Fyn Phosphorylates Human MAP-2c on Tyrosine 67*

S. Pilar Zamora-Leon‡, Anne Bresnick§, Jonathan M. Backer†, and Bridget Shafit-Zagardo‡|

From the Departments of ‡Pathology, §Biochemistry, and ¶Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

The Src homology 3 (SH3) domain of Fyn binds to a conserved PXXP motif on microtubule-associated protein-2. Co-transfections into COS7 cells and in vitro kinase assays performed with Fyn and wild-type, or mutant MAP-2c, determined that Fyn phosphorylated MAP-2c on tyrosine 67. The phosphorylation generated a consensus sequence for the binding of the SH2 domain of Grb2 (pYSN). Pull-down assays with SH2-Grb2 from human fetal brain homogenates, and co-immunoprecipitation of Grb2 and MAP-2 confirmed the interaction in vivo, and demonstrated that MAP-2c is tyrosine-phosphorylated in human fetal brain. Filter overlay assays confirmed that the SH2 domain of Grb2 binds to human MAP-2c following incubation with active Fyn. Enzyme-linked immunosorbent assays confirmed the interaction between the SH2 domain of Grb2 and a tyrosine-phosphorylated MAP-2 peptide spanning the pY67SN motif. Thus, MAP-2c can directly recruit multiple signaling proteins important for central nervous system development.

Microtubule-associated protein-2 (MAP-2)1 is a member of the family of structural MAPs that binds microtubules (MTs). Multiple MAP-2 isoforms are expressed in the central nervous system and are the result of alternative splicing of a single gene. These include the high molecular weight MAP-2a, MAP-2b, and MAP-2e, and the low molecular weight MAP-2c and MAP-2d isoforms (1–9). All MAP-2 isoforms are heat-stable phosphoproteins that are developmentally regulated in the central nervous system (7–9), and contain a number of functional domains. These include, at the amino terminus, the sequence for binding the regulatory subunit (RII) of cAMP-dependent protein kinase A (10, 11), and at the carboxyl terminus, the repeats of the microtubule-binding domain (MTBD) (12–14). Adjacent to the first repeat of the MTBD is the sequence RTPPKSP. We previously demonstrated that this motif on MAP-2c binds the SH3 domain of the non-receptor tyrosine kinase Fyn, and that the interaction is regulated by ERK2 phosphorylation (15).

Fyn is expressed in neurons, oligodendrocyte progenitors, and differentiatied oligodendrocytes (16–19). Fyn localizes to the cell body and processes and is the only active non-receptor tyrosine kinase in early oligodendrocyte development (18). It has also been demonstrated that Fyn phosphorylates and binds p190 RhoGAP, and in that manner, regulates oligodendrocyte differentiation (20). MAP-2c is expressed early in development, in neuronal cell bodies and dendrites; and in human fetal spinal cord is also expressed in the axon (8, 21, 22).

Whereas our earlier study demonstrated a Fyn/MAP-2 interaction (15), our present study focuses on defining which of the 5 tyrosine residues on MAP-2c is phosphorylated by Fyn. We present biochemical evidence that tyrosine 67 (TYr67) on MAP-2c is phosphorylated by Fyn creating a consensus motif (pYSN) for the binding of the SH2 domain of Grb2 that exists at low levels in the human central nervous system. In addition, we note that an indirect interaction between MAP-2 and the SH3 domains of Grb2 also exists.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Expression and Purification of GST Fusion Proteins—cDNAs from full-length Grb2-GST and GST protein without insert (pGEX vector) were obtained from Dr. Hamid Band (Brigham and Women’s Hospital, Boston, MA). SH2-Grb2-antennapedia-GST cDNA (antennapedia amino acid sequence: RQIKIWFQNRRMKWKK) was obtained from Dr. Bruce Terman (Albert Einstein College of Medicine, Bronx, NY). N-SH3-Grb2-GST cDNA (amino-terminal SH3 domain of Grb2) was obtained from Dr. Brian Kay (University of Wisconsin, Madison, WI). Expression and purification of the fusion proteins was performed as described previously (23). Purification of the protein was confirmed by Western blot analysis with the mAb DT-12 (IgG1), which recognizes GST. Human wild type (WT) Fyn cDNA and K299M mutant Fyn cDNA (pCMV5 vector), which is inactive because of a point mutation in the ATP binding pocket, were obtained from Dr. Marilyn Heish (Memorial Sloan-Kettering Cancer Center, New York, NY).

Expression and Purification of Recombinant Human WT and Mutant His Tag MAP-2c—The procedure was performed as previously described (15). The eluted proteins were concentrated and buffer exchanged to 25 mM Tris–HCl, pH 7.4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM EDTA, and 1 mM diithiothreitol using the ultrafree-15 centrifugal device (30K, Millipore). Protein quantification was performed at 280 nm considering the respective His-tag MAP-2c extinction coefficients obtained at ProtoParam tool (ExPASy), and calculated according to the equation: A = εc/l, where A = absorbance at 280 nm, ε = extinction coefficient, c = concentration, and l = length of the cuvette.

Mutagenesis—Mutations of tyrosine residues of human MAP-2c (accession number I67798) were made by PCR cloning as described under the QuikChange™ Site-directed Mutagenesis kit instruction manual (Stratagene). The primers used to mutate either the first or second tyrosine residues (underlined) to phenylalanine on MAP-2c were: mut1 (mutation of Tyr-50): GCCAATGATTCCATCCGAGGAGATGAAG (sense), and mut2 (mutation of Tyr-67): GGGTCAACAGGGCACCTTTTCAATAACAAAAAGAG (sense), respectively.

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The abbreviations used are: MAP-2, microtubule-associated protein-2; GST, glutathione S-transferase; His tag-MAP-2c, histidine-tagged MAP-2c; HRP, horseradish peroxide; IP, immunoprecipitation; mAb, monoclonal antibody; MT, microtubule; MTBD, microtubule-binding domain; WT, wild-type; SH, Src homology domain; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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† To whom correspondence should be addressed: Dept. of Pathology, Albert Einstein College of Medicine, Bronx, NY 10461. Tel.: 718-430-2189; Fax: 718-430-8541; E-mail: zagardo@necom.yu.edu.

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last cycle. The constructs were sequenced to verify the reading frames and the mutation sites.

Heat-stable Protein Extractions—Heat-stable protein extraction of the in vitro kinase reactions was performed by adding NaCl (0.34 mM final) and β-mercaptoethanol (0.28 mM final) to the reaction mixtures, and boiling the samples for 5 min. Then, the samples were centrifuged at 14,000 rpm in an Eppendorf centrifuge for 5 min at 4 °C, and the supernatants were retained.

In Vitro Kinase Assay—Bacterially expressed human His-tag MAP-2c (3 μg) was incubated with 0.2 units of Fyn kinase (Upstate Biotechnology Inc.). The reaction conditions were performed as described in the manufacturer’s protocol, in a reaction volume of 25 μl, either in the presence of [γ-32P]ATP (10 μCi/reaction; PerkinElmer Life Sciences, NEG002A) and cold ATP (0.5 mM), or cold ATP only (0.5 mM). Although at 15 min MAP-2c was phosphorylated, the amount of radioactivity increased with time reaching a plateau at 90 min. Therefore, the samples were incubated at 37 °C for 90 min. The samples were then placed on ice and mixed with 6× sample buffer (350 mM Tris, pH 6.8, 0.2% 2-mercaptoethanol, 3% SDS, 60% glycerol, 1.7 mM β-mercaptoethanol). The samples were then loaded either in a 10 or 10–20% gradient SDS-PAGE.

When the kinase reaction was performed with [γ-32P]ATP, the gel was stained with Coomassie (0.25% Brilliant Blue, 45% methanol, and 10% glycerol) and visualized on a UVP gel imager system. Western blotting was performed with 7M Urea, pH 9.5, 10% SDS, 15% glycerol, 0.075 M Tris, pH 6.8, 0.02% bromphenol blue, 3% SDS, 60% glycerol, 1.7 mM β-mercaptoethanol). The samples were then loaded either in a 10 or 10–20% gradient SDS-PAGE.

Filter Overlay Assays—Proteins from the in vitro kinase reactions performed with cold ATP were loaded in a 10% SDS-PAGE. Following protein transfer, the nitrocellulose membranes were incubated overnight at 4 °C with 10 μg/ml GST fusion proteins in 5% milk, 1× TBS, washed 3 times (10 min each) with 1× TBS and 0.1% Tween 20, incubated the anti-GST mAb DT-12 (anti-GST) and the mAb anti-His, provided by Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY) the supernatants, predominantly in 2% bovine serum albumin, 1× TBS for the phosphotyrosine mAb PY-100 (Cell Signaling) and 4×G10 (Upstate Biotechnology); and HRP-conjugated secondary antibodies (Southern Biotechnology Associates, Inc.).

Enzyme-linked Immunosorbent Assay (ELISA)—The MAP-2 and the irrelevant synthetic peptides (Table I) were synthesized with a biotin tag at the amino terminus (45% glycerol, 0.075 M Tris, pH 6.8, 0.02% 2-mercaptoethanol, and dried, and exposed to Kodak Bio-Max MR film. The radioactive antibodies were counted in a 4×G10 (Upstate Biotechnology); and HRP-conjugated secondary antibodies (Southern Biotechnology Associates, Inc.).

Preparation of Human Fetal Brain Homogenates—Fetal brain tissue (19 gestational weeks) was homogenized in extraction buffer at 3 times weight/volume, and centrifuged at 22,900 × g at 4 °C for 20 min. The supernatant was saved and the protein concentration was determined (Bio-Rad protein assay). The brain protein homogenates were aliquoted in 5% bovine serum albumin and 0.1% Tween 20. 24 g of GST fusion proteins in 5% milk, 1× TBS, washed 3 times (10 min each) with 1× TBS and 0.1% Tween 20, incubated the anti-GST mAb DT-12 (anti-GST) and the mAb anti-His, provided by Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY) and stored at –80 °C. The Clinical Investigation and the Health and Hospital Corporation of the City of New York and the Institutional Review Committee approved all studies.

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RESULTS

Fyn Phosphorylates the Second Tyrosine Residue on MAP-2c—To determine which tyrosine residue is phosphorylated by Fyn, sequential mutagenesis of individual tyrosine residues were performed beginning with the N-end of MAP-2c (Fig. 1A). Human His tag-MAP-2c, mut1-MAP-2c, consisting a mutation of the first tyrosine (Tyr92) to phenylalanine, or mut2-MAP-2c, consisting of the second tyrosine (Tyr100) were incubated with cold ATP at 37 °C in the presence or absence of active Fyn kinase. A heat-stable protein extraction was performed after the kinase reaction to eliminate the Fyn from the reaction mixture. Following electrophoresis and protein transfer, the membranes were incubated with the MAP-2 c-Ab Tau-46 (Fig. 1B lanes 1–3), an anti-His mAb (lanes 4–6), and the phosphotyrosine mAb PY-100 (lanes 7–13). Tau-46 detected only the full-length MAP-2c (lanes 1–3), whereas the anti-His mAb detected full-length MAP-2c and lower bands representative of degradation products from the carboxyl terminus of MAP-2c (lanes 4–6). The pY100 mAb (lanes 7–13) confirmed that WT MAP-2c and mut1-MAP-2c were tyrosine phosphorylated when incubated with active Fyn kinase (compare lanes 11 and 12, and lanes 9 and 10, respectively). Lane 8, containing mut2-MAP-2c and Fyn, was faintly positive with the phosphotyrosine mAb, and that band co-migrated with Fyn in the Fyn only sample (lane 13). Blots previously incubated with pY100 mAb were stripped and incubated with the anti-Fyn mAb. As shown in the lower panel, residual Fyn was detected in some of the supernatants, predominantly in lanes 8 and 13. This supported our claim that the faint phosphorylation detected in the Fyn/mut2-MAP-2c lane corresponded to autophosphorylation on Fyn and not mut2-MAP-2c. Because we determined that the phosphorylation was on Tyr100, mutagenesis of the last 3 tyrosine residues was not performed.

To confirm that the second tyrosine (Tyr100) on MAP-2c is phosphorylated by Fyn, [γ-32P]ATP in vitro kinase reactions were performed in duplicate. Following the kinase reactions,
the samples were placed on ice, and MAP-2c was added as a carrier. Again, heat-stable proteins were extracted and examined for the incorporation of radioactivity into full-length MAP-2c and the degradation products. As shown in Fig. 2A, WT MAP-2c (lanes 6 and 7) and mut1-MAP-2c (lanes 4 and 5) were tyrosine phosphorylated, but mut2-MAP-2c was not (lanes 2 and 3). There was not a radioactive band for the Fyn only sample in lane 1, indicating that although we observed variability in various preparations, this time Fyn was not retained in the heat-stable fraction. The Coomassie-stained gel (lower panel) showed that there were equal amounts of MAP-2c protein in each reaction mixture. Because MAP-2c was added as a carrier, the Fyn only sample (lane 1) contains non-radioactive MAP-2c. The table in Fig. 2B contains the total counts/min incorporated into WT and mutant MAP-2c proteins. Background levels of Fyn were obtained after performing the heat-stable protein extraction, thereby precipitating the majority of Fyn. This data confirms that only WT MAP-2c and mut1-MAP-2c were phosphorylated by Fyn, and mut2-MAP-2c was not.

To further confirm this result in vivo, WT, or mut 2 MAP-2c, and WT Fyn were co-transfected into COS7 cells and 24 h post-transfection, the protein homogenate was immunoprecipitated with the MAP-2-specific mAb HM2, transfected into nitrocellulose, and the blot was incubated with the phosphotyrosine mAb pY100. As shown in the top panel of Fig. 2C, lanes 2 and 6, WT MAP-2c and WT Fyn were positive with the pY100 mAb. WT or mut-2 forms of MAP-2c transfected alone (not shown); with WT Fyn and mut 2 (lane 3), mut Fyn and WT MAP-2c (lane 4), or mut Fyn and MAP-2c mut 2 (lane 5) were negative with mAb pY100. The middle and lower panels of Fig. 2C demonstrate the efficiency of co-transfection and immunoprecipitation. Note that lane 1 was immunoprecipitated with an irrelevant IgG1 mAb and was negative for both MAP-2c and Fyn. Lane 7 contains an aliquot of the WT Fyn, WT MAP-2c transfection prior to IP. Fig. 2D representing immunoprecipitation experiments with MAP-2 mAb HM-2 demonstrated that when WT Fyn was co-transfected with WT MAP-2c (lanes 1), and mut-1 (lanes 2, 4) MAP-2c was tyrosine phosphorylated. By contrast, no phosphorylation on tyrosine was observed on MAP-2c when Fyn was co-transfected with mut-2 (lanes 3 and 5) further confirming that Tyr67 is the sole site on MAP-2c phosphorylated by Fyn. As shown in lane 6, an irrelevant IgG1 mAb failed to immunoprecipitate MAP-2c.

MAP-2 Interacted with Grb2 in Human Fetal Brain Homogenates—Fyn phosphorylation of Tyr67 on MAP-2c creates a consensus sequence for the recruitment of the SH2 domain of Grb2, pY75Sh. Although Tyr67 was phosphorylated in COS7 cells, a pull-down assay with SH2-Grb2 was negative for MAP-2c. In addition, immunoprecipitation of MAP-2c from COS7 cells following the triple transfection of Fyn, MAP-2c, and YFP-SH2-Grb2 fusion protein inconsistently co-immunoprecipitated YFP-SH2-Grb2 (data not shown). To explore whether MAP-2 and Grb2 interact in the central nervous system, immunoprecipitations and a pull-down assay with human fetal brain homogenates were performed. As shown in Fig. 3A, Grb2 co-immunoprecipitated with MAP-2c. Incubation of the membrane with the anti-MAP-2 mAb confirmed MAP-2 in the pellet (data not shown). The MAP-2/Grb2 interaction was further confirmed by an immunoprecipitation performed with a Grb2 polyclonal antibody. As shown in Fig. 3B, the Grb2 antibody co-immunoprecipitated high and low molecular weight MAP-2. Incubation of the membrane with the anti-Grb2 polyclonal Ab confirmed Grb2 in the pellet (data not shown). These results indicated that MAP-2c and Grb2 interact in vivo but it did not establish that the interaction was via the Grb2-SH2 domain. To address this, Grb2 was immunoprecipitated from the brain homogenate and the Grb2 antibody-protein-G-agarose complex was extensively washed, resuspended in IP buffer, and incubated with 50 and 100 μg of GST-SH2-Grb2 for 30 min at 4 °C. The pellet was washed, boiled, and the supernatant was subject to electrophoresis and transfer to nitrocellulose. Fig. 3C shows that when GST-SH2-Grb2 was added to the immunoprecipitated complex there was a decrease in phosphotyrosine on MAP-2, detected with mAb4G10 (compare lanes 2 and 4), demonstrating that an interaction between MAP-2c and the SH2 domain of Grb2 exists in vivo.
FIG. 2. In vitro kinase assays and co-transfection assays demonstrate that Fyn phosphorylates the second tyrosine residue on human MAP-2c. Bacterially expressed human His tag-MAP-2c (wt, mut1, and mut2; 2 μg each) were incubated with 0.2 units of Fyn kinase in the presence of [γ-32P]ATP. Aliquots corresponding to 0.6 μg of MAP-2c were loaded in a 10–20% gradient SDS-PAGE and, in A, upper panel, exposed to Kodak Bio-Max MR film. Lane 1, reaction performed only with Fyn; lanes 2 and 3, mut2-MAP-2c and Fyn; lanes 4 and 5, mut1-MAP-2c and Fyn; lanes 6 and 7, WT MAP-2c and Fyn. A, lower panel, shows the Coomassie staining of the above gel. B, table corresponding to the average radioactivity incorporated into all the MAP-2c proteins during the kinase reactions from the gel in A. The bands were cut and processed as described under “Experimental Procedures.” C, MAP-2c was immunoprecipitated with mAb HM-2 (4 μg; Sigma) following co-transfected of WT or mut-2 with WT or kinase-dead Fyn (lanes 2–6). The blot was incubated with mAb pY100 (top panel); or MAP-2 recognizing mAb Tau-46 (middle panel) or Fyn (bottom). IP with an irrelevant IgG, mAb DT-12, to GST, is shown in lane 1. D, co-transfection of WT Fyn with WT MAP-2c (lane 1), mut-1 (lanes 2 and 4), mut-2 (lane 3 and 5), or WT MAP-2c into COS7 cells was immunoprecipitated with HM2 (lanes 1–5), or an irrelevant antibody (lane 6), and blotted with the Tyr(P) mAb 4G10 (upper panel), or the MAP-2 mAb Tau-46 (lower panel). WB, Western blot.
FIG. 3. Grb2 interacts with MAP-2 in fetal human brain homogenates. A and B, co-immunoprecipitations of MAP-2 and Grb2 from human fetal brain protein homogenates. One mg of total human fetal brain protein homogenate was incubated with either 10 μg of the anti-MAP-2 mAb HM-2 (A), or 10 μg of Grb2 polyclonal antibody (B). Total protein homogenates prior to immunoprecipitations (homog; A, 100 μg; B, 50 μg), and pellets following immunoprecipitation were separated in 10–20% SDS-PAGE. A, membrane containing pellets and homogenate from IPs performed with the anti-MAP-2 mAb HM-2 or an irrelevant control mAb was incubated with a Grb2 affinity purified polyclonal antibody (1:200). B, membrane containing pellets and homogenate from IPs performed with the Grb2 polyclonal antibody or rabbit serum (control) was incubated with the anti-MAP-2 mAb HM-2 (1:2000). C, 0.5 mg of total brain homogenate was incubated with 10 μg of rabbit serum (lane 1) or a Grb2 polyclonal antibody (lanes 2–4). The precipitate was incubated with 100 μg of GST only (lane 2), or 50 or 100 μg of GST-SH2-Grb2 fusion protein (lanes 3 and 4). Again the immunoprecipitated pellet was washed 5 times, boiled in 2X dye mixture, and the supernatant was loaded on the gel. Densitometry was performed in NIH Image (rsb.info.nih.gov/nih-image/index.html). Each lane was scanned and the value in lane 1 was subtracted from lanes 2–4. The ratio of MAP-2c/Grb2 is: lane 2, 1.15; lane 3, 1.29; lane 4, 1.07. The ratio of 4G10/Grb2 is: lane 2, 0.44; lane 3, 0.40; lane 4, 0.32. D, MAP-2c interacts with Grb2 in human fetal brain pull-down assays. One mg of human fetal brain protein homogenate was incubated with full-length Grb2-, SH2-Grb2-, N-SH3-Grb2-GST fusion proteins, or GST protein without insert. Homogenates prior to pull-down (homog; 10 μg) and the pellets were separated in 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the anti-MAP-2 mAb HM-2 (upper panel) or with the GST-specific mAb DT-12 (lower panel, 1:500).
To further examine the domain that bound MAP-2c, pull-down assays were performed with human fetal brain homogenates and N-SH3-Grb2-, SH2-Grb2-, full-length Grb2-GST fusion proteins, and GST protein without insert coupled to glutathione-agarose beads. Following the assay, the proteins were separated by electrophoresis, transferred to nitrocellulose membranes, and incubated with the anti-MAP-2 mAb HM-2. As shown in Fig. 3D (upper panel), fetal brain MAP-2c interacted with full-length Grb2-, the SH3-Grb2-, and the SH2-Grb2-GST fusion proteins but not the GST-only fusion protein. This data supports an interaction between MAP-2c and the SH3 domain of Grb2, and the SH2 domain of Grb2. Because MAP-2c recruits the SH2 domain of Grb2 this supports our data that endogenous MAP-2c is tyrosine phosphorylated in vivo within the YSN motif. To confirm that all lanes contained GST fusion proteins, the blots were stripped and re-incubated with the GST-specific DT-12 mAb (Fig. 3D, lower panel).

Phosphorylation of MAP-2c by Fyn Recruits the SH2 Domain of Grb2—To further confirm that the SH2 domain of Grb2 binds to the pYSN motif on MAP-2c, a direct filter overlay assay was performed following an in vitro kinase assay. The kinase reaction was performed with cold ATP, Fyn kinase, and WT MAP-2c or mut2-MAP-2c; WT MAP-2c without Fyn was included as a control. Following the kinase reactions, total protein homogenates were separated by electrophoresis and transferred to nitrocellulose. Filter overlay assays were performed by incubating the membranes with 10^6 H9262 g/ml full-length Grb2-, lanes 10–12 with SH2-Grb2-GST, lanes 13–15 with N-SH3-Grb2-GST, and lanes 16–18 with GST without insert. To identify the input proteins in the reaction mixtures, the blot was incubated with an anti-Fyn mAb (Fig. 4A, lanes 1–3), and anti-MAP-2 mAb HM-2 (lanes 4–6). As shown in Fig. 4A, WT MAP-2c bound Grb2 only when tyrosine phosphorylated by Fyn (compare lanes 8 and 9, and 11 and 12). N-SH3-Grb2-GST fusion protein did not bind to WT MAP-2c (lanes 13–15). Although lanes 7 and 10 containing the mut2-MAP-2c and Fyn showed interactions with full-length Grb2- and SH2-Grb2-GST fusion proteins, respectively, the bands co-migrate with Fyn indicating an interaction between Fyn and the SH2 domain of Grb2.

To corroborate that the SH2 domain of Grb2 binds to Fyn, an in vitro kinase assay with cold ATP and only Fyn kinase was performed. A 10% SDS-polyacrylamide gel with varying

![Fig. 4. Following phosphorylation by Fyn, bacterially expressed human His tag-MAP-2c interacts with the SH2 domain of Grb2 in a concentration-dependent manner.](http://www.jbc.org/)

A. wt MAP-2c

| Lane | MAP-2c Fragment | Fyn |
|------|-----------------|-----|
| 1    | +               | -   |
| 2    | +               | -   |
| 3    | +               | -   |
| 4    | +               | -   |
| 5    | +               | -   |
| 6    | +               | -   |
| 7    | +               | -   |
| 8    | +               | -   |

B. filter overlay

| Lane | 65.8 KDa | 83.8 KDa |
|------|---------|---------|
| 1    | Fyn     | SH2-Grb2 |
| 2    | GST     |         |

C. filter overlay

| Lane | 65.8 KDa | 83.8 KDa |
|------|---------|---------|
| 1    | Fyn     | SH2-Grb2 |
| 2    | GST     |         |
Fyn Phosphorylation of MAP-2c Recruits Grb2

Table 1

Synthetic peptides spanning the YSN motif on MAP-2

| Synthetic peptides | O.D. (405 nm) |
|--------------------|--------------|
| 1466: GSQGYTSNKT  | 1.0          |
| 1466-P: GSQGTYSNKT| 1.2          |

Irrelevant phosphotyrosine peptide

PY: GGYMDMSKDE (human PDGF receptor β)

DISCUSSION

Although in vitro tyrosine phosphorylation of high molecular weight MAP-2 has been previously reported, the sites phosphorylated by specific tyrosine kinases have not been described (26, 27). In our previous study, we have demonstrated that Fyn binds and tyrosine phosphorylates MAP-2c (15). In this study we have determined that Fyn phosphorylates MAP-2c solely on Tyr67. We provide evidence that MAP-2c is tyrosine phosphorylated in vivo and that the SH2 domain of Grb2 can pull-down tyrosine-phosphorylated MAP-2 from human fetal brain homogenate. In vivo and in vitro results demonstrated that only WT- and mut1-MAP-2c were phosphorylated by Fyn. Kinase assays performed with [γ-32P]ATP determined that counts incorporated into mut2-MAP-2c were slightly above the background values obtained from the kinase reaction with Fyn only, indicating that Tyr67 on MAP-2c is the only site phosphorylated by Fyn. The higher amount of radioactivity incorporated into mut1-MAP-2c relative to the WT MAP-2c is not totally unexpected, as a mutation of an adjacent phosphorylation site can increase the phosphorylation of the specific site, by making the site more accessible for phosphorylation.

The stimulus responsible for the binding of Fyn to MAP-2c and its subsequent tyrosine phosphorylation is not known. Fyn binds to tubulin via its SH2 and SH3 domains (28, 29); however, whether MAP-2c is bound and phosphorylated by Fyn when these proteins interact with tubulin, is not known. Previous studies determined that in vitro tyrosine phosphorylation of high molecular weight MAP-2 by the epidermal growth factor receptor kinase decreased MT polymerization and actin filament cross-linking activities of MAP-2 (26), however, it has not been determined whether epidermal growth factor directly phosphorylates MAP-2c or signals to Fyn in vivo. Furthermore, whether the MAP-2c/Fyn interaction enhances their dissociation or association with MTs remains to be elucidated. In the central nervous system, Fyn binds and phosphorylates several proteins associated with the cytoskeleton including p190 RhoGAP and Tau (15, 20, 29, 30, 31). Fyn is the only active non-receptor tyrosine kinase in early oligodendrocyte develop-
ment and shortened processed were observed in central nervous system cultures in which Fyn was inactivated (18). Fyn and MAP-2c are abundantly expressed early in neurons and oligodendrocytes, especially during process extension (8, 16–19, 32, 33). MAP-2 knock-out mice are viable, but have shortened processes (34). During synaptogenesis, MAP-2c is down-regulated; however, its expression continues in the olfactory system and in photosensitive cells of the adult retina, implying that MAP-2c might be important for plasticity in neural cells (35–38). Scaffolds between MAPs and Fyn likely form signaling complexes at specific locales such as dendrites, axons, and synapses and may function to fine-tune process outgrowth and cell-cell communication (15, 30, 31).

Grb2 is an adaptor protein that consists of a SH2 domain flanked by SH3 domains. SH2 domains bind proteins that contain a phosphorylated tyrosine as part of the motif and the amino acids following the phosphotyrosine residue are critical for the recruitment and binding of specific SH2 domain containing proteins (39–42). In the case of the SH2 domain of Grb2, the presence of asparagine at the +2 position following the phosphorylated tyrosine (pYXN), gives the selectivity. Grb2, the presence of asparagine at the +2 position following the phosphorylated tyrosine (pYXN), gives the selectivity. Grb2

MAP-2 in fetal brain, suggesting that additional phosphorylation of MAP-2c is small when compared with the overall abundance of the SH3 domain of Grb2, and that the interaction between the SH3 domain of Grb2 and MAP-2c is indirect.

MAP-2 synthetic peptides spanning the YSN motif in ELISAs demonstrated that the SH2 domain of Grb2 bound specifically to MAP-2 when the tyrosine within the motif was phosphorylated. The SH2-Grb2-GST fusion protein bound non-specifically to the unphosphorylated MAP-2 and irrelevant peptides only at high concentrations (0.2 and 0.1 μg/ml). SH2-Grb2 at concentrations of 0.5–0.013 μg/ml specifically bound the 1466-P MAP-2 phosphopeptide. Thus, the affinity of the interaction between the MAP-2-phosphorylated 1466-P peptide and the SH2 domain of Grb2 is high, and concentration dependent (50, 51). In conclusion, we postulate that in fetal human brain there is an intracellular signaling pathway that recruits Grb2 to MAP-2c following Fyn phosphorylation of Tyr(P)67 on MAP-2c. In addition, an indirect interaction between MAP-2c and the SH3 domain of Grb2 also exists. The significance of both Grb2/MAP-2c interactions warrants further study.

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