Peptide EphB2/CTF2 Generated by the γ-Secretase Processing of EphB2 Receptor Promotes Tyrosine Phosphorylation and Cell Surface Localization of N-Methyl-D-aspartate Receptors*

Jindong Xu1, Claudia Litterst1,2, Anastasios Georgakopoulos, Ioannis Zaganas3, and Nikolaos K. Robakis4

From the Center for Molecular Biology and Genetics of Neurodegeneration, Departments of Psychiatry and Neuroscience, Mount Sinai School of Medicine, New York, New York 10029

Presenilin 1, a protein involved in the development of familial Alzheimer disease, is an important functional component of the γ-secretase complex that processes many cell surface receptors including the EphB2 tyrosine kinase receptors (Litterst, C., Georgakopoulos, A., Shiio, J., Ghersi, E., Wisniewski, T., Wang, R., Ludwig, A., and Robakis, N. K. (2007) J. Biol. Chem. 282, 16155–16163). Recent evidence reveals that cytosolic peptides produced by the combined metalloproteinase/γ-secretase processing of cell surface proteins function in signal transduction and protein phosphorylation. Here we show that peptide EphB2/CTF2 released to the cytosol by the γ-secretase processing of EphB2 receptor, has tyrosine kinase activity, and directly phosphorylates the N-methyl-d-aspartate receptor (NMDAR) subunits in both cell lines and primary neuronal cultures. This phosphorylation occurs in the absence of Src kinases and is resistant to Src inhibitors revealing a novel pathway of NMDAR tyrosine phosphorylation independent of Src activity. EphB2/CTF2, but not a kinase-deficient mutant of EphB2/CTF2, promotes the cell surface expression of NMDAR. Because NMDAR plays central roles in synaptic plasticity and function, our results provide a potential link between the γ-secretase function of presenilin 1 and learning and memory.

N-Methyl-D-aspartate receptor (NMDAR) is a subtype of ionotropic glutamate receptors expressed in the mammalian central nervous system. Because of its high calcium permeability, this receptor plays crucial roles in the development of the central nervous system, neuroplasticity, and excitotoxicity. In the adult forebrain NMDAR consists mainly of NR1, NR2A, and NR2B subunits, and its functions are regulated by tyrosine phosphorylation events in these subunits. For example, up-regulation of NMDAR channel functions following subunit phosphorylation by the Src family of nonreceptor tyrosine kinases plays crucial roles in cellular signaling, learning, and memory. In addition, tyrosine phosphorylation of NMDAR is important in pathological conditions including stroke, epilepsy, and chronic pain (for review see Ref. 2).

Eph receptors constitute the largest family of transmembrane receptor tyrosine kinases. This family is divided into EphA and EphB subclases based on their ability to be activated by ephrinA and ephrinB ligands, respectively. EphB-ephrinB interactions have been implicated in brain ontogenesis, repulsive axonal guidance, and dendritic spine morphogenesis (reviewed in Refs. 3 and 4). Mounting evidence suggests that interactions between EphB receptors and NMDARs regulate synapse formation, maturation, and plasticity. For example, activation of EphB2 receptor (EphB2R) by ephrinB ligand leads to the formation of complexes between EphB2R and NMDAR, resulting in increased synaptogenesis (5). In contrast, EphB2R knock-out mice show reduced current activity of synaptic NMDAR and a decreased number of glutamate receptors in excitatory synapses (6, 7). Synaptic plasticity, e.g. long term potentiation and depression, is also impaired in these mice (6, 8). Other evidence suggests that the NR2B subunit is tyrosine-phosphorylated by Src kinases in response to EphB2R activation (9, 10).

PS1, a protein involved in the development of familial Alzheimer disease (FAD), is expressed in embryonic and adult brains where it is enriched in neurons (11, 12). Previous work revealed that PS1 is a necessary component of the proteolytic γ-secretase complex that promotes production of the Aβ peptides of Alzheimer disease amyloid by processing APP at the γ-cleavage sites (13, 14). Recent evidence, however, revealed that in addition to APP, the PS/γ-secretase system facilitates the proteolytic processing and signaling of many cell surface transmembrane proteins. Almost all γ-secretase substrates are first processed through a pathway that involves extracellular cleavages, usually by a metalloproteinase (MP), and shedding of their ectodomain, whereas the remaining membrane-associated fragments are cleaved at the epsilon site (ε-site) by the PS1/γ-

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secretase system to produce cytosolic peptides containing the cytoplasmic sequence of the receptor. Many of these peptides have important signal transduction properties including regulation of gene expression and protein phosphorylation (for review see Ref. 15).

Recently we reported that EphB2R is processed by the MP/γ-secretase system (1). This processing involves cleavage of the EphB2R extracellular domain close to the transmembrane sequence by MP ADAM10 (a disintegrin and metalloproteinase 10) to produce EphB2/N-terminal fragment that is released to the extracellular medium. The remaining membrane-bound C-terminal fragment, termed EphB2/CTF1, is further processed by the PS/γ-secretase system at the ε-site to release cytosolic peptide EphB2/CTF2 containing the cytoplasmic sequence of the receptor where its kinase activity resides (1). We reported recently that processing of EphB2R and production of peptide EphB2/CTF2 is stimulated by ephrinB ligands (1). Here we report that EphB2/CTF2 has tyrosine kinase activity, phosphorylates the NMDAR subunits in the absence of Src activity, and promotes their surface localization.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—Lactacystin, L-685,458, and NHSS-LC-biotin were purchased from Calbiochem. Polyclonal and monoclonal anti-EphB2R as well as anti-NR2B phosphoryrosine 1472 antibodies were obtained from Zymed Laboratories Inc.; anti-NR1 antibody was from BD Bioscience Pharmingen; anti-NR2A, NR2B, Src, and phosphoryrosine (clone 4G10) antibodies were from Upstate Biotechnology; anti-Src phosphoryrosine 418 was from Cell Signaling; and anti-histone 3 and anti-tubulin were from Santa Cruz Biotechnology. Magnesium/ATP mixture and recombinant active EphB2R C-terminal kinase were acquired from Upstate Biotechnology (catalog number 14-553) and [γ-32P]ATP was from PerkinElmer Life Sciences, and streptavidin-agarose was from Sigma.

**Recombinant Constructs and Cell Culture Transfection**—Retroviral EphB2R expression constructs were described previously (1). To generate FLAG-tagged EphB2/CTF2, the respective fragment was amplified from EphB2R-FLAG (1) using PCR (sense primer, 5’-gcgcattatggctgcatctgatg-3’; antisense primer, 5’-ctcttaagctttgcatctgttgtagtgcactacacgactcagactg-3’), phosphorylated, digested with BamHI, and then subcloned into the BamHI and the blunted EcoRI sites of pQCXIP retroviral vector. To generate a kinase-deficient FLAG-tagged EphB2/CTF2-K664M, EphB2 K664M (1) was used as template for PCR (same primer sequences as above). The PCR fragment was then dephosphorylated and subcloned into the BamHI and blunted EcoRI sites of pQCXIP vector. SYF cell line was purchased from ATCC, and HEK293T and SYF cell cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). Transient transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science) as per the manufacturer’s instruction.

**Primary Neuronal Culture and Transfection**—Cortical neuronal cultures were prepared from embryonic brains of Wistar rats (embryonic day 17–18) as described (18). Briefly, neocortices and hippocampi were dissected out, treated with trypsin, and mechanically dissociated. The neurons were suspended in neurobasal medium supplemented with B27 (Invitrogen) and plated on poly-d-lysine-coated 6-well dishes at 1 × 10^6 cells/well. For transfection with Nucleofector electroporation (Amaza), dissociated neurons were suspended in Nucleofector solution and electroporated with either adenovirus vector using rat neuron protocol O-003 as per the manufacturer’s instruction. Electroporated neurons were then plated in neurobasal medium supplemented with B27 and harvested at 7 DIV.

**Subcellular Fractionation**—Subcellular fractionation of transfected cells was performed as described (16). Briefly, the cells were harvested in a buffer of 10 mM Tris/HCl (pH 7.5), 0.25 mM sucrose, and 1 mM EDTA. Cell lysates were centrifuged at 500 × g for 10 min, and pellets (nuclear fraction) were solubilized by sonication in RIPA buffer. The supernatants were centrifuged at 120,000 × g for 45 min at 4 °C to separate the cytosolic and crude membrane fractions that were solubilized in 1% SDS. A 50-μg protein aliquot from each fraction was used in WB analysis.

**Cell Lysates, Immunoprecipitation (IP), and Western Blot Analysis**—The cells were lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 0.5% deoxycholate, 1% Brij35, 0.1% SDS, 1 mM EDTA) containing protease inhibitor mixture and phosphatase inhibitor mixture II (Sigma). For IP, solubilized proteins were incubated overnight at 4 °C with antibodies as indicated. The immune complexes were collected with protein G-Sepharose beads (Pierce) for 2 h at 4 °C. The bound proteins were washed three times with modified RIPA buffer and three times with MOPS buffer (20 mM MOPS, pH 7, 0.01% Brij). The blotting analysis was performed by probing with indicated antibodies. For separation of EphB2/CTF1 and EphB2/CTF2, 12% Protean II XL SDS-PAGE gels (Bio-Rad) were used.

**In Vitro Kinase Assay**—Purified proteins were immediately subjected to kinase assay (90% of the purified protein) or used for WB analysis (10% of the purified protein). Kinase reaction was started by addition of 3.3 μCi of [γ-32P]ATP, 10 mM MnCl₂, and magnesium/ATP mixture in kinase buffer (8 mM MOPS, pH 7, 0.2 mM EDTA). After 20 min of incubation at 30 °C, phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography. For phosphorylation of NR2B by EphB2R in vitro, a DNA sequence encoding C-terminal fragment of NR2B was amplified (forward primer, GAGGATCGGACCTCAGCTGTTC; and reverse primer, CCGCTCGAGACATCAGACTC) and subcloned into the BamHI/XhoI sites of pet30A. His-tagged NR2B C-terminal protein was synthesized in BL21DE3, and the bacteria were lysed in lysis buffer containing 50 mM Tris/HCl (pH 8.0), 250 mM NaCl, 10% (v/v) glycerol, and 1% (v/v) Triton X-100. The lysates were sonicated and cleared by centrifugation at 14,000 rpm for 30 min. NR2B C-terminal proteins were bound to nickel-nitrioltriacetic acid-agarose beads (Qiagen), washed with lysis buffer, and equilibrated in
MOPS buffer. Human kinase active EphB2R (amino acids 573 to the end, corresponding to mouse EphB2R) was purchased from Upstate Biotechnology (catalog number 14-553) and is only four amino acids shorter than EphB2/CTF2 (1). The recombinant NR2B C-terminal protein was then incubated with kinase active EphB2R in kinase buffer and subjected to SDS-PAGE and autoradiography as mentioned above.

Surface Biotinylation—The cells were incubated with 1 mg/ml SulfoNHS-LC-biotin in PBS at 4 °C for 30 min, washed with PBS and PBS with 0.1 M glycine followed by PBS, and lysed in modified RIPA buffer (see above). The cell extracts were incubated with 40 μl of streptavidin-agarose/reaction for 3–5 h. The bound proteins were washed three times with RIPA buffer and eluted with SDS sample loading buffer.

RESULTS

EphB2/CTF2 Is Localized in the Cytosol—We recently showed that EphB2R is processed sequentially by MP ADAM10 and the PS1/γ-secretase system to produce peptides EphB2/CTF1 and EphB2/CTF2, respectively, deriving from the C-terminal sequence of the receptor (1). To gain further insights into the potential functions of these cleavage products, we first analyzed their subcellular localization. HEK293T cell cultures transduced with EphB2R were treated with γ-secretase inhibitor L-685,458 and proteasome inhibitor LC to accumulate cleavage products EphB2/CTF1 and EphB2/CTF2 as described previously (1). Cell extracts from these cultures were fractionated into cytosolic, membrane, and nuclear fractions. The purity of nuclear and cytosolic fractions was tested with anti-histone 3 (panel IV) and anti-tubulin (panel III) antibodies, respectively. f. l. EphB2, full-length EphB2 receptor; DMSO, dimethyl sulfoxide.

PS/γ-Secretase Regulates Phosphorylation of NMDAR Subunits

FIGURE 1. Peptide EphB2/CTF2 is localized in the soluble fraction. EphB2R-transduced HEK293T cells were treated with 5 μM LC or L-685,458 (γ-inh., 1 μM) overnight as indicated. The cell extracts were fractionated into cytosolic (soluble), membrane, and nuclear fractions and then subjected to WB with monoclonal anti-EphB2R antibody (panels i and ii). The purity of nuclear and cytosolic fractions was tested with anti-histone 3 (panel IV) and anti-tubulin (panel III) antibodies, respectively. f. l. EphB2, full-length EphB2 receptor; DMSO, dimethyl sulfoxide.

CTF1 nor EphB2/CTF2 was found in the nuclear fractions (Fig. 1, panel II, lanes 7–9). These data suggest that PS1/γ-secretase cleavage releases EphB2/CTF2 from membrane to the cytosol. EphB2/CTF2 Released by PS1/γ-Secretase Cleavage Retains Its Kinase Activity—It is known that recombinant peptides containing the cytoplasmic C-terminal sequence of EphB2R display intrinsic kinase activity when prepared in Escherichia coli or other gene expression systems (20, 21). To examine whether peptide EphB2/CTF2 released from EphB2R by the γ-secretase cleavage retains its kinase activity, HEK293T cell cultures transfected with EphB2R were treated with LC and produced EphB2/CTF2 was then immunopurified and analyzed for kinase activity in vitro using [γ-32P]ATP. Obtained products were analyzed for EphB2 protein using anti-EphB2 receptor antibodies (left panel) and for incorporation of [γ-32P]ATP (right panel). B, HEK293T cells were transfected with EphB2/CTF2 or vector (V). EphB2/CTF2 was i.p. from soluble fraction of cell extracts, and IPs were probed in vitro for kinase activity using [γ-32P]ATP. The obtained products were analyzed for EphB2 receptor (left panel) and for [γ-32P]ATP activity (right panel) as above. C, HEK293T cells were transfected with vector alone (V), EphB2/CTF2, or kinase-deficient EphB2/CTF2-K664M (KD). EphB2/CTF2 peptides were IPed, and the obtained IPs were probed on WBs using anti-phosphotyrosine (right panel) or anti-EphB2R (left panel) antibodies. The faster migration of the KD mutant (EphB2/CTF2-KD) is likely due to the lack of massive autophosphorylation.

EphB2/CTF2 Released by PS1/γ-Secretase Cleavage Retains Its Kinase Activity—It is known that recombinant peptides containing the cytoplasmic C-terminal sequence of EphB2R display intrinsic kinase activity when prepared in Escherichia coli or other gene expression systems (20, 21). To examine whether peptide EphB2/CTF2 released from EphB2R by the γ-secretase cleavage retains its kinase activity, HEK293T cell cultures transfected with EphB2R were treated with LC and produced EphB2/CTF2 was then immunopurified and analyzed for kinase activity in vitro using [γ-32P]ATP (see “Experimental Procedures”). Fig. 2A shows that isolated EphB2/CTF2 is phosphorylated in our assays. No phosphorylation is observed in similarly prepared samples from vector-transfected cultures (Fig. 2A). These data show that peptide EphB2/CTF2 can be phosphorylated in vitro and suggest that EphB2/CTF2, the product of the γ-secretase cleavage of EphB2R, retains its kinase activity after it is released to the cytosol. To further examine the kinase activity of this peptide, we took advantage of the exact γ-secretase cleavage site of the EphB2R (1) to prepare

FIGURE 2. Peptide EphB2/CTF2 released by the γ-secretase cleavage of EphB2R retains its kinase activity. A, EphB2R-transduced (EphB2) or vector-transduced (V) HEK293T cells were treated with 5 μM LC overnight. EphB2/CTF2 was IPed from the soluble fraction of cell extracts, and the obtained IPs were probed in vitro for kinase activity using [γ-32P]ATP. Obtained products were analyzed for EphB2 protein using anti-EphB2 receptor antibodies (left panel) and for incorporation of [γ-32P]ATP (right panel). B, HEK293T cells were transfected with EphB2/CTF2 or vector (V). EphB2/CTF2 was IPed from soluble fraction of cell extracts, and IPs were probed in vitro for kinase activity using [γ-32P]ATP. The obtained products were analyzed for EphB2 receptor (left panel) and for [γ-32P]ATP activity (right panel) as above. C, HEK293T cells were transfected with vector alone (V), EphB2/CTF2, or kinase-deficient EphB2/CTF2-K664M (KD). EphB2/CTF2 peptides were IPed, and the obtained IPs were probed on WBs using anti-phosphotyrosine (right panel) or anti-EphB2R (left panel) antibodies. The faster migration of the KD mutant (EphB2/CTF2-KD) is likely due to the lack of massive autophosphorylation.
a cDNA construct that expresses recombinant EphB2/CTF2 (rEphB2/CTF2). This peptide, which contains EphB2R residues 569–792 (1), was then overexpressed in HEK293T cells. Similar to the subcellular localization of peptide EphB2/CTF2 generated in vivo by the γ-secretase cleavage of EphB2R, rEphB2/CTF2 was also located in the cytosol (data not shown), and following immunopurification it was autophosphorylated in our in vitro kinase assays (Fig. 2B), corroborating previous findings that peptides containing the C terminus of EphB2R retain the kinase activity of the receptor (20, 21).

In a different approach, we used WBs and anti-phosphotyrosine antibodies to analyze the in vivo tyrosine phosphorylation of rEphB2/CTF2. As a negative control, we included a kinase-deficient (KD) EphB2/CTF2 construct that harbors mutation K664M in the kinase domain. This mutant contains all of the potential tyrosine phosphorylation sites that may be phosphorylated in trans, but it cannot be autophosphorylated because of the mutation (1). Construct rEphB2/CTF2K664M migrates faster than wild type (WT) rEphB2/CTF2 (Fig. 2C, left panel, compare lanes 2 and 3), probably because of the lack of autophosphorylation (22). Fig. 2C shows that cell extract from cultures expressing WT rEphB2/CTF2, but not extract from cultures expressing the KD variant rEphB2/CTF2-K664M, contains EphB2/CTF2 that is tyrosine-phosphorylated (Fig. 2C, right panel, lanes 2 and 3). Taken together, our data show that peptide EphB2/CTF2 produced by the γ-secretase cleavage of full-length EphB2R maintains the tyrosine kinase activity of the receptor.

EphB2/CTF2 Phosphorylates NMDAR Subunits—It has been shown that co-expression of EphB2R with NMDAR subunit NR2B in HEK293T cells is sufficient to induce tyrosine phosphorylation of NR2B (9). Because our data indicate that EphB2/CTF2 has tyrosine kinase activity, we investigated whether rEphB2/CTF2 is able to phosphorylate NMDAR subunits. Fig. 3A shows that co-expression of rEphB2/CTF2 and NMDAR subunit NR2B in HEK293T cells was sufficient to induce tyrosine phosphorylation of this subunit. Similarly, rEphB2/CTF2 was able to promote tyrosine phosphorylation of subunit NR2A (Fig. 3B). Neither NR2A nor NR2B were phosphorylated when either the KD rEphB2/CTF2-K664M or control vectors were co-expressed with NMDAR subunits (Fig. 3, A, lanes 1 and 2, and B, lanes 1 and 3). Similar observations were made for NMDAR subunit NR1 that was tyrosine-phosphorylated in cell cultures overexpressing rEphB2/CTF2 but not in control cells transfected with vectors or with the KD rEphB2/CTF2 (Fig. 3C, lanes 1 and 3, respectively). It has been shown that tyrosine 1472 is the major tyrosine phosphorylation site of NR2B and that phosphorylation at this site correlates with the physiolog-
co-IP experiments. Furthermore, co-IPed NMDAR subunits were consistently tyrosine-phosphorylated in the presence of exogenous WT rEphB2/CTF2 but not in the presence of exogenous KD rEphB2/CTF2 or in vector transfected cultures (Fig. 3, D and E). These results show that functional NMDAR subunits can be tyrosine-phosphorylated by EphB2/CTF2.

Our data raise the possibility that the PS1/γ-secretase system contributes to the EphB2R-dependent phosphorylation of NMDAR by promoting the cleavage of EphB2R and release of peptide EphB2/CTF2. To examine this possibility, we tested the effect of γ-secretase inhibitor L-685,458 on the tyrosine phosphorylation of NMDAR subunits. Co-expression of EphB2R with subunit NR1 resulted in the phosphorylation of the subunit (Fig. 4A, lane 2). However, in the presence of γ-secretase inhibitor L-685,458, phosphorylation of NR1 was clearly decreased (Fig. 4A, lane 3). Similarly, overexpression of EphB2R stimulated tyrosine phosphorylation of subunit NR2B (Fig. 4B, lane 2), a process inhibited by γ-secretase inhibitors (Fig. 4B, lane 3). In contrast, these inhibitors had no effect on the rEphB2/CTF2-dependent phosphorylation of NR2B subunits.

FIGURE 4. γ-Secretase inhibitor decreases the EphB2R-induced tyrosine phosphorylation of NMDAR subunits. A, HEK293T cells were transfected with EphB2R (EphB2) or vector (V) along with NR1. The cells were co-IPed from cell extracts, and the obtained IPs were probed on WBs using anti-phosphotyrosine or anti-NR1 antibodies. B, HEK293T cells transfected with EphB2R (EphB2) or vector (V) along with NR2B were treated overnight with either dimethyl sulfoxide or L-685,458 (γ-inh., 1 μM) and NR2B was IPed from cell extracts. IPs were probed on WBs with anti-phosphotyrosine (middle panel) or anti-NR2B (top panel) antibodies. The inset shows the statistical analysis from three independent experiments. *p < 0.05 (Student’s t test). The cell extracts were also probed with anti-EphB2R antibodies to show accumulation of EphB2/CTF1 in the presence of γ-secretase inhibitor (bottom panel). DMSO denotes controls. C, HEK293T cells were co-transfected with NR2B and EphB2R/CTF2. KD EphB2R/CTF2 (KD), or vector alone (V) as indicated. The cultures were then treated overnight with either dimethyl sulfoxide or L-685,458 (γ-inh., 1 μM), and the cell extracts were IPed with anti-NR2B antibody. The obtained IPs were analyzed on WB with anti-phosphotyrosine or anti-NR2B antibodies. γ-Secretase inhibitor had no effect on EphB2R/CTF2-induced tyrosine phosphorylation (lanes 2 and 3).
tion, the Src-induced phosphorylation of NR2B was significantly inhibited by Src inhibitor SU6656. Decreased Src activity in the presence of SU6656 is also indicated by the reduced phosphorylation of Src residue Tyr418 (Fig. 5B, right panels). These data indicate that EphB2/CTF2 is able to phosphorylate NR2B subunit independent of Src kinases, a suggestion further supported by experiments in HEK293T cells where Src inhibitor SU6656 was unable to inhibit the EphB2/CTF2-induced tyrosine phosphorylation of NR2B (Fig. 5B). To further examine the ability of EphB2/CTF2 to phosphorylate directly subunit NR2B, we used a commercially available kinase, termed EphB2 kinase, derived from the cytoplasmic sequence of EphB2R (see “Experimental Procedures”). EphB2 kinase was able to phosphorylate the C terminus of NR2B in an in vitro assay (Fig. 5C), suggesting that the free cytoplasmic domain of EphB2R is able to directly phosphorylate this NMDAR subunit. Together, these data indicate that the γ-secretase product EphB2/CTF2 is able to directly phosphorylate the NR2B subunit of the NMDAR.

EphB2/CTF2 Phosphorylates Endogenous NMDARs in Primary Neurons—To examine the potential role of γ-secretase on the tyrosine phosphorylation of endogenous neuronal NMDARs, we treated rat cortical neuronal cultures of either 7 or 14 DIV with γ-secretase inhibitors and monitored the tyrosine phosphorylation of NR2B. Fig. 6A shows that tyrosine phosphorylation of this NMDAR subunit was significantly decreased in the presence of γ-secretase inhibitors (Fig. 6A). In contrast, phosphorylation of Src kinase was not altered by γ-secretase inhibitors (Fig. 6A), indicating that the effect of γ-secretase inhibitor on the phosphorylation of NR2B is specific and independent of Src activity. These data show that inhibition of γ-secretase activity decreases the tyrosine phosphorylation of NR2B, possibly by inhibiting processing of EphB2R and production of EphB2/CTF2. We next asked whether EphB2/CTF2 may indeed promote tyrosine phosphorylation of endogenous neuronal NMDAR. To this end, we overexpressed EphB2/CTF2 in rat primary neurons (see “Experimental Procedures”) and monitored phosphorylation of NR2B 7 days later. We observed a significant increase in NR2B tyrosine phosphorylation in EphB2/CTF2-transfected neurons compared with vector-transfected con-

**FIGURE 5.** EphB2/CTF2 directly phosphorylates NMDARs. A, SYF cells were co-transfected with NR2B and either vector (V, left panels, lane 1), rEphB2/CTF2 (left panels, lanes 2 and 3), or Src (right panels). The cells were treated with dimethyl sulfoxide (−) or 2 μM SU6656 (+) for 2 h. NR2B was then IPed from cell extracts, and the obtained IPs were probed on WBs with anti-phosphotyrosine and anti-NR2B antibodies. The input panels show the expression of transfected EphB2/CTF2 (left panel) and Src (upper right panel) as well as the Src kinase autophosphorylation (lower right panel). Histograms show the quantification of NR2B tyrosine phosphorylation from three independent experiments. *p < 0.05 (Student’s t test). B, HEK293T cells were transfected with rEphB2/CTF2 or vector (V) along with NR2B as indicated. The cells were treated with 2 μM SU6656 for different periods of time. NR2B was IPed, and the obtained IPs were probed with anti-NR2B and anti-phosphotyrosine antibodies. The input panels show the levels of transfected EphB2/CTF2. The histogram shows the quantification of NR2B tyrosine phosphorylation from three independent experiments. C, His-tagged intracellular C terminus of NR2B (NR2B C-term) was expressed in E. coli and purified using nickel-nitrilotriacetic acid-agarose beads (see “Experimental Procedures”). 2 μg of recombinant NR2B C-terminal protein was incubated with or without kinase active EphB2R (EphB2 kinase, see “Experimental Procedures”) in the presence of (γ-32P)ATP. 32P-Labeled NR2B C-terminal protein was separated by SDS-PAGE and detected by autoradiography. Upper panel, autoradiogram of 32P-labeled NR2B C-terminal protein. Lower panel, Coomassie Blue staining of NR2B C-terminal proteins.
expression is an indicator of the surface expression of the receptor (35–37). As shown in Fig. 7A, the levels of cell surface NR1 increased significantly in the presence of exogenous WT rEphB2/CTF2. In contrast, KD mutant rEphB2/CTF2-K664M was unable to increase cell surface NR1. These data indicate that EphB2/CTF2 promotes the cell surface expression of NMDAR, possibly by stimulating the tyrosine phosphorylation of its subunits.

We then tested whether EphB2/CTF2 is able to increase the surface localization of NMDAR in primary neurons. Rat cortical neurons were transfected with vector or EphB2/CTF2, and cell surface proteins were biotinylated at 7 DIV. Similar to the data obtained in cell lines, EphB2/CTF2 overexpression significantly increased surface localization of NR1 in primary neurons (Fig. 7B, panels I and II). As a negative control, cytoplasmic protein tubulin was not biotinylated (Fig. 7B, panels III and IV). Together, the above data suggest that the product of the y-secretase cleavage of EphB2R promotes the cell surface localization of NMDAR.

**DISCUSSION**

Recent reports show that the EphB2R tyrosine kinase is processed first by MPs, including ADAM10, and then by y-secretase to release cytosolic peptide EphB2/CTF2 which contains the entire cytoplasmic sequence of the receptor where its kinase activity resides (1, 38). This processing of EphB2R is stimulated by ephrinB ligands but is inhibited by FAD-linked mutations of PS1, an important functional component of the y-secretase complex (1). EphB2R interacts functionally with NMDAR and stimulates its tyrosine phosphorylation through the activity of Src kinases (5, 9). Tyrosine phosphorylation of NMDARs has been associated with increased receptor cell surface expression and enhanced channel activity. For over a decade, the Src family of kinases, especially Src and Fyn, have been identified as the only tyrosine kinases that directly phosphorylate NMDARs. This phosphorylation is considered of primary importance for many physiological and pathological conditions as well as for the convergence of signaling pathways that regulate the functions of NMDAR (2). Here we report the novel observation that in HEK293T cells, peptide EphB2/CTF2, the product of the MP/y-secretase processing of EphB2R, is able to directly phosphorylate the NMDAR subunits at tyrosine resi-
Multiple tyrosine residues at the cytosolic sequence of NMDAR subunits NR2A and NR2B are phosphorylated by the Src family of kinases. Although different studies have yielded different phosphorylation profiles of these tyrosines, NR2B tyrosine residue 1472 is consistently phosphorylated in vivo (23). In addition, this phosphorylation event correlates with increased cell surface localization of the NMDAR where it functions as a calcium channel (33, 39, 40). Interestingly, we found that NR2B Tyr^{1472} is phosphorylated by EphB2/CTF2, suggesting a potential role of EphB2/CTF2 in regulating cell surface expression of NMDAR. The cytoplasmic domain of NMDAR subunit NR1 contains only one tyrosine residue at position 837, but it remains unclear whether this tyrosine is phosphorylated. It has been suggested, however, that phosphorylation of this tyrosine by Src kinases may be important for NMDAR trafficking (32). Our data show that peptide EphB2/CTF2 stimulates tyrosine phosphorylation of NR1, suggesting that this phosphorylation takes place at tyrosine 837. Accordingly, we obtained data that mutation of this tyrosine to phenylalanine inhibits the EphB2/CTF2-induced tyrosine phosphorylation of NR1 (data not shown). Our data show that in addition to NR2B and NR1, peptide EphB2/CTF2 is also able to promote phosphorylation of subunit NR2A. The functional significance of the tyrosine phosphorylation of the NMDAR subunits by EphB2/CTF2 awaits further investigation. We obtained evidence, however, that in both primary neuronal cultures and cell lines, EphB2/CTF2 increases cell surface expression of NMDAR, a process promoted by the tyrosine phosphorylation of its subunits (31–34). Together our observations suggest that peptide EphB2/CTF2, the product of the γ-secretase processing of EphB2R, stimulates cell surface expression of the NMDAR by promoting phosphorylation of its subunits.

Our data that peptide EphB2/CTF2 stimulates phosphorylation and cell surface localization of NMDAR supports the suggestion that by promoting processing of EphB2R and production of EphB2/CTF2, the PS/γ-secretase system may regulate the channel activity as well as cell surface localization and ultimately the function of NMDAR, a critical factor involved in memory formation. Furthermore, abnormalities in the cell surface localization of NMDAR and synaptic deficiencies have been implicated in the development of Alzheimer disease (for review see Ref. 41). Interestingly, it has been shown that γ-secretase processing of EphB2R and production of EphB2/CTF2 is inhibited by FAD-linked mutations of PS1 (1), an important functional component of γ-secretase. Together these observations suggest a mechanism by which PS FAD mutations may affect NMDAR localization, synaptic function, and memory by interfering with the processing of EphB2R and production of the tyrosine kinase activity-containing peptide EphB2/CTF2. Further work will be needed to examine the potential involvement of these pathways in the memory dysfunctions associated with Alzheimer disease.

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