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Fyn Promotes Phosphorylation of Collapsin Response Mediator Protein 1 at Tyrosine 504, a Novel, Isoform-Specific Regulatory Site

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ABSTRACT

In vertebrates the collapsin response mediator proteins (CRMPs) are encoded by five highly related genes. CRMPs are cytosolic phosphoproteins abundantly expressed in developing and mature mammalian brains. CRMPs are best understood as effectors of Semaphorin 3A signaling regulating growth cone collapse in migratory neurons. Phosphorylation in the carboxyl-terminal regulatory domain of CRMPs by several serine/threonine kinases has been described. These phoshorylation events appear to function, at least in part, to disrupt the interaction of CRMPs with tubulin heterodimers. In a large-scale phosphoproteomic analysis of murine brain, we recently identified a number of in vivo tyrosine phosphorylation sites on CRMP isoforms. Using biochemical approaches and quantitative mass spectrometry we demonstrate that one of these sites, CRMP1 tyrosine 504 (Y504), is a primary target of the Src family of tyrosine kinases (SFKs), specifically Fyn. Y504 is adjacent to CDK5 and GSK-3β sites that regulate the interaction of CRMPs with tubulin. Although Y504 is highly conserved among vertebrate CRMP1 orthologs, a residue corresponding to Y504 is absent in CRMP isoforms 2–5. This suggests an isoform-specific regulatory role for CRMP1 Y504 phosphorylation and may help explain the observation that CRMP1-deficient mice exhibit neuronal migration defects not compensated for by CRMPs 2–5. J. Cell. Biochem. 111: 20–28, 2010.

KEY WORDS: COLLAPSIN RESPONSE MEDIATOR PROTEIN (CRMP); FYN; PHOSPHORYLATION; TYROSINE KINASE; QUANTITATIVE MASS SPECTROMETRY; QUANTITATIVE PROTEOMICS; NEURONAL POSITIONING; GROWTH CONE; ABSOLUTE QUANTIFICATION (AQUA); STABLE ISOTOPE LABELING WITH AMINO ACIDS IN CELL CULTURE (SILAC)

During embryonic development, neurons in the central nervous system (CNS) are receptive to numerous extracellular guidance cues that allow for accurate migration and axonal pathfinding. This pathfinding is largely mediated by the growth cone, which responds to diffusible or tethered attractive and repulsive cues in the environment [Lowery and Van Vactor, 2009]. Collapsin Response Mediator Proteins (CRMPs) form a family of intracellular proteins that play important roles in transducing responses to some extracellular guidance cues. There are five genes encoding CRMP isoforms (CRMP1-5), with CRMP1-4 sharing ~75% amino acid sequence identity. CRMP5 shares ~50% sequence identity with the other isoforms [Schmidt and Strittmatter, 2007]. CRMP isoforms show a strong interaction with each other and can be found as heterotetramers when purified from brain [Wang and Strittmatter, 1997].

The CRMPs were originally named for their role in mediating the response of the inhibitory guidance cue Semaphorin 3A (Sema3A), also called Collapsin-1 [Goshima et al., 1995]. In the canonized Sema3A pathway, secreted Sema3A binds a receptor complex consisting of the transmembrane proteins Neuropilin-1 (NP-1) and plexin A1 (PlexA1). In the absence of Sema3A, NP-1 antagonizes CRMP-PlexA1 interactions. Upon Sema3A binding to the receptor

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complex, the CRMP tetramer is recruited to the cytoplasmic domain of PlexA1 where it can be phosphorylated and regulated by CDK5 and GSK-3β [Schmidt and Strittmatter, 2007]. Growth cones may achieve directional growth by responding to intersecting gradients of guidance cues, with one cue stabilizing and another cue destabilizing distinct sides of a growth cone's cytoskeleton [Bentley and O'Connor, 1994; Lin et al., 1994; Buck and Zheng, 2002]. Sema3A is best characterized as a signal that triggers redistribution of Fibrillar (F) actin resulting in collapse of a responsive growth cone's lamellipodium [Fan et al., 1993; Fournier et al., 2000; Schmidt and Strittmatter, 2007]. Although the exact mechanisms have not been elucidated, this Sema3A-dependent growth cone collapse is greatly accelerated by CRMP proteins [Deo et al., 2004; Schmidt and Strittmatter, 2007].

Strong evidence has been provided that phosphorylation of CRMP proteins within the carboxyl-terminal regulatory region is important in transducing Sema3A signaling. Best understood in the context of CRMP2, multiple studies have found Sema3A- and Fyn-dependent CDK5 phosphorylation of S522 of CRMP2 (and presumably its counterparts in CRMPs 1 and 4). This phosphorylation acts to "prime" the CRMP protein for sequential phosphorylation by GSK-3β at S518, T514, and T509 [Sasaki et al., 2002; Uchida et al., 2005; Yoshimura et al., 2005; Cole et al., 2006]. Rho kinase has also been shown to phosphorylate CRMP2 at T555 in response to lysophosphatidic acid (LPA) but not in response to Sema3A [Arimura et al., 2000]. Whereas significant evidence shows Sema3A-dependent reorganization of the actin cytoskeleton, the role of CRMPs in Sema3A signaling is mechanistically better understood as modulating microtubules. CRMP1-4 all can bind tubulin in the brain, and at least CRMP2 can induce microtubule assembly by binding to α- and β-tubulin heterodimers [Schmidt and Strittmatter, 2007]. Phosphorylation of T514 by GSK-3β, or of T555 by Rho Kinase, disrupts the interaction of CRMP2 with tubulin heterodimers preventing increases in axon length and branching [Schmidt and Strittmatter, 2007].

Although there has been significant research done on the role of serine/threonine phosphorylation of CRMP isoforms, comparatively less is known regarding CRMP tyrosine phosphorylation. A few studies have shown Fes/Fps, Fer and Fyn tyrosine kinases to induce phosphorylation of certain CRMP isoforms, [Mitsui et al., 2002; Yamashita et al., 2006; Shapovalova et al., 2007] although the specific phosphorylated residues were not identified. However, two recent reports have identified SFK-regulated tyrosine phosphorylation sites on CRMP2. Uchida et al. [2009] identified CRMP2 Y32 phosphorylation to be induced by active Fyn and Sema3A signaling, and Varrin-Doyer et al. [2009] suggested the chemokine CXCL12 leads to Yes-dependent Y479 phosphorylation of CRMP2. In the present study, we identify Y504 in CRMP1 as a novel, isoform-specific, Fyn-induced phosphorylation site. Y504 is highly conserved among vertebrate CRMP1 orthologs, but is not conserved in CRMPs 2–4. Given its proximity to serine/threonine phosphorylation sites known to regulate CRMP activity, Y504 phosphorylation may serve as a mechanism whereby tyrosine kinases such as Fyn can directly influence CRMP1 or CRMP1-containing complexes.

EXPERIMENTAL PROCEDURES

CELL CULTURE, STABLE ISOTOPE LABELING, PLASMIDS, TRANSFECTIONS, STIMULATIONS, AND CELL LYSIS

Primary embryonic murine forebrain cultures were generated and maintained as described previously [Arnaud et al., 2003]. E1A-transformed human embryonic kidney 293 (HEK 293) cells were cultured in DMEM (Mediatech, Manassas, VA) with 10% fetal bovine serum or 10% calf serum (Hyclone, Logan, UT) and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA). For experiments employing stable-isotope labeling by amino acids in cell culture (SILAC, [Ong et al., 2002]) cells were grown essentially as described [Ballif et al., 2005; Matsuoka et al., 2007]; briefly, HEK 293 cells were grown for at least seven days in DMEM prepared deficient in L-arginine and L-lysine (Cambridge Isotope Laboratories, Inc., Andover, MA), but supplemented with 10% dialyzed fetal bovine serum (Hyclone); 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen); and either 73 mg/L unlabeled L-lysine and 42 mg/L unlabeled L-arginine, or 73 mg/L $^{13}C_6$-L-lysine and 42 mg/L $^{13}C_6$-, $^{15}N_4$-L-arginine (Cambridge Isotope Laboratories, Inc.). All transfections were performed using calcium phosphate precipitation. Expression constructs CRMP1a, CRMP2a, CRMP3a, and CRMP4a in pcDNA 3.1 V5-His [Alabed et al., 2007] were gifts of Dr. Alyson Fournier (McGill University). The V5-CRMP1 Y504F mutant was generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenesis was confirmed by DNA sequencing at the Vermont Cancer Center. The pRK5-Fyn construct was acquired through Addgene (Addgene plasmid 16032), originally constructed in the laboratory of Filippo Giancotti [Mariotti et al., 2001]. PP2 pretreatment was performed at 1, 5, or 10 μM for 20 min prior to the addition of H_2O_2. H_2O_2 stimulation was performed at a concentration of 8.1 mM for 15 min (20 min for primary embryonic forebrain cultures) before cells were washed with ice-cold phosphate-buffered saline (Hyclone) and lysed on ice with lysis buffer (25 mM Tris, pH 7.2, 137 mM NaCl, 10% glycerol, 1% IGEPAL (Nonidet P-40), 25 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 1 mM dithiothreitol) containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, and 10 μg/ml leupeptin. Cell extracts were centrifuged for 15 min at 4°C and at 14,000*g* and the supernatant was isolated for Western blotting and/or immunoprecipitation.

IMMUNOPRECIPITATIONS, SDS-PAGE, IMMUNOBLOTS, AND ANTIBODIES

Immunoprecipitations were performed with α-V5 rabbit polyclonal antibodies (Chemicon/Millipore, Billerica, MA) and protein-A sepharose (Zymed, South San Francisco, CA), or α-CRMP1 goat polyclonal antibodies (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) and protein-G sepharose (Zymed). For every 800 μg of whole cell extract, 2 μg of antibody, and 20 μl of a 50% slurry of protein A or G beads were used and immunoprecipitations were conducted at 4°C overnight. Immune complexes were washed five times with lysis buffer (that had PMSF as the only protease inhibitor) prior to being heated to 100°C for 5 min in denaturing sample buffer and subjected to SDS-PAGE on 7.5%, 19:1 polyacrylamide/bis-acrylamide gels. The 19:1 gels were used to increase the separation between the

CRMP proteins and the immunoglobulin heavy chain. We note that these gels also increased the apparent molecular weight of proteins compared to our pre-stained markers. Immunoblots were performed using 0.2 μm nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Following transfer, membranes were blocked with 5% milk in tris-buffered saline (TBST). The primary antibodies used were: rabbit polyclonal α-V5 (Chemicon/Millipore), α-CRMP1 goat polyclonal (C-18, Santa Cruz Biotechnology), α-phosphotyrosine (4G10, Upstate Biotechnology/Millipore, Billerica, MA), α-Fyn rabbit polyclonal (FYN3/SC-16, Santa Cruz Biotechnology). Primary antibodies were diluted in 1.5% bovine serum albumin in TBST and were incubated with the membrane overnight at 4°C. Appropriate HRP-conjugated secondary antibodies diluted in TBST were used prior to enhanced chemoluminescence (Pierce/Thermo Fisher Scientific, Rockford, IL) and exposure to Hyblot CL film (Denville Scientific, Metuchen, NJ). Approximation of intensity for bands A(−) and A(+) in Figure 1C was done using Adobe Photoshop CS2 using an inverted histogram of the lassoed bands. The A(−) band had a mean intensity of 233 within 10471 pixels, and the A(+) band had a mean intensity of 221 within 18141 pixels.

IN-GEL DIGESTION AND MASS SPECTROMETRY

Gels used in mass spectrometry analyses were stained directly with coomassie blue and gel bands of V5-CRMP1 were subjected to in-gel tryptic digestion as described previously [Ballif et al., 2006]. For SILAC experiments, mass measurements were made in an LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) which was setup with a liquid chromatography interface essentially as described [Ballif et al., 2008]. An initial analysis was conducted with a precursor MS1 full scan followed by 10 MS/MS scans on the most abundant ions identified in the precursor scan using a dynamic exclusion of 30 s and a repeat count of 3. Sequest analysis of tandem mass spectra was conducted using the rat CRMP1 amino acid sequence, requiring no enzyme specificity, allowing a 2 Da precursor mass tolerance, and allowing differential mass additions of 80 Da for serine, threonine, and tyrosine; 16 Da for methonine; 6 Da for lysine; and 10 Da for arginine. A static modification of 71 Da (acrylamide adduct) on cysteine was required. The doubly charged peptide ions harboring tyrosine Y504 in its phosphorylated form (GMYDGPV(pY)EVPATPK) and its unphosphorylated form (GMYDGPVYEVPATPK) as well as other selected peptides (GVNSFQVYMAYK, MDENQFVAVTSTNAAK, and IFNLYPR) were chosen for targeted, quantitative analysis. To accomplish this zoom scans were conducted throughout the run, encompassing 15–20 m/z units and the isotopic envelopes for both the unlabeled and labeled peptide ions. In the same run, targeted MS/MS scans were performed after each zoom scan on the m/z values of the doubly charged peptide ion precursors to verify the identity of the precursor ions measured in the zoom scans. Peptide ions were observed with methionine residues both oxidized and non-oxidized, but quantitative analyses were performed on the non-oxidized forms. Absolute quantification (AQUA, [Gerber et al., 2007]) analysis was conducted in a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) with a liquid chromatography interface as described for the LTQ-XL. Extracted peptides from CRMP1 in-gel digests were dried and resuspended in 8.5 μl of 2.5% formic acid, 2.5% acetonitrile containing 50 fmol/μl of both GMYDGPV(pY)E($^{13}C_5$-, $^{15}N_1$-V)PATPK and GMYDGPVYE($^{13}C_5$-, $^{15}N_1$-V)PATPK synthetic peptides. Synthetic AQUA peptides with $^{13}C_5$-, $^{15}N_1$-V-labels were generated at Cell Signaling Technology (Beverly, MA). Duplicate samples of 4 μl were subjected to LC-MS/MS analysis. MS1 precursor scans were performed in the Orbitrap at 30,000 resolution, and the MS/MS scans were performed in the LTQ. The relative abundance of the phosphorylated and non-phosphorylated species was determined based on the reference of 200 fmol of the introduced synthetic peptide in each run.

RESULTS

IDENTIFICATION OF ISOFORM-SPECIFIC CRMP TYROSINE PHOSPHORYLATION

As reversible phosphorylation involves the antagonizing enzymatic activities of kinases and phosphatases, a general inhibition of cellular phosphatases is a non-specific approach to increase the phosphorylation of substrates by cellular kinases. This approach was used to determine if CRMP isoforms 1–4 might have specific sites of regulated tyrosine phosphorylation. Cells transfected with constructs encoding V5-tagged CRMP isoforms were either untreated or stimulated with H_2O_2 for 15 min just prior to cell lysis. As H_2O_2 is a general inhibitor of tyrosine phosphatases [Denu and Tanner, 1998] H_2O_2 stimulation was used to facilitate phosphotyrosine accumulation within cells. Following cell lysis, CRMP proteins were immunoprecipitated using α-V5 antibodies. Immune complexes were separated via SDS-PAGE and subjected to immunoblotting using α-phosphotyrosine antibodies. Strikingly, only CRMP1 showed a dramatic increase in tyrosine phosphorylation, unveiling the presence of a CRMP1-specific regulatory site phosphorylated by kinase(s) endogenous to HEK 293 cells (Fig. 1A). To determine if such an endogenous kinase also existed in primary neuronal cells, murine embryonic forebrain cultures were treated with or without H_2O_2, and cell extracts were subjected to immunoprecipitation using CRMP1-specific antibodies followed by SDS-PAGE. Immunoblotting with α-phosphotyrosine antibodies showed a significant increase in CRMP1 tyrosine phosphorylation induced by H_2O_2 in primary forebrain cultures (Fig. 1B) as was seen in HEK 293 cells.

IDENTIFICATION OF Y504 AS AN H_2O_2-INDUCED, CRMP1-SPECIFIC PHOSPHORYLATION SITE

To identify specific H_2O_2-regulated, CRMP1 tyrosine phosphorylation sites, we employed a quantitative mass spectrometry approach as described previously [Ballif et al., 2005]. This approach employs differential protein mass-tagging using metabolic labeling with native amino acids (“light”) or amino acids containing ^{13}C and ^{15}N stable isotope tags (“heavy”). In these experiments, both “heavy” and “light” cell cultures were transiently transfected with V5-tagged CRMP1 constructs. In one experiment “light” cells were stimulated with H_2O_2 and “heavy” cells were left untreated. In the reciprocal experiment, the “heavy” cells were stimulated while “light” cells were untreated. Equal portions of whole cell extract

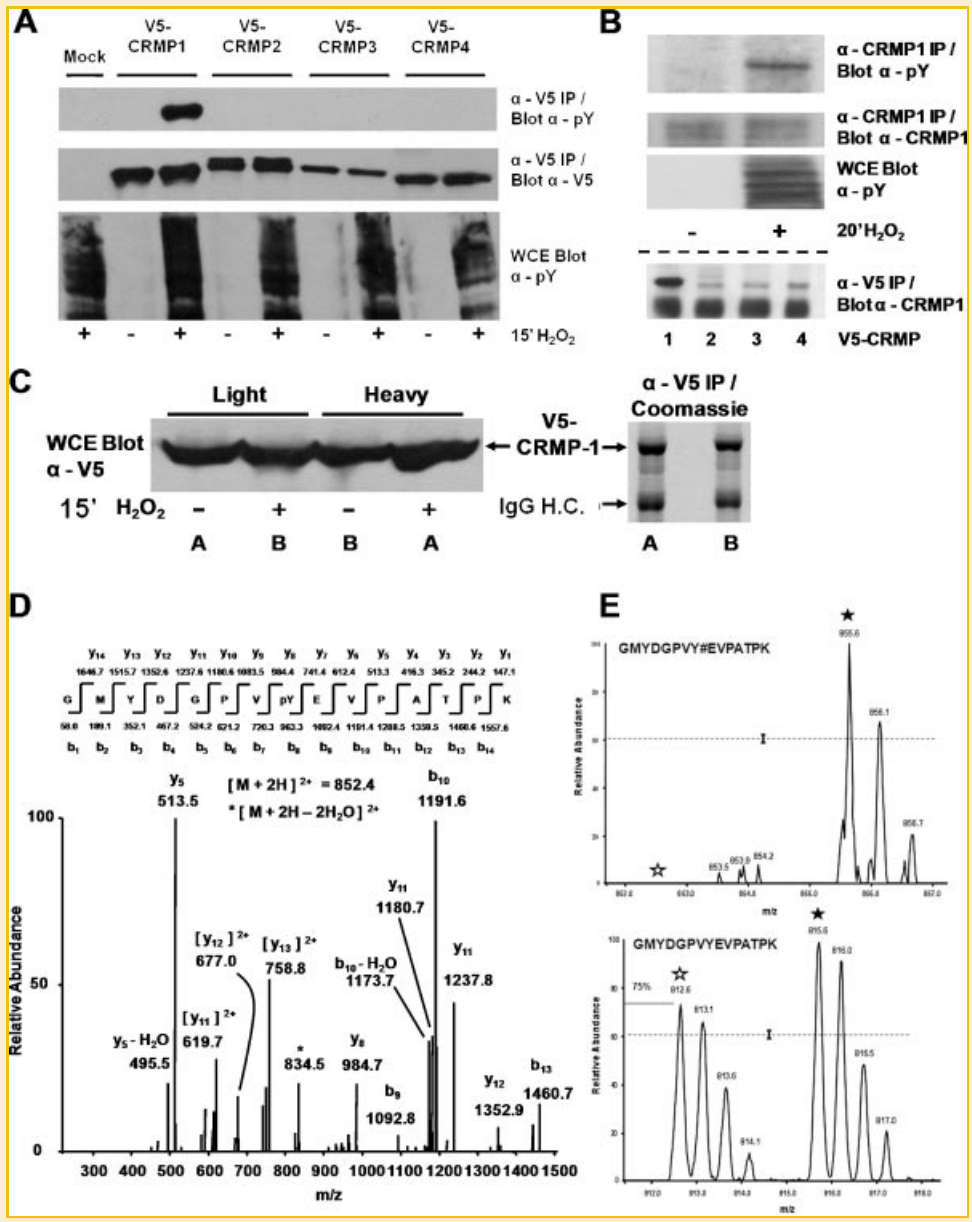

Fig. 1. Isoform-specific phosphorylation of CRMP1 at Y504. A: Isoform-specific regulation of CRMP1 tyrosine phosphorylation. Constructs encoding V5-tagged CRMP isoforms 1–4 were expressed in HEK 293 cells. Cells were stimulated with 8.1 mM H_2O_2 for 15 min as indicated. Following α-V5 immunoprecipitation and SDS-PAGE, immunoblotting was performed first with α-phosphotyrosine antibodies (top panel) and the blot was then re-probed with α-V5 antibodies (middle panel). Whole cell extracts (WCE) were separated by SDS-PAGE and immunoblotted with α-phosphotyrosine antibodies. A portion of this blot is shown to verify the effect of H_2O_2 treatment (bottom panel). B: H_2O_2-induces CRMP1 tyrosine phosphorylation in embryonic murine forebrain cultures. Embryonic stage 16.5 murine forebrain cultures were treated with or without 8.1 mM H_2O_2 for 20 min. Following immunoprecipitation with α-CRMP1 antibodies and SDS-PAGE, immune complexes were immunoblotted first using α-phosphotyrosine antibodies (top panel) and were then re-probed with α-CRMP1 antibodies (upper middle panel). The α-phosphotyrosine reactivity in the top panel aligns perfectly with the upper band of the CRMP1 apparent doublet. Whole cell extracts (WCE) were separated by SDS-PAGE and immunoblotted with α-phosphotyrosine antibodies. A portion of this blot is shown to verify the effect of H_2O_2 treatment (lower middle panel). A small portion of immunoprecipitated V5-tagged CRMP isoforms from (A) were immunoblotted with the α-CRMP1 antibodies to verify their specificity (lower panel). C: HEK 293 cells grown in "heavy" and "light" SILAC media were transfected with V5-CRMP1 constructs and stimulated with H_2O_2 as indicated. Whole cell extracts (WCE) were immunoblotted with α-V5 antibodies (left panel). Extracts labeled "A" were combined, and extracts labeled "B" were combined. "A" and "B" extracts were subjected to α-V5 immunopreciptation and immune complexes were separated by SDS-PAGE and visualized by coommassie blue (right panel). D: Identification of phosphorylation at Y504 by LC-MS/MS. The immunoprecipitated V5-CRMP1 bands from "A" and "B" extracts were digested in-gel with trypsin. Peptides from band "B" ("light"-stimulated) were subjected to LC-MS/MS analysis in an LTQ linear ion trap. One phosphotyrosyl-containing peptide was identified and the phosphorylation site was Y504 on the "light" peptide. The low energy collision-induced dissociation MS/MS spectrum of this peptide is shown with observed and theoretical masses for b- and y-type ions. E: Quantitative MS shows H_2O_2-induced Y504 phosphorylation. Peptides from band "A" ("heavy"-stimulated) were subjected to quantitative LC-MS/MS analyses using zoom scans in the LTQ that targeted three random CRMP1 peptides as well as the phosphorylated and unphosphorylated forms of the peptide containing Y504 (see Experimental Procedures Section for details). The upper panel shows the averaged zoom scans encompassing the mass range of the "light" and "heavy" phosphopeptide pair (Y# indicates phosphotyrosine). Anticipated monoisotopic peaks for the "light" (open star) and "heavy" (filled star) peptide ions are indicated. The dashed line indicates the average "light"/"heavy" relative abundance (60.7%) for the three quantified random peptides (Supplementary Fig. 1) with the error bars representing the standard deviation of the mean. The lower panel is as above, except data are for the unphosphorylated Y504-containing peptide.

were first analyzed by SDS-PAGE and immunoblotting (Fig. 1C) and extracts from the "light" stimulated and "heavy" unstimulated cells were then mixed equally. Extracts from the reciprocal experiment were also mixed equally. Mixed extracts were subjected to immunoprecipitation with α-V5 antibodies. Immune complexes were separated by SDS-PAGE and stained with coomassie blue (Fig. 1C). Bands corresponding to V5-CRMP1 were subjected to in-gel tryptic digestion and resultant peptides were extracted and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS). The stimulated "light" and unstimulated "heavy" sample was used first for shotgun sequencing to obtain maximal initial identification of "heavy" and "light" peptides. MS/MS analysis identified a single phosphotyrosyl peptide ion corresponding to the "light" peptide GMYDGPV(pY)EVPATPK in which the phosphorylated tyrosine was Y504 (Fig. 1D). Importantly, we previously found this site to be an in vivo phosphorylation site in a large-scale phosphoproteomic analysis of murine brain [Ballif et al., 2008]. Next the stimulated "heavy" and unstimulated "light" sample was analyzed and precursor MS1 zoom scans were performed to encompass three distinct CRMP1 peptide ions pairs to establish the baseline "heavy" to "light" peptide ratios. These analyses indicated that the level of protein in the unstimulated "light" samples was approximately 60.7% (measurements for the three peptides were 60%, 62%, and 60%) of the "heavy" protein level (Fig. 1E and Supplementary Fig. 1), consistent with a similar ratio (60.8%) approximated from the immunoblot in Figure 1C (see Experimental Procedures Section). Zoom scans were also performed to quantify the phosphorylated and unphosphorylated Y504-containing "light" and "heavy" peptide ion pairs, with measurements of the unphosphorylated pair indicating the "light" peptide was about 75% of its "heavy" counterpart (Fig. 1E). This could be explained by an increase in the amount of "heavy" pY504-phosphopeptide, as Y504 phosphorylation in the "heavy" peptide would concomitantly decrease the amount of unphosphorylated Y504 "heavy" peptide. This explanation is supported by the comparison of the amount of "light" and "heavy" pY504-phosphopeptide; there was a clear isotopic envelope corresponding to the mass of the "heavy" phosphopeptide, but there was no peptide ion found at the expected monoisotopic mass of the "light" phosphopeptide ion (Fig. 1E), consistent with low CRMP1 tyrosine phosphorylation prior to stimulation (Fig. 1A). Although zoom scans to obtain precise quantification were not used on the "light" stimulated, "heavy" unstimulated sample, the ratios of heavy/light peptide ions (not shown) were consistent with the results from the reciprocal experiment using zoom scans. Together these data indicated that in these overexpression studies, endogenous kinase activity was able to phosphorylate approximately 10–14% of the overexpressed CRMP1 molecules following H_2O_2 treatment.

CRMP1 Y504F DRAMATICALLY DIMINISHES H_2O_2-INDUCED CRMP1 TYROSINE PHOSPHORYLATION

To verify that Y504 is the main H_2O_2-regulated tyrosine phosphorylation site on CRMP1, we employed site-directed mutagenenesis to generate a Y504F mutation in the V5-tagged CRMP1 construct. HEK 293 cells were transfected with either V5-CRMP1 or V5-CRMP1 Y504F and either treated with H_2O_2 or left untreated. Following V5-immunoprecipitation and SDS-PAGE, the extracts were immunoblotted with α-phosphotyrosine antibodies. As expected, the wildtype V5-CRMP1 showed a strong increase in tyrosine phosphorylation, but the V5-CRMP1 Y504F showed minimal, if any, increase in tyrosine phosphorylation (Fig. 2A).

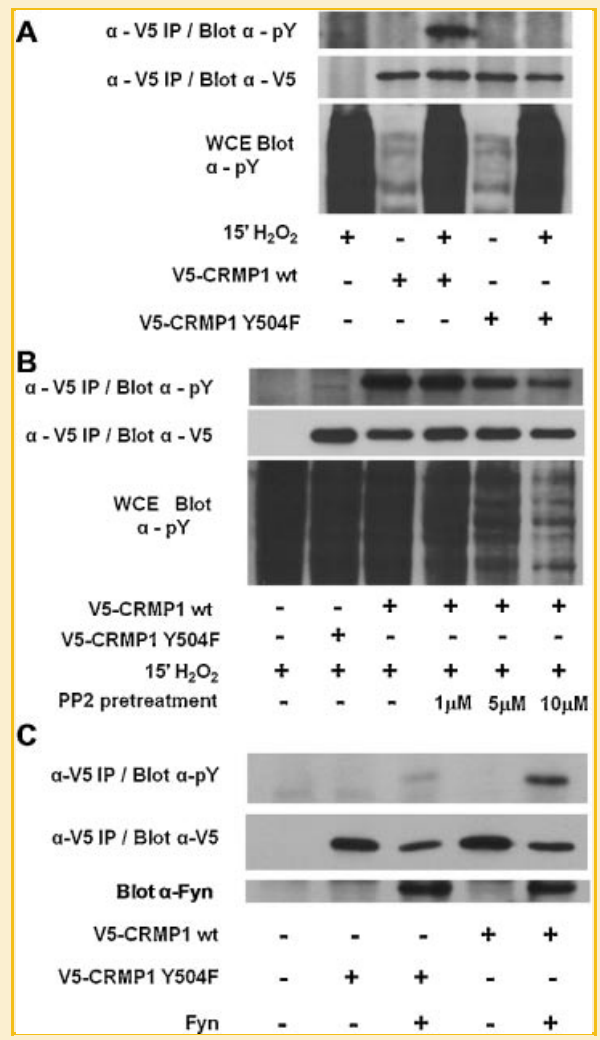

Fig. 2. Y504 is the major CRMP1 phosphorylation site induced by SFKs, specifically Fyn. A: Y504F blocks H_2O_2-induced CRMP1 tyrosine phosphorylation. HEK293 cells were transfected with constructs of either V5-CRMP1 or V5-CRMP1 Y504F and stimulated with H_2O_2 for 15 min as indicated. Following V5-immunoprecipitation, immune complexes were resolved by SDS-PAGE and immunoblotted first with α-phosphotyrosine antibodies (top panel) and the blot was then re-probed with α-V5 antibodies (middle panel). Whole cell extracts (WCE) were separated by SDS-PAGE and immunoblotted with α-phosphotyrosine antibodies. A portion of this blot is shown to verify H_2O_2 treatment (bottom panel). B: PP2 greatly reduces H_2O_2-induced CRMP1 tyrosine phosphorylation. HEK293 cells were transfected and treated as in (A) except prior to stimulation, cells were pretreated with the indicated concentrations of PP2 for 20 min. C: Wildtype Fyn induces Y504 phosphorylation. HEK293 cells were transfected as in (A) with or without co-transfection of a construct encoding wildtype Fyn. Immunoprecipitations and immunoblots were as in (A) except that whole cell extracts (WCE) were blotted with α-Fyn antibodies.

SFKs, SPECIFICALLY Fyn, PROMOTE PHOSPHORYLATION OF CRMP1 AT Y504

Given Fyn is known to be activated downstream of Sema3A signaling and activates CDK5 by phosphorylation at Y15 [Sasaki et al., 2002], and given an activated allele of Fyn has been shown to induce CRMP1 tyrosine phosphorylation [Yamashita et al., 2006], we asked if PP2, a pharmacological inhibitor of SFKs, would block H_2O_2-induced CRMP1 tyrosine phosphorylation. HEK 293 cells were transiently transfected with V5-CRMP1, and cells were pretreated with 1, 5, or 10 μM PP2 prior to H_2O_2 treatment. Following V5-immunoprecipitation and SDS-PAGE, immune complexes were immunoblotted with α-phosphotyrosine antibodies. The level of CRMP1 tyrosine phosphorylation decreased as the amount of inhibitor increased (Fig. 2B). However, at 10 μM PP2, when the vast majority of H_2O_2-induced tyrosine kinase activity was blocked in the whole cell extract, some CRMP1 tyrosine phosphorylation remained. It is therefore likely that although SFKs may be the primary Y504 kinases, additional PP2-insensitive kinases, such as those of the Fes/Fps and Fer family, may also contribute to CRMP1 Y504 phosphorylation.

To determine if overexpression of Fyn kinase would induce CRMP1 Y504 phosphorylation, we performed co-transfection of plasmids encoding wildtype Fyn and either wildtype V5-CRMP1 or V5-CRMP1 Y504F. Following α-V5-immunoprecipitation and SDS-PAGE, immune complexes were blotted with α-phosphotyrosine antibodies. There was a significant increase in tyrosine phosphorylation on V5-CRMP1 in Fyn-co-transfected cells, and only a slight increase on the V5-CRMP1 Y504F mutant (Fig. 2C). This suggests that the major Fyn phosphorylation site on CRMP2 is Y504, but also suggests the presence of another minor Fyn-regulated tyrosine phosphorylation site on CRMP1.

ABSOLUTE QUANTIFICATION OF CRMP1 Y504 PHOSPHORYLATION

To further support the SILAC data and to determine the stoichiometry of CRMP1 Y504 phosphorylation induced by either H_2O_2 or Fyn co-transfection, we employed another quantitative mass spectrometry approach, absolute quantification or AQUA [Gerber et al., 2007]. Using this approach, dried tryptic peptides of immunoprecipitated V5-CRMP1 from either H_2O_2-stimulated or Fyn-cotransfected cells were resuspended in a solution containing a known concentration of synthetic AQUA peptides corresponding to the phosphorylated and unphosphorylated tryptic peptides harboring CRMP1 Y504. V5-CRMP1 from unstimulated cells, and V5-CRMP1 Y504F cotransfected with Fyn, were used as negative controls. Each AQUA peptide is mass-tagged with ''heavy'' isotopes of carbon and nitrogen. Samples were subjected to duplicate LC-MS/MS analyses in an LTQ-Orbitrap. Quantification of the relative abundance of each peptide pair was done manually using Xcalibur software as previously described [Ballif et al., 2005]. Given each analysis contained 200 fmol of each AQUA peptide, the precise fmol amount of the native peptide could be determined from the relative abundance of each peptide pair. The stoichiometry of phosphorylation was then calculated as the fmol of phosphorylated peptide/(fmol of the phosphorylated peptide + the fmol of the unphosphorylated peptide). The results are shown in Figure 3. Consistent with the SILAC data, approximately 10% of over-expressed CRMP1 molecules were phosphorylated following H_2O_2 stimulation. Wildtype Fyn co-transfection resulted in 3% of CRMP1 molecules being phosphorylated. As a true negative control, no signal was detected for the Y504F mutant for the phosphorylated or unphosphorylated peptide as the mutation to phenylalanine alters its mass. Following stimulation, absolute phosphorylation stoichiometries of endogenous proteins can show significant temporal variability in any given cell, or in any given subcellular space. These factors can dramatically influence ultimate cellular outcomes. Such considerations for MAPK (ERK1 and 2) activation have recently been reviewed [Murphy and Blenis, 2006]. We previously reported approximately 10% phosphorylation stoichiometry of over-expressed HA-ERK2 in HEK 293 cells following stimulation with epidermal growth factor [Ballif et al., 2005], a stimulation that can typically lead to more than 50% phosphorylation stoichiometry of the endogenous kinase in the same cells type as judged by electrophoretic mobility shifts [Ballif and Blenis, 2001]. Thus, the 3–14% CRMP1 Y504 phosphorylation stoichiometry measured in these overexpression studies is within a significant range.

DISCUSSION

The main findings of this study are the identification of Y504 as a novel, regulated CRMP1 tyrosine phosphorylation site and its regulation by SFKs, specifically Fyn. We show Y504 is the major Fyn phosphorylation site on CRMP1. It is known that the Sema3A receptors PlexA1 and A2 are constitutively bound to Fyn and that Fyn is activated downstream of Sema3A signaling. Fyn also directly phosphorylates CDK5 at Y15, a critical event in the regulation of CRMPs by Sema3A [Sasaki et al., 2002]. It has also been shown that GSK-3β, which is normally regarded as an enzyme that is constitutively active, is maintained in an inactive pool co-localized with F-actin in the leading edge of the neuronal growth cone. Upon Sema3A stimulation, GSK-3β from the inactive pool is activated [Eickholt et al., 2002]. This concurrent activation of CDK5 and GSK-3β allows for sequential phosphorylation and inactivation of CRMPs. Figure 4 illustrates CRMP domain structure and provides a sequence alignment of the carboxyl-terminal regulatory region of isoforms 1–4, highlighting CDK5, GSK3-β, and Fyn phosphorylation sites. Since the phosphorylation of CRMPs by GSK-3β and Rho Kinase at residues adjacent to Y504 serves to disrupt the interaction between CRMPs and tubulin heterodimers, Y504 phosphorylation may also disrupt this interaction. We are currently testing this hypothesis and deem it to be the most likely functional consequence of Y504 phosphorylation. Alternatively, Y504 phosphorylation may alter the interaction of CRMP1 with other CRMP isoforms or may serve as a docking site for proteins with phosphotyrosine-binding domains. As Fyn is activated by Sema3A stimulation, Fyn may be able to phosphorylate CRMP1 at Y504 downstream of Sema3A signaling, and directly inhibit the interaction between CRMP1 and tubulin heterodimers. Alternatively, guidance cues that activate Fyn but are not known to activate CDK5, such as Reelin [Ballif et al., 2003; Yamashita et al., 2006], may be able to regulate CRMP1 directly.

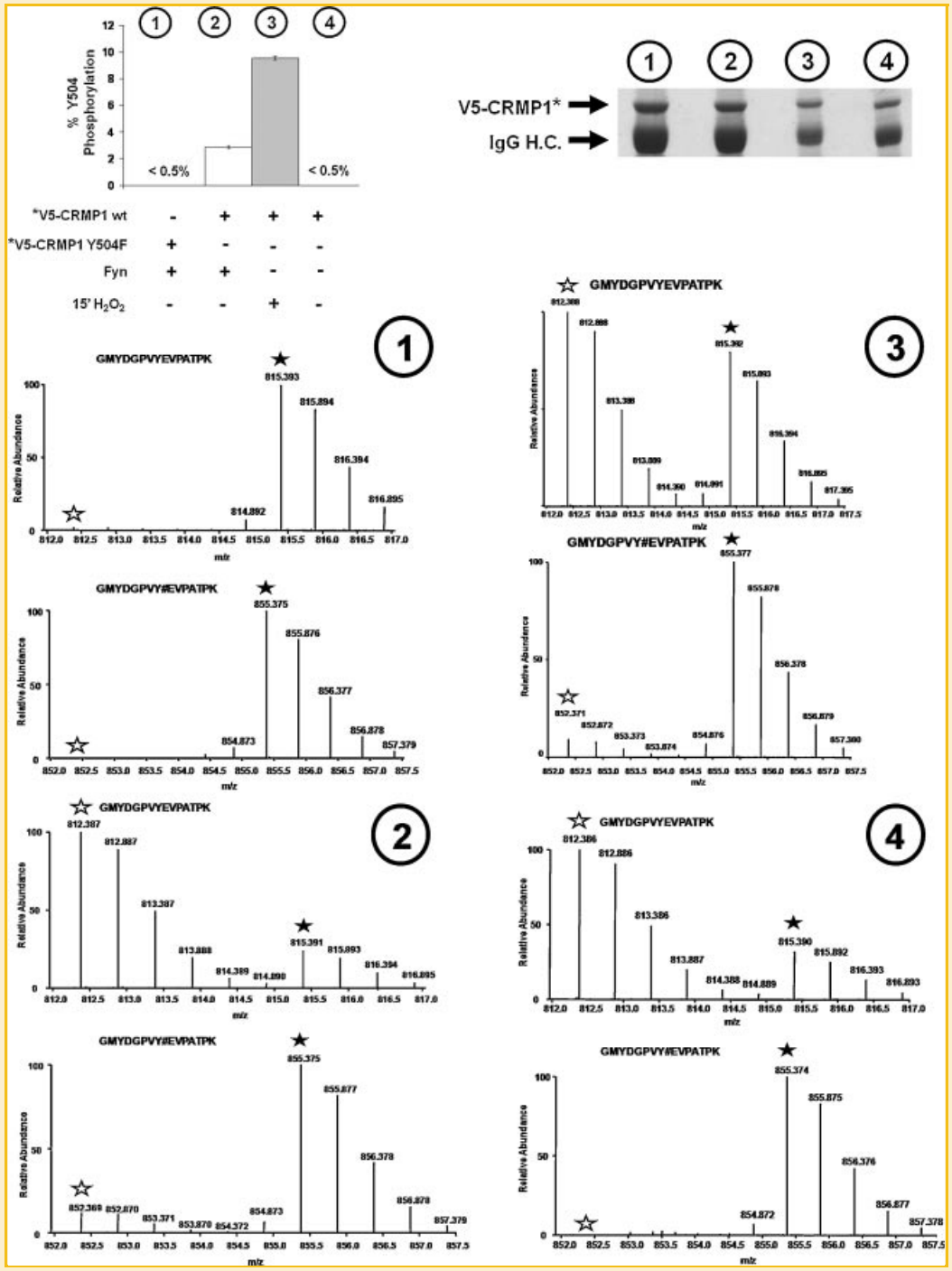

Fig. 3. Absolute quantification of Y504 phosphorylation. V5-CRMP1 was immunoprecipitated from HEK 293 cells transfected with the indicated constructs. Immune compexes were resolved by SDS-PAGE and were stained with coomassie blue (upper right panel). Immunoprecipitations 1 and 2 show higher amounts of V5-CRMP1 and antibody. This is due simply to the fact that samples 1 and 2 were each derived from a proprtionately larger amount of cell extract and antibody. Dried tryptic peptides extracted from in-gel digests of the V5-CRMP1 bands were resuspended in a solution containing 450 fmol (50 fmol/μl) each of the synthetic "heavy" peptides (GMYDGPVY#E($^{13}C_5$-, $^{15}N_1$-V)PATPK and GMYDGPVYE($^{13}C_5$-, $^{15}N_1$-V)PATPK). Y# denotes phosphotyrosine. Duplicate injections of 4 μl were analyzed by LC-MS/MS in a LTQ-Orbitrap as described in the Experimental Procedures Section. MS1 measurements for each sample type are shown, and were used to determine phosphorylation stoichiometry at Y504 (upper left histograms) as described in experimental procedures. Error bars represent the standard deviation of the replicate means. "IgG H.C." indicates the antibody heavy chain. Open and filled stars correspond to the monoisotopic peaks of the "light" and "heavy" isotopic envelopes respectively.

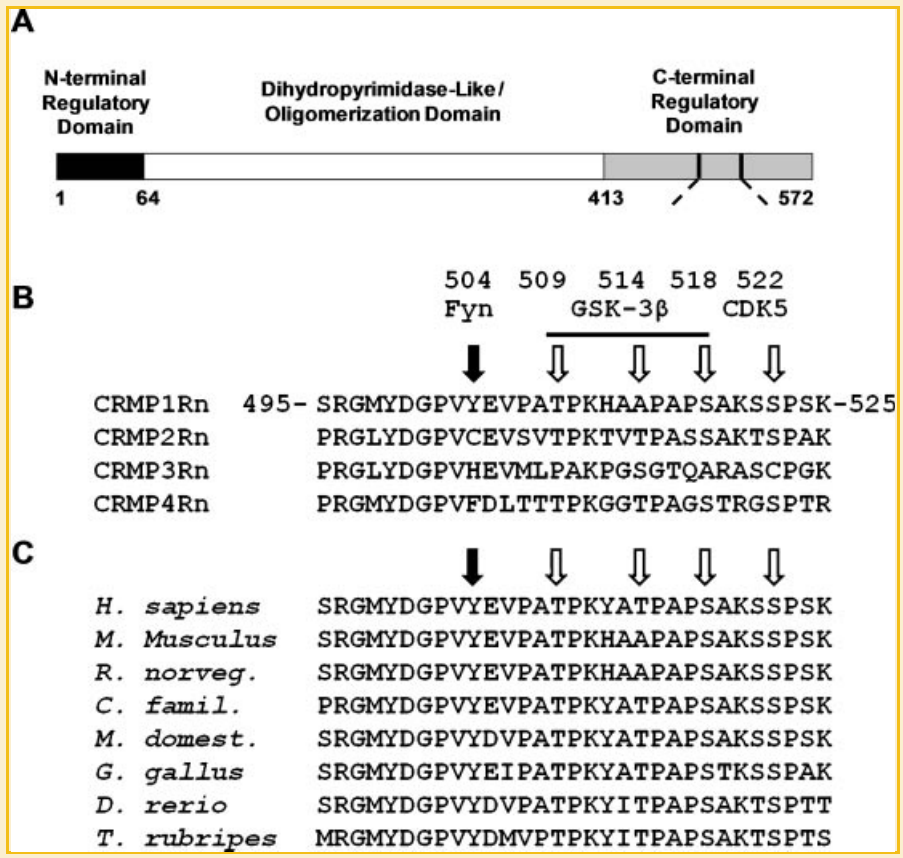

Fig. 4. CRMP structure and regulation. A: CRMP isoforms have N-terminal and a C-terminal regulatory domains surrounding their oligomerization or dihydropyrimadase-like domain. Numbering indicates amino acid residues at domain boundaries. B: Sequence alignment of CRMP isoforms 1–4 shows high sequence homology in the region of Y504. CDK5 and GSK-3β serine/threonine phosphorylation sites (open arrows) are relatively conserved in isoforms 1, 2, and 4. Y504 (filled arrow) is only present in CRMP1. C: Sequence alignment (residues 495–525) of CRMP1 in various organisms shows high conservation, and Y504 is present in all eight species surveyed (human, mouse, rat, dog, opossum, chicken, zebrafish, and pufferfish).

Since Y504 is conserved only in CRMP1 orthologs (Fig. 4C), Y504 phosphorylation may be a way to regulate CRMP binding to tubulin heterodimers based on the relative levels of the CRMP isoforms present in a given cell. For example, a cell may become more sensitive to Sema3A signaling if it preferentially expresses CRMP1 relative to other isoforms. As neuronal migration is reduced in the cerebral cortex of CRMP1 knock-out mice [Yamashita et al., 2006], CRMP1 appears to have a unique function for which CRMPs2-4 cannot be substituted, highlighting the importance of identifying CRMP1-specific regulatory mechanisms.

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