Ligand Binding and Calcium Influx Induce Distinct Ectodomain/γ-Secretase-processing Pathways of EphB2 Receptor

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Binding of EphB receptors to ephrinB ligands on the surface of adjacent cells initiates signaling cascades that regulate angiogenesis, axonal guidance, and neuronal plasticity. These functions require processing of EphB receptors and removal of EphB-ephrinB complexes from the cell surface, but the mechanisms involved are poorly understood. Here we show that the ectodomain of EphB2 receptor is released to extracellular space following cleavage after EphB2 residue 543. The remaining membrane-associated fragment is cleaved by the presenilin-dependent γ-secretase activity after EphB2 residue 569 releasing an intracellular peptide that contains the cytoplasmic domain of EphB2. This cleavage is inhibited by presenilin 1 familial Alzheimer disease mutants. Processing of EphB2 receptor depends on specific treatments: ephrinB ligand-induced processing requires endocytosis, and the ectodomain cleavage is sensitive to peptide inhibitor N-benzoyloxycarbonyl-Val-Leu-leucinal but insensitive to metalloproteinase inhibitor GM6001. The ligand-induced processing takes place in endosomes and involves the rapid degradation of the extracellular EphB2. EphrinB ligand stimulates ubiquitination of EphB2 receptor. Calcium influx- and N-methyl-D-aspartic acid-induced processing of EphB2 is inhibited by GM6001 and ADAM10 inhibitors but not by N-benzoyloxycarbonyl-Val-Leu-leucinal. This processing requires no endocytosis and promotes rapid shedding of extracellular EphB2, indicating that it takes place at the plasma membrane. Our data identify novel cleavages and modifications of EphB2 receptor and indicate that specific conditions determine the proteolytic systems and subcellular sites involved in the processing of this receptor.

The Ephrin (Eph)² receptors are the largest family of receptor tyrosine kinase proteins. They bind membrane ligand proteins, called ephrins, on adjacent cells forming multimeric clusters that bridge juxtaposed cells. These binding interactions trigger signaling cascades in both the receptor-expressing cells (forward signaling) and the ligand-expressing cells (reverse signaling) stimulating functions that modulate cell morphogenesis, tissue patterning, and angiogenesis (1–4). In the developing central nervous system, binding of ephrin ligands to Eph receptors regulates axon guidance and synapse formation (5, 6). Paradoxically, depending on specific conditions such as the expression levels of Eph receptors and their ligands, signaling events initiated by the Eph-ephrin interactions can lead either to increased cell-cell adhesion or to repulsion and separation of the involved cells (4). In the adult brain, the Eph-ephrin systems regulate memory-related functions, including synaptic structure and long term potentiation (5, 7, 8). There are two subclasses of Eph receptors, EphA and EphB, which are selectively activated by ephrinA and ephrinB ligands, respectively, although exceptions to this rule have been observed (4).

The EphB-ephrinB system regulates the development of many tissues, including the vasculature and the central nervous system, where EphB-ephrinB interactions control axonal path-finding and dendritic spine morphogenesis (reviewed in Refs. 5 and 6). Furthermore, there is evidence that EphB-ephrinB binding regulates the function of excitatory synapses and synaptic plasticity by initiating forward signaling cascades that regulate phosphorylation of Src kinases and N-methyl-D-aspartic acid (NMDA) receptor (NMDAR) activity (5, 7, 9). The cytosolic region of EphB receptors contains a tyrosine kinase domain (10) and a highly conserved sequence motif that is a major site for autophosphorylation by the EphB tyrosine kinase. This EphB motif interacts with Src-homology domain-containing factors, including Ras GTPase-activating proteins and Src kinases (4).

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‡ The abbreviations used are: ADAM, a disintegrin and metalloproteinase; BACE, β-secretase; CTF, C-terminal fragment; NTF, N-terminal fragment; Eph, ephrin receptor; FAD, familial Alzheimer disease; LC, lactacystin; MP, metalloproteinase; NMDA, N-methyl-D-aspartic acid; NMDAR, NMDA receptor; PS, presenilin; WB, Western blot; ZVLL, N-benzoyloxycarbonyl-Val-Leu-leucinal; APP, amyloid precursor protein.
Recent reports indicate that mechanisms by which adhesive and signaling interactions between EphB2 receptor and ephrinB2 ligands are terminated include endocytosis of the cell surface EphB-ephrinB complexes and cleavage of the ectodomain of ephrinB ligands (11–13). These processes reduce contact between the receptor and ligand-expressing cells and convert the initial cell-cell adhesion into a repulsion mediated by Rac signaling and actin polymerization. Ligand-induced endocytic vesicles internalized in the EphB-expressing cells contain EphB-ephrinB complexes and surrounding membranes deriving from both cells, but the fate of the internalized protein complex is not clear (11, 12). Like other substrates of the metalloproteinase (MP)/γ-secretase system (14), Eph receptors are cell surface proteins containing an extensive extracellular (ectodomain) region, a transmembrane sequence, and a cytoplasmic domain. We thus asked whether a MP/γ-secretase system participates in the processing of EphB2 receptor and its internalized complexes. Here we present evidence that two distinct pathways, one stimulated by calcium influx and the other by ephrinB2 ligands, regulate proteolytic processing of EphB2 receptor and its complexes. Both pathways involve a cleavage within the extracellular domain and processing of the remaining membrane bound fragment by the presenilin1 (PS1)-dependent γ-secretase system. Only ephrinB ligands, however, stimulate ubiquitination of EphB2 receptor.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—The γ-secretase inhibitor L-685,458, lactacystin (LC), bafilomycin, and epoxomicin were obtained from Calbiochem. MP inhibitor GM6001 was from Chemicon International and chloroquine from Sigma. Goat anti-human-Fc was obtained from Jackson ImmunoResearch Laboratories. Monoclonal antibody 33B10 against the C-terminal fragment of PS1 (PS1/CTF) and polyclonal antisera 222 against the N-terminal fragment of PS1 (PS1/NTF) have been described (15). Polyclonal and monoclonal anti-EphB2 antibodies were purchased from Zymed Laboratories (San Francisco, CA). Anti-N-cadherin (C32) and anti-phospho-tyrosine (clone 4G10) antibodies were from BD Biosciences and Upstate Biotechnology (Charlottesville, VA), respectively. Anti ubiquitin antibody P4D1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated antibodies against extracellular EphB2 and recombinant mouse ephrin-B2/Fc Chimera were from R&D Systems (Minneapolis, MN). Streptavidin-Cy3 conjugate and anti-FLAG M2 affinity gels were obtained from Sigma.

**Recombinant Plasmids and Constructs**—Murine EphB2 cDNA was kindly provided by Dr. G. Yancopoulos (Regeneron Pharmaceuticals). For retroviral gene expression, EphB2 cDNA was subcloned into the XhoI sites of pMX-IRES-GFP (provided by Dr. Kitamura, Tokyo, Japan). PMX/EphB2 construct carrying mutation K664M was generated by site-directed mutagenesis (sense primer, 5′-catcaaggctctggccctggagagag-3′; antisense primer, 5′-ctcgggtcatcggctgatgctg-3′), and mutation was verified by sequencing. The sequence encoding a FLAG tag was inserted at the end of the EphB2 cDNA, and the resulting FLAG-EphB2 sequence was subcloned into the NotI sites of pQCXIP (Clontech).

**Mouse Embryo Preparation, Cell Cultures, Transfections, and Transduction**—PS1+/+ and PS1−/− mouse embryos were collected at embryonic day 16 (E16), and brains were solubilized in radioimmune precipitation assay buffer as described (16). Eighty micrograms of extract was analyzed on Western blots (WBs). Primary neuronal cultures were prepared from E16 rat brains as described (17). Neurons were maintained 8 days in vitro in NeuroBasal medium (Invitrogen). Fibroblasts from PS1+/+ or PS1−/− mice were as described (16). Transient transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science). Retroviral gene expression was performed as described (18).

**Treatment with Inhibitors and Clustering of Ligand**—Inhibitors were used at the following concentration in the indicated vehicle: L-685,458 (0.5 μM in Me2SO), ionomicin (2.5 μM in Me2SO), GM6001 (25 μM in Me2SO), ZVLL (30 μM in Me2SO), LC (10 μM in water), chloroquine (100 μM in water), epoxomicin (5 μM in Me2SO), bafilomycin (1.0 μM in Me2SO), monodansylcadaverine (125 μM in Me2SO and ethanol 1:1). Clustering of recombinant mouse ephrin-B2/Fc ligands and anti-Fc were performed as described and used at 1 μg/ml ephrin-B2/Fc for stimulation (19, 20).

**Cell Lysates, Immunoprecipitation, SDS-PAGE, and Immunoblotting**—Cell lysates for Western blotting (WB) were prepared in SDS lysis buffer (100 mM Tris/HCl, 20 mM NaCl, 10 mM EGTA, 10 mM EDTA, 1% SDS) containing complete protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixture I (Sigma). For immunoprecipitation, cells were lysed in immunoprecipitation buffer (20 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Triton X-100 plus protease inhibitor, and phosphatase inhibitor mixtures). Lysates were processed for immunoprecipitation as described (15). For separation of EphB2/CTF1 and EphB2/CTF2, 12% Protein II XL SDS-PAGE gels (Bio-Rad) were used. EphB2 fragments CTF1 and CTF2 were purified for sequencing from EphB2-transfected HEK293T cultures in the absence of ephrinB ligand.

**RESULTS**

**EphB2 Is Cleaved by PS1-dependent γ-Secretase**—To investigate whether EphB2 receptor is processed by the γ-secretase system, we used WB to probe embryonic brain extract from PS1 null (PS1−/−) and WT (PS1+/+) mice. Antibodies against the cytoplasmic sequence of EphB2 receptor detected a peptide of an apparent molecular mass of ~50 kDa (designated EphB2/CTF1) in the brains of PS1−/− but not in the brains of PS1+/+ mice (Fig. 1A). The immunoreactivity and apparent molecular mass of this peptide suggest that it is derived from EphB2 receptor and may contain all of its cytoplasmic and transmembrane sequences. Accumulation of EphB2/CTF1 in PS1 null mice suggests that this peptide is metabolized by the PS1-dependent γ-secretase system and may be produced following cleavage of the extracellular region of EphB receptor similar to a number of type I transmembrane proteins (14). A peptide similar to brain EphB2/CTF1 accumulates in EphB2 receptor-transfected PS1−/−, but not in PS1+/+, cells (Fig. 1B), further confirming the identity of EphB2/CTF1 and its degradation by the PS-γ-secretase system.
Ectodomain/γ-Secretase Cleavages of EphrinB2 Receptor

To detect the fragment expected from the γ-secretase cleavage of EphB2/CTF1 (termed EphB2/CTF2), we employed the in vitro γ-secretase assay (17, 21) using membranes from EphB2-transfected PS1−/− and PS1+/+ fibroblasts. As expected, EphB2/CTF2 is produced in membranes from PS1+/+ but not from PS1−/− cells (Fig. 1C, lanes 2 and 4), and its production is sensitive to γ-secretase inhibitor L-685,458 (γ-inh.) overnight as indicated, and cell extract was analyzed on large gels as in C, E, conditioned media (50 μl) from overnight HEK293T cultures transduced with vector (V) or EphB2 were probed with an antibody against the ectodomain sequence of EphB2. Numbers at the right indicate apparent molecular mass in kilodaltons.

FIGURE 1. EphB2 receptor is cleaved by the PS1-dependent γ-secretase system. A, brain tissue extract from PS1 knock-out (PS1−/−, lane 1) or wild type (PS1+/+, lane 2) mouse embryos were prepared in the presence of protease inhibitors (see “Experimental Procedures”) and then probed on Western blots either with anti-cytoplasmic EphB2 antibodies (upper two panels) or anti-PS1/NTF antibodies 222 (lower panel). Arrows identify full-length EphB2 (f.l. EphB2), EphB2/CTF1, and PS1/NTF peptides as indicated. B, EphB2-transfected PS1−/− or PS1+/+ mouse fibroblasts were analyzed by WB using anti-cytoplasmic EphB2 antibody (upper panels) or anti-PS1 antibody 33810 (lower panel). C, membranes from PS1−/− or PS1+/+ mouse fibroblasts transduced with pMX/EphB2 were incubated at 37 °C in the presence or absence of γ-secretase inhibitor L-685,458 (γ-inh.) as indicated (see also “Experimental Procedures”). To resolve the EphB2 fragments, proteins were separated on large gels and probed on WB with monoclonal anti-EphB2 antibody. D, EphB2-transduced HEK293T cells were treated with LC or L-685,458 (γ-inh.) overnight as indicated, and cell extract was analyzed on large gels as in C, E, conditioned media (50 μl) from overnight HEK293T cultures transduced with vector (V) or EphB2 were probed with an antibody against the ectodomain sequence of EphB2. Numbers at the right indicate apparent molecular mass in kilodaltons.

FIGURE 2. Identification of the proteolytic processing sites of EphB2 receptor. A, schematic view of the ectodomain and transmembrane cleavage sites of EphB2 receptor. Sequence numbers denote amino acid residues of full-length EphB2 receptor, including the leader peptide. MP, metallopeptinase; TM, transmembrane domain; γ-cleavage, site of epsilon cleavage catalyzed by the γ-secretase activity. B, effects of PS1 FAD mutants on EphB2/CTF2. PS1−/− fibroblasts were transduced as described (18) with EphB2 and either vector, WT, or mutant PS1 as indicated. Cells were collected and fractionated. Obtained membranes were incubated and produced EphB2/CTF2 peptide was detected as in Fig. 1C. Levels of EphB2/CTF2 were determined by densitometric quantitation and normalized to EphB2/CTF2 produced from WT PS1-transduced cells. Bars represent the mean ± S.E. of three independent experiments.

Determination of the Processing Sites of EphB2 Receptor—EphB2/CTF1 and EphB2/CTF2 were affinity-purified from HEK293T cells overexpressing EphB2 receptor (Fig. 1D). Edman sequencing of EphB2/CTF1 through 10 cycles showed the following major sequence: SIKKELPLIV. This is a unique sequence that corresponds to mouse EphB2 residues 544–553. These data show that the ectodomain cleavage site of EphB2 receptor is located seven amino acids upstream of its predicted transmembrane sequence (Fig. 2A). Thus, the 50-kDa EphB2/CTF1 is produced by a cleavage within the ectodomain region of EphB2 receptor after residue 543 (Fig. 2A, MP site) and contains the 451 C-terminal residues of EphB2 receptor, including all transmembrane and cytoplasmic sequence (Fig. 2A). Mass spectrometric analysis of lysine-C digests of EphB2/CTF2 (48.3 kDa) yielded peptide AIVCNRRGERADESTD (m/z 2287.00, +0.07). This is a unique sequence corresponding to mouse EphB2 receptor residues 570–588 located at the cytoplasmic juxtamembrane region of the receptor. The mass spectrometric analysis of EphB2/CTF2 yielded additional peptides all derived from the cytoplasmic sequence of EphB2 receptor (not shown). These results show that the γ-secretase-dependent cleavage of EphB2 receptor takes place between residues 569 and 570, three amino acids upstream of the C-terminal end...
of the predicted transmembrane sequence (Fig. 2A). This cleavage produces peptide EphB2/CTF2 containing the 425 C-terminal amino acids of EphB2 receptor (Fig. 2A). The close proximity of this cleavage site to the membrane/cytosol interface indicates it is analogous to the γ-secretase-catalyzed epsilon (e)- cleavage sites observed in many PS-γ-secretase substrates (23).

**PS1 Familial Alzheimer Disease Mutants Inhibit the γ-Secretase Cleavage of EphB2/CTF1**—Recent evidence shows that PS1 FAD mutations may inhibit the PS1-dependent e-cleavage of many substrates suggesting that FAD mutations cause a loss of cleavage function at this site (17, 18, 24). Fig. 2B shows that several PS1 FAD mutants, including P117L, ΔE9, A260V, P264L, and E280G as well as the γ-secretase-negative dominant negative PS1 mutant D257A (25), are impaired in their ability to stimulate production of EphB2/CTF2 compared with WT PS1. PS1 FAD mutants A246E and M146L, however, showed no inhibition in their ability to increase EphB2/CTF2. These data show that certain PS1 FAD mutations interfere with the ability of PS1 to mediate cleavage of EphB2/CTF1 at the e-site.

**Calcium Influx and Ligand Binding Stimulate Distinct Processing of EphB2 Receptor**—Calcium influx stimulates a MP/γ-secretase processing of transmembrane proteins (26–28). To investigate the effect of calcium influx on the processing of EphB2, cell cultures expressing this receptor were treated with the calcium ionophore ionomycin as described (26). This treatment resulted in the rapid decrease of full-length EphB2 and in a concomitant increase of both cellular EphB2/CTF1 and medium EphB2/NTF (Fig. 3, left panel), suggesting that extracellular EphB2 is rapidly released to the conditioned media following cleavage of cell surface receptor. The half-life of EphB2 in the presence of ionomycin was <10 min, and by 15 min most receptor was degraded (Fig. 3, lanes 1–5).

To further explore the physiological significance of the EphB2 processing, we asked whether it is regulated by ligand-receptor interactions. To this end, we treated our cultures with a clustered ephrinB2-Fc construct containing the extracellular domain of ephrinB2 ligand fused to Fc portion of IgG. This construct binds to and activates the EphB2 receptor mimicking the ligand effects (19). Following 2 h of treatment, the levels of cellular full-length receptor decreased while EphB2/CTF1 increased suggesting that ephrinB ligand stimulates the extra-
cellular cleavage of EphB2. By 4 h of ligand treatment, most EphB2 receptor had been degraded. Examination of the conditioned medium, however, failed to show any increase in EphB2/NTF, the extracellular counterpart of EphB2/CTF1 (Fig. 3, right panel, lanes 6–10). Furthermore, examination of cell extract from ligand-treated cultures failed to detect any cell-associated EphB2/NTF (data not shown) suggesting that EphB2/NTF produced in response to ligand binding is rapidly degraded. Together, these data show that ephrinB ligands as well as calcium influx induce processing of EphB2 receptor, but the kinetics of EphB2 decrease and the appearance of degradation products differ significantly between the two conditions suggesting that these two factors stimulate distinct processing pathways.

**Distinct Enzymes Cleave Extracellular EphB2 in Response to Calcium Influx or Ligand Treatment**—Cleavage within the ectodomain sequence of a number of cell surface transmembrane proteins, including cadherins, APP, and Notch1, is often catalyzed by enzymes sensitive to the broad spectrum MP inhibitor GM6001. In addition, the ectodomain of APP is cleaved by β-secretase (BACE), an aspartyl protease sensitive to peptide inhibitor ZVLL (29). Fig. 4A (left panels) shows that GM6001 inhibits the ionomycin-stimulated metabolism of EphB2 and the production of both cell-associated EphB2/CTF1 and released EphB2/NTF suggesting that GM6001 blocks a MP cleavage of EphB2 ectodomain. GM6001, however, has no effect on the ligand-stimulated metabolism of EphB2 receptor (Fig. 4A, right panels). In contrast, peptide ZVLL inhibits the ligand-stimulated metabolism of EphB2 and the appearance of EphB2/CTF1 (Fig. 4B, right panels), but it has no effect on the ionomycin-stimulated metabolism of EphB2 and production of EphB2/CTF1 (Fig. 4B, left panels). Because ZVLL is not specific to BACE (29), we probed two additional BACE inhibitors, including Statin and the cell-permeable BACE inhibitor IV (30). None of these was able to inhibit the ligand-induced processing of EphB2 (data not shown), suggesting that the activity responsible for the ligand-induced processing of EphB2 may differ from BACE. Together, these results show that, depending on the inducing agent, cleavage of the extracellular region of EphB2 involves two distinct activities, one sensitive to MP inhibitor GM 6001 and one sensitive to peptide inhibitor ZVLL.

Because EphB2 interacts closely with NMDAR and regulates its activity (31), we asked whether calcium influx through NMDAR affects EphB2 processing. Fig. 4C (left panels) shows that treatment of rat embryo primary neuronal cultures (see “Experimental Procedures”) with NMDA, an agonist of NMDAR, increased production of EphB2/CTF1, and this increase was augmented by γ-secretase inhibitors (lanes 1–3). NMDA-induced production of EphB2/CTF1 was inhibited by the specific NMDAR antagonist d(−)-2-amino-5-phosphonono- 
valerate (APV) and by GM6001 indicating that stimulation of NMDAR induces a MP-like cleavage of EphB2 receptor (Fig. 4C, left lanes, lanes 4–7). Treatment of primary neurons with ephrinB ligand results in the degradation of EphB2 and in the accumulation of EphB2/CTF1 in the presence of γ-secretase inhibitor (Fig. 4C, right panels, lanes 1–3). As expected, this degradation was blocked by ZVLL (Fig. 4C, right panels, lane 4). Absence of EphB2/CTF1 accumulation upon ligand treatment may be due to rapid degradation of this peptide in primary
Distinct enzymes cleave extracellular EphB2 in response to ligand or calcium influx. EphB2-transduced fibroblasts grown overnight on 0.5% serum were treated for 30 min with either GM6001 (A) or ZVLL (B) and then stimulated with ionomycin for further 10 min (left panels) or with clustered ephrinB2 ligand for 4 h (right panels). Lysates and culture media were analyzed for the indicated proteins as in Fig. 1. In C: Left panels: Primary rat cortical neuronal cultures (8 days in vitro) were washed and then incubated for 30 min in Hanks’ balanced salt solution media plus calcium chloride without magnesium in the presence of (-)-2-amino-5-phosphonovalerate, GM6001, or L-685,458 as indicated. Then NMDA and glycine were added, and cells were collected 2 h later. Lysates were analyzed using a monoclonal antibody against cytoplasmic EphB2. Right panels: Rat cortical neurons (4 days in vitro) were pretreated with γ-secretase inhibitor L-685,458 and ZVLL as indicated for 30 min. Following treatment, clustered ephrinB ligand was added, and cultures were further incubated for another 4 h. Cell extract was then probed as indicated. Absence of EphB2/CTF1 increase in lane 2 suggests an efficient removal of this peptide in primary neurons. D: EphB2-transduced fibroblasts grown overnight on 0.5% serum were treated for 30 min with either GW280264X or GI254023X and then stimulated with ionomycin for 10 min. Extract was then probed for EphB2 and EphB2/CTF1 as indicated.

FIGURE 4. Distinct enzymes cleave extracellular EphB2 in response to ligand or calcium influx.
Ligand-induced cleavage of EphB2 depends on endocytosis. A, mouse neuronal N2a cells transduced with EphB2 were grown overnight on 0.5% serum and then media were made 0.45 M in sucrose and 10 μM in LC as indicated. Cultures were incubated for another 30 min, and then clustered ephrinB ligand was added for 4 h more. Cells were then collected, and extracts were probed for the proteins indicated at the left. B, mouse neuronal N2a cells transduced and grown as above were treated with 125 μM monodansylcadaverine in the presence of LC and then clustered ephrinB ligand was added for 4 h. Cultures were harvested, and extracts were probed for the indicated proteins. C, vector (mock), EphB2 (WT), or EphB2K664M-transduced N2a cells were grown overnight on 0.5% serum and then stimulated with clustered ephrinB-ligand for 30 min. EphB2 proteins were immunoprecipitated and probed with anti-ephrinB antibody. Detected antigens are indicated at the right. D, membranes from N2a cells transduced with EphB2 or EphB2/K664M were used for in vitro γ-secretase assays. Samples were analyzed on large gels to separate closely migrating fragments and probed with anti-EphB2 antibody. Detected antigens are indicated at the left of panel E. N2a cells transduced either with EphB2 or EphB2 mutant K664M were grown overnight on 0.5% serum and then pretreated with LC or L-685,458 as indicated for 30 min. Following this, clustered ephrinB ligand was added, and cells were harvested after 4 h. Prepared extracts were probed with anti-EphB2 antibody and detected antigens are indicated at the left.

FIGURE 5. Ligand-induced cleavage of EphB2 depends on endocytosis. A, mouse neuronal N2a cells transduced with EphB2 were grown overnight on 0.5% serum and then media were made 0.45 M in sucrose and 10 μM in LC as indicated. Cultures were incubated for another 30 min, and then clustered ephrinB ligand was added for 4 h more. Cells were then collected, and extracts were probed for the proteins indicated at the left. B, mouse neuronal N2a cells transduced and grown as above were treated with 125 μM monodansylcadaverine in the presence of LC and then clustered ephrinB ligand was added for 4 h. Cultures were harvested, and extracts were probed for the indicated proteins. C, vector (mock), EphB2 (WT), or EphB2K664M-transduced N2a cells were grown overnight on 0.5% serum and then stimulated with clustered ephrinB-ligand for 30 min. EphB2 proteins were immunoprecipitated and probed with anti-ephrinB antibody as indicated. D, membranes from N2a cells transduced with EphB2 or EphB2/K664M were used for in vitro γ-secretase assays. Samples were analyzed on large gels to separate closely migrating fragments and probed with anti-EphB2 antibody. Detected antigens are indicated at the left of panel E. N2a cells transduced either with EphB2 or EphB2 mutant K664M were grown overnight on 0.5% serum and then pretreated with LC or L-685,458 as indicated for 30 min. Following this, clustered ephrinB ligand was added, and cells were harvested after 4 h. Prepared extracts were probed with anti-EphB2 antibody and detected antigens are indicated at the left.

Ectodomain/γ-Secretase Cleavages of EphrinB2 Receptor

Ligand Binding Stimulates the Ubiquitination of EphB2 Receptor—We noticed that, in addition to inhibiting the metabolism of EphB2, sucrose treatment in the presence of ligand slightly decreased the SDS-PAGE mobility of the receptor (Fig. 5A, upper panel, compare lane 3 to 4 and lane 7 to 8). This shift in apparent molecular mass is consistent with the ligand-induced tyrosine phosphorylation of surface EphB2 (19). To examine whether ligand treatment might also induce ubiquitination of this receptor, EphB2 was immunoprecipitated from samples treated as in Fig. 5A and obtained immunoprecipitates were probed with anti-ubiquitin antibodies. Fig. 6 (lower panel) shows that ligand treatment strongly increases ubiquitination of EphB2 (lanes 1–4). As expected (19), these treatments also increased the levels of tyrosine phosphorylation of EphB2 (Fig. 6, middle panel). These data indicate that ligand binding induces phosphorylation and ubiquitination of cell surface EphB2 receptor.

Ligand-induced Cleavage of EphB2 Takes Place in the Endosomal System—That the ligand-induced processing of EphB2 requires internalization raises the possibility that the endosomal proteolytic system might be involved in the processing of the endocytosed receptor. To test this possibility we examined the effects of endosomotropic drugs on the ligand-induced processing of EphB2. Fig. 7 shows that inhibition of endosomal acidification by either chloroquine or bafilomycin blocks the ligand-induced metabolism of EphB2 and abolishes production of EphB2/CTF1 (lanes 1–6). In contrast, proteasomal inhibitors, including LC and epoxomycin, had no effect on the ligand-induced metabolism of EphB2 but led to the accumulation of both EphB2/CTF1 and EphB2/CTF2 (lanes 7–10) consistent with the suggestion that EphB2 metabolites EphB2/CTF1 and EphB2/CTF2 are degraded in the proteasome. Together, these data indicate that the ephrinB ligand-induced endocytosis and γ-secretase cleavages of EphB2 receptor take place in the endosomal/lysosomal system and that the products of this processing, EphB2/CTF1 and EphB2/CTF2, are degraded in the proteasome.
Ectodomain/γ-Secretase Cleavages of EphrinB2 Receptor

FIGURE 6. Ligand binding stimulates ubiquitination of EphB2 receptor. Mouse neuronal N2a cells were transduced, grown, and treated as in as Fig. 5A, lanes 1–4. Extracts were prepared after 4 h of incubation, and EphB2 proteins were immunoprecipitated with polyclonal EphB2 antibody. Immunoprecipitated proteins were detected with anti-phosphotyrosine, anti-ubiquitin, or anti-EphB2 antibodies as indicated.

FIGURE 7. Ligand-induced processing of EphB2 takes place in the endosomal system. EphB2-transduced mouse N2a cultures were grown over-night on 0.5% serum and then incubated for 30 min with chloroquine, bafilomycin, LC, or epoxomycin as indicated. Following this, clustered ephrinB ligand was added for 4 h as indicated. Extracts were probed on WBs for the indicated antigens.

DISCUSSION

Forward signaling of EphB2 receptor, a type I transmembrane protein, regulates development and function of many tissues and organs, including the vascular and nervous systems (1, 2, 4–6). Recent research shows that several type I transmembrane receptors are processed through a pathway that involves cleavage and shedding of their ectodomain, usually catalyzed by a MP, whereas the remaining membrane-associated fragment is processed by the PS-dependent γ-secretase system producing cytosolic peptides containing the cytoplasmic sequence of the receptor (14). The biological significance of the MP/γ-secretase processing of cell surface receptors is still being explored, but evidence suggests that a number of cytosolic peptides produced by this processing have important signal transduction properties regulating transcription and protein phosphorylation (14, 18, 35). In addition, the MP/γ-secretase processing of E-cadherin mediates disassembly of adherens junctions promoting cell-cell dissociation and cytosolic release of the transcription factor β-catenin (26). The ectodomain of certain substrates, including APP, can be cleaved by both a MP and an aspartyl protease activities (BACE) (36, 37), and recent reports show that processing of cell surface receptors is stimulated by calcium influx or ligand binding (18, 26), but it is unknown whether these treatments stimulate identical or distinct processing pathways.

Here we show that EphB2 receptor is cleaved at its ectodomain between residues 543 and 544 (residue count includes the leader peptide of EphB2) to produce fragment EphB2/NTF released to the extracellular space and membrane-bound C-terminal fragment EphB2/CTF1. Production of EphB2/CTF1 fragment used for sequencing was performed in the absence of ephrinB ligands, and its synthesis was sensitive to GM6001 but not to ZVLL (data not shown) suggesting that we identified the MP cleavage site of EphB2. EphB2/CTF1 is further processed by the PS-γ-secretase system between EphB2 residues 569 and 570 to release cytosolic peptide EphB2/CTF2. We obtained a unique N-terminal sequence of CFT2 indicating that γ-secretase cleaves EphB2/CTF1 at one main ε-site. Our data indicate that most PS1 FAD mutations tested here inhibit production of this peptide in agreement with recent reports that these mutations cause a loss of cleavage function at the ε-site of γ-secretase substrates (17, 18, 24). It is not clear why PS1 mutants A246E and M146L show no impaired ability to produce EphB2/CTF2 (Fig. 2B). These mutants however, showed loss of cleavage function when tested with N-cadherin as a substrate (17) suggesting that specific PS1 mutations may affect cleavage of a limited number of substrates.

Processing of EphB2 is greatly stimulated by ephrinB ligands and by calcium influx. Ligand-induced processing requires endocytosis and results in increased production of both EphB2/CTF1 and EphB2/CTF2. We obtained evidence that inhibition of the ligand-induced ectodomain cleavage decreases production of EphB2/CTF2 suggesting that full-length EphB2 is not an efficient substrate for PS1-γ-secretase and that increased levels of EphB2/CTF2 result from increased production of γ-secretase substrate EphB2/CTF1. This conclusion is in agreement with the processing of other substrates of γ-secretase (18, 28) and with models suggesting ectodomain shedding as the rate-limiting step for the PS1-dependent proteolysis (38). Although the ligand-induced ectodomain cleavage of EphB2 is sensitive to BACE inhibitor ZVLL, other BACE inhibitors failed to inhibit this cleavage suggesting that an activity distinct from BACE of APP may be involved in the ligand-induced cleavage of EphB2.

Endosomal/lysosomal inhibitors block the ligand-induced metabolism of EphB2 and inhibit production of both EphB2/CTF1 and EphB2/CTF2 suggesting that ligand-induced processing of EphB2 to EphB2/CTF1 and possibly to EphB2/CTF2 takes place in endosomal/lysosomal vesicles. Production of EphB2/CTF2 in these vesicles is in agreement with recent data indicating that γ-secretase is a lysosomal protease (39, 40). Ligand-induced processing of EphB2 failed to produce detectable levels of EphB2/NTF, the extracellular counterpart of EphB2/CTF1, suggesting that this processing involves the rapid degradation of extracellular EphB2 presumably within the

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lumen of vesicles where it should be released. In contrast, the intracellular products of the ligand-induced processing, EphB2/CTF1 and EphB2/CTF2, are degraded in the proteasome.

The second processing pathway of EphB2 is stimulated by calcium influx and NMDA treatment and is sensitive to broad spectrum MP inhibitor GM6001 suggesting that calcium stimulates a MP cleavage of EphB2. Interestingly, this cleavage is also sensitive to inhibitor GI254023X specific to ADAM10, an enzyme involved in the ectodomain processing of APP (37), E-cadherin (27), and N-cadherin (28). Together, these data identify ADAM10 as the protease involved in the calcium-induced processing of EphB2 receptor. Similar to the ligand-induced processing, fragment EphB2/CTF1 produced by the MP cleavage is further metabolized by the PS1-γ-secretase system to produce peptide EphB2/CTF2. In contrast to ligand-induced processing, however, calcium-induced processing requires no endocytosis and results in the rapid shedding of extracellular EphB2 to the medium suggesting that the ectodomain and γ-secretase cleavages of this pathway take place at or near the plasma membrane. This suggestion is further supported by the cell surface localization of PS1 (15) and evidence that γ-secretase processing of cadherins and Notch1 takes place at the cell surface (26, 28, 41). Extracellular EphB2 released to the medium may have functional roles. For example, it may compete with cell surface EphB2 receptor for binding to ephrinB ligands and may thus regulate the extent of the EphB2-ephrinB binding and signaling. Such regulation of ligand-receptor interactions involving released ectodomain is well characterized for cytokine receptors (42).

It is interesting that the ectodomain cleavage of EphB2 is stimulated by NMDAR agonists. In the central nervous system, ephrinB ligands localized at pre-synaptic terminals activate trans-synthaptically their receptors at post-synaptic membranes (31, 43). Activated EphB receptors at post-synaptic spines associate and functionally interact with the NMDAR. Furthermore, recent works indicate that neuronal ephrinB-EphB interactions promote tyrosine phosphorylation of NMDAR and regulate NMDAR-dependent calcium influx and synaptic plasticity (31, 44–47). Our data predict that neuronal ephrinB-EphB2 interactions may promote EphB2 processing through two pathways: the ephrinB ligand-induced endocytic processing and the calcium-induced processing stimulated by increased activity of NMDA receptor. This degradation of EphB2 may serve to terminate or control signaling cascades initiated by the EphB2-ephrinB interactions. Furthermore, similar to other intracellular peptides produced by the PS-γ-secretase activity, which have been shown to have signaling properties (14, 18), peptide EphB2/CTF2 may also function in the signaling cascades initiated by the EphB2-ephrinB binding or by the activation of NMDAR. Such potential functions may be impaired by FAD mutations that inhibit production of EphB2/CTF2.

EphB-ephrinB interactions initiate cell-cell contacts and bidirectional signaling that, depending on signal intensity, can result in either stabilization of cell-cell adhesion or in repulsion and cell separation (3, 48). Due to the relatively high affinity of the Eph-ephrin binding (11, 13) repulsive signaling outcomes that promote cell-cell disengagement involve removal of the cell surface EphB-ephrinB complexes rather than dissociation of the bound proteins. Cellular mechanisms used for this purpose include internalization of vesicles containing the intact Eph-ephrin complexes (11, 12, 49) or cleavage and release of the extracellular domains of ephrin ligands engaged to their receptors (13, 50). Our data suggest a novel mechanism of cell-cell disengagement where adjacent cells held together through EphB2-ephrinB complexes may dissociate following cleavage of the extracellular domain of EphB2 receptor. Furthermore, our data, that binding of ephrinB ligand to EphB2 stimulates internalization and endosomal/lysosomal degradation of EphB2, suggest that EphB-ephrinB complexes internalized to facilitate disengagement of neuronal surfaces (11) may be processed in endosomes through ectodomain and cytoplasmic cleavages and then degraded in the proteasome. We observed, for the first time, that ligand binding stimulates the ubiquitination of EphB2 receptor. Because this modification may promote receptor internalization (51), it will be interesting to determine whether ligand-induced ubiquitination of EphB2 receptor is important for its internalization and signaling.

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