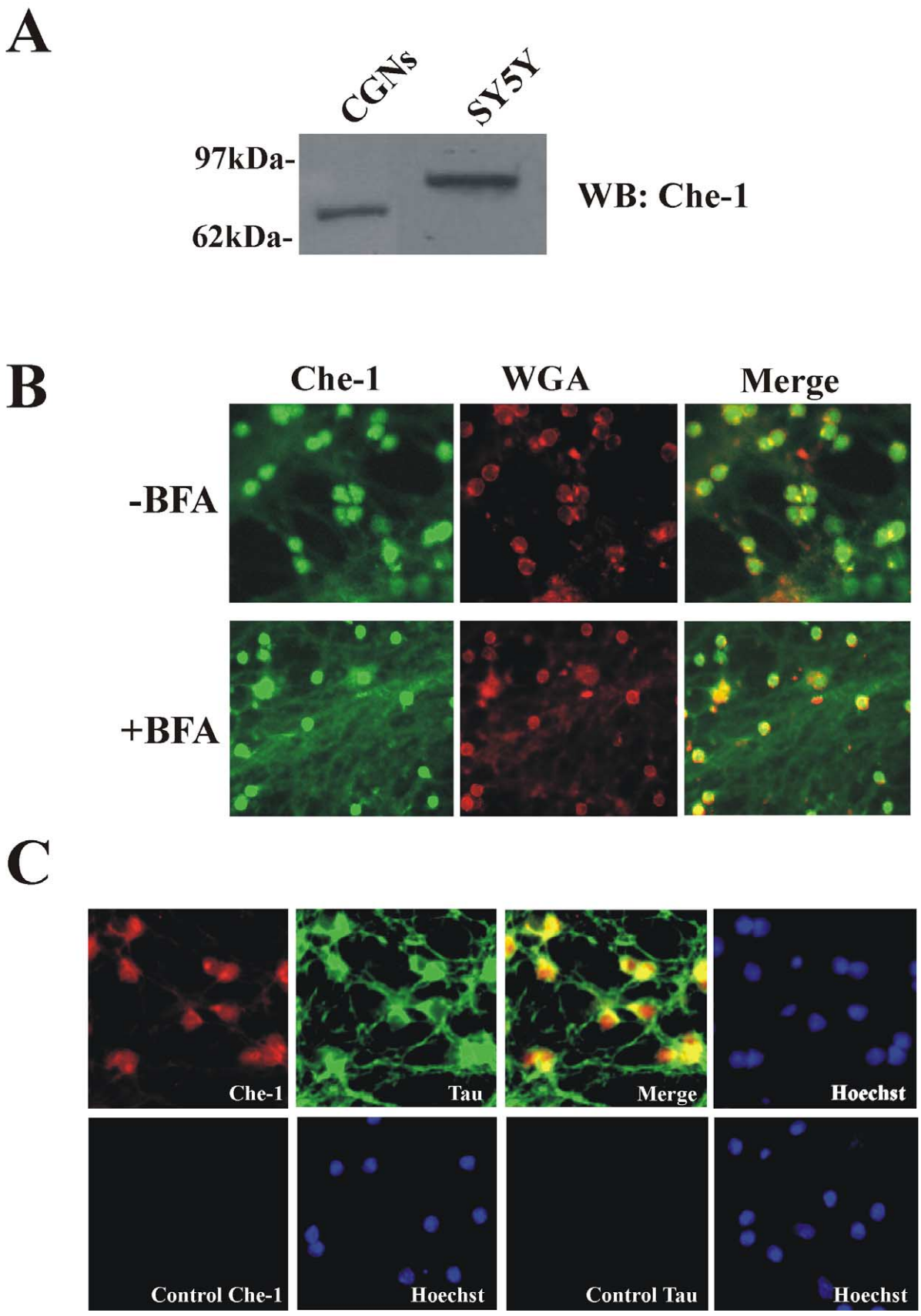


Fig. 1. Che-1 expression in neuronal cells. (A) Western blot analysis of Che-1 in total lysates from rat cerebellar granule cells (CGNs) and SY5Y human neuroblastoma cell line. (B) Indirect immunofluorescence in CGNs treated (where indicated) with 5 μg/ml Brefeldin A (BFA) for 30 min, fixed and processed with anti-Che-1, anti-WGA, and combined double-labeled images (merge). (C) Double immunofluorescence in CGNs, grown in normal condition (S + K25) for 24 h, stained with rabbit polyclonal anti-Che-1 and monoclonal anti-Tau antibodies, and combined double-labeled merge; nuclei were stained with Hoechst (upper panel). Che-1 negative control (preimmune serum) and nuclei stained with Hoechst; Tau negative control (secondary antibody) and nuclei stained with Hoechst (lower panel).

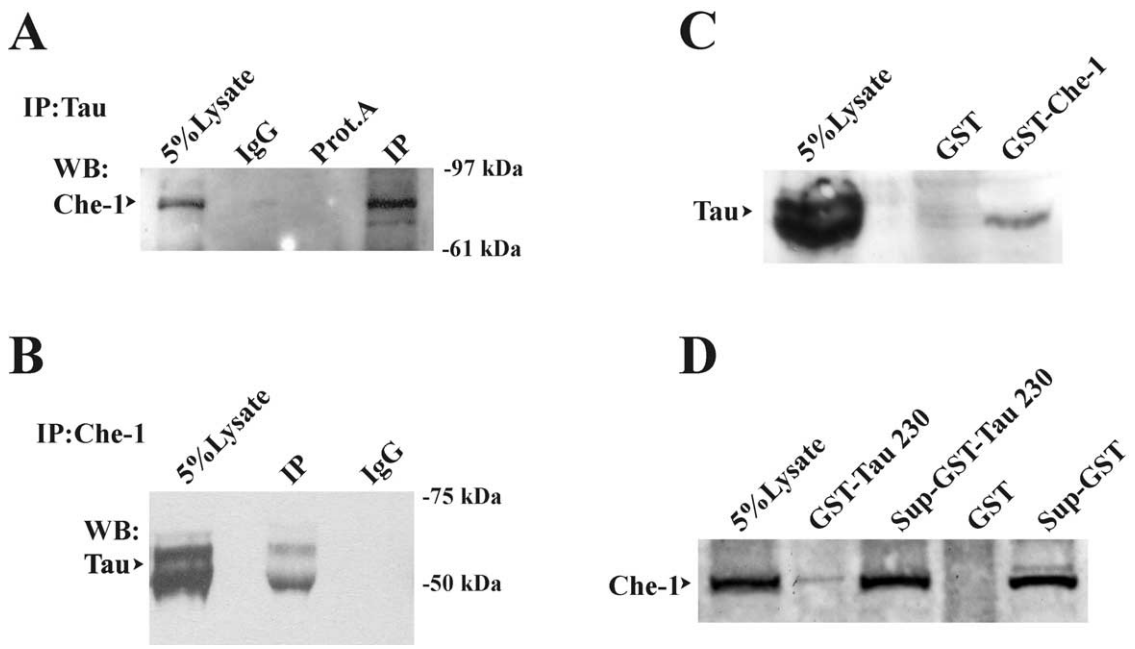


Fig. 2. Tau interacts with Che-1. (A) Lysate from CGNs was coimmunoprecipitated with anti-Tau goat polyclonal antibody and nonspecific goat IgG and protein A as control. Immunoprecipitates were blotted with anti-Che-1 rabbit polyclonal antibody. (B) Lysate from CGNs was coimmunoprecipitated with anti-Che-1 rabbit polyclonal antibody or nonimmune rabbit IgG as negative control. Immunoprecipitates were immunoblotted with anti-Tau monoclonal antibody. (C) Rat cerebellar granule cell extract was incubated in NETN buffer in the presence of GST and GST-Che-1 beads as indicated. Recovered interacting proteins were then blotted with the Tau monoclonal antibody. (D) Binding of Che-1 by Tau affinity chromatography. Purified GST-Tau N-terminal fusion protein (GST-Tau 230) were coupled to CNBr-activated Sepharose 4B. An equal amount of GST-purified protein was coupled to CNBr-activated Sepharose 4B as control. Whole cell extract of rat cerebellar granule neurons was pull-down assayed with GST-Tau 230 and GST. Recovered interacting proteins were then blotted and incubated with Che-1 rabbit polyclonal antibody.

action and to efficiently coimmunoprecipitate with overexpressed Flag-Che-1 protein.

Similarly, using the coimmunoprecipitation approach, we mapped the Che-1 protein portions involved in driving Tau contacts. Fig. 4A shows a schematic view of the Myc-tagged Che-1 (Myc-Che-1) constructs coexpressed with full-length Tau protein. Two Che-1 regions appear to be responsible for Tau interaction. The first region spans from amino acid in position 1 to 163 (Myc-Che-1-B) (Fig. 4B), whereas the second region is localized in the middle of Che-1 protein and spans from amino acid portion 371 to 470 (Myc-Che-1-E) (Fig. 4C).

FRET analysis of Che-1/Tau interaction

The Che-1/Tau protein-protein interaction was further investigated using confocal microscopy-based fluorescence resonance energy transfer (FRET) techniques (Fig. 5), which permits to detect protein interactions within 10 nm. The binding between Che-1/Tau was studied both in COS-7 cells double transfected with Tau and Che-1 constructs (Fig. 5A), and in CGNs (Fig. 5B). Che-1 was immunostained with the rabbit polyclonal anti-Che-1, for Tau was used the monoclonal anti-Tau, and then were labeled with TRITC-conjugated anti-rabbit antibody and FITC-conjugated anti-mouse antibody, respectively. After photobleaching of acceptor molecules (TRITC), the average increase in fluorescence (FITC), was measured. For Cos-7 cells, an increase of 7.01% in donor fluorescence was observed within the discrete area that was photobleached, while the increase in donor fluorescence outside this area was of −0.91%. In CGNs an increase of 5.82% in donor fluorescence was observed within the discrete area that was photobleached, while the increase in donor fluorescence outside this area was of 0.05%. Positive controls for FRET experiments were performed both in cells expressing either endogenous or exogenous Tau. As Tau interaction control, we used a monoclonal antibody anti-tubulin since tubulin is known to bind Tau. (Weingarten et al., 1975). In these samples, the percentage of increase in donor fluorescence after complete photobleaching of the acceptor molecules was about 5.78% (data not shown). Thus, we conclude that the donor dequenching observed after photobleaching of the acceptor reflects FRET positive values for Che-1/Tau interaction.

Coimmunolocalization of Tau and Che-1 proteins in CGNs during apoptosis

In an attempt to identify the biological significance of Che-1/Tau interaction, we investigated their possible involvement during neuronal apoptosis. In vitro cultured

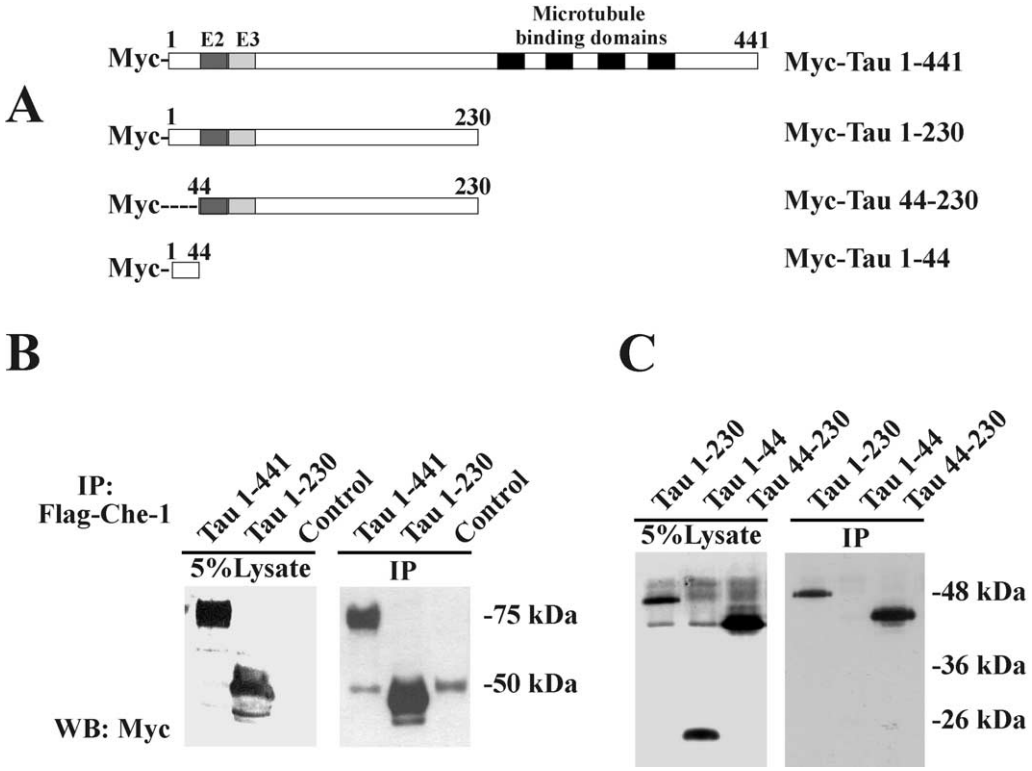


Fig. 3. Identification of Tau region that binds Che-1 protein. (A) Schematic representation of myc-Tau constructs used to identify the portion of Tau that binds Che-1. The schematic whole structure of Tau is represented at the top of the figure, and the deletion mutants are aligned below. Tau is characterized by two inserts (E2 and E3) and by four microtubule-binding domains. (B and C) Lysate from COS-7 cells doubly transfected with both Myc-Tau indicated constructs and Flag-Che-1 was immunoprecipitated with anti-Flag monoclonal antibody, agarose conjugated. Immunoprecipitates were then blotted with anti-myc monoclonal antibody.

CGNs undergo a massive process of apoptosis, when are deprived of serum (S-) and the extracellular concentration of KCl employed for their culturing is shifted from 25 mM to 5 mM (switch from S-K25 mM to S-K5 mM) (D'Mello et al., 1993; Galli et al., 1995). Recently, we have demonstrated that in CGNs undergoing apoptosis, Tau is rapidly degraded and the microtubule network collapses (Canu et al., 1998). Moreover, Che-1 protein was shown to be involved in cell-cycle regulation (Fanciulli et al., 2000; Bruno et al., 2002) and the rat Che-1 orthologous interferes with apoptosis (Page et al., 1999).

We performed indirect immunofluorescence assays using Che-1 antibody and an anti-Tau monoclonal antibody in CGNs undergoing apoptosis. As shown in Fig. 6, in control neurons, after serum deprivation (S-K25), Che-1 immunostaining showed a typical pattern of nuclear and cytoplasm/Golgi-network localization, as reported in Fig. 1B. After the KCl shift (S-K5), cells exhibit a small compacted nucleus, strongly stained by Hoechst, and the Che-1 localization is lost in favor of redistribution in the condensed cytoplasm, while Che-1 nuclear immunostaining remains substantially unchanged. As previously shown (Canu et al., 1998), the immunostaining of Tau protein has a typical distribution in the neuritic network and in the cell body of neurons cultured in serum-deprived conditions (S-K25), while in apoptotic neurons (S-K5) Tau immunostaining appears to decrease and to redistribute in the soma. The merge analysis of Che-1 and Tau proteins by double indirect immunofluorescence, presented in Fig. 6, indicates a colocalization in the cytoplasm/Golgi network, in the control CGNs, whereas during apoptosis, the Che-1/Tau colocalization was reduced in intensity and coincided with condensed cytoplasm.

The binding of Che-1/Tau is modulated during cerebellar granule cell death

The results evidenced by indirect immunofluorescence were confirmed by western blot analysis of coimmunoprecipitated Tau and Che-1 proteins during a time course of apoptosis in CGNs. CGNs were maintained in high-potassium and serum-free medium (S-K25) for 24 h or switched for different times to K5 and serum-free medium (S-K5). As shown in Fig. 7A, total cell lysates and anti-Che-1 immunoprecipitate proteins were analysed by western blot using both monoclonal anti-Tau and Che-1 antibodies. In Fig. 7B, densitometric analysis of the results clearly demonstrated that the amount of Tau protein coimmunoprecipitated with Che-1 decreases following the onset of apoptosis. These data indicate that Che-1/Tau association is finely modulated during neuronal apoptosis, and that Che-1 pro-

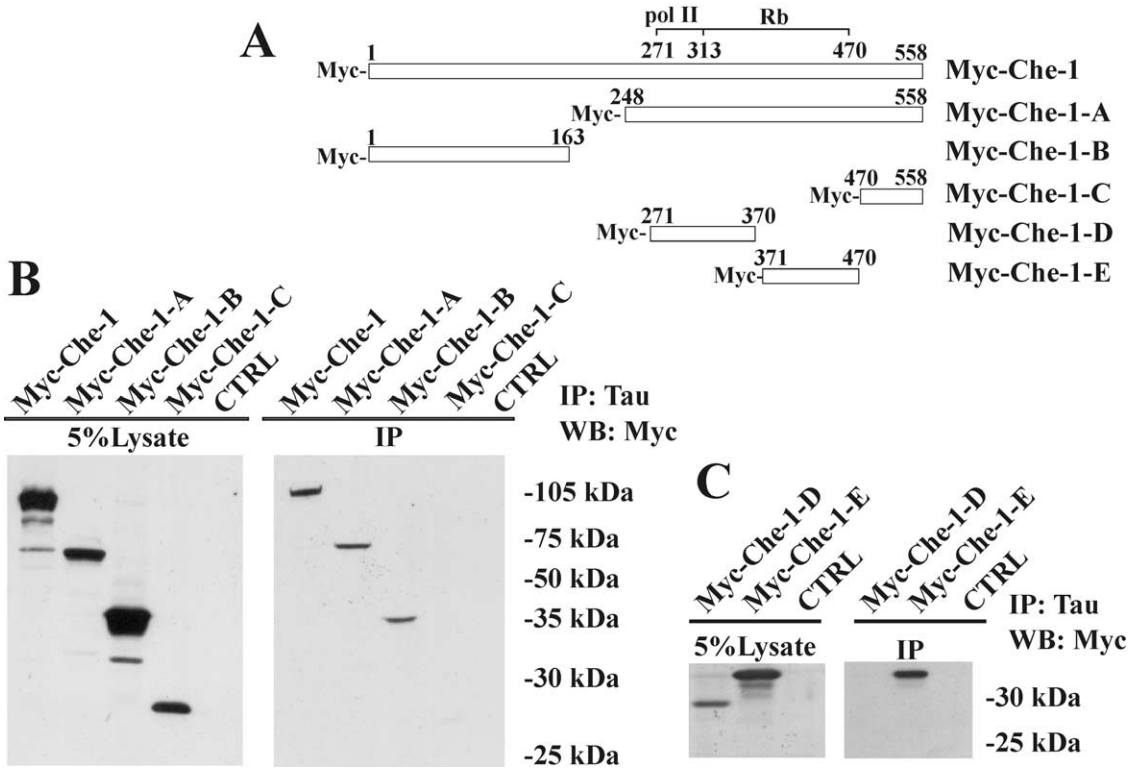


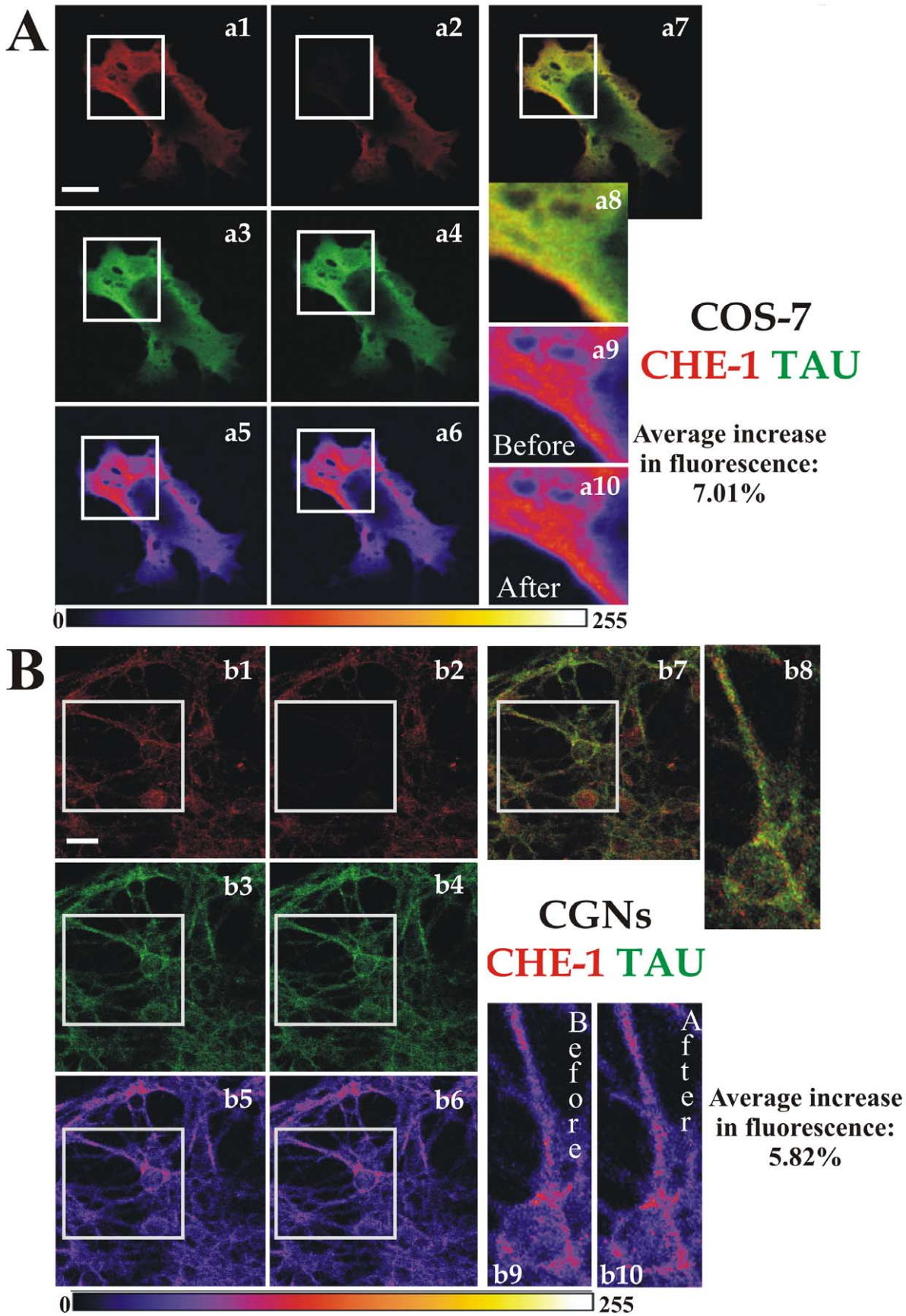
Fig. 4. Identification of Che-1 region that binds Tau protein. (A) Schematic representation of myc-Che-1 constructs used to identify portions of Che-1 that bind Tau. The schematic structure of Che-1 is represented at the top of the figure and the deletion mutants are aligned below. Che-1 is characterized by the presence of the regions involved in the binding of RNA polymerase B (pol II) and retinoblastoma gene product (Rb). (B and C) Coimmunoprecipitation of myc-Che-1 molecules with full-length Tau1–441 construct expressed in COS-7 cells. Immunoprecipitates were blotted with anti-myc monoclonal antibody.

tein profile is not modified, suggesting a functional role for this interaction during execution of the apoptotic program.

Discussion

Unbalanced expression of cell cycle regulatory proteins might be an important mechanism involved in apoptotic death of postmitotic neurons (Liu and Greene, 2001). Looking for gene products potentially involved in the regulation of neuronal cell cycle, we characterized, in primary CGNs, the expression of the recently isolated Rb-binding Che-1 protein (Fanciulli et al., 2000). Che-1 protein was described to be involved in cell cycle, interacting with Rb, and removing HDAC1 from the Rb/E2F cell cycle-regulated promoters (Fanciulli et al., 2000; Bruno et al., 2002). In cycling cells, Che-1 was described to have a nuclear dotted localization. In CGNs, we showed an additional presence of Che-1 in the cytoplasm. In this compartment Che-1 appeared to colocalize with a vesicles Golgi-derived marker, WGA, indicating that it was confined in the Golgi area. On the other hand, the yeast gene, more closely related to the human Che-1, named BFR2, was isolated as a high-copy suppressor of the growth defects induced by the fungal metabolite Brefeldin A (BFA) that disrupts the Golgi apparatus (Chabane et al., 1998). This finding is also in good agreement with our data that showed a cytoplasmic dispersed localization of Che-1 after BFA treatment in CGNs. On the basis of these observations, we further investigated the potential associations of Che-1 with neuronal cytoskeletal proteins in CGNs. A Che-1/Tau colocalization was discovered during these investigations. Tau is a neuronal

Fig. 5. FRET analysis of Tau/Che-1 interaction. The interaction between microtubule-associated protein Tau and Che-1 was investigated by FRET (fluorescence resonance energy transfer), in COS-7 cells (A) and cerebellar granule neurons (B; CGNs). Cells were immunostained with monoclonal anti-Tau and rabbit polyclonal antibody anti-Che-1 and visualized with FITC- and TRITC-conjugated antibodies, respectively. (a1 and b1) TRITC signal after 568-nm excitation. (a2 and b2) A discrete area of the cell was photobleached using intense 568-nm laser. (a3 and b3) FITC signal using 488-nm excitation before photobleaching. (a4 and b4) FITC signal using 488-nm excitation after photobleaching of the acceptor fluorophore (TRITC) with intense 568-nm laser light. (a5 and b5, and a6 and b6) FITC signal using 488-nm excitation before (a5 and b5) and after (a6 and b6) photobleaching observed employing pseudo-colour representation based on the colour bar reported on the bottom of each panel and relative to increasing fluorescence intensity; a detail of each image is shown in a9 and b9, and a10 and b10, respectively. (a7 and b7) Combined image of FITC and TRITC fluorescence signals obtained before photobleaching, in which colocalization of the two signals is shown as a yellow hue; a detail is shown in a8 and b8.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Average increase in fluorescence: 7.01%
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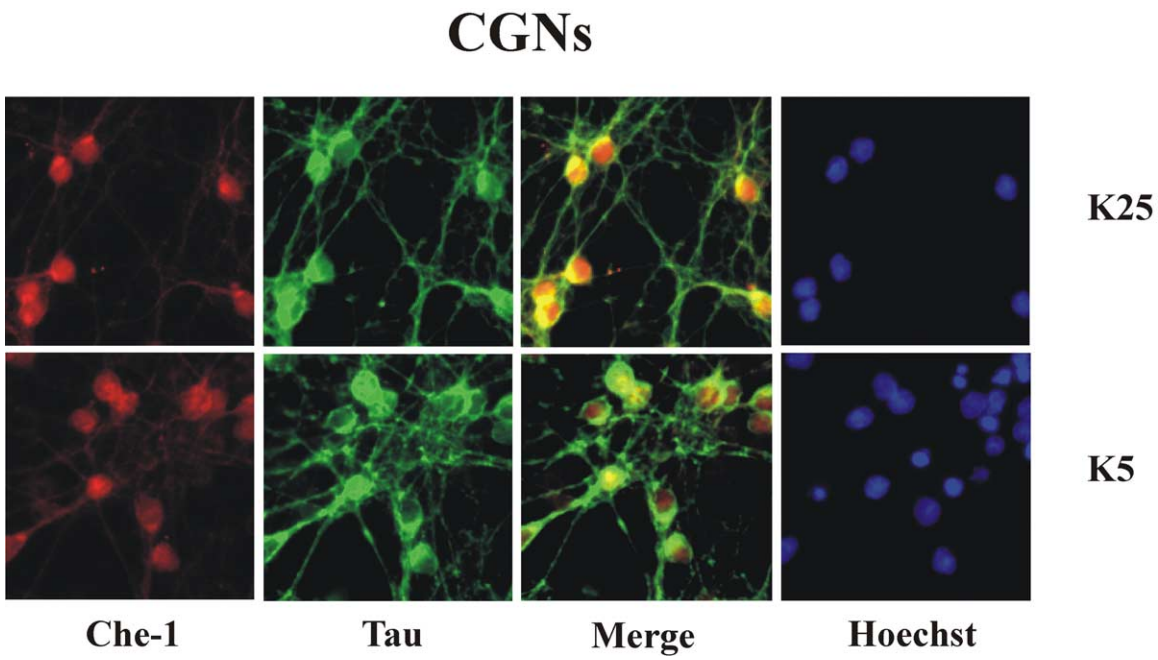

Fig. 6. Immunofluorescence of Tau and Che-1 proteins in CGNs. From the left, first column: indirect immunofluorescence using rabbit polyclonal anti-Che-1; second column: indirect immunofluorescence using monoclonal anti-Tau; third column: merge of Che-1 and Tau double indirect immunofluorescence; fourth column; nuclei were stained with Hoechst. From the top, first row: CGNs were cultured with 25 mM potassium and serum deprivation; second row: CGNs were induced to undergo apoptosis in 5 mM potassium and serum deprivation. All cell cultures were growth for 12 h.

specific protein associated to microtubules (Goedert et al., 1989). It plays a pivotal role in the maintenance of neuronal cytoskeleton, in traffic of organelles, neurofilaments, and Golgi-derived vesicles (Hirokawa et al., 1988; Ebneth et al., 1998; Stamer et al., 2002). In addition, Tau interacts with various proteins, including tubulin (Weingarten et al., 1975), spectrin (Carlier et al., 1984), actin (Correas et al., 1990), PP2A phospatase (Sontag et al., 1999), Presenilin1 (Takashima et al., 1998), alfa-synuclein (Jensen et al., 1999), S100β (Yu and Fraser, 2001), phospholipase C-γ (Hwang et al., 1996), Fyn (Lee et al., 1998), and with plasma membrane costituents (Brandt et al., 1995). To investigate the interaction between a cell cycle-regulating protein and a microtubule-associated protein in neurons, we focused on the cellular and molecular characterization of Che-1/Tau interaction. By affinity cromatography we have found that the N-terminal portion of Tau protein binds Che-1. Thereafter, endogenous Che-1 and Tau proteins were successfully coimmunoprecipitated from CGNs extracts. These data demonstrate that Che-1 and Tau proteins directly interact in vitro and in vivo. In addition, we showed that the N-terminal portion of the longest human isoform of Tau mediates the interaction with two portions of Che-1 molecule. The N-terminus of Tau is characterized by the presence of two highly acidic domains (E1, 44–72; and E2, 73–101) and a proline-rich region defined as the "projection domain" that mediates interactions with neural plasma membrane and cytoskeletal components (Brandt et al., 1995; Hirokawa et al., 1988). A Tau deletion construct, lacking the proline-rich region (1–156), was still able to contact Che-1 (data not shown), suggesting that the minimal Tau region required to associate Che-1 spans from amino acid position 44 to 156. Importantly, this Tau region is regulated by alternative splicing both during development and axonal injury (Collet et al., 1997; Halverson et al., 2001; Arikan et al., 2002).

Two distinct portions of Che-1 protein have been described to mediate interaction with retinoblastoma gene product (Rb) (Fanciulli et al., 2000), and here we show that two other distinct portions of Che-1 protein mediate interaction with Tau. Significantly, Myc-Che-1B, located in the amino terminal, contains a relevant homology with the "J domain" of SV40 large T, that was defined to be sufficient to block apoptosis, induced by growth factor withdrawal, in a neuronal stem cell line (Slinskey et al., 1999). Myc-Che-1E is located in the carboxyl terminal half of the molecules and it is characterized by an elevated number of leucine residues. We used the confocal microscopy, named Fluorescence Resonance Energy Transfer technique (FRET) to investigate the intimate cellular interactions between Che-1 and Tau. FRET permits to investigate protein-protein interaction at a distance between 10 and 30 nm. FRET was detected as an increase in donor fluorescence, after complete photobleaching of the acceptor molecules. We showed that both in COS-7 cells double transfected with Tau and Che-1, and in CGNs, we detected positive FRET values of 7.01% and 5.82%, respectively. These data, in addition to the biochemical demonstration of Che-1/Tau interaction, confirmed a close proximity between Che-1 and Tau inside the

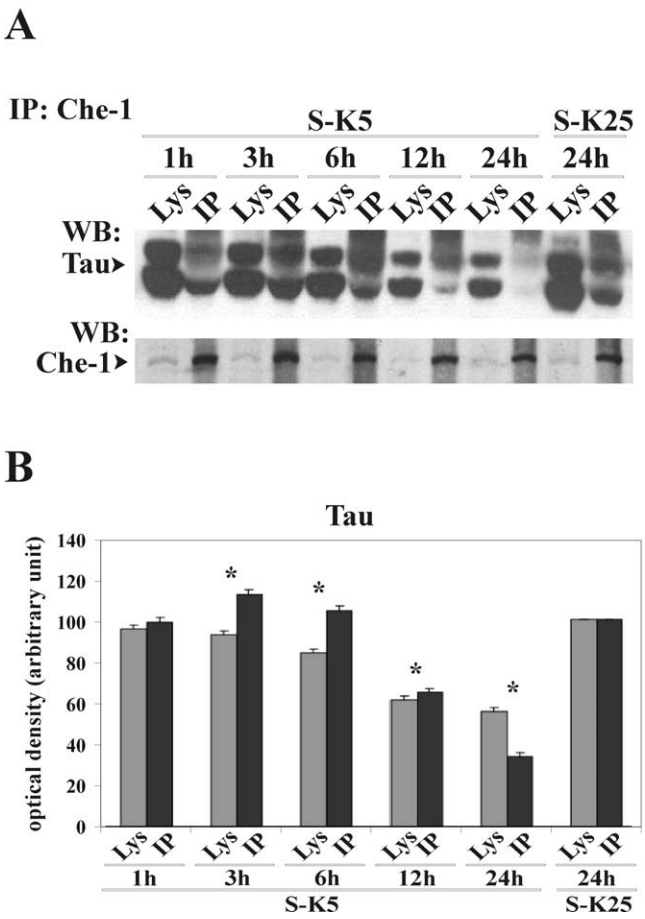

Fig. 7. Coimmunoprecipitation of Tau and Che-1 proteins during time course of neuronal apoptosis. (A) Western blot analysis of Tau profile in CGNs cultured in serum-free medium containing 25 mM KCl (S-K25) and during onset of apoptosis induced by potassium and serum deprivation (S-K5) using anti-Tau monoclonal antibody (upper panel) and anti-Che-1 rabbit polyclonal antibody (lower panel). One representative experiment (of three) is shown. (B) Densitometric analysis of the different bands recognized by anti-Tau monoclonal antibody (upper panel). The absolute scanning values are given for total lysates (Lys) and for immunoprecipitate (IP). Densitometric values are expressed as the percentage of optical density of control cells (S-K25), which have been given a value of 100. Data represent means (± SEM) of experiments from three separate CGN preparations. $*P < 0.05$ mean values ± SEM compared to the control (S-K25).

cell. Importantly, we showed for the first time a FRET analysis performed in primary neuronal culture, as CGNs.

With the intent to study the biological significance of Che-1/Tau association in CGNs, we have investigated their interaction during neuronal apoptosis. CGNs deprived of potassium and serum represent a powerful model system to study apoptotic cell death (D'Mello et al., 1993; Galli et al., 1995). Using indirect double immunofluorescence, we observed that Che-1 protein was not delocalized, but a very light nuclear diffusion appeared during the time course of neuronal apoptosis. Importantly, the amount of Che-1 protein during neuronal apoptosis was not reduced. While, as previously described and herein confirmed, the amount of Tau protein present in CGNs total lysates decreases during neuronal apoptosis (Canu et al., 1998). In addition, as revealed by densitometric analysis, we observed that the amount of Tau protein coimmunoprecipitating with Che-1, after a moderate early increase, was drastically reduced during induction of apoptosis. These observations suggest that Che-1/Tau proteins are decoupled during the proceeding of neuronal cell death. Previously, we described that Tau was cleaved, dephosphorylated, and redistributed from neurites to cell body in CGNs undergoing apoptosis (Canu et al., 1998). We have not revealed evident Tau apoptotic fragments in Che-1 immunoprecipitate complexes. Several mechanisms can be evoked to explain the modulation of Che-1/Tau interaction during apoptosis, such as posttranslation modifications (phosphorylation) of the two proteins and/or of other components eventually associated. Both Che-1 and Tau proteins appear to be involved in programmed cell death (Page et al., 1999; Canu et al., 1998; Lorio et al., 2001). Cyclin-dependent kinases, E2F, and a concomitant modification of Rb were described to be associated in cell death evoked by low K^+ in CGNs (Padmanabhan et al., 1999; O'Hare et al., 2000; Martin-Romero et al., 2000; Trinh et al., 2001). Moreover, it was described that E2F-dependent gene transcription was mediated by HDAC activity during neuronal apoptosis (Boutillier et al., 2003). Che-1 protein was described to be involved in cell cycle, interacting with Rb and removing HDAC1 from the Rb/E2F cell cycle-regulated promoters (Fanciulli et al., 2000; Bruno et al., 2002). Tau overexpression was described to be involved in neuronal dysfunction and neuronal toxicity of Drosophila and postmitotic human central nervous system model neurons (Grundke-Iqbal and Iqbal, 1999; DeSilva and Farrer, 2002; Fath et al., 2002). During neuronal apoptosis Tau undergoes several modifications (Canu et al., 1998; Lorio et al., 2001; Mookherjee et al., 2001). In addition, since Tau is a substrate for apoptotic protease, the caspase-3 cleavage products of Tau may contribute, as effectors of apoptosis, to the progression of neuronal cell death in Alzheimer's disease (Fasulo et al., 2000; Chung et al., 2001). Our upcoming research work could be supported by the speculation that in rat postmitotic CGNs, the Rb-binding protein Che-1 can be one of the proteins involved in maintaining the balance between cell cycle and apoptosis, and the microtubule-associated protein Tau is its neuronal cytoplasmic partner that participates at Che-1 compartmentalization, modulating in this way specific or general functions involved in apoptotic death and survival.

Experimental methods

Cell cultures, transfections, and treatments

Cultures enriched in granule neurons were obtained from dissociated cerebella of 8-day-old Wistar rats (Charles River, Calco, Italy) as described by Levi et al. (1984). Cells

were plated in basal medium Eagle (BME; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 25 mM KCl, and 2 mM glutamine (Life Technologies) on dishes (Nunc, Roskilde, Denmark) coated with poly-L-lysine. Cells were plated at 2.5×10^6 per 35-mm dish or 7×10^6 per 60-mm dish. 1β-Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of nonneuronal cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. COS-7 cell transfections were performed in 60- or 100-mm dishes using Lipofectamine (Life Technologies) according to the manufacturer's direction. Cultures of CGNs at 6–7 days in vitro (DIV) were treated with 5 μg/ml of Brefeldin A (BFA) (Sigma) for 30 min.

Induction of CGNs apoptosis

Cultures of CGNs at 6–7 DIV were washed two times and switched to serum-free BME containing 5 mM KCl supplemented with glutamine and gentamicin. Control cells were washed with serum-free BME and maintained in serum-free medium containing 25 mM KCl (D'Mello et al., 1993).

Affinity chromatography and pull-down assay

As a matrix to immobilize proteins, CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) was used. The purified human GST-Tau fusion proteins (1–230), were coupled to CNBr-activated Sepharose 4B at the concentration of 5 mg of GST-Tau/ml of gel according to the recommendations of the manufacturer. An equal amount of GST-purified protein was coupled to CNBr-activated Sepharose 4B as control. CGNs were rinsed three times with ice-cold PBS and lysed on plates at 0°C for 30 min in 500 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, NP40 1%, SDS 0.1%, 5 mM DTT, 10 mM NaF, 1 mM $NaOV_4$, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin). Neuronal cells were harvested, centrifuged at 4°C for 10 min at 3000 rpm, and the supernatants were precleared using 20 μl of Sepharose 4B for 1 h at 4°C. The precleared protein extracts was incubated in lysis buffer with GST-Tau fusion protein coupled with CNBr-activated Sepharose 4B for 20 h at 4°C, GST coupled with CNBr-activated Sepharose 4B served as control. The sample of pull-down assay was centrifuged at 4°C for 10 min at 3000 rpm, the pellet washed with lysis buffer and added to SDS-reducing sample buffer, heated for 5 min at 95°C, and subjected to electrophoresis and electrotransfer for western blot analysis.

BL21 bacteria were transformed with GST fusion protein constructs and the proteins purified on glutathione-Sepharose resin (Pharmacia). For protein-protein interaction assays, comparable amounts of resin-bound GST fusion proteins were incubated for 1 h at 4°C with CGNs lysates prepared in RIPA buffer. The resins were then pelleted and extensively washed in the same buffer. The bound proteins were subjected to SDS-PAGE on 10% gels, electroblotted, and visualized by western blot analysis.

Western blot analysis

Equal amounts of proteins were subjected to SDS-PAGE on 10% gels (Laemmli, 1970). After electroblotting to nitrocellulose (Hybond-C), proteins were visualized using appropriate primary antibodies. All primary antibodies were diluted in 0.5% (wt/vol) nonfat dry milk and incubated with the nitrocellulose blot overnight at 4°C. Incubation with secondary peroxidase-coupled anti-mouse or anti-rabbit antibodies was performed at room temperature for 45 min. Immunoreactivity was detected by ECL chemiluminescence reaction (Amersham). The following antibodies have been described: rabbit polyclonal anti-Che1 (1:1000) (Fanciulli et al., 2000); monoclonal anti-tau (1:1000) (BD, Transduction Laboratories, 610672); monoclonal anti-myc (1:1000) (9E10; SantaCruz).

Immunoprecipitation and coimmunoprecipitation

Cultured cells were rinsed three times with ice-cold PBS and lysed on plates at 0°C for 20 min in 500 μl of immunoprecipitation (IP) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM NaF, 1 mM $NaOV_4$, 1% NP40) supplemented with protease inhibitor cocktail (Sigma). Lysates were centrifuged at 4°C for 10 min at 3000 rpm, and the supernatants were precleared using 20 μl of protein A/protein G beads (Boerhinger) for 1 h at 4°C. The precleared protein extracts was immunoprecipitated by standard procedures using anti-Che-1 rabbit polyclonal antibody and anti-Tau goat polyclonal antibody (C-17) (Santa Cruz), or monoclonal anti-myc (9E10) (SantaCruz) and anti-FLAG M2 monoclonal antibody agarose affinity gel (Sigma, Life Science, A1205).

Immunofluorescence

CGNs were fixed with 4% (wt/vol in PBS) paraformaldehyde for 15 min at room temperature, washed in PBS, pH 7.4, and then permeabilized with 0.2% Triton X-100–Tris/HCl, pH 7.4, for 5 min. The coverslips were treated with monoclonal anti-tau (1:100) (BD, Transduction Laboratories, 610672) and anti-Che-1 rabbit polyclonal antibody (1:100), for 1 h in a moist chamber at room temperature, rinsed in PBS, and stained with rhodamine and fluorescein-conjugated secondary antibodies (Sigma) for 30 min. Nuclei were stained with Hoechst 33258 (Sigma) 0.5 mg/ml in PBS for 5 min. For Golgi apparatus labeling, cells were incubated for 30 min with rhodamine-conjugated wheat germ agglutinin (WGA; 1:200).

Plasmid construction

Plasmids encoding full-length or deleted human tau cDNA were generated by PCR amplifications using tau40pSG5 vector as template (Novak et al., 1993). The numbering of tau fragment is according to the longest human tau isoform, tau40. The 5′ primer for tau 1–441, 1–230, and 1–44 was 5′-AGCTGAATTCAATGGCTGAGCCCCGCCAG-3′. The 3′ primers were: 5′-GACCGCTCGAGTCACAAACCCTGCTTGGC-3′, 5′-GACCGCTCGAGTCAACGGACCACTGCCAC-3′, and 5′-GACCGCTCGAGTCACAGGCCAGCGTCCGTGTC-3′, respectively. Construct encoding tau 44–230 was made using 5′-AGCTGAAATTCAAAAGAATCTCCCCTGCAG-3′ as 5′ primer and 5′-GACCGCTCGAGTCAACGGACCACTGCCAC-3′ as 3′ primer. PCR primers contained *Eco*RI (5′) and *Xho*I (3′) for insertion into *Eco*RI-*Xho*I cut pCS2+MT myc-tagged mammalian expression vector and into pGEX4T3 (BioRad, Hercules, CA), to generate a glutathione-*S*-transferase (GST)-Tau fusion protein. Che-1 myc-tagged constructs and GST-Che-1 fusion protein were generated as described (Fanciulli et al., 2000).

Fluorescence resonance energy transfer (FRET) analysis

Fluorescently labeled samples were imaged by a confocal LEICA TCS 4D microscope (Leica, Heidelberg, Germany) equipped with an argon/krypton laser. The excitation and emission wavelengths were 488 and 510 nm, respectively, for FITC labeling, and 568 and 590 nm, respectively, for TRITC labeling. FRET was measured using a method developed for laser-scanning confocal microscopy (Knowles et al., 1999; McLean et al., 2000; Kinoshita et al., 2001). The energy transfer was detected as an increase in donor fluorescence (FITC) after complete photobleaching of the acceptor molecules (TRITC). The amount of energy transfer was calculated as the percentage of increase in donor fluorescence after acceptor photobleaching. Initial scan was obtained scanning the cells at low-laser energy (laser power about 25%) using the 488-nm excitation wavelength to record the fluorescein signal. A second scan was performed using the 568-nm excitation wavelength to record the TRITC signal, and a combined image was obtained. A small part of the cells, in which colocalization of the two signals was noted, was then photobleached with the intense 568-nm light (laser power 100%) to destroy the acceptor molecules. The cells were then rescanned using 488-nm light, at the same laser power (about 25%) used to obtain the initial image of the fluorescein signal. The increase in the fluorescein signal within the photobleached area was used as a measure of the amount of FRET present. To evaluate the specificity of the results obtained, the ratio of donor fluorescence after photobleaching to donor fluorescence before photobleaching within the photobleached area was compared with the same ratio obtained outside the photobleached area in the same cell. As reported by other authors (Kinoshita et al., 2001), FRET can be detected only if the two fluorophores are in close physical proximity (less that ~10 nm): consequently, FRET provides a >10-fold increase in resolution compared with double staining with conventional confocal microscopy, which has a resolution of ~500 nm. Positive control for FRET experiments was performed on cells (Madine-Darby canine kidney and COS7) double-stained with monoclonal antibody anti-tubulin (1:2000; Sigma) and rabbit polyclonal anti-tau (1:100; Dako), which subunits have been reported to interact (Weingarten et al., 1975). Negative control for FRET experiments was performed on COS-7 cells expressing Tau and Che-1 mutant constructs unable to interact. As expected, no FRET was observed between these Tau and Che-1 mutant proteins. Moreover, no FRET was observed when either the primary or the secondary antibodies were omitted. We conclude that the donor dequenching observed after photobleaching of the acceptor reflects FRET.

Acknowledgments

This work is dedicated to the memory of Dr. Giusy Arpaia. We thank Annalisa Onori and Leila Tilia for precious technical assistance and Laura Micheli for her help with the statistical analysis. We thank Dr. A. Levi for critical reading of this manuscript. This work was supported by Ministero della Salute-“Progetto Strategico Malattia di Alzheimer” and Telethon grant no. E0855 to P.C., Ministero della Salute Grant ICS 110.1/RA00.54 to N.C., and Ministero della Salute, AIRC and Telethon project A160 to C.P. Dr. Nicoletta Corbi is the recipient of a F.I.R.C. fellowship.

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