Regulation of X11L-dependent Amyloid Precursor Protein Metabolism by XB51, a Novel X11L-binding Protein*  

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We isolated a cDNA encoding a novel protein, XB51, that interacts with the amino-terminal domain of the neuron-specific X11-like protein (X11L). The protein XB51 inhibited the association of X11L with amyloid precursor protein through a non-competitive mechanism and abolished the suppression of β-amyloid production by X11L. The majority of XB51 is localized around the nucleus and recovered in 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) buffer-insoluble fraction when XB51 is expressed in cells. Association of XB51 with X11L changed the intracellular distribution of XB51 and resulted in redistribution of XB51 into the CHAPS buffer-soluble fraction. These observations suggest that XB51, together with X11L, plays an important role in the regulatory system of amyloid precursor protein metabolism and β-amyloid generation.

The protein X11L-like (X11L) was originally shown to interact with the cytoplasmic domain of the β-amyloid precursor protein (APP)†† (1), a protein molecule central to the pathogenesis of Alzheimer’s disease (AD) (reviewed in Ref. 2). The expression of X11L is neuron-specific, and X11L has the ability to regulate β-amyloid (Aβ) production (1, 3). Therefore, X11L is thought to play an important role in regulating the amyloidogenic pathway of APP metabolism in neuronal tissues and thus contributing to the pathogenesis of AD.

X11L has 53.5% amino acid homology with X11 protein (4). X11 and X11L are equivalent to Mint 1 and Mint 2, which are thought to contribute to synaptic vesicle exocytosis (5, 6). X11L and X11 contain a phosphotyrosine interaction (PI) domain in their central regions and two repeated sequences in the brain-specific protein ESDL, the Drosophila septate junction protein diskin-large, and the epithelial tight junction protein ZO-1 (PDZ) domains in their carboxy-terminal regions (1). These multiple protein-protein interaction domains suggest that X11L and/or X11 proteins are engaged in yet unknown neuronal function(s) through interactions with other proteins (7, 8). The amino acid sequences of the PI domains (82.4%) and PDZ domains (85.6%) of X11L and X11 are highly homologous, but the amino-terminal domains are poorly conserved between the two proteins (18.3%). Furthermore, Northern blot hybridization revealed neuron-specific expression of X11L but expression of X11 in both neuron and testis of adults.†† These results suggest that the distinct functions of X11L and/or X11, if both proteins function independently, may be mediated by protein-protein interactions involving their divergent amino-terminal domains.

In this study, we isolated XB51 (name derived from X11L-binding-protein of clone number 51) as a protein that interacts with the amino-terminal domain of human X11L. XB51 is expressed in a neuron-specific manner in mouse and inhibits the association of X11L with APP. Therefore, the interaction between XB51 and X11L may regulate the function of either or both of these two molecules. Furthermore, association of XB51 with X11L abolished the suppression of Aβ40 production by X11L. These observations suggest that X11L and XB51 play a role in the regulation of metabolism and function of APP.

EXPERIMENTAL PROCEDURES
cDNA Cloning of Human and Mouse XB51 and Plasmid Construction—The yeast two-hybrid system used in this study was described previously (1). The pGBT9hX11-LN+PI plasmid, a cDNA encoding hX11L196–555, was used as bait. The nucleotide sequence of positive clones was determined. The cDNA, clone number 51, encoding the full-length version of a novel protein was named human XB51 (hXB51).

Mouse homologous cDNA (mXB51) of hXB51 was isolated from a cDNA library of adult mouse brain (CLONTECH) by cross-hybridization using hXB51 cDNA as a probe. Human and mouse cdNA were inserted into pcdNA3 (Invitrogen) with (pcDNA3-FLAG-hXB51) or without (pcDNA3-hXB51) FLAG tag on the amino-terminal end of the insert.

The cDNA clones encoding the amino-terminal domain (pcDNA3hX11L-N; 1–367) and amino-terminal domain attached to the PI domain (pcDNA3hX11L-N+PI; 1–555) of hX11L were described (1). The cDNA clones encoding the amino-terminal leucine zipper-like domain (pcDNA3FLAG-hXB51-LZ; 1–51) and carboxyl-terminal domain (pcDNA3FLAG-hXB51-C; 52–273) of hXB51 were constructed with a FLAG tag at the amino-terminal end of the insert.

Antibodies—The rabbit polyclonal anti-hXB51 antibody UT-58 was raised against a peptide, hXB51127–139. This epitope is specific for hXB51 (see Fig. 1a). Another polyclonal anti-XB51 antibody, UT-63, was raised against a peptide, hXB51235–252 plus Cys. The UT-63 can recognize both human and mouse XB51 proteins. Anti-X11L UT-29 and UT-30 antibodies and anti-APP UT-18 antibody were described (1). These antibodies were affinity-purified before use (9). Anti-FLAG monoclonal antibody M2 was purchased from Eastman Kodak.

Co-immunoprecipitation—CO87 cells (~1 × 107 cells) were doubly transfected with 2 μg of each of the indicated plasmids as described (1). The cells were harvested and lysed in CHAPS buffer (phosphate-buff-

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¶ These abbreviations used are: APP, β-amyloid precursor protein; Aβ, β-amyloid; AD, Alzheimer’s disease; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; kb, kilobase; PI, phosphotyrosine interaction; h, human; m, mouse; ConA, concanavalin A; IP, immunoprecipitation; ER, endoplasmic reticulum.

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ered saline containing 10 mM CHAPS, 5 µg/ml chymostatin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 1 mM Na3VO4, and 1 mM NaF) for 1 h on ice and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant of the CHAPS buffer lysate contained the majority of the soluble cytoplasmic and some membrane proteins. The precipitate of the CHAPS buffer lysate contained crude membrane and cytoskeletal components. The affinity-purified polyclonal (50 µg) antibodies were added to the supernatant of the CHAPS lysate, and immunoprecipitation studies were analyzed by Western blot analysis as described (1).

**Immunocytochemistry**—COS7 cells that stably expressed FLAG-hXB51 were further transfected with or without pcDNA3hX11L. The cells were fixed and stained with the indicated antibodies or rhodamine-conjugated ConA as described (1). The protein distribution was observed under a confocal laser scanning microscope.

**Quantitation of Aβ with a Sandwich ELISA**—HEK293 cells stably expressing human APP695 were transfected with 2 µg each of indicated plasmids. Aβ40 and Aβ42 peptides secreted into the medium (10 µl) were quantified by sandwich ELISA as described (10, 11).

**RESULTS**

**Molecular Cloning of cDNAs Encoding Human and Mouse XB51 Protein: Primary Structure and Gene Expression**—cDNAs encoding proteins that interact with the N+PI construct of hX11L were isolated from a human brain cDNA library. Of 60 positive clones, termed hXB51, contained 1.8 kb of full-length cDNA and encoded a novel protein of 273 amino acids (Fig. 1a) with a leucine zipper-like motif. We also isolated mXB51 cDNA. The mXB51 gene encodes a protein of 239 amino acids with 75.5% homology to hXB51 but lacks the middle 34-amino acid sequence of hXB51.

The expression of XB51 was analyzed by Northern blot hybridization (Fig. 1, b and c). Interestingly, the ~1.9-kb hXB51 mRNA was found mainly in heart and skeletal muscle and only moderately in brain and pancreas, although mXB51 mRNA, which is in almost identical size, was expressed specifically in brain. Cross-probe hybridization studies using the human probe with the mouse membrane and the mouse probe with the human membrane also gave the same expression pattern (Fig. 1, b and c) (data not shown). Thus, we concluded that the expression of XB51 is brain-specific in mouse but not in human.

**Interaction of hXB51 with hX11L in Vivo**—The hXB51 binding domain of hX11L (Fig. 2a–c) and the hX11L binding domain of hXB51 (Fig. 2, d–f) were determined by co-immunoprecipitation studies. COS7 cells that transiently expressed the domain constructs derived from hX11L and FLAG-tagged hXB51 (FLAG-hXB51) were lysed with CHAPS buffer, and the supernatant of the CHAPS lysate (non-IP in Fig. 2, b and c) was subjected to immunoprecipitation (IP) using anti-FLAG monoclonal antibody M2. The same quality of non-immune mouse IgG was used as a control. The immunoprecipitates were analyzed by Western blot using anti-X11L UT-29 (Fig. 2b) or M2 (Fig. 2c) antibodies. All constructs containing the amino-terminal domain of hX11L were co-immunoprecipitated with the M2 (Fig. 2b) antibody together with FLAG-hXB51 (Fig. 2c). The result indicated that hXB51 interacts with the amino-terminal domain of hX11L in mammalian cells.

FLAG-tagged hXB51 constructs and hX11L were also expressed transiently in COS7 cells, and the co-immunoprecipitation experiment was performed (Fig. 2, d–f). The hX11L protein was co-immunoprecipitated with the M2 antibody in the presence of hXB51 (FLAG-hXB51) or the hXB51-C construct (FLAG-hXB51-C) (Fig. 2e). A construct, hXB51-LZ (FLAG-hXB51-LZ), failed to be expressed as a protein in the cells (non-IP in Fig. 2f). Thus, we could not examine co-immunoprecipitation of hX11L and hXB51-LZ directly (IP in Fig. 2, e and f). However, these studies indicate that the carboxyl-terminal half of hXB51 interacts with hX11L in mammalian cells and that the leucine zipper-like motif of hXB51 is dispensable for this interaction.

In Fig. 2, full-length hX11L and FLAG-hXB51 could not be detected in the sample immunoprecipitated using non-immune IgG, indicating that the co-immunoprecipitation of hX11L and hXB51 was specific.

**Intracellular Translocation of XB51 upon Expression of X11L**—We analyzed the intracellular distribution of hXB51 by biochemical fractionation (Fig. 3a). COS7 cells that transiently expressed FLAG-hXB51 and/or hX11L were lysed in CHAPS buffer. Contrary to our expectations, the majority of hXB51 was not recovered from the soluble fraction containing the cytosolic components (sup in Fig. 3a) but from the insoluble fraction (ppt in Fig. 3a) when FLAG-hXB51 was expressed alone. Co-expression of hX11L with FLAG-hXB51 resulted in redistribution of hXB51 protein into the CHAPS buffer soluble fraction (sup in Fig. 3a).
The change in the distribution of XB51 upon expression of X11L was examined by immunocytochemical staining (Fig. 3b). COS7 cells that stably expressed FLAG-hXB51 were double-stained with anti-FLAG antibody M2 and ConA (ER plus cis-Golgi marker) (12). The majority of hXB51 was localized around the nucleus, and the position coincided with that of ConA staining, clearly demonstrating that the location of hXB51 seemed to depend on the expression of X11L.

**Fig. 2. In vivo interaction between hXB51 and hX11L.** a—c, interaction of hXB51 and the constructs derived from hX11L protein by co-immunoprecipitation. a, schematic structure of the hX11L construct used in this study. N, amino-terminal region of the PI domain; PI, phosphotyrosine interaction domain; PDZ, PDZ domain. Amino acid numbers of hX11L are indicated. b, the supernatant of a CHAPS lysate (non-IP, 100-μg protein) of COS7 cells transiently expressing constructs derived from hX11L and FLAG-hXB51 was used for IP with antibodies (C, non-immune mouse IgG; M, anti-FLAG monoclonal antibody M2). Vector indicates a study using pcDNA3 without hX11L cDNA. The crude sample (non-IP) and immunoprecipitates (IP) were subjected to Western blot analysis using anti-hX11L antibody UT-29 (b) and M2 (c) following SDS-PAGE (12.5% polyacrylamide (w/v)). Arrows indicate full-length hX11L, the amino-terminal domain attached to the PI domain (N+PI), and the amino-terminal domain (N) or hXB51 tagged with the FLAG sequence (FLAG-hXB51). d—f, interaction of hX11L and the constructs derived from hXB51 protein by co-immunoprecipitation. d, schematic structure of the hXB51 construct used in this study. LZ, a leucine zipper-like coiled coil domain. Amino acid numbers of hXB51 are indicated. e, the supernatant of a CHAPS buffer lysate (non-IP, 100-μg protein) of COS7 cells transiently expressing FLAG-hXB51 and hX11L was used for IP with antibodies (C, non-immune mouse IgG; M, anti-FLAG monoclonal antibody M2). Vector indicates a study using pcDNA3 without FLAG-hXB51 cDNA. The crude sample (non-IP) and immunoprecipitates (IP) were subjected to Western blot analysis with UT-29 (e) and M2 (f) following SDS-PAGE (12.5% polyacrylamide (w/v)). Arrows indicate full-length hX11L, the amino-terminal domain attached to the PI domain (N+PI), and the carboxy-terminal domain tagged with the FLAG sequence (FLAG-hXB51-C) (f). A leucine zipper domain tagged with the FLAG sequence (FLAG-hXB51-LZ) was not expressed in cells. H and L indicate heavy and light chains from IgG, respectively. 170, 116, and 76 and 43, 30, and 20 refer to the molecular masses (kDa) of the protein standards.

**Fig. 3. Intracellular distribution of hXB51.** a, fractionation of hXB51 protein. COS7 cells were transiently transfected with pcDNA3-FLAG-hXB51 (FLAG-hXB51) or pcDNA3 alone (vector) in the presence (hX11L) or absence (vector) of pcDNA3hX11L. Cells were lysed with CHAPS buffer and centrifuged at 15,000 × g for 10 min at 4°C. The resulting pellet (ppt, 100 μg of protein) and supernatant (sup, 100 μg of protein) were subjected along with the unfraccionated lysate (lysate, 100 μg of protein) to Western blot analysis using anti-hXB51 antibody UT-58 following SDS-PAGE (12.5% polyacrylamide (w/v)). Arrow indicates hXB51 protein. 43 and 30 refer to the molecular masses (kDa) of the protein standards. b, localization of hXB51 protein. Intracellular localization of hXB51 (FLAG-hXB51 in left panels), ER and cis-Golgi (ConA in right panels), and hX11L (hX11L in right panels) are shown. Non-transfected COS7 cells (COS7), COS7 cells that stably express FLAG-hXB51 (COS7/FLAG-hXB51), COS7 cells that stably express FLAG-hXB51 and transiently express hX11L (COS7/FLAG-hXB51-hX11L), and COS7 cells that transiently express hX11L (COS7/hX11L) were stained with anti-FLAG antibody M2. The cells were also double-stained with rhodamine-conjugated ConA to identify the localization of the ER and cis-Golgi (Con A) or with anti-hX11L antibody UT-29 (hX11L). Scale bar, 20 μm.
XB51 Interferes with the Association of X11L with APP—

X11L interacts with APP through its PI domain and with XB51 through its amino-terminal domain. Our previous results suggested that the amino-terminal domain of X11L might modulate the interaction of X11L with APP (1). Therefore, we next examined whether XB51 association with X11L enhances or suppresses the interaction of X11L with APP (Fig. 4). HEK293 cells that stably expressed human APP695 were transiently co-transfected with cDNA encoding X11L and XB51. At 72 h, Aβ40 and Aβ42 secreted into the medium was quantified (Fig. 5). The negative control consisted of the Aβ released by cells containing vector alone. This amount was set at 100%, and levels in test samples related to this. As reported previously (1), expression of X11L slightly decreased the level of Aβ42 but did not alter the level of Aβ40. However, when XB51 was co-transfected with X11L, X11L-dependent suppression of Aβ40 generation was abolished. The secretion level of Aβ42 was not altered in this situation. Because expression of XB51 alone in the absence of X11L induced insolubility of XB51 protein (Fig. 3) and induced a remarkable decrease of cell number (data not shown), the amount of Aβ secreted into medium was not qualified in this case. This result suggests that the dissociation of X11L from APP in the presence of XB51 (Fig. 4) results in the invalidation of the suppressive effect by X11L on Aβ generation.

DISCUSSION

The protein X11L is thought to regulate Aβ generation (1, 3). Its PI and PDZ domains are important for the interaction with APP and the regulation of Aβ generation. We also found that the amino-terminal domain can regulate the binding affinity of X11L for APP (1). To elucidate the physiological role of the amino-terminal domain of X11L, we first isolated proteins that interact with the amino-terminal domain of X11L.

In the present study, we focused on XB51, an X11L-binding protein. The mXB51 protein lacks the middle 34-amino acid sequence of hXB51, but the sequence otherwise shows strong homology with hXB51. However, the tissue specificity of hXB51 expression differs from that of mXB51 expression, which is brain-specific. The difference in the expression of XB51 between human and mouse is not artificial, as an autoradiogram of a membrane blotted with human RNA and probed with mXB11 (a cross-hybridization study) gave a result identical to that observed in Fig. 1b and vice versa (data not shown).

We observed that XB51 efficiently inhibited the interaction of Aβ40, and the amino-terminal domain of X11L regulated the association of X11L through the PI domain to APP (1). Therefore, we examined the effect of XB51 on Aβ generation. HEK293 cells that stably expressed human APP695 were transiently co-transfected with cDNA encoding X11L and XB51. At 72 h, Aβ40 and Aβ42 secreted into the medium was quantified (Fig. 5). The negative control consisted of the Aβ released by cells containing vector alone. This amount was set at 100%, and levels in test samples related to this. As reported previously (1), expression of X11L slightly decreased the level of Aβ40 but did not alter the level of Aβ42. However, when XB51 was co-transfected with X11L, X11L-dependent suppression of Aβ40 generation was abolished. The secretion level of Aβ42 was not altered in this situation. Because expression of XB51 alone in the absence of X11L induced insolubility of XB51 protein (Fig. 3) and induced a remarkable decrease of cell number (data not shown), the amount of Aβ secreted into medium was not qualified in this case. This result suggests that the dissociation of X11L from APP in the presence of XB51 (Fig. 4) results in the invalidation of the suppressive effect by X11L on Aβ generation.
of X11L with APP. Association of XB51 with X11L may alter the conformation of X11L so that it can no longer interact with APP. XB51 was also found to inhibit the interaction between X11L and APP using a yeast three-hybrid assay (data not shown). It has already been reported that X11L slightly suppresses the production of Aβ40 (1). We observed that the suppression of Aβ40 production in cells expressing X11L was abolished by the concomitant expression of XB51. Our present and previous results (1) suggest that X11L suppresses Aβ40 generation by direct interaction with the cytoplasmic domain of APP. This suppression of Aβ40 generation was abolished by dissociation of X11L from APP resulting from association of XB51 with X11L. Although the mechanisms regulating the metabolism of APP and secretion of Aβ are as yet not well revealed, the present results suggest the importance of X11L and XB51 in the regulatory system of APP metabolism and Aβ generation. Further characterization of protein regulators in the APP metabolic pathways should increase our understanding of pathogenesis of the sporadic types of AD.

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