Novel Procedure To Investigate the Effect of Phosphorylation on Protein Complex Formation in Vitro and in Cells[†]

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ABSTRACT: The identification of phosphorylation state-dependent interacting proteins provides clues as to the function of the phosphorylation. Techniques such as yeast two hybrid and co-immunoprecipitation do not employ a single species of fully phosphorylated proteins. This is a particular problem for substrates of glycogen synthase kinase-3 (GSK3), where multiple Ser/Thr residues can be targeted, almost always subsequent to a priming phosphorylation by an alternative kinase. We previously identified the brain enriched collapsin response mediator proteins (CRMP2 and CRMP4) as physiological substrates of GSK3. Cdk5 phosphorylates CRMP2 at Ser522, priming for subsequent phosphorylation at three residues by GSK3 in vitro and in vivo. It is clear that phosphorylation of CRMP2 influences axonal growth; however, the molecular processes underlying this action are not fully established. In addition, the role of phosphorylation in other actions of CRMPs has not been elucidated. We developed a novel procedure to isolate CRMP2 and CRMP4 fully phosphorylated at four sites, namely, Ser522 (by CDK5), Ser518, Thr514, and Thr509 (by GSK3). These phosphoproteins were then used to identify binding partners in rat brain lysates in direct comparison with the non-phosphorylated isoforms. We validated the approach by confirming that a previously reported interaction with tubulin-β is regulated by phosphorylation. We also show that CRMPs (CRMP1, CRMP2, and CRMP4) form heteromers and found that these complexes may also be regulated by phosphorylation. We identified DYRK and Pin1 as novel CRMP4 binding proteins with DYRK interacting preferentially with dephospho-CRMP4 and Pin1 with phospho-CRMP4. Finally, we used this approach to identify the mitochondrial protein ANT as a novel CRMP2 and CRMP4 binding protein. We believe that this approach could be applied generally to the study of phosphorylation-dependent interactions.

Protein phosphorylation is a reversible post-translational modification found in around 30% of all mammalian proteins (*1*, *2*). It can alter protein activity, localization, and/or stability and thus is a key regulatory mechanism in signal transduction, gene expression, cell cycles, protein transport, and cell structure (*3*). In many cases, the introduction of a phosphate into the protein structure prevents or induces protein−protein interactions. Therefore, the identification of phosphorylation-dependent binding partners is a potential method for the characterization of novel protein function. Classical approaches for the identification of binding partners (e.g., yeast two hybrid, co-immunoprecipitation) can be modified to characterize potential effects of protein phosphorylation. For example, mutation of phosphorylatable residues to negatively charged amino acids such as glutamate can often partially mimic phosphorylation. Equally, co-expression of upstream protein kinases or the use of kinase inhibitors can shift the equilibrium of phosphorylation of the protein of interest. However, these approaches seldom produce fully phosphorylated or dephosphorylated proteins and can be complicated by specificity issues. This is of particular concern for substrates of glycogen synthase kinase-3 (GSK3),[1] most of which require priming by a distinct protein kinase prior to recognition by GSK3 and which are often phosphorylated on a series of residues by GSK3 (*4*).

We recently identified a family of neuron specific proteins called collapsin response mediator proteins (CRMP) as excellent substrates for GSK3 in vitro and in vivo (*5*, *6*). CRMPs (CRMP1, CRMP2, CRMP3, and CRMP4) are approximately 572 amino acids in length, with an approximate molecular weight of 62 kDa, and share a high level of sequence homology. They are members of the amidohydrolase family, although they do not have any amidohydrolase enzymatic activity (*7*−*11*). CRMP2 (also called DRP2) is required for Sema 3A induced growth cone collapse in dorsal root ganglia (*12*), while CRMP4 expression is increased in mature dentate gyrus by transient global ischemia, indicating enhanced neurogenesis (*13*), and this

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[1] Abbreviations: CRMP, collapsin response mediator protein; AD, Alzheimer's disease; GSK3, glycogen synthase kinase 3; Cdk5, cyclin-dependent kinase 5.

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protein is sometimes used as a marker of neurogenesis. A phosphorylated form of CRMP2 is found in neurofibrillary tangles (NFTs) from human Alzheimer's disease (AD) brain (*14*). We established that the residues that exhibit increased phosphorylation in AD are those targeted by GSK3 (Thr509, Thr514, and Ser518), as well as the priming residue (Ser522) (*5*). Phosphorylated CRMP2 is also increased in the soluble fraction of the neuron and in animal models of AD (*15*). CRMP2 is primed for GSK3 recognition in vivo by phosphorylation by CDK5 (*6*).

One physiological function of the phosphorylation of these residues is the regulation of axon growth and neuronal polarity (*5*, *16*, *17*). It has been reported that CRMP-2 can bind to tubulin heterodimers and that this promotes microtubule assembly in vitro (*18*). Interestingly, phosphorylation reduces this interaction with the tubulin dimers. CRMP2 is also reported to bind to kinesin-1 (*19*), the specifically Rac1-associated protein (Sra-1)/WASP family verprolin-homologous protein (WAVE1) complex (*20*), and the protein Numb (*21*), although the role of phosphorylation in the interactions was not addressed.

CRMP4 (also known as Ulip (Unc-33-like phosphoprotein) (*8*)) has the same conserved phosphorylation motif as CRMP2 and is also an excellent substrate of GSK3, although the priming kinase remains unknown (*5*, *6*). It is reported to co-localize with F-actin in regular rib-like structures within the lamellipodia of B35 neuroblastoma cells, and the distribution of GFP-CRMP-4 is changed by depolymerization of actin fibers in vivo (*22*). Moreover, it has been reported that recombinant CRMP-4 forms homo-oligomers (*22*) and, like CRMP2, can interact with tubulin in vitro (*18*). Recently, CRMP4 was found to regulate neurite outgrowth through an interaction with RhoA (*23*). Again, the effect of phosphorylation on such interactions remains unclear. Finally, although CRMP isoforms are thought to exist as oligomers, the role of phosphorylation in complex formation has not been established.

In this study, we developed a procedure to identify proteins that bind to CRMP2 and CRMP4 in a phosphorylation-dependent fashion. This is designed to not only help clarify the physiological function of these CRMPs but also to establish potential pathological effects of hyperphosphorylation of CRMP2 in AD.

MATERIALS AND METHODS

Materials. CL-exposure film was purchased from Pierce. Protein G Sepharose, nitrocellulose membranes, and glutathione Sepharose were purchased from Amersham Biosciences. Bradford Reagent was purchased from Sigma.

Antibodies and Peptides. Antibodies to CRMP2 phosphorylated at Thr 514/Thr 509, CRMP4 phosphorylated at Thr 509, and total CRMP2 were raised in sheep and purified by the Division of Signal Transduction Therapy, University of Dundee as described previously (*6*).

Anti-CRMP4 (Chemicon Int.); anti-FLAG, anti-FLAG agarose, sheep anti-mouse IgG-HRP, anti-tubulin-α, and anti-tubulin-β (Sigma); monoclonal anti-HA and anti-Pin1 (Cell Signaling Technology); anti-DYRK (BD Biosciences Pharmingen); anti-ANT (Santa Cruz Biotechnology Inc.); and rabbit anti-sheep IgG and goat anti-rabbit IgG, conjugated to horseradish peroxidase (HRP) (Pierce) were purchased from the sources in parentheses. Phospho-514/509-CRMP2 (^{504}CEVSVpTPKTVpTPAS^{517}), phospho-509-CRMP4 (^{504}FDLTTpTPKGGT^{517}), dephospho-CRMP2 (^{504}CEVSVTPKTVTPAS^{517}), and dephospho-CRMP4 (^{504}FDLTTTPKGGT^{517}) peptides (where pT is phospho-threonine) were generated by the Division of Signal Transduction Therapy, University of Dundee (*6*).

Recombinant Proteins. GST/FLAG-CRMPs and His-GSK3β were prepared as described previously (*5*). Recombinant Cdk-5/p35 complex was purchased from Upstate Biotechnologies, Inc. (Cat. No. 14−-477).

Buffers. Cell lysis buffer contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 1 mM EDTA, 1% Triton-X100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, complete protease inhibitor tablets (Roche Diagnostics), and 0.1% β-mercaptoethanol. Kinase assay buffer contained 50 mM Tris-HCl (pH 7.5), 0.03% Brij-35, and 0.1% β-mercaptoethanol. Homogenization buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, complete protease inhibitor tablets, and 0.1% β-mercaptoethanol.

Protein Phosphorylation Reactions. We purified stoichiometrically phosphorylated (phosphorylated at all four residues, i.e., 4 mol of phosphate/1 mol of of CRMP) CRMP species as follows. Recombinant GST-tagged CRMP proteins (125 μg/1.39 nmol) were phosphorylated using a Cdk5/p35 complex (750 mU) in kinase buffer containing 10 mM $MgCl_2$ and 0.1 mM ATP at 30 °C for 1 h in a total of 135 μL. GSK3β (750 mU) was then added, and the reaction was incubated for an additional 1 h (final volume of 150 μL, final CRMP concentration of 9.3 μM). Small scale reactions were similar except that 1.25 μg/13.9 pmol of CRMP and 75 mU of CDK5 and GSK3 in 30 μL of reaction (0.46 μM CRMP) solution were used. Finally, the reactions were terminated by the addition of EDTA (final concentration of 50 mM). These reaction conditions were selected following detailed radiolabeling experiments to maximize the incorporation of phosphate into the residues of interest, with minimum utilization of protein kinases.

Affinity Purification of Phosphorylated CRMP. Phospho-CRMP4 or phospho-CRMP2 reactions stated previously were incubated with anti-phospho-CRMP-4 (pThr 509) or anti-phospho-CRMP-2 (pThr 509/514) antibody, respectively, conjugated to Protein G Sepharose, in the presence of the appropriate dephospho-peptide (2 μM) at 4 °C for 2 h. The phosphorylated proteins were then pelleted at 4 °C, 10 000*g* for 5 min, and washed with assay buffer, and the immunoprecipitates were incubated with the appropriate phosphopeptide as indicated in the figure legends. The Protein G Sepharose antibody beads were then pelleted, and the supernatants containing the released phospho-CRMP were transferred into new tubes. The purity, concentration, and yield of the final phospho-CRMP were estimated by immunoblot by comparison with known amounts of dephospho-CRMP.

Homogenization of Rat Brain. A complete rat brain hemisphere was diced with scissors and homogenized in 4 mL of ice-cold homogenization buffer using a glass Dounce homogenizer. The solution was centrifuged at 10 000*g* at 4 °C for 20 min. The protein concentration of the supernatant

was measured by the method of Bradford (*24*), and the cell lysate was snap frozen on liquid N_2 and stored at −80 °C.

Isolation of CRMP Binding Proteins. Large Scale. An equivalent amount of the purified fully phosphorylated GST-CRMP (pCRMP2 or pCRMP4) or dephospho-GST-CRMP (approximately 30 μg, 0.33 nmol) was precipitated following incubation with glutathione Sepharose at 4 °C for 1 h. These pellets (plus a control pellet containing GST bound to glutathione Sepharose) were washed with homogenization buffer twice and then incubated with 50 mg of rat brain lysate (5 mg/mL protein) at 4 °C for 4 h, with gentle mixing. The Sepharose beads were pelleted once more and washed 3 times with 1 mL of homogenization buffer and then once with homogenization buffer without Triton-X100 or NaCl. Proteins retained in the immunocomplexes were released by incubation with SDS sample buffer (Invitrogen) plus 10 mM DTT at 70 °C for 15 min. The supernatants were treated with 50 mM iodoacetamide at room temperature for 30 min in the dark, prior to analysis by SDS-PAGE. The gels were stained with Coomassie G-250 (Simply Blue Safe Stain, Invitrogen) for 1 h.

Pilot Scale. The isolation of binding proteins for analysis by Western blot was performed on a smaller scale. The same protocol as stated previously was used but starting with 3 μg of CRMP (33 pmol) and incubating with 5 mg of rat brain lysate in 1 mL final volume (i.e., all concentrations were kept the same as stated previously).

Gel Piece Isolation and Peptide Release for Mass Fingerprinting. Protein bands for analysis were cut from the gel using a clean scalpel. The gel pieces were washed sequentially in water, 50% acetonitrile (v/v), 0.1 M NH_4HCO_3, then 0.05 M NH_4HCO_3/50% acetonitrile (v/v), and finally 100% acetonitrile for 15 min. The washed gel pieces were dried under vacuum and re-swelled in 30 μL of 25 mM triethylammonium bicarbonate containing 5 μg/mL trypsin before incubation overnight at 30 °C with shaking. Next, an equivalent volume of acetonitrile was added, and the mixture was shaken for 15 min and centrifuged. The supernatants containing released peptides were collected and dried. The remaining gel pieces were incubated with 60 μL of 50% acetonitrile/2.5% formic acid for 15 min. The resultant supernatants were combined with the first extract and also dried. The dried peptides were resuspended in 0.1% formic acid and separated by HPLC on a pepmap C_{18} reversed phase column coupled to a 4000QTRAP mass spectrometer for LC/MS/MS analysis.

Mass Fingerprinting. The mass fingerprinting analysis was performed at the University of Dundee Proteomics Facility (http://www.dundee.ac.uk/biocentre/services_proteomics.htm). The peptide results were searched against the Uniprot database (all species) with a peptide and fragment mass tolerance of ±0.8 Da. Only positive results returning three or more peptides from a single polypeptide and a protein score of 100 or above were considered to be significant.

Cell Culture. Human embryonic kidney 293 (HEK293) and SH-SY5Y cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v/v) fetal calf serum, penicillin, and streptomycin in 5% CO_2 atmosphere at 37 °C.

Transient Transfection. HEK293 cells were transfected by calcium phosphate precipitation with 1.5 μg of pRK5-FLAG-CRMP4 ± 3 μg of pCMV5-HA-CRMP2. Cells were washed and incubated at 37 °C for 48 h prior to the addition of lysis buffer. Following lysis, insoluble material was removed by centrifugation, and protein concentrations were determined using the Bradford assay (*24*).

Immunoprecipitation of Transfected Proteins for SDS-PAGE. FLAG-tagged or HA-tagged proteins were immunoprecipitated following incubation of the cell lysate (1 mg/mL, 200 μL) with anti-FLAG agarose or anti-HA antibody (conjugated to Protein G Sepharose) for 16 h at 4 °C. Immunoprecipitates were washed twice with cell lysis buffer containing 150 mM NaCl, then once with lysis buffer prior to heating at 70 °C for 15 min in 150 μL of 1xSDS sample buffer. Released proteins were chromatographed on 4−12% Bis-Tris NuPage Gels (Invitrogen), prior to protein staining or transfer to nitrocellulose for immunoblotting.

Immunoblotting. After transfer of proteins to nitrocellulose, the membranes were blocked for 1 h at room temperature or overnight at 4 °C with 5% (w/v) milk in 0.1% (v/v) Tween20 in Tris buffered saline (TTBS) prior to a 16 h incubation at 4 °C with primary antibodies. Washed membranes were incubated for 1 h with the appropriate peroxidase-linked secondary antibodies (1: 2500), and specific proteins were detected using enhanced chemiluminescence reagents (ECL; Amersham Biosciences).

Subcellular Fractionation. Mitochondria and cytosol were isolated using the mitochondria isolation kit (Pierce, Cat. No. 89874) as per manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation of Stoichiometrically Phosphorylated CRMPs. A three step procedure was developed to isolate CRMPs phosphorylated at all four residues (522/518/514/509) from a CRMP preparation containing a mixture of phospho-forms of CRMP (Figure 1A). Bacterially expressed GST-CRMP2 and GST-CRMP4 fusion proteins were not phosphorylated and thus served both as the non-phosphorylated controls and as the starting points for the isolation of the stoichiometrically phosphorylated CRMPs. Each isoform was incubated with CDK5, GSK3, and MgATP under conditions previously established to result in a high efficiency of phosphorylation of Ser522, Ser518, Thr514, and Thr509 (Materials and Methods and ref *5*). Since GSK3 phosphorylates CRMP sequentially (518, then 514, then 509), we monitored full phosphorylation by Western blot analysis using antibodies to phospho-Thr509 (or 509 plus 514), and the reaction was terminated when no further increase in phosphorylation was observed.

Next, the fully phosphorylated CRMP2 (Figure 1B) and CRMP4 (Figure 1C) molecules present in the reaction mixture were specifically immunoprecipitated with the phospho-Thr509/514-C2 and phospho-Thr509-C4 antibodies, respectively, bound to Protein G Sepharose. Incubation of CRMPs with GSK3 alone resulted in no phosphorylation and therefore no precipitation (lanes 7 and 8, Figure 1B,C). A small amount of phosphorylation of Thr509 can be obtained by incubation of CRMP with CDK5 alone in vitro (lanes 5 and 6, Figure 1B,C); however, this is minor as compared to incubation with CDK5 plus GSK3 (lanes 9 and 10, Figure 1B,C and ref *6*). Therefore, as expected, only incubation with both kinases resulted in efficient precipitation of the CRMPs.

The beads were then washed extensively as described in the Materials and Methods prior to incubation with excess

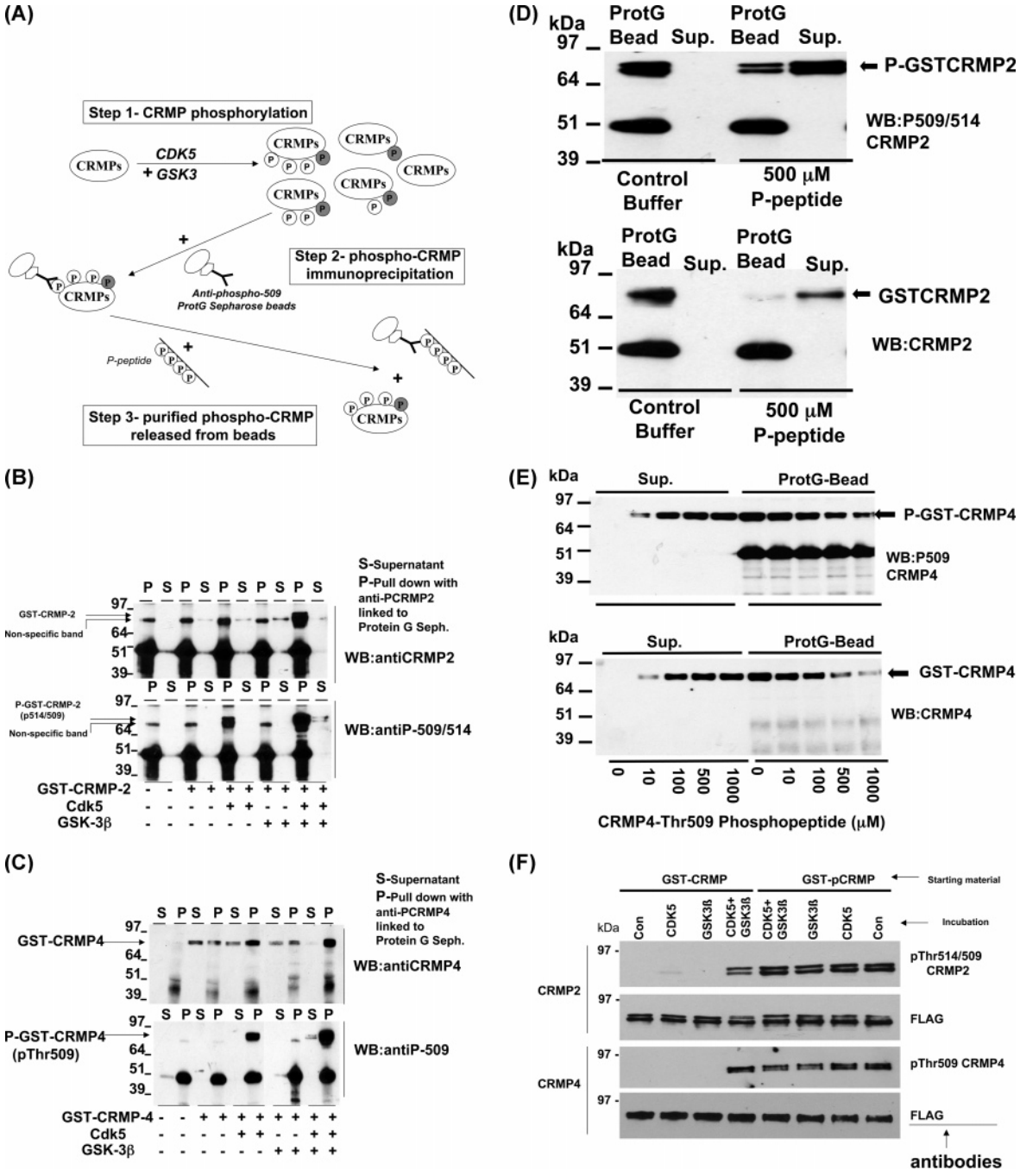

FIGURE 1: Three step purification of fully phosphorylated CRMPs. (A) A schematic representation of the purification procedure to isolate fully phosphorylated CRMP. Shaded phosphates indicate those incorporated by CDK5, and clear phosphates indicate those incorporated by GSK3. (B) GST-CRMP2 was phosphorylated sequentially at Ser522, Ser518, Thr514, and Thr509 by incubation with CDK5, GSK3β, and MgATP in vitro as described in the Materials and Methods for large scale phosphorylation. The phosphorylated CRMP2 (with phosphate at all four residues) was separated from other forms of CRMP2 (non-phosphorylated or single, double, or triple phospho-forms) by immunoprecipitation using an antibody that recognizes CRMP2 only when phosphorylated at all four residues (anti-phospho509/514 linked to Protein G Sepharose). CRMP2 and phospho-CRMP2 were visualized in the pull-downs (P) and supernatants (S). Thr509 is weakly phosphorylated by CDK5 in vitro; for phosphorylation at all four residues, CDK5 plus GSK3 is required. (C) GST-CRMP4 was phosphorylated as for GST-CRMP2 as stated previously, and fully phosphorylated CRMP4 was separated from other forms of CRMP4 by immunoprecipitation using an antibody that recognizes CRMP4 only when it is phosphorylated at all four residues (anti-phospho509) linked to Protein G Sepharose. CRMP4 and phospho-CRMP4 were visualized in the pull-downs (P) and supernatants (S). As with CRMP2, CDK5 weakly phosphorylates Thr509 in vitro, but for phosphorylation of all four residues, both CDK5 and GSK3 are required. (D) Phosphorylated CRMP2 was released from the anti-Thr509/Thr514 Protein G Sepharose (Bead) by competition with CRMP2 phospho-peptide for 3 h at room temperature. Released CRMP2 was visualized in the supernatant (Sup.) with both anti-CRMP2 and anti-phospho-CRMP2. (E) Phosphorylated CRMP4 was released from the anti-Thr509 Protein G Sepharose (Bead) by competition with the CRMP4 phospho-peptide at the concentrations indicated for 1 h at 4 °C. Released CRMP4 was visualized in the supernatant (Sup.) with both anti-CRMP4 and anti-phospho-CRMP4. (F) GST-FLAG-CRMP (0.5 μM), either bacterially expressed (GST-CRMP, no phosphorylation) or phospho-CRMP (GST-pCRMP) that had been phosphorylated and purified as in panels A−E, was incubated for 1 h at 30 °C in the presence of p35-Cdk5 (5 mU/μL) and His-GSK3β (5 mU/μL) plus MgATP as indicated. Reactions were terminated by boiling in SDS containing buffer, prior to separation by SDS-PAGE. Total CRMP (FLAG), pCRMP2 (Thr509/514), or pCRMP4 (Thr509) were visualized by Western blot analysis. The data signify that purified phospho-CRMPs are fully phosphorylated at all four residues.

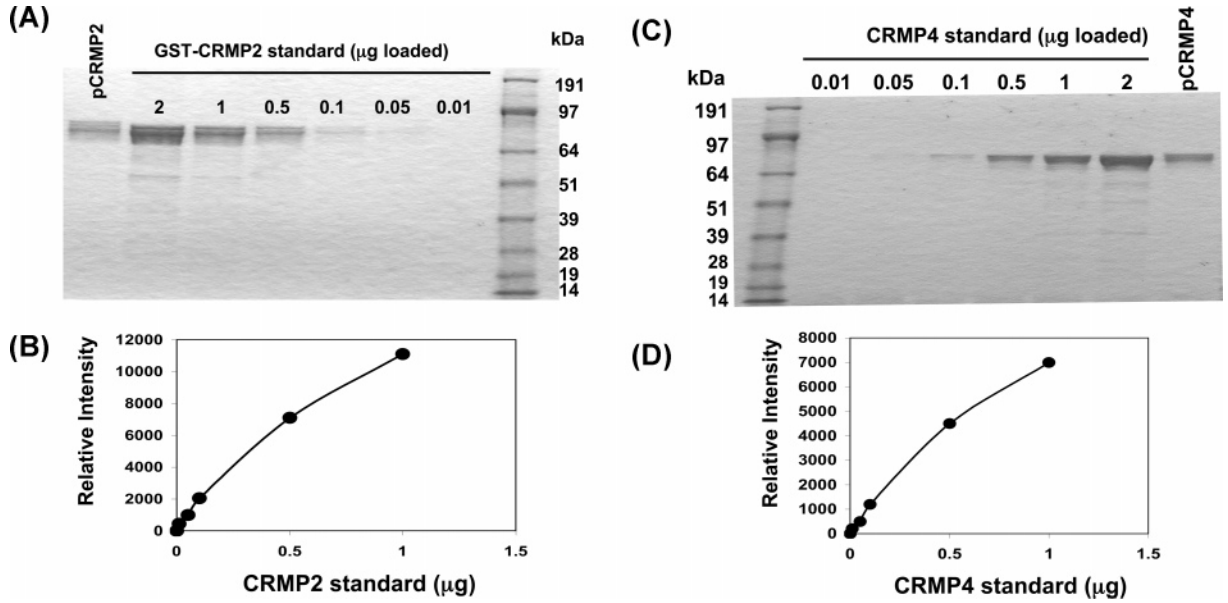

FIGURE 2: Quantitation of recovery and concentration of the phospho-CRMPs. Western blot analyses of known amounts of GST-CRMP2 (A and B) or GST-CRMP4 (C and D) were used to calculate the concentration and recovery of the purified phospho-CRMPs (P-CRMP) from Figure 1. A representative blot (A and C) together with a standard curve calculated from the blot (B and D) are shown.

phospho-peptide (specific to each isoform) to elute the phospho-CRMP from the beads (Figure 1D,E). The optimal conditions for release of each isoform were established by performing a series of incubations at different temperatures, peptide concentrations, and time. Optimal release conditions were slightly different for each isoform, 3 h at room temperature (CRMP2) and 1 h at 4 °C (CRMP4), although 500 μM peptide was sufficient to release >85% of both CRMPs (Figure 1D,E). Finally, the released phosphoproteins were separated from the phospho-peptides by purification on glutathione Sepharose beads (see Materials and Methods).

Importantly, subsequent incubation of the isolated phosphorylated CRMP2 or CRMP4 with CDK5, GSK3, and Mg ATP did not result in any further phosphorylation (Figure 1F), demonstrating stoichiometric phosphorylation of the 522−509 region of the purified protein (4 mol of phosphate to 1 mol of CRMP). Therefore, the anti-phospho-CRMP antibodies specifically immunoprecipitated the fully phosphorylated forms of CRMP with a high efficiency. To our knowledge, this is the first example of purification of a fully phosphorylated substrate of GSK3 using this approach.

Identification of CRMP Binding Proteins by Mass Fingerprinting. The recovery and concentration of the purified phospho-CRMPs was estimated following SDS-PAGE analysis, by comparison with a standard curve comprised of the dephospho-CRMPs (Figure 2). Approximately 30% of GST-CRMP in the initial phosphorylation reaction could be purified as the fully phosphorylated protein using this method. Equal amounts of unphosphorylated or phospho-CRMP fusion protein linked to glutathione Sepharose, along with GST control beads alone, were then incubated for 4 h at 4 °C with 50 mg of rat brain lysate. Beads were isolated by centrifugation, and the precipitates were washed extensively (Materials and Methods). Binding proteins and GST-CRMPs were released by heating in 1X SDS sample buffer, chromatographed on a 4−12% SDS-PAGE gradient gel, and visualized by Coomassie staining (Figure 3A,B). Protein bands present in the phospho- or dephospho-CRMP lanes, but not in the GST alone lanes, were excised, digested by trypsin, and identified by mass fingerprinting. In each case, the equivalent gel piece from the GST alone lane was analyzed as a control.

Bands of apparent molecular mass of 90, 62, 53, 49, 43, 42, 40, 38, 35, and 31 kDa were analyzed for CRMP2 interacting proteins (Figure 3A, Supporting Information tables, and Table 1) and 66, 64, 62, 53, 51, 50, 48, 42, 40, 38, 35, 31, 18, and 16 kDa for CRMP4 interacting proteins (Figure 3B, Supporting Information tables, and Table 1). Fragments of the bacterially expressed GST-CRMP proteins were present in many of these fractions, presumably due to co-purification with the full length protein. However, this approach also identified tubulin-β (53 kDa), CRMP4, CRMP1 (both 62 kDa), and ANT-1 (35−38 kDa) as CRMP2 interacting proteins (see Supporting Information tables for peptide identification data). Meanwhile, tubulin-β (53 kDa), CRMP2, CRMP1 (both 62 kDa), ANT-1 (35−38 kDa), and ANT-2 (35−38 kDa) were identified as CRMP4 interacting proteins (see Supporting Information tables for peptide mass data). Interestingly, tubulin-β was present in both the phospho- and the dephospho-CRMP lanes but not the GST control lane. However, a different pattern of tubulin-β chains were identified in each case (Table 1 and Supporting Information tables). For example, only tubulin-β15 interacted with phosphorylated CRMP4, while β15, β5, and β3 polypeptides were pulled down with dephospho-CRMP4. The data suggest that phosphorylation of CRMP4 reduces the binding of specific tubulin-β chains to CRMP4. Only the tubulin-β5 polypeptide was identified by mass fingerprinting as a CRMP2 interacting protein; hence, the data indicate that although both CRMP isoforms can interact with tubulin-β, each exhibits a distinct preference for specific tubulin polypeptides.

Of particular note was the identification of other CRMP isoforms interacting with the dephosphorylated CRMPs but not the phosphorylated forms (Table 1). CRMPs have been reported to exist as higher order structures including tetramers (*11*, *22*). The data suggest that it is likely that CRMP1,

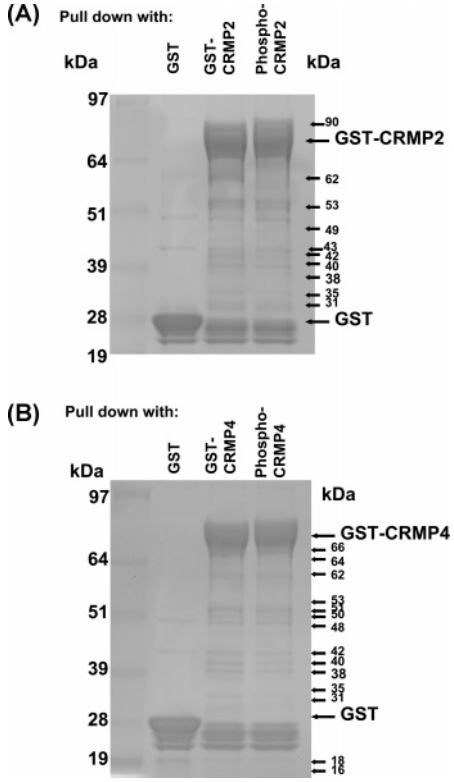

FIGURE 3: Isolation of proteins that interact with CRMP and/or phospho-CRMP. (A) GST, GST-CRMP2, or phospho-GST-CRMP2 Sepharose beads (30 μg, 0.33 nmol) were individually incubated with 50 mg of rat brain lysate at 4 °C for 4 h (total volume: 10 mL). Beads were pelleted and washed 3 times with homogenization buffer prior to protein release by heating in SDS containing buffer and separation by SDS-PAGE. Proteins were visualized by Coomassie staining. (B) GST, GST-CRMP4, or phospho-GST-CRMP4 Sepharose beads (30 μg, 0.33 nmol) were individually incubated with 50 mg of rat brain lysate at 4 °C for 4 h (total volume: 10 mL). Beads were pelleted and washed 3 times with homogenization buffer prior to protein release by heating in SDS containing buffer and separation by SDS-PAGE. Proteins were visualized by Coomassie staining.

CRMP2, and CRMP4 all have the ability to form complexes with each other and that phosphorylation reduces the hetero-oligomerization.

To investigate the interaction of distinct CRMP isoforms further, we incubated GST-CRMP2 (phospho and dephospho versions) with bacterially expressed CRMP4 (lacking GST). CRMP4 could be isolated by coprecipitation with GST-CRMP2 (on glutathione beads) but not with phosphorylated GST-CRMP2 (Figure 4A). This is a relatively weak interaction (lane 3, Figure 4A), perhaps because the recombinant protein has already formed homotetramers; however, it is significantly weaker following phosphorylation (lane 1, Figure 4A).

Co-transfection of HEK293 cells with HA-tagged CRMP2 ± FLAG-tagged CRMP4 also produced heterocomplexes (Figure 4B). HA-CRMP2 immunoprecipitated with FLAG-CRMP4 on anti-FLAG agarose and vice versa. Almost all of the CRMP present could be depleted from a lysate through co-immunoprecipitation with the other CRMP isoform, suggesting that very little monomer or homo-oligomers exist, at least when isoforms are co-transfected. Of course, this ability to form heteromers means that any proteins found to interact with both isoforms of CRMP may only interact with one isoform directly but be part of a complex including more than one isoform of CRMP.

Table 1: Summary of Interacting Proteins[a]

dephospho-CRMP2 specific binding proteins	phospho-CRMP2 specific binding proteins	CRMP2 binding proteins (with and without phosphorylation)
CRMP4 (MF, Co-IP) CRMP1 (MF) ANT-1 (MF, WB)	none identified	tubulin-β (MF, WB) β5 chain only
dephospho-CRMP4 specific binding proteins	**phospho-CRMP4 specific binding proteins**	**CRMP4 binding proteins (with and without phosphorylation)**
CRMP2 (MF, Co-IP) CRMP1 (MF) ANT-1, -2 (MF, WB) DYRK (WB)	Pin1 (WB)	tubulin-β (MF, WB) β15—phospho β15, β3, and β5—dephospho

[a] Binding proteins identified are grouped by the CRMP isoforms with which they interact, while the method used to indicate an interaction is provided in parenthesis. MF: mass fingerprinting; WB; Western blot analysis; and Co-IP: co-immunoprecipitation.

A novel CRMP interacting protein, ADP/ATP translocase (also called adenine nucleotide translocator; ANT, Q05962), was identified in this study. The isoforms ANT-1 and ANT-2 bound to CRMP4, but only ANT-1 was identified in the CRMP2 purification. These mitochondrial proteins were not detected in the phospho-CRMP pull-downs.

Validation of Potential CRMP Interacting Proteins. The mass fingerprinting data established that we could identify novel as well as previously proposed CRMP interacting proteins. We next attempted to establish the validity of these identifications by alternative analysis.

The purified CRMPs and phospho-CRMPs were incubated with rat brain lysate, and interacting proteins were isolated as before. However, after separation by SDS-PAGE, the proteins were transferred to nitrocellulose and analyzed with antibodies specific for each potential interacting protein (Figure 5). Tubulin-β was present in relatively equal amounts in the P-CRMP2 and dephospho-CRMP2 pull-downs (Figure 5A) but not in the GST control beads. Interestingly, comparatively less tubulin-β appeared to be purified with P-CRMP4 than with dephospho-CRMP4 (Figure 5B). This is consistent with the mass fingerprinting data that identified a greater number of tubulin-β chains associated with the dephospho-CRMP4 (Table 1). Therefore, both analyses suggested that phosphorylation regulates the CRMP4-tubulin interaction. Previous work suggested that phosphorylation of CRMP2 reduces its interaction with tubulin (*18*). We did not detect differences in the absolute binding of tubulin to this isoform after phosphorylation; however, Western blot analysis, like mass fingerprinting, is not fully quantitative. Therefore, we cannot rule out that phosphorylation of CRMP2 regulates the affinity of binding to tubulin-β.

ANT1 and -2 were detected in the CRMP pull-downs by mass fingerprinting (Supporting Information tables). These are mitochondrial carrier proteins found in the inner mitochondrial membrane (*25*, *26*); however, CRMPs have never been reported to be present in this subcellular compartment. Therefore, we probed the GST-CRMP2 (Figure 5C) and -CRMP4 (Figure 5D) pull-downs from rat brain lysates with an antibody to ANT and found that a protein of the correct molecular weight was present in both the CRMP2 and the

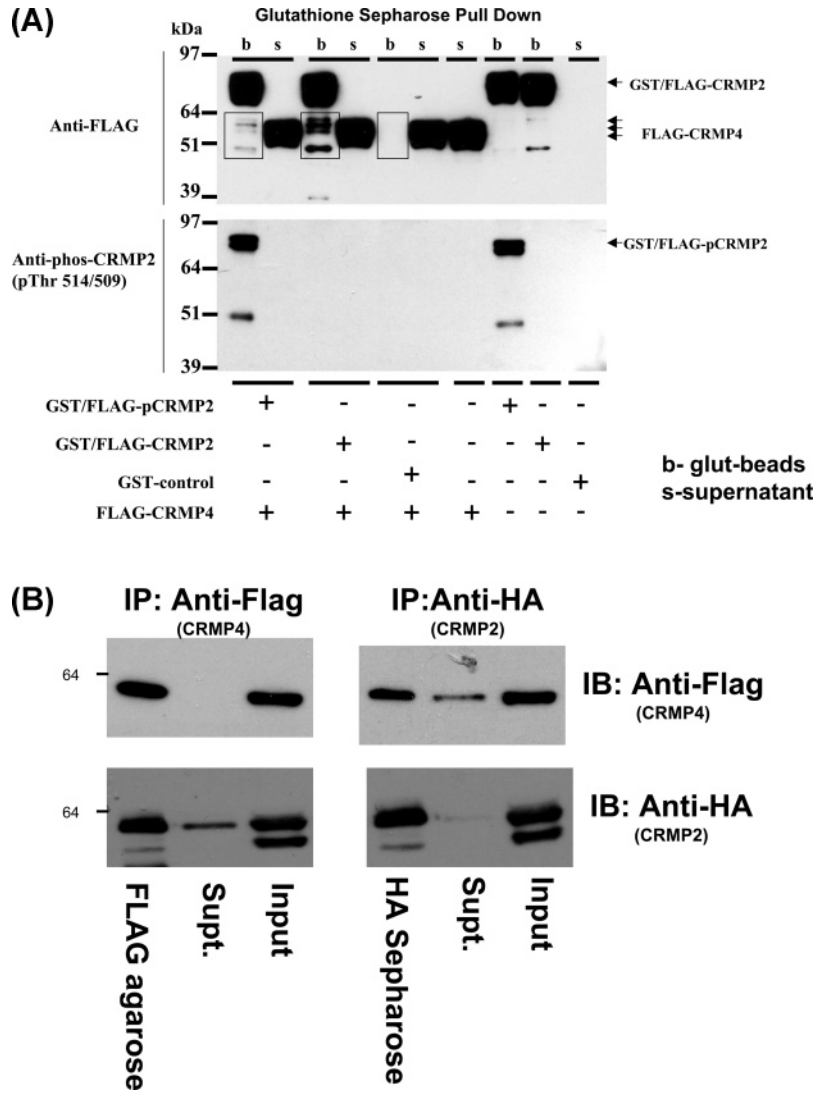

FIGURE 4: CRMP2 and CRMP4 interact in vitro and in cells. (A) GST, GST-FLAG-CRMP2, or purified phospho-GST-FLAG-CRMP2 (prepared as described in the Materials and Methods for small scale phosphorylation) were incubated in vitro with or without FLAG-tagged CRMP4 (lacking GST). The CRMP2 was then isolated using glutathione Sepharose. The beads (b) and supernatant (s) were analyzed by Western blot analysis using anti-FLAG (upper panel) and anti-phospho-509/514-CRMP2 (lower panel). The boxes indicate where CRMP4 would be found if it interacted with GST-CRMP2 or GST. (B) HEK293 cells were co-transfected with pRK5-FLAG-CRMP4 ± pCMV5-HA-CRMP2, and 48 h later, protein lysates were prepared. A total of 200 μg of lysate was incubated overnight with either anti-FLAG-agarose or anti-HA-Sepharose, and each tagged protein was isolated by centrifugation. Cell lysate (Input), unbound protein (Supt.: supernatant), and protein bound to each bead were chromatographed by SDS-PAGE and analyzed by Western blot analysis using anti-HA or anti-FLAG as indicated. Representative blots of three separate experiments are presented. HA-CRMP2 is not precipitated by anti-FLAG unless FLAG-CRMP4 is co-transfected, while FLAG-CRMP4 is not precipitated by anti-HA unless HA-CRMP2 is co-transfected (data not shown).

CRMP4 complexes, although it was much more apparent with CRMP4. The mitochondrial fraction of neuronal cells was also probed for the presence of CRMP2 and CRMP4 (Figure 5E). These proteins were primarily cytoplasmic; however, both could be detected in the mitochondrial fraction.

The function of ANT is to exchange ADP and ATP between the extra- and inner-mitochondrial spaces, thereby linking ATP production and ATP utilization. This process is closely regulated. For example, the ANT activity is enhanced by Bcl2 and inhibited by Bax (*27*), suggesting that ANT plays a role in the balance between apoptosis and proliferation. This position as key regulators of mitochondrial function places them as potential mediators of neurodegeneration and provides a mechanistic link to the correlation between CRMP hyperphosphorylation and AD (*15*). This clearly requires more study.

Purification of Fully Phosphorylated CRMPs Allows More Specific Analysis of Phosphorylation-Dependent Interacting Proteins. The mass fingerprinting approach is a non-biased method to identify proteins that interact with the CRMPs. However, this method is unlikely to detect low abundance interacting proteins, many of which may contribute to CRMP function. Therefore, we also probed the CRMP pull-downs with antibodies to proteins present in cells at relatively low abundance but with a potential regulatory role in CRMP function.

For example, our previous work had identified CDK5 as the priming kinase (Ser522 kinase) for CRMP2, but we were not able to detect CDK5/p35 (or GSK3) in the CRMP2 or

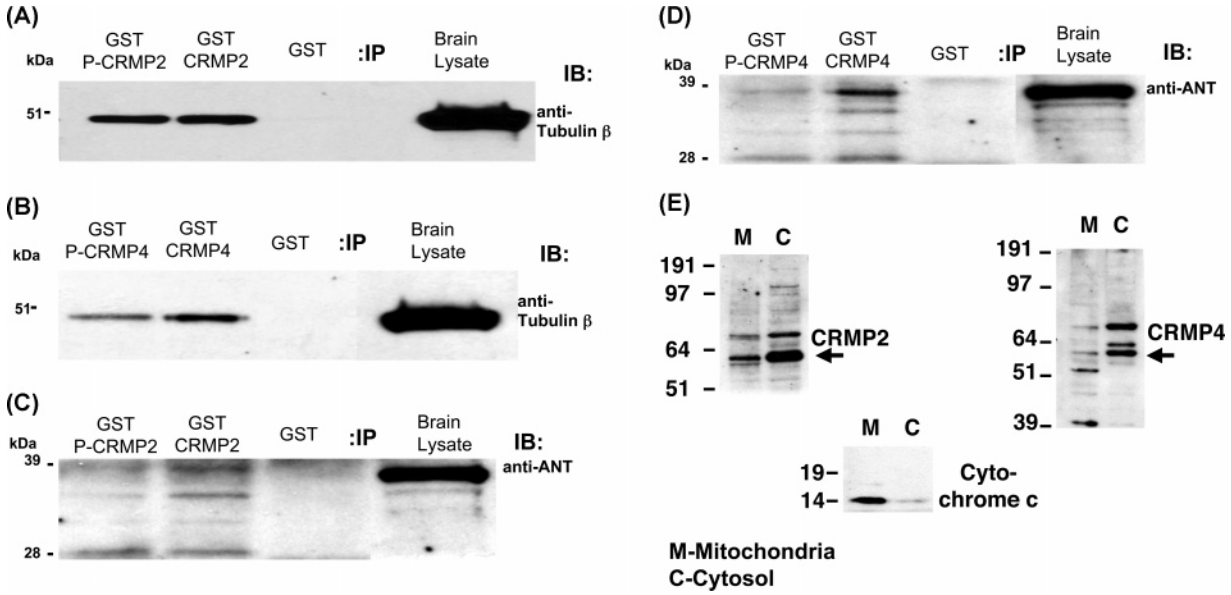

FIGURE 5: Validation of identified CRMP interactors. (A) GST-CRMP2 and phospho-GST-CRMP2 (phosphorylated and purified as in Materials and Methods for small scale) attached to glutathione Sepharose were incubated with rat brain lysates, prior to purification of the CRMP2 binding proteins by centrifugation and separation by SDS-PAGE. Proteins were transferred to nitrocellulose, and the presence of tubulin-β was assessed using a specific antibody. A representative blot is shown. Neat rat brain lysate was examined simultaneously. (B) GST-CRMP4 and phospho-GST-CRMP4 were incubated with rat brain lysate as stated previously. Proteins were transferred to nitrocellulose, and the presence of tubulin-β was assessed. A representative blot is shown. Neat rat brain lysate was examined simultaneously. (C) GST-CRMP2 and phospho-GST-CRMP2 were incubated with rat brain lysates as stated previously. Proteins were transferred to nitrocellulose, and the presence of ANT was assessed using a specific antibody. A representative blot is shown. Rat brain lysate was examined simultaneously. (D) GST-CRMP4 and phospho-GST-CRMP4 were incubated with rat brain lysates as stated previously. Proteins were transferred to nitrocellulose, and the presence of ANT was assessed using a specific antibody. A representative blot is shown. Neat rat brain lysate was examined simultaneously. (E) Human neuroblastoma SH-SY5Y cells were fractionated to separate mitochondria from cytoplasm as described in the Materials and Methods. Each fraction was then probed by Western blot analysis for CRMP2, CRMP4, and cytochrome *c* (a mitochondrial marker).

CRMP4 pull-downs (data not shown). Interestingly, we previously found that although CDK5 could prime CRMP4 at Ser522 in vitro, it was not required for Ser522 phosphorylation of CRMP4 in vivo (*6*). The protein kinase DYRK also phosphorylates CRMP4 (but not CRMP2) at Ser-522 in vitro (*5*). Therefore, we examined the pull-downs of GST-CRMP4 for the presence of DYRK (Figure 6A). Interestingly, DYRK was found in the dephospho- but not phospho-GST-CRMP4 precipitates (and not to any significant level in either of the CRMP2 pull-downs (Figure 6A)). This strengthens the possibility that DYRK is the in vivo Ser522 kinase for CRMP4.

Meanwhile, Tau is a microtubule-associated neuronal enriched substrate of GSK3 and CDK5. Like CRMP2, it is also hyperphosphorylated in tangles of Alzheimer's brain. Interestingly, the dephosphorylation of Tau (*28*), and another Alzheimer's related protein APP (*29*), is regulated by the peptidyl prolyl isomerase Pin1. In addition, Pin1 modification and activity is altered in mild cognitive impairment and AD. Therefore, it has been hypothesized that Pin1 defects may contribute to the progression to dementia (reviewed in ref *30*). We examined the CRMP pull-downs for the presence of Pin1 (Figure 6B). Interestingly, Pin1 only interacted with phosphorylated CRMP4 and not dephospho-CRMP4. We could not detect it in either of the CRMP2 pull-downs (Figure 6B). This suggests that Pin1 may specifically regulate CRMP4 dephosphorylation, although it must be noted that we did not observe hyperphosphorylation of this isoform in human AD brain (*15*). The relative phosphorylation status of these proteins in Pin1 knockout neurones would help answer as to whether this interaction has a physiological function.

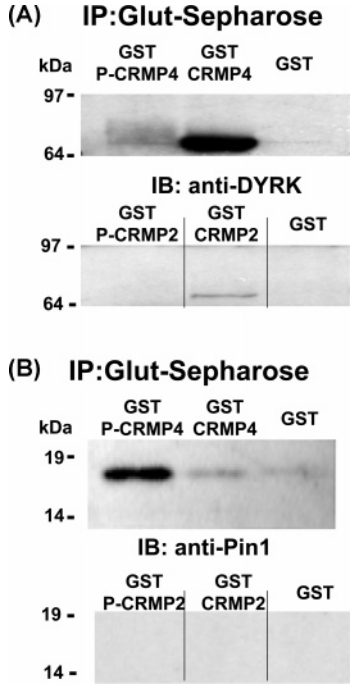

FIGURE 6: CRMP4 interacts with DYRK and Pin1 in a phosphorylation-dependent fashion. (A) GST-CRMP and phospho-GST-CRMP were incubated with rat brain lysates, prior to purification and separation by SDS-PAGE as described in Figure 5. Proteins were transferred to nitrocellulose, and the presence of DYRK (A) and Pin1 (B) was assessed using specific antibodies.

CONCLUSION

We developed a procedure to identify phosphorylation-dependent (and possibly regulated) CRMP2 and CRMP4 binding proteins. The technique could be developed for any phospho-protein provided that pure protein was available, the kinase was available, and finally a phosphorylation-dependent antibody was produced. This work has validated some previously proposed CRMP interacting proteins and identified two potential novel interactors. These require further validation, but the data suggest a regulation of CRMP4 by the kinase DYRK and a novel function of CRMPs in the mitochondria. It is of course highly unlikely that this represents a comprehensive list of CRMP interactors. Various modifications of the binding conditions could be used to search for less stringent binding partners. Alternatively, high abundance interactors (e.g., tubulin) could be pre-cleared from the interacting pool, or more specialized protein mixtures (e.g., mitochondrial fraction, different brain areas, and developmental stages) could be investigated. Therefore, we believe that this technique is applicable to the study of many biochemical systems and molecules where phosphorylation is a key regulatory component.

SUPPORTING INFORMATION AVAILABLE

Mass fingerprinting results that led to the identification of the interacting proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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