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GSK-3 Mediates the Okadaic Acid-Induced Modification of Collapsin Response Mediator Protein-2 in Human SK-N-SH Neuroblastoma Cells

Mei-Hui Ni,[1] Chih-Ching Wu,[2] Wen-Hsiung Chan,[5] Kun-Yi Chien,[1,3] and Jau-Song Yu[1,4]*

[1]Graduate Institute of Basic Medical Sciences, Medical College of Chang Gung University, Tao-Yuan, Taiwan, Republic of China
[2]Molecular Medicine Research Center, Medical College of Chang Gung University, Tao-Yuan, Taiwan, Republic of China
[3]Department of Biochemistry, Medical College of Chang Gung University, Tao-Yuan, Taiwan, Republic of China
[4]Department of Cell and Molecular Biology, Medical College of Chang Gung University, Tao-Yuan, Taiwan, Republic of China
[5]Department of Bioscience Technology and Center for Nanotechnology, Chung Yuan Christian University, Chung Li, Taiwan, Republic of China

Abstract Collapsin response mediator protein-2 (CRMP-2), a phosphoprotein involved in axonal outgrowth and microtubule dynamics, is aberrantly phosphorylated in Alzheimer's disease (AD) brain. Alteration of glycogen synthase kinase-3 (GSK-3) activity is associated with the pathogenesis of AD. Here, we show that CRMP-2 is one of the major substrates for GSK-3 in pig brain extracts. Both GSK-3α and 3β phosphorylate purified pig brain CRMP-2 and significantly alter its mobility in SDS-gels, resembling the CRMP-2 modification observed in AD brain. Interestingly, this modification can be detected in SK-N-SH neuroblastoma cells treated with a phosphatase inhibitor, okadaic acid (OA), and GSK-3 inhibitors completely block this OA-induced event. Knockdown of both GSK-3α and 3β, but not either kinase alone, impairs OA-induced modification of CRMP-2. Mutation of Ser-518 or Ser-522 of CRMP-2, which are highly phosphorylated in AD brain, to Ala blocks the OA-induced modification of CRMP-2 in SK-N-SH cells. Ser-522 prephosphorylated by Cdk5 is required for subsequent GSK-3α-mediated phosphorylation of CRMP-2 in vitro. Collectively, our results demonstrate for the first time that OA can induce phosphorylation of CRMP-2 in SK-N-SH cells at sites aberrantly phosphorylated in AD brain, and both GSK-3α and 3β and Ser-522 kinase(s) are involved in this process. J. Cell. Biochem. 103: 1833–1848, 2008.

Key words: GSK-3; phosphorylation; CRMP-2; okadaic acid; Alzheimer's disease

Alzheimer's disease (AD) is characterized by two prominent pathological traits; one is amyloid plaque containing mainly amyloid-β (Aβ) peptides, and the other is accumulation of intraneuronal neurofibrillary tangles (NFTs). The hyperphosphorlyated tau protein is the main component of NFTs [Grundke-Iqbal et al., 1986; Lee et al., 2001]. In addition to tau,

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Abbreviations used: CRMP-2, collapsin response mediator protein 2; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; GSK-3, glycogen synthase kinase-3; OA, okadaic acid; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; PKA, catalytic subunit of cAMP-dependent protein kinase.

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*Correspondence to: Jau-Song Yu, PhD, 259, Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan, Taiwan.
E-mail: yusong@mail.cgu.edu.tw

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microtubule-associated protein-2 was found to present in NFTs [Kosik et al., 1984]. Meanwhile, hyperphosphorylated neurofilament [Perry et al., 1985; Sternberger et al., 1985] and collapsin response mediator protein-2 (CRMP-2) [Yoshida et al., 1998; Gu et al., 2000] were also reported to be associated with NFTs. The presence of tau protein kinase, glycogen synthase kinase-3 (GSK-3) in isolated NFTs [reviewed in Jope and Johnson, 2004] and decreased activities of protein phosphatases PP1 and PP2A [Gong et al., 1993] have been reported to link to NFT formation and pathogenesis of AD.

GSK-3 was originally identified as one of several protein kinases that phosphorylated and inactivated glycogen synthase, the enzyme responsible for the final step in glycogen biosynthesis [Embi et al., 1980; Hemmings et al., 1981]. This kinase was independently identified as an activating factor of Mg.ATP-dependent protein phosphatase termed protein kinase FA [Vandenheede et al., 1980; Yang et al., 1980]. GSK-3 was further characterized as a multi-substrate protein kinase that could phosphorylate many other proteins [Frame and Cohen, 2001]. Besides its role in regulating glycogen metabolism, GSK-3 has been demonstrated as a critical component of the Wnt-signaling pathway that regulates body patterning during embryonic development [Ferkey and Kimelman, 2000]. Other studies also showed that, by phosphorylating its substrates, GSK-3 can modulate protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, and cell motility [Doble and Woodgett, 2003]. Two classes of GSK-3 cDNA have been cloned termed GSK-3α and GSK-3β [Woodgett, 1990]. These two isoforms are encoded by distinct genes: the α-type encodes a 51–53 kDa polypeptide identical to kinase FA and the β-type encodes a 47 kDa polypeptide with 85% amino acid identity to GSK-3α.

Although multiple functions of GSK-3 have been described in a variety of physiological settings, investigation of its role in the nervous system is still limited. Moreover, for unknown reasons, the role of GSK-3α, in sharp contrast to GSK-3β, has long been neglected in most studies about GSK-3. Previously, we have shown that GSK-3α exists most abundantly in the brain [Yu and Yang, 1993a] and can be detected in several subcellular fractions such as myelin, microtubules, and coated vesicles [Yang et al., 1990, 1991; Yu and Yang, 1993b]. In these fractions, several key proteins involved in the regulation of brain functions could serve as substrates for GSK-3, including myelin basic protein [Yu and Yang, 1994c], microtubule-associated protein tau and MAP-2 [Yang et al., 1991, 1993, 1994], and coated vesicle proteins [Yu and Yang, 1993b]. The phosphorylation sites of myelin basic protein and tau protein by GSK-3 match well with the in vivo phosphorylation sites of the two proteins [Martenson et al., 1983; Deibler et al., 1990; Hasegawa et al., 1992; Lichtenberg-Kraag et al., 1992; Yang et al., 1993, 1994; Yu and Yang, 1994c]. These observations suggest that GSK-3 might be an important brain kinase participating in the control of diverse brain functions.

To facilitate the study of functional roles of GSK-3 in brain, we tried to identify more substrates for GSK-3 in the brain. We fractionated pig brain extracts by a serial column chromatography and found a set of 60–70 kDa proteins as very good substrates for GSK-3. In the present study, we identify these substrate proteins as CRMP-2, and show the unusual molecular weight shift of CRMP-2 in SDS-gels after phosphorylation by GSK-3α and 3β, which resembles the hyperphosphorylated form of CRMP-2 found in AD brain and in human neuroblastoma SK-N-SH cells treated with phosphatase inhibitor okadaic acid (OA). Using pharmacological inhibitors and siRNA pools as tools, we further show that both GSK-3α and 3β are involved in the OA-induced phosphorylation of CRMP-2 in SK-N-SH cells. We also identify both Ser-522 and Ser-518 of CRMP-2 as essential residues for this OA-induced event in SK-N-SH cells, and provide evidence that Ser-522 kinase(s) might participate in this event.

EXPERIMENTAL PROCEDURES

Materials

Lithium chloride, SB216763 and SB415286 were from Sigma (St. Louis, MO). Recombinant human GSK-3α, GSK-3β, and Cdk5 were from Upstate (Charlottesville, VA). BCA protein assay reagent was from Pierce (Rockford, IL). OA was from Boehringer Mannheim (Mannheim, Germany). [γ-^{32}P]ATP, IPG strips (pH 3–10) and protein A-Sepharose CL-4B were from Amersham Biosciences (Buckinghamshire,

England). Anti-GSK-3 antibody (0011-A) and anti-c-Myc antibody (A-14) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Purification of Enzymes and Proteins

GSK-3 was purified from pig brain as the activating factor of Mg.ATP-dependent protein phosphatase (kinase FA) according to previous reports [Yu and Yang, 1993a,b]. Briefly, Triton X-100-extracts of pig brain particulate fractions were loaded onto a DEAE-cellulose column, and the flow-through fractions were absorbed onto a phosphocellulose column. Kinase FA activity eluted from the column at 0.35 M NaCl was collected and then purified sequentially by chromatography on Blue Sepharose CL-4B, casein-Sepharose 4B, and hydroxyapatite columns. The purified GSK-3 showed a major protein band at ~53 kDa in SDS-gel when stained with Coomassie Brilliant Blue. In some preparations of the purified GSK-3, a minor band at ~47 kDa could also be detected in addition to the 53-kDa protein. Western blot analysis using antibodies against GSK-3α and 3β showed that the 53- and 47-kDa proteins represent the GSK-3α and 3β isoforms. The purified kinase preparations containing both GSK-3α and 3β isoforms were used as the kinase source to search GSK-3 substrates in the pig brain (see below). Microtubule-associated tau protein was purified from pig brain as previously described [Yang et al., 1993, 1994]. For purification of substrates for GSK-3 from pig brains, pig brains (500 g) freshly obtained from a local slaughterhouse were homogenized in 1.5 L ice-cold buffer A (0.32 M sucrose, 50 mM Tris-HCl pH 7.4, 30 mM 2-mercaptoethanol, 4 mM EDTA, and 1 mM EGTA and 0.1 mM PMSF). All subsequent operations were performed at 4°C. The homogenate was centrifuged at 8,000*g* for 30 min, and the resulting supernatant was used as the starting material for purification of substrate(s) for GSK-3 (see Results).

Assay for Substrate Phosphorylation

Phosphorylation in the column fractions was performed in 20 μl of phosphorylation buffer (20 mM Tris-HCl pH 7.0, 0.5 mM dithiothreitol, 10 mM NaF, 0.2 mM [γ-^{32}P]ATP and 20 mM $MgCl_2$) containing 10 μl of column fraction and 20 ng of GSK-3 at 30°C for 30 min. The reaction products were resolved in 10% SDS-gels, stained by Coomassie blue, and air-dried. ^{32}P-labeled proteins were identified by autoradiography of the dried gels. For some experiments, ^{32}P-incorporation into substrates was determined by paper assay as previously described [Yu et al., 1998].

Two-Dimensional (2D) Gel Electrophoresis, In-Gel Digestion of Proteins, and Mass Spectrometric Analysis

2D gel electrophoresis of purified proteins, in-gel digestion of proteins, and mass spectrometric analysis was performed as previously described [Tsai et al., 2005; Wu et al., 2005]. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis was performed on an Ultraflex™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Searches were performed without constraining protein molecular weight or isoelectric point, and allowed for carbamidomethylation of cysteine, partial oxidation of methionine residues, and one missed trypsin cleavage. MS/MS sequencing analyses were carried out using the same MALDI-TOF/TOF mass spectrometer. Sequence analysis with TOF-TOF technology was performed using the software of the instrument (Biotools; Bruker Daltonik GmbH, Bremen, Germany).

Production of Anti-CRMP-2, Anti-GSK-3α, and Anti-GSK-3β Antibodies

The anti-CRMP-2 (N16 and C17) antibodies were produced in rabbits using the N16 peptide (MSYQGKKNIPRITSDRC) and the C-17 peptide (NIPRRTTQRIVAPPGGR), corresponding to the N-terminal region from amino acids 1–16 and the C-terminal region from amino acids 549–565 of the sequence of human CRMP-2 [Kitamura et al., 1999] as the antigen. The two peptides were synthesized by Kelowna International Scientific (Taipei, Taiwan) and AnaSpec (San Jose, CA). A cysteine residue was added to the C-terminus to the N16 peptide to facilitate coupling the peptide to bovine serum albumin. Anti-GSK-3α and anti-GSK-3β antibodies were produced in rabbits using the peptides CQAPDATPTLTNSS and CSDAN-TGDRGQTNNAASASA, respectively. The methods used for production and affinity-purification of the anti-peptide antibody were detailed in previous reports [Yu et al., 1997, 1998].

Cell Culture, Transfection, Drug Treatment, Western Blot, and Immunoprecipitation

The human neuroblastoma SK-N-SH cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was performed by using Lipofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For drug treatment, lithium chloride, SB216763, SB415286, or OA (dissolved in H_2O or DMSO) was added to the medium, and cells were incubated at 37°C in a CO_2 incubator for the indicated time periods. The cells were washed twice with ice-cold PBS, and cell lysates were prepared and subjected to Western blot and immunoprecipitation using antibody against CRMP-2, GSK-3α, or GSK-3β as previously described [Yu et al., 1997, 1998]. Kinase activity assay in the immunoprecipitates using myelin basic protein as the substrate was performed as previously described [Yu et al., 1997, 1998].

Plasmids Construction

Full-length human CRMP-2 cDNA was amplified by polymerase chain reaction (PCR) using cDNAs derived from SK-N-SH cells as the template and oligonucleotides, 5′-GGAATTCGGATGTCTTATCAGGGGAAGAAAAA-3′ and 5′-GGAATTCGGCTAGCCCAGGCTGGTGATGT-3′, as primers. The PCR product was purified from agarose gel, digested with *EcoRI*, and ligated with *EcoRI*-treated pCMV-Myc vectors (BD Biosciences Clontech, Palo Alto, CA). The resulting plasmid was designated as pCMV-Myc-CRMP-2-FL. To generate the expression plasmid pET-32a-CRMP-2-F3, which could produce recombinant fusion protein F3 containing the C-terminal fragment of CRMP-2 and both thioredoxin and His tags, the full-length CRMP-2 sequence was digested with *BamHI* and separated by electrophoresis. The fragment containing the most 3′-end sequence (510 bp) was excised, purified, and subcloned into *BamHI*- and *EcoRI*-treated pET-32a vector (Novagen, Madison, WI). The resulting expression plasmid was designated as pET-32a-CRMP-2-F3, which encodes the C-terminal portion of CRMP-2 from amino acid 404–572. The single site F3 mutants (T509A, S518A, or S522A) and multiple site F3 mutants 3A (T509A/T514A/S518A) and 4A (T509A/T514A/S518A/S522A) were generated by PCR using pET-32a-CRMP-2-F3 as the template and the following oligonucleotides as primers. Forward primer: 5′-TGTGGGATCCGATGCCGACCTGGTCATCTG-3′; reverse primers: 5′-AGGTTCCGGACAGGTGGGGCCTGCTGCTTGGCAGGAGACGTCTTGGCCGAGGAGGCTGGAGTGACTGTCTTGGGCGCCACAGACACTTCACACAC-3′ (for T509A); 5′-AGGTTCCGGACAGGTGGGGCCTGCTGCTTGGCAGGAGACGTCTTGGCCGCGGAGGCTGGAGTGACTGTC-3′ (for S518A); 5′-AGGTTCCGGACAGGTGGGGCCTGCTGCTTGGCAGGAGCCGTCTTGGCCGAGGAGGCTG-3′ (for S522A); 5′-AGGTTCCGGACAGGTGGG-GCCTGCTGCTTGGCAGGAGACGTCTTGGCCGCGGAGGCTGGAGCGACTGTCTTGGGCGCCACAGACACTTCACACAC-3′ (for 3A); 5′-AGGTTCCGGACAGGTGGGGCCTGCTGCTTGGCAGGAGCCGTCTTGGCCGCGGAGGCTGGAGCGACTGTCTTGGGCGCCACAGACACTTCACACAC-3′ (for 4A). The PCR products were purified from agarose gel, digested with *BseAI* and *EcoRI*, and put back into *BseAI*- and *EcoRI*-treated pET32a-CRMP-2-F3. The resulting plasmids were designated as pET-32a-CRMP- 2-F3-T509A, S518A, S522A, 3A and 4A, respectively. For the production of pCMV-Myc-CRMP-2-FL mutants, pET-32a-CRMP-2-F3 mutants were digested with *XhoI* to excise the ~460 bp, mutation-containing fragments near the 3′ end of the coding sequence. The plasmid pCMV-Myc-CRMP-2-FL was also treated with *XhoI* to remove a ~450 bp *XhoI* fragment. The ~460 bp mutation-containing fragments were purified and ligated with *XhoI*-treated pCMV-Myc-CRMP-2-FL. The resulting plasmids were designated as pCMV-Myc-CRMP-2-FL-T509A, S518A, S522A, 3A, and 4A, respectively. The DNA sequences of all the constructs were confirmed by auto-sequencing. Among them, a single nucleotide (G) insertion occurred just next to the nucleotides coding for the amino acid K525 in the 4A construct. Such a mutation causes a frame shift and introduces a termination codon 62 bp downstream of the mutation. This mutation leads to a premature stop of the translation, and the resulting Myc-tagged CRMP-2-FL-4A protein was found to have smaller molecular weights than the wild-type protein as judged by SDS–PAGE and Western blotting. However, it would not affect our experimental design because all the four Ser or Thr to Ala mutations were indeed preserved and the rest of the sequence except the insertion was verified.

Production of Recombinant Proteins

Recombinant thioredoxin and His tags fusion proteins harboring the C-terminal portion of CRMP-2 from amino acid 404–572, either wild-type or mutated at different phosphorylation sites, were expressed in *E. coli* BL21 (DE3) pLysS cells transformed with pET-32a-CRMP-2-F3 or F3 mutant plasmids. The transformed cells were incubated with IPTG (1 mM) at 37°C for 4 h, collected by centrifugation, and lysed in Binding Buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9) containing 1% Triton X-100. His-tagged recombinant proteins were then purified by Ni^{2+}-Chelating Sepharose-Fast Flow Resin (GE Healthcare, Buckinghamshire, England) followed by extensive dialysis against buffer containing 20 mM Tris-HCl, pH 7.0 and 0.5 mM dithiothreitol. The purified proteins were confirmed by Western blotting using His-probe (Santa Cruz, CA) and anti-CRMP-2 (C-17) antibody.

RNA Interference and siRNA Transfection

GSK-3α and GSK-3β ON-TARGETplus SMARTpool siRNA reagents and non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO). Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated horse serum, plated on 24-well plates, and co-transfected with plasmid pCMV-Myc-CRMP-2-FL and 10–50 nM of indicated siRNA for 48 h using Lipofectamine 2000 (Invitrogen) according to the manual of the manufacturer.

RESULTS

Purification of a Major Substrate for GSK-3 From Pig Brain

In order to identify potential GSK-3 substrates, extracts of pig brain were fractionated by liquid chromatography and the separated fractions were phosphorylated in the absence or presence of GSK-3. After fractionation by DEAE-Sepharose CL-4B column, a set of 60–70 kDa proteins emerged as the major substrates for GSK-3 (Fig. 1A). These proteins were subsequently purified by gel filtration, polylysine-Sepharose 4B, and protamine-Sepharose 4B columns, respectively (Fig. 1B–D). After chromatography on the polylysine-Sepharose 4B column, endogenous phosphorylation of these substrates could no longer be detected (Fig. 1C). After purification by the protamine-Sepharose 4B column, three protein bands (~58, 62, and 66 kDa) represented the major species that could be phosphorylated by GSK-3 (Fig. 1D). Approximate 12 mg of substrate proteins with >90% purity could be obtained from 500 g of pig brain by this purification procedure.

The molecular weights of these purified proteins are very close to those of microtubule-associated tau proteins, which are also abundant in the brain tissue and had been demonstrated as substrates for GSK-3 [Yang et al., 1991, 1993; Hanger et al., 1992; Mandelkow et al., 1992]. To rule out the possibility that these purified substrate proteins might represent the heat-stable tau proteins [Weingarten et al., 1975; Herzog and Weber, 1978], both tau and these purified substrate proteins were phosphorylated by GSK-3 and subjected to heat treatment. In contrast to tau proteins, which mainly remained in the supernatant fraction after heat treatment, most of the purified substrates were denatured by heat treatment and detected in the pellet fraction (Fig. 1E). This result indicates that the purified substrates are not tau proteins.

Molecular Weight Shift of the Purified Substrates in SDS–PAGE After Phosphorylation by GSK-3

When both unphosphorylated and phosphorylated substrates were simultaneously analyzed by SDS–PAGE, it was found that the GSK-3-catalyzed phosphorylation caused a significant molecular weight shift of the purified substrates. Before phosphorylation two protein species with molecular weights at ~62 and 58 kDa represented the major components in the purified substrates (Supplementary Fig. S1A, arrows 2 and 3). However, another protein species at ~66 kDa emerged after phosphorylation, which corresponded to the major ^{32}P-labeled product (Supplementary Fig. S1A, arrow 1). The purified substrates could also be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (PKA). PKA phosphorylated exclusively on the 62-kDa species (Supplementary Fig. S1A, arrow 2), and the phosphorylation did not alter the protein pattern in SDS–PAGE. Approximately 0.6 mol of phosphate can be incorporated into one mol of purified substrates by GSK-3 (Supplementary Fig. S1B), and both serine and threonine residues are phosphorylated (Supplementary Fig. S1C).

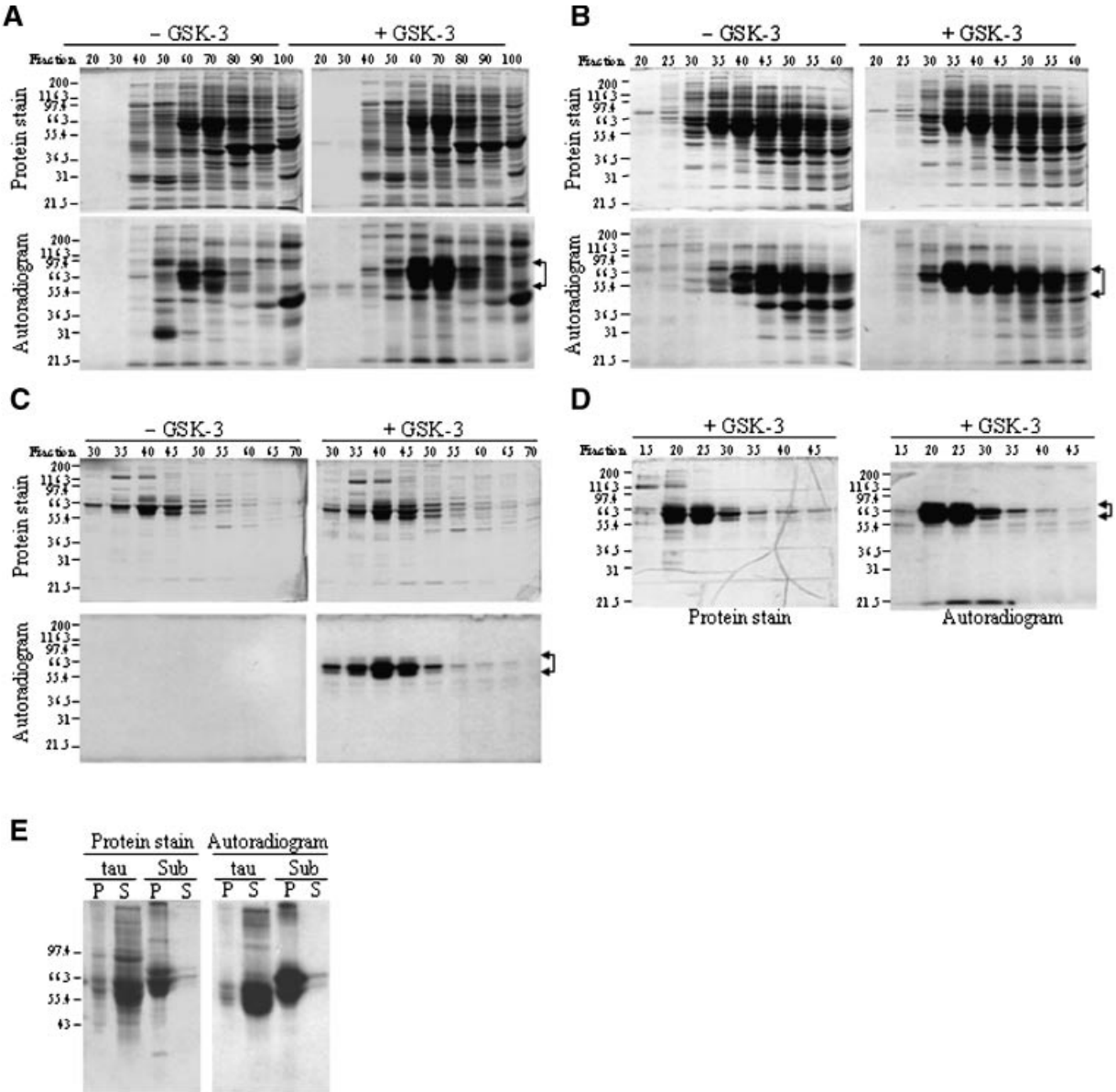

Fig. 1. Chromatography of GSK-3 substrates from pig brain extracts. **A–D**: Pig brain extracts were fractionated sequentially by DEAE-Sepharose CL-4B (A), AcA-34 Ultrogel (B), polylysine-Sepharose 4B (C), and protamine-Sepharose 4B (D) chromatography. Ten microliters of fractions collected from columns described above were phosphorylated by GSK-3 and the reaction products were analyzed by 10% SDS–PAGE, followed by Coomassie blue staining and autoradiography. Arrows denote the major substrates for GSK-3. **E**: Ten micrograms of purified microtubule-associated protein tau (tau) or the substrates for GSK-3 (Sub) was phosphorylated by 40 ng of GSK-3 in the presence of [γ-^{32}P]ATP at 30°C for 30 min. The reaction products were then incubated at 100°C for 5 min and centrifuged at 10,000*g* for 20 min. The supernatants (S) were carefully removed from pellets (P), and both fractions were analyzed by 10% SDS–PAGE, followed by Coomassie blue staining and autoradiography. The pellets were derived from the residual proteins stuck to the test tubes after removing the supernatants. Proteins that were presented in the pellets might be those denatured after heat treatment or those bound non-specifically to the tubes.

Identification of the Purified Substrates as Collapsin Response Mediator Protein-2 (CRMP-2)

To address the identity of the purified substrates for GSK-3, they were first resolved by 2D gel and the resulting protein spots were excised for tryptic peptide fingerprint analysis using a MALDI-TOF mass spectrometer. Before phosphorylation two major groups of spots (a and b) and one minor spot (c) were observed

in 2D gel, whereas in addition to these three groups of spots another group of spots (d) were detected after phosphorylation by GSK-3 (Fig. 2A). Spots of each group were selected and excised for tryptic peptide mass fingerprint analysis by MALDI-TOF/TOF mass spectrometry (Table I). All of the fingerprints from spots b1 (62 kDa), b′1 (62 kDa), and d2 (66 kDa) are almost identical and match to peptides of human CRMP-2 with ~40% sequence coverage, ranging from amino acid residue 44 to 565. Human CRMP-2 is composed of 572 amino acids, and has a predicted molecular weight of 62,711 [Hamajima et al., 1996; Kitamura et al., 1999]. Direct amino acid sequence analysis of the tryptic peptide selected from spot b1 with m/z value of 2169.2 (see Table I) by MALDI-TOF/TOF mass spectrometry demonstrated the presence of predicted amino acid sequences: NLHQSGFSLSGAQIDDNIPR (Fig. 2B). Although this peptide sequence is highly conserved in all the five isoforms of CRMP found in human, it is specific for the CRMP-2 isoform (Fig. 2B). The results indicate spot b as the intact form of pig CRMP-2. Furthermore, because spot d2 could only be detected after phosphorylation by GSK-3 (Fig. 2A) and phosphorylation catalyzed by GSK-3 caused mobility shift of the purified substrates in SDS–PAGE (Supplementary Fig. S1), our finding implicates spot d2 as the phosphorylated form of pig CRMP-2. Although with lower sequence coverage, the fingerprints from both spots a1 and a2 (58 kDa) also match to CRMP-2 significantly (Table I). The lack of detecting three peptides (residues 526–532, 533–552, and 558–565) in spot a, as compared to spots b and d, suggests that it should represent the degraded form of pig CRMP-2.

To further confirm the mass spectrometric analysis data, anti-peptide antibodies against the C- and N-terminal regions of human CRMP-2 were produced, affinity-purified, and used for Western blot analysis of the purified substrates, as well as extracts from mouse brain and human neuroblastoma SK-N-SH cells. Both antibodies recognized a major 62-kDa species in the mouse brain extracts, SK-N-SH cell lysates and purified substrates, respectively (Fig. 2C). Moreover, a minor 58-kDa species could also be detected in the purified substrate by the anti-CRMP-2 (N16) antibody, but not the anti-CRMP-2 (C17) antibody (Fig. 2C, lanes 6, 9). This observation supports the notion that the

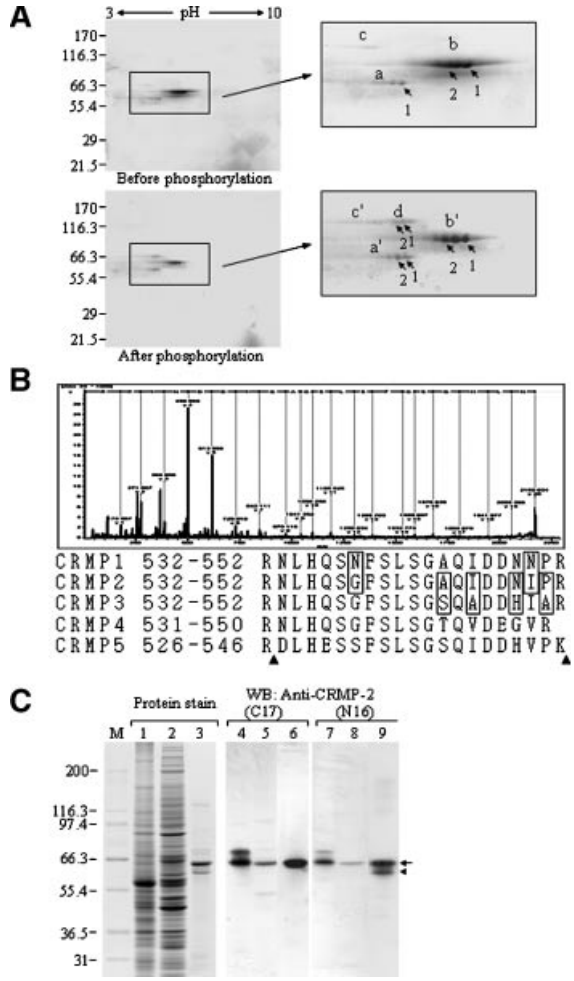

Fig. 2. Identification of the purified substrates as CRMP-2. **A**: The purified substrates (20 μg) were incubated without or with 80 ng GSK-3 in phosphorylation buffer (where the [γ-^{32}P]ATP was replaced by ATP) at 30°C for 1 h. Reaction products were then analyzed by 2D gel electrophoresis, and the protein-staining pattern is shown. **B**: Amino acid sequence analysis of one tryptic peptide selected from spot b1 (**upper panel**). The tryptic peptide with m/z value of 2169.2 from spot b1 (see Table I) was selected for MS/MS sequencing analysis. Shown here are its mass spectrum and the deduced amino acid sequences from the spectrum. The sequence obtained from the peptide with m/z value of 2169.2 matched completely to that of human CRMP-2 (**lower panel**). The peptide sequences that cover this region from human CRMP-1 to -5 are shown. Boxes highlight the residues that can be used to differentiate CRMP-1, -2, and -3. Arrowheads indicate the trypsin-cutting site. **C**: Extracts of mouse brain (80 μg, **lanes 1, 4, 7**) or human neuroblastoma SK-N-SH cells (80 μg, **lanes 2, 5, 8**) and the purified substrates (5 μg, **lanes 3, 6, 9**) were resolved in 10% SDS-gel and stained with Coomassie blue or electroblotted onto PVDF membrane. The membrane was then probed with anti-CRMP-2 (C17) (lanes 4–6) or anti-CRMP-2 (N16) (lanes 7–9) antibody as described. Intact CRMP-2 and its degraded product were indicated by an arrow and arrowhead, respectively. **Lane M**, marker proteins.

TABLE I. Analysis of Tryptic Peptide Mass Fingerprints of Protein Spots From 2D Gels

Mass observed	Mass matched $[M+H]^+$	Spot b1	b′1	a′1	a′2	d2	Start–end	Peptide sequence[a]
1323.833	1323.764	+	+	−	+	+	44–56	QIGENLIVPGGVK
813.418	813.422	+	+	−	−	+	57–63	TIEAHSR
1294.725	1294.694	+	+	+	+	+	64–75	MVIPGGIDVHTR
2377.371	2377.175	+	+	+	+	+	190–211	DIGAIAQVHAENGDIIAEEQQR
2900.771	2900.512	+	−	+	+	+	212–238	ILDLGITGPEGHVLSRPEEVEAEAVNR
1015.592	1015.554	+	+	+	+	+	259–268	SAAEVIAQAR[b]
2553.174	2553.263	+	−	−	−	−	270–293	GTVVYGEPITASLGTDGSHYWSK
2147.964	2148.057	+	−	−	+	−	342–361	AVGKDNFTLIPEGTNGTEER
1792.972	1792.835	+	+	+	−	+	346–361	DNFTLIPEGTNGTEER
908.523	908.500	+	+	+	+	+	391–397	VFNLYPR
1900.070	1899.970	+	+	−	+	+	401–418	IAVGSDADLVIWDPDSVK
1084.652	1084.637	+	+	−	+	+	441–451	GSPLVVISQGK
1683.010	1682.871	+	+	+	+	+	452–467	IVLEDGTLHVTEGSGR

Tryptic peptide mass fingerprints of protein spots from 2D gels corresponding to Figure 2 were analyzed by MALDI-TOF mass spectrometry. The peptide masses observed and shown here are derived from spot b1. This fingerprint matches 17 (16) peptides of bovine (human) CRMP-2, with 40.5% (38.8%) sequence coverage. Using this fingerprint as a reference, the presence (+) or absence (–) of individual peptide mass in the tryptic fingerprint of other spots is also indicated. The corresponding amino acid residue number (start-end) of matched peptides in bovine CRMP-2 is denoted.

[a]Peptide sequences are derived from bovine CRMP-2. All peptides sequences listed are of the same between bovine and human CRMP-2, except for the peptide SAAEVIAQAR.

[b]The sequence of CRMP-2 for human is SSAEVIAQAR.

58-kDa species represents the degraded product of CRMP-2 lacking its most C-terminal region. The results taken together confirm CRMP-2 and its proteolytic products as the major substrates for GSK-3 purified from pig brain.

Okadaic Acid Induces Modification of CRMP-2 in Human SK-N-SH Neuroblastoma Cells

CRMP-2 is known as a phosphoprotein in vivo [Byk et al., 1996]. Highly phosphorylated CRMP-2 has been found in NFTs associated with AD, and the highly phosphorylated CRMP-2 has a higher apparent molecular weight of about 66 kDa [Gu et al., 2000]. To investigate whether the phosphorylation state of CRMP-2 can be modulated in culture cell model, the human neuroblastoma SK-N-SH cells were treated with OA, a potent inhibitor for protein phosphatases type 1 and 2A (PP1 and PP2A) [Bialojan and Takai, 1988], for various time periods, and the electrophoretic pattern of CRMP-2 in the resulting cell extracts was examined by Western blot. Without OA treatment, the anti-CRMP-2 (N16) antibody recognized a major 62-kDa band in SK-N-SH cells (Fig. 3A). In addition to this major band, however, two minor but clear bands (~64–68 kDa) appeared after OA treatment in a time course-dependent fashion (Fig. 3A). This finding strongly suggests that OA can induce phosphorylation of CRMP-2 in SK-N-SH cells, and this modification can cause a significant molecular weight shift of CRMP-2 in SDS–PAGE. When the purified pig CRMP-2 was simultaneously analyzed in the same gel, it migrated to the same position as the major 62-kDa band from SK-N-SH cells (Fig. 3B). Interestingly, after phosphorylation by GSK-3α or 3β the molecular weight shift of CRMP-2 was almost identical to that observed in OA-treated SK-N-SH cells (Fig. 3B). To examine if the mobility shift of CRMP-2 observed in OA-treated SK-N-SH cells was caused by phosphorylation, CRMP-2 was immunoprecipitated by anti-CRMP-2 (C17) antibody from extracts of OA-treated cells, incubated with the catalytic subunit of PP2A (PP2Ac) in the absence or presence of OA and then analyzed by Western blot using anti-CRMP-2 (N16) antibody. As shown in Figure 3C, the modified form of CRMP-2 diminished after PP2Ac treatment, and inhibition of PP2Ac by OA prevented this diminishment. This result demonstrates that the OA-induced mobility shift of CRMP-2 in SK-N-SH cells is caused by the phosphorylation reaction.

GSK-3 Inhibitors Prevent the OA-Induced Phosphorylation of CRMP-2 in SK-N-SH Cells

The observations described above might imply GSK-3 as the candidate kinase responsible for the OA-mediated phosphorylation and unusual mobility shift of CRMP-2 in SK-N-SH cells. To explore further, the effect of OA on the

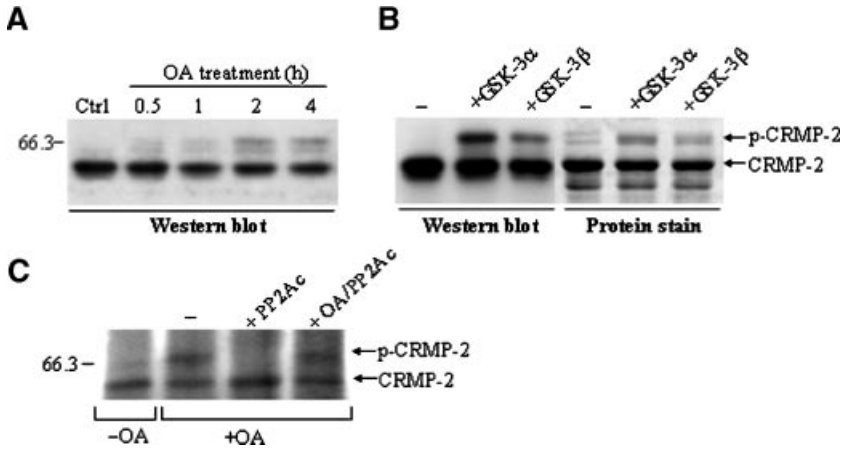

Fig. 3. OA treatment induces phosphorylation of CRMP-2 in SK-N-SH neuroblastma cells. **A**: SK-N-SH cells were treated with DMSO vehicle as control (Ctrl) or with 400 nM OA for 0.5–4 h. Cell extracts (80 μg) were then analyzed by Western blot with anti-CRMP-2 (N16) antibody. **B**: Purified CRMP-2 (5 μg) was incubated without or with 40 ng GSK-3α or GSK-3β in phosphorylation buffer at 30°C for 30 min. The reaction products were resolved in 8% SDS–PAGE and stained by Coomassie blue or transferred to PVDF membrane for Western blot using anti-CRMP-2 (C17) antibody. p-CRMP-2 indicates the phosphorylated CRMP-2. **C**: SK-N-SH cells were left untreated or treated with 400 nM OA for 4 h. Cell extracts (1 mg) were subjected to immunoprecipitation by anti-CRMP-2 (C17) antibody. The immunoprecipitates were incubated without or with the purified PP2Ac (5 μg/ml) in a 20-μl reaction mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, and 10 mM $MnCl_2$ at 30°C for 30 min. OA (1 μM) was included in one sample prior to the addition of PP2Ac. The reaction was stopped by the addition of 2× Laemmli sample buffer, and then analyzed by Western blot with anti-CRMP-2 (N16) antibody.

modification of CRMP-2 in SK-N-SH cells was re-examined when the activity of endogenous GSK-3α and 3β in SK-N-SH cells were inhibited, prior to OA treatment, by incubating the cells with lithium chloride, SB-216763 or SB-415286, three potent pharmacological inhibitors of GSK-3 [Klein and Melton, 1996; Coghlan et al., 2000; Cross et al., 2001]. All three inhibitors could significantly reduce the activities of GSK-3α and 3β in SK-N-SH cells without affecting their protein levels (Fig. 4A). Under this circumstance, OA treatment could no longer elicit phosphorylation/modification of CRMP-2 in SK-N-SH cells (Fig. 4B). The results strongly suggest GSK-3 as the kinase responsible for the OA-induced phosphorylation and mobility shift of CRMP-2 in SK-N-SH cells.

GSK-3α Is as Important as GSK-3β in OA-Induced Modification of CRMP-2 in Cells

The role of GSK-3β in CRMP-2 phosphorylation has been demonstrated in several studies [Brown et al., 2004; Cole et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005], but the role of GSK-3α in regulating CRMP-2 remains unclear. To clarify the role of the two GSK-3 isoforms in OA-induced phosphorylation of CRMP-2, siRNA-mediated gene silencing was used to knock down GSK-3α, 3β, or both in CRMP-2-expressing cells prior to OA treatment. To ensure efficient knockdown of the target genes by RNA interference, we chose HEK293 cells as the cell model due to its superior transfection efficiency. HEK293 cells were transfected simultaneously with the expression plasmid of Myc-tagged CRMP-2 and specific siRNA pools against GSK-3α or 3β to examine the effects of differential knockdown of GSK-3 isoforms on the OA-induced modification of ectopically expressed Myc-tagged CRMP-2. As shown in Figure 5, a clear expression of Myc-CRMP-2 and efficient reduction of GSK-3α or 3β expression to ~20–30% of the level in control cells could be observed in HEK293 cells. OA treatment could also enhance the mobility shift of Myc-CRMP-2 in control siRNA-transfected cells (the percentage of phospho-CRMP-2/total CRMP-2 increased from 41% to 54%), and efficient knockdown of either GSK-3α or 3β alone didn't affect the OA-induced modification of Myc-CRMP-2 (the percentage of phospho-CRMP-2/total CRMP-2 was 50% and 54%, respectively). In contrast, knockdown of both GSK-3α and 3β severely impaired the OA-induced mobility shift of Myc-CRMP-2 (the

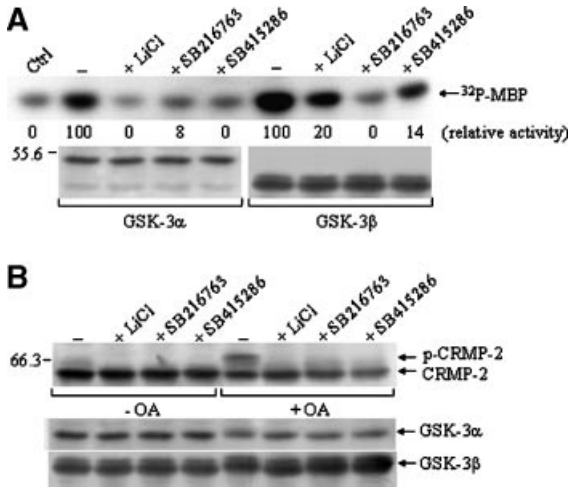

Fig. 4. GSK-3 mediates the OA-induced phosphorylation of CRMP-2 in SK-N-SH neuroblastma cells. **A**: SK-N-SH cells were left untreated or treated with LiCl (30 mM), SB216763 (10 μM), or SB415286 (30 μM) for 2 h. Cell extracts (1 mg) were subjected to immunoprecipitation by anti-GSK-3α or GSK-3β antibody, and the kinase activity in the immunoprecipitates was assayed as described. Ctrl denotes the background activity from cell extracts incubated with protein A-beads alone. The activity of GSK-3α or GSK-3β immunoprecipitated from cells without any drug treatment was taken as 100%. The protein levels of GSK-3α and GSK-3β in cell extracts (80 μg) from each condition were examined by Western blot and shown at **lower panel**. **B**: SK-N-SH cells were left untreated or treated with GSK-3 inhibitors for 2 h and further incubated with or without OA (400 nM) for another 4 h. Cell extracts (80 μg) were then analyzed by Western blot with anti-CRMP-2 (C17), GSK-3α, or GSK-3β antibody.

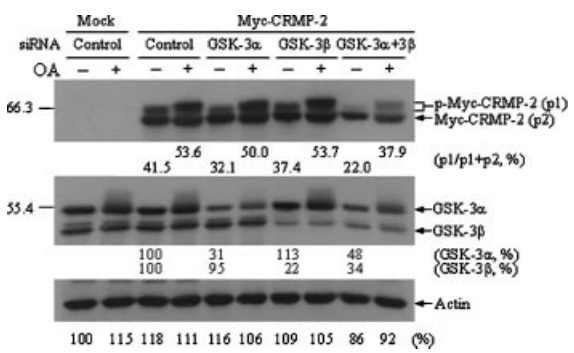

Fig. 5. Knockdown of both GSK-3α and 3β, but not either kinase alone, impairs OA-induced modification of CRMP-2. HEK293 cells were co-transfected with Myc-tagged full-length CRMP-2 and 10 nM of the indicated GSK-3 siRNA for 48 h and then treated without (–) or with (+) 400 nM OA for 2 h. Cell extracts were analyzed by Western blot using anti-CRMP-2 (C17) or anti-c-Myc antibody (**upper panel**), anti-GSK-3 antibody (**middle panel**), and anti-actin antibody (**lower panel**). The intensity of target proteins was quantitated by densitometry and then normalized by the level of actin. The percentage of phospho-CRMP-2/total CRMP-2 and the knockdown efficiency of siRNA are shown below the western blot.

percentage of phospho-CRMP-2/total CRMP-2 reduced from 54% to 38%). Taken together, these results indicate that GSK-3 is indeed the kinase responsible for the OA-induced modification of CRMP-2. Furthermore, neither GSK-3α nor GSK-3β silencing affected the OA-induced modification of CRMP-2 in cells, suggesting that GSK-3α and GSK-3β play important but redundant roles in regulating the OA-induced phosphorylation of CRMP-2.

Both Ser-522 and Ser-518 Are Required for OA-Induced Phosphorylation-Dependent Mobility Shift of CRMP-2 in Neuroblastoma Cells

Induction of modification (hyperphosphorylation) of tau and other cytoskeletal proteins by OA in neuroblastoma cells has been well documented in several studies [Arias et al., 1993; Dupont-Wallois et al., 1995; Wang et al., 2001]. In addition, the NFT-associated CRMP-2 was found to be highly phosphorylated on Thr-509, Ser-518, and Ser-522 in the peptide segment $T^{509}PKTVT^{514}PASS^{518}AKTS^{522}P$ located at the C-terminal region [Gu et al., 2000]. This peptide segment is well conserved in CRMP-2 proteins from various species (Fig. 6A, upper panel); it has three Ser/Thr-Pro motifs and contains several Ser/Thr residues that are tandem spaced by three amino acid residues in the format Ser/Thr-X-X-X-Ser/Thr, which match well with the substrate consensus sequence motif for GSK-3 [Dent et al., 1989; Fiol et al., 1990; Ramakrishna et al., 1990; Yang et al., 1993; Yu and Yang, 1994c; Dajani et al., 2001; Woodgett, 2001]. To examine the role of Thr-509, Ser-518, and Ser-522 in the OA-induced modification of CRMP-2 in neuroblastoma cells, the Myc-tagged CRMP-2 expression plasmids, either wild-type or mutants (T509A, S518A, S522A, 3A, or 4A) were generated (Fig. 6A, lower panel), transfected into SK-N-SH cells and then treated with or without OA. Results showed that OA could also induce modification of the ectopically expressed wild-type CRMP-2, as well as the T509A mutant in SK-N-SH cells (Fig. 6B). On the other hand, such modification was not observed on S518A, S522A, 3A, and 4A mutants after OA treatment (Fig. 6B). This finding indicates that both Ser-518 and Ser-522 are required for the OA-induced phosphorylation-dependent modification of CRMP-2 in neuroblastoma cells.

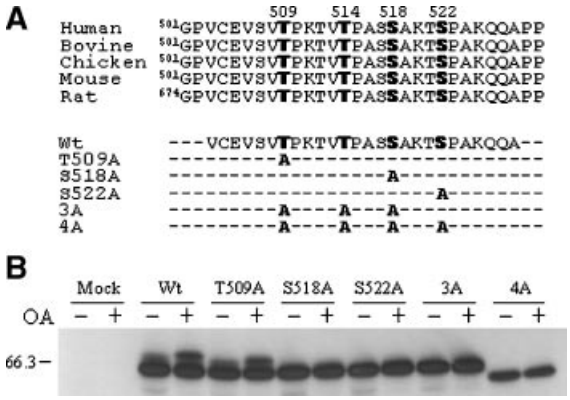

Fig. 6. Effect of mutations on the OA-induced modification of CRMP-2 in cells. **A**: **Upper panel**: Comparison of potential GSK-3 phosphorylation sites in CRMP-2 sequence from different species. **Lower panel**: Schematic illustration of the CRMP-2 mutants, in which the potential GSK-3 phosphorylation sites were either singly or multiply mutated to alanine residue. **B**: SK-N-SH cells were transfected with indicated plasmids for 24 h and then treated without (–) or with (+) 400 nM OA for 2 h. Cell extracts (80 μg) were then analyzed by Western blot using anti-c-Myc antibody (A-14).

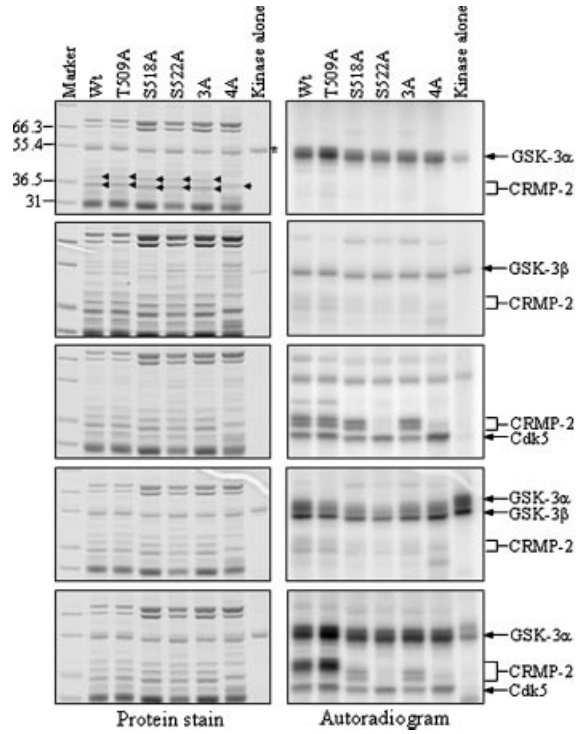

Fig. 7. Phosphorylation of recombinant CRMP-2 fusion proteins by GSK-3α, GSK-3β, and Cdk5. (**Top** to **bottom**) Recombinant fusion proteins (5 μg) harboring the C-terminal portion of CRMP-2 from amino acid 404–572, either wild-type or mutated at different phosphorylation sites as indicated, were incubated with GSK-3α (immunoprecipitated from 100 ng of the purified GSK-3 preparation by anti-GSK-3α antibody and protein A-beads), GSK-3β (100 ng), Cdk5/p35 (25 ng), GSK-3α plus GSK-3β, or GSK-3α plus Cdk5/p35 in phosphorylation buffer at room temperature for 30 min. The reaction products were resolved in 12.5% SDS–PAGE, stained, and air-dried for autoradiography. Arrowheads indicate the positions of different recombinant CRMP-2 fusion proteins. The protein band denoted by asterisk represents the IgG heavy chain of the antibody used in the immunoprecipitation of GSK-3α.

Prephosphorylation of CRMP-2 by Cdk5 Is Required for Subsequent GSK-3α-Catalyzed Phosphorylation and Mobility Shift of CRMP-2 In Vitro

Cdk5 has been shown to phosphorylate Ser-522 in vitro and phosphorylation of Ser-522 is required for subsequent GSK-3β-mediated CRMP-2 phosphorylation in cells [Brown et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005; Cole et al., 2006]. We thus performed experiments to examine the phosphorylation of recombinant CRMP-2 by GSK-3α, in the absence and presence of GSK-3β or Cdk5. The recombinant proteins were produced in *E. coli* as the C-terminal portion of CRMP-2 from aa 404–572, either in a wild-type format or mutated at different phosphorylation sites, fused to thioredoxin and His tags. As shown in Figure 7, GSK-3α and 3β, either alone or in combination, could hardly phosphorylate these recombinant proteins; in contrast, Cdk5 could significantly act on the wild-type, T509A, S518A, and 3A mutants but not on S522A or 4A mutants, which is consistent with the previous observations that Cdk5 can act as a Ser-522 kinase for CRMP-2. When these recombinant proteins were phosphorylated simultaneously by GSK-3α and Cdk5, the wild-type and T509A mutant, but not S518A and 3A mutants, showed a dramatic phosphorylation-dependent mobility shift in SDS-gels as compared to those phosphorylated by Cdk5 alone (Fig. 7). Taken together, these observations indicate that (1) GSK-3α, like GSK-3β, can phosphorylate CRMP-2 and causes its mobility shift in SDS-gels only when Ser-522 has been prephosphorylated by another kinase such as Cdk5, and (2) Ser-518 is also important for this phosphorylation-dependent mobility shift of CRMP-2 in vitro mediated by the cooperated action between Cdk5 and GSK-3α.

DISCUSSION

By an in vitro screening method, we identified CRMP-2 as one major substrate for GSK-3 in pig brain. CRMP-2 was initially identified as a

mediator required for semaphorin-triggered growth cone collapse [Goshima et al., 1995] and is a member of the CRMP family proteins exclusively expressed in the nervous system [Wang and Strittmatter, 1996; Inatome et al., 2000]. It plays an important role in the axonogenesis as it is enriched in the growing axon and transfection of wild-type or deleted mutants of CRMP-2 significantly affected the axon formation in neurons [Inagaki et al., 2001]. The ability of CRMP-2 to bind to tubulin heterodimers and promote microtubule assembly [Gu and Ihara, 2000; Fukata et al., 2002] may account for its unique functional role.

CRMP-2 is a phosphoprotein in vivo [Byk et al., 1996]. The role of phosphorylation on the biological functions of CRMP-2 is emerging recently. Phosphorylation of CRMP-2 at Thr-555 by Rho-associated kinase could be observed in and required for the lysophosphatidic acid-induced collapse of growth cone of chicken dorsal root ganglion neurons [Arimura et al., 2000]. However, this Rho-associated kinase-mediated pathway is not involved in the semaphorin 3A-triggered growth cone collapse of dorsal root ganglion neurons. Instead, Ser-522 phosphorylation of CRMP-2, possibly by Cdk 5/p35, is involved in the semaphorin 3A-triggered growth cone collapse [Brown et al., 2004]. Moreover, Ser-522 phosphorylation was detected in CRMP-2 protein isolated from rat brain, and mutation of this residue to non-phosphorylatable Ala impaired the ability of CRMP-2 to promote axon elongation in primary hippocampal neurons or SH-SY5Y neuroblastoma cells [Cole et al., 2004]. In addition, decreased phosphorylation of CRMP-2 at Thr-514 was found in neurotrophin-3 and brain-derived neurotrophic factor induced axon outgrowth in hippocampal neurons [Yoshimura et al., 2005].

Besides the role of phosphorylation in the normal physiological functions of CRMP-2 in neurons, recent studies have correlated abnormal phosphorylation of CRMP-2 with human brain disease. Using a monoclonal antibody 3F4, which was raised against partially purified paired helical filaments (PHFs), Yoshida et al. [1998] reported CRMP-2 as the antigen of 3F4 and its association with NFTs isolated from the brain of AD patients. The NFT-associated CRMP-2 has a higher apparent molecular weight (~64 and 66 kDa) in SDS–PAGE and is highly phosphorylated on Thr-509, Ser-518, and Ser-522 in the C-terminal region, supposedly by an unknown kinase with a molecular mass of 45–50 kDa [Gu et al., 2000]. These observations taken together indicate the importance of phosphorylation in controlling the role of CRMP-2 at both normal physiological and abnormal pathological settings. In this report, we provide evidence for the first time that the phosphatase inhibitor OA can induce phosphorylation-dependent mobility shift of CRMP-2 in human neuroblastoma cells (Fig. 3). This result corresponds with a recent finding from Hill et al. [2006] that the phosphorylation level of Thr-555 of CRMP2/DRP-2 is enhanced in primary rat cortical neurons after OA treatment. Since OA is a potent inhibitor for protein phosphatases PP1 and PP2A, this observation indicates the direct involvement of PP1 and/or PP2A in the dephosphorylation of CRMP-2 in neuroblastoma cells. We further showed that PP2Ac can efficiently dephosphorylate the phosphorylated CRMP-2 immunoprecipitated from OA-treated cells and reverse its mobility shift (Fig. 3C). This suggests that PP2A might act as the CRMP-2 phosphatase in vivo. Moreover, using several selective inhibitors for GSK-3 as tools, we also show that GSK-3 can be the candidate kinase required for the OA-induced phosphorylation of CRMP-2 in human neuroblastoma cells (Fig. 4). Because GSK-3 has a high basal activity in cells, and most extracellular stimuli and pharmacological agents including OA that affect GSK-3 can downregulate its activity [Welsh and Proud, 1993; Saito et al., 1994; Stambolic and Woodgett, 1994; Yu and Yang, 1994a,b; Eldar-Finkelman et al., 1995; Yu et al., 1997; Ding et al., 2000], our findings seem to implicate that protein phosphatase activity play a more dominant role than kinase activity in maintaining the low phosphorylation level of CRMP-2 in cells under resting condition.

Recently, several studies demonstrated the roles of GSK-3β and Cdk5 in CRMP-2 phosphorylation. GSK-3β phosphorylates CRMP-2 at Ser-509 [Cole et al., 2004; Uchida et al., 2005], Thr-514 [Brown et al., 2004; Cole et al., 2004; Yoshimura et al., 2005], and Ser-518 [Brown et al., 2004; Cole et al., 2004] in vitro and in cells. Cdk5 was shown to phosphorylate Ser-522 in vitro and phosphorylation of Ser-522 is required for subsequent GSK-3β-mediated CRMP-2 phosphorylation in cells [Brown et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005;

Cole et al., 2006]. Co-expression of GSK-3β with CRMP-2 in cells induces mobility shift of CRMP-2 [Brown et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005]. Mutation of Thr-514 to Ala impairs GSK-3β-induced mobility shift of CRMP-2 [Brown et al., 2004; Yoshimura et al., 2005]. Inhibition of GSK-3β by applying specific GSK-3β siRNA to rat cells results in enhanced axon elongation which mimics effects of non-phosphorylated CRMP-2 [Yoshimura et al., 2005]. Furthermore, treatment of hippocampal neurons with neurotrophin-3 and brain-derived neurotrophic factor leads to GSK-3β inactivation and subsequently decreases phosphorylation levels of CRMP-2 at Thr-514 and then promotes axon outgrowth [Yoshimura et al., 2005]. In the present study, we show that both GSK-3α and 3β can phosphorylate purified pig brain CRMP-2 in vitro, leading to a dramatic mobility shift of CRMP-2 in SDS-gels, a phenomenon resembling the phosphorylation-dependent modification of CRMP-2 in neuroblastoma cells induced by OA treatment (Figs. 3, 4, 6) and in Alzheimer-diseased brain [Gu et al., 2000]. In agreement with Cole et al. [2004] that Ser-522 of CRMP-2 has already been phosphorylated in intact brain and serves as a priming phosphorylation site for subsequent GSK-3 phosphorylation, our data also show that purified pig brain CRMP-2 can be phosphorylated by GSK-3α and 3β directly in vitro. We also provide evidence that GSK-3α, like GSK-3β, is not able to directly act on non-phosphorylated CRMP-2; it can only phosphorylate CRMP-2 that had been prephosphorylated at Ser-522 by another kinase such as Cdk5 (Fig. 7). The role of GSK-3α, in sharp contrast to GSK-3β, has long been neglected in most studies about GSK-3. Our finding that knockdown of both GSK-3α and 3β, but not GSK-3α or GSK-3β alone, impairs the OA-induced mobility shift of CRMP-2 (Fig. 5) suggests that both GSK-3α and 3β may act as in vivo CRMP-2 kinases to trigger the phosphorylation of CRMP-2 in OA-treated cells. As both GSK-3α and 3β can hardly act on non-phosphorylated CRMP-2, it is apparent that the action of certain Ser-522 kinase(s) for CRMP-2 is a prerequisite for the observed GSK-3-mediated phosphorylation of CRMP-2 in OA-treated cells.

From a biochemical viewpoint, the characteristics of abnormal phosphorylation of CRMP-2 found in AD brain is quite similar to that detected in tau protein, the first protein component directly identified from the PHF core protein [Goedert et al., 1988; Lee et al., 1991]. Both are tubulin-binding proteins, exist in highly phosphorylated forms with apparently higher molecular weights when isolated from PHFs of AD brain, and serve as good substrates for various protein kinases. Among these kinases, GSK-3 appears to be the most interesting one investigated so far because it can phosphorylate both tau and CRMP-2 at sites abnormally phosphorylated in AD brain and significantly causes their mobility shift in SDS-gels [Mandelkow et al., 1992; Ishiguro et al., 1993; Yang et al., 1993, 1994; Brown et al., 2004; Cole et al., 2004]. Phosphorylation of tau protein by GSK-3 reduced its microtubule assembly promoting and microtubule-binding activities [Wang et al., 1998]. Recently, two studies also showed similar scenario when CRMP-2 was phosphorylated by GSK-3 [Uchida et al., 2005; Yoshimura et al., 2005]. Although the biological functions of CRMP-2 is not fully understood, the observations in tau protein would provide valuable clues to further investigate this important issue.

In conclusion, we identify CRMP-2 as a major substrate for GSK-3 in the brain and show that OA can induce phosphorylation of CRMP-2 in SK-N-SH cells, which involves the actions of both GSK-3α and 3β. Both Ser-522 and Ser-518, two of the three sites highly phosphorylated in CRMP-2 from AD brain, are essential residues for this OA-induced event in SK-N-SH cells.

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