XB51 isoforms mediate Alzheimer's β-amyloid peptide production by X11L (X11-like protein)-dependent and -independent mechanisms

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XB51 (derived from X11-like binding protein of clone number 51) was isolated by yeast two-hybrid cDNA screening using the N-terminal domain of X11L (X11-like protein) as a bait. X11L is a neuron-specific adaptor protein that is known to down-regulate APP (β-amyloid precursor protein) metabolism by associating with the cytoplasmic domain of APP, but the detailed mechanisms are still unknown. Thus the X11L-associated protein XB51 is believed to regulate APP metabolism by modifying X11L function through its interaction with X11L. Here we report that the *hXB51* (human *XB51*) gene can yield two transcripts, one with exon 9 spliced out (resulting in the hXB51β isoform) and the other containing exon 9 (yielding the hXB51α isoform). hXB51α binds to X11L to form a tripartite complex composed of hXB51α, X11L and APP. Complex-formation results in blocking X11L's suppression of Aβ (β-amyloid) generation from APP. hXB51β associates with X11L and inhibits its interaction with APP. However, hXB51β suppresses Aβ generation and secretion in an X11L-independent manner. Thus the hXB51 isoforms regulate Aβ generation differently, either enhancing it by modifying the association of X11L with APP or suppressing it in an X11L-independent manner. These observations advance our understanding of the molecular mechanisms regulating intracellular Aβ production and the pathogenesis of Alzheimer's disease.

Key words: Alzheimer's disease, β-amyloid, amyloid precursor protein, RNA splicing, X11-like protein, XB51.

INTRODUCTION

The production of Aβ (β-amyloid) is believed to be an initial step in the pathogenesis of AD (Alzheimer's disease) [1]. Aβ is generated by the intracellular processing of APP (β-amyloid precursor protein) [2]. The cytoplasmic domain of APP regulates the metabolism and function of APP by interacting with cytoplasmic proteins and by phosphorylation of APP [3–5]. One such cytoplasmic protein is neuron-specific adaptor protein X11L (X11-like protein), which binds to the ^{681}GYENPTY^{687} motif (human APP695 isoform numbering) and thereby stabilizes the intracellular metabolism of APP and suppresses Aβ generation [6,7]. Two other members of the X11 protein family, X11 and X11-like 2 (X11L2), also interact with the cytoplasmic domain of APP and regulate APP metabolism [8,9]. X11 was originally identified through its possible link to Friedreich's ataxia [10], while X11L2 was found to be expressed in a variety of tissues [9]. X11, X11L and X11L2 are equivalent to Mint1, Mint2 and Mint3, which are involved in vesicle exocytosis [11]. However, it remains unclear how the X11 protein family (X11s) regulate APP metabolism, including Aβ generation.

The X11s consist of a poorly conserved N-terminal region, a highly conserved PI domain (phosphotyrosine-interaction domain), and two C-terminal PDZ domains [6]. The structure of the X11s, together with the presence of their multiple protein–protein interaction domains, suggest that their function(s) may be regulated through interactions with other proteins. One of these proteins may be XB51 (derived from X11-like binding protein of clone number 51), which we previously isolated through its interaction with the N-terminal region of X11L [12]. We reported that the interaction of XB51 with X11L could block the association of X11L with APP and thereby enhance Aβ generation [12].

The hXB51 (human XB51) protein that we found previously was denoted hXB51αshort, because progressive cDNA and human genome sequencing projects revealed that it might be a truncated or spliced product of the *hXB51* gene. Here we show that two larger isoforms of XB51, denoted XB51α and XB51β, do exist. The XB51β isoform is generated by the splicing of exon 9, whereas XB51α is unspliced. Aβ generation is enhanced by XB51α because it forms a tripartite complex together with APP and X11L and thereby abolishes the suppressive effect of X11L. In contrast, XB51β decreases Aβ generation in an X11L-independent mechanism. Thus *hXB51*-encoded proteins appear to regulate APP metabolism in different ways. These observations are useful in understanding AD pathogenesis and provide a novel approach for the development of AD drugs.

MATERIALS AND METHODS

Identification of longer cDNA containing the hXB51 sequence

cDNA encoding part of the human X11L protein (amino acids 129–555) was used as bait in the yeast two-hybrid screening [12]. Ultimately, 60 clones were isolated from an adult human brain cDNA library (approx. 10^6 independent clones) that tested positive for nutrient (His) selection and β-galactosidase activity. The positive clones were numbered and their nucleotide sequences were determined. Nucleotide sequences of the two clones, clones 31

Abbreviations used: APP, β-amyloid precursor protein; Aβ, β-amyloid; X11L, X11-like protein; XB51, X11-like binding protein of clone number 51; h, human; m, mouse; AD, Alzheimer's disease; HBST, Hepes-buffered saline with Triton X-100; PI domain, phosphotyrosine-interaction domain; RT-PCR, reverse transcriptase PCR; HA, haemagglutinin-epitope tag.

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and 51, revealed novel protein sequence. Proteins encoded by these clones were named hXB31 and hXB51. The XB51 contained 273 amino acids.

We found a partial cDNA clone (AK023706 of NEDO human cDNA sequencing project; http://www.genome.ad.jp), that contained sequences identical with the N-terminal region of the hXB51 protein discovered previously (GenBank accession no. AB039947) and also had additional 5′ sequences. This clone suggests that a larger protein exists, containing the 273 amino acids of the XB51 protein that we identified earlier (designated hXB51αshort) [12]. To investigate this, we isolated the cDNA sequence of the larger XB51 protein. The larger cDNAs were cloned by RT-PCR (reverse transcriptase PCR) using total RNA prepared from the human neuroblastoma cell line SH-SY5Y and the following primers: 5′-GTGGCGGCGCCATGGCGT-3′ (forward; 5′ sequence of AK023706) and 5′-CCCTCGGCGTGTGCAGGTCT-3′ (reverse, 3′ sequence of AB039947). The resulting 1225- and 1123-bp products encoded the 396- and 362-amino-acid proteins, denoted hXB51α and hXB51β respectively.

Plasmid construction

By using the *Nhe*I and *Not*I sites, the cDNA encoding hXB51α was cloned into pcDNA3.1 (Invitrogen) that had the FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) or HA (haemagglutinin-epitope tag) sequences inserted into its 5′-end, thereby producing pcDNA3.1-FLAG-hXB51α and pcDNA3.1-HA-hXB51α. By using the *Nhe*I and *Not*I sites, hXB51α cDNA was also inserted into a pcDNA3.1 construct that had FLAG inserted into its 3′-end. This produced pcDNA3.1-hXB51α-FLAG. The hXB51β cDNA was inserted into pcDNA3.1 constructs in the same manner as hXB51α.

When FLAG-tagged hXB51α was expressed in cells, a small amount of lower-molecular-mass product was detected. We identified this as the product of the short splicing of exon 9, hXB51β (results not shown).

To prevent the production of the hXB51β by the short splicing of exon 9, we altered the nucleotide sequences AAG of Lys-241 and GTG of Val-242 to AAA and GTC, respectively [13] (results not shown). These mutations did not change the amino acid sequence of hXB51α, but prevented the production of hXB51β from the hXB51α transcript by eliminating the splicing sites. The altered plasmids were designated pcDNA3.1-FLAG-hXB51α[no-sp], pcDNA3.1-HA-hXB51α[no-sp] and pcDNA3.1-hXB51α-FLAG[no-sp]. Several constructs encoding deletion mutants of hXB51α were also made using PCR techniques. These included the N-terminally truncated constructs pcDNA3.1-FLAG-hXB51α[no-sp]NΔ173, pcDNA3.1-FLAG-hXB51α[no-sp]NΔ220 and pcDNA3.1-FLAG-hXB51α[no-sp]NΔ246, the internally deleted construct pcDNA3.1-FLAG-hXB51α[no-sp]Δ174–253, and the N- and C-terminally truncated constructs pcDNA3.1-FLAG-hXB51α[no-sp]NΔ123–CΔ323. Human X11L and its N-terminal and internally deleted constructs were cloned into pcDNA3.1-Myc using *Nhe*I and *Not*I sites. This resulted in pcDNA3.1-Myc-hX11L, pcDNA3.1-Myc-hX11LNΔ140, pcDNA3.1-Myc-hX11-LNΔ160, pcDNA3.1-Myc-hX11LNΔ180, pcDNA3.1-Myc-hX11LNΔ220, pcDNA3.1-Myc-hX11LΔ31–220, pcDNA3.1-Myc-hX11LΔ61–220 and pcDNA3.1-Myc-hX11LΔ91–220. The pcDNA3-hX11L and pcDNA3APP695 constructs have been described previously [6,12].

Determining the expression profiles of hXB51α and hXB51β

hXB51α and hXB51β expression was determined by RT-PCR of total human tissue RNA (Human Total RNA Master Panel II; Clontech) using a forward primer (5′-CCCGGCTCTTCTGACACA-3′) in exon 8 and a reverse primer (5′-GCGGACACATGCAGACA-3′) in exon 11. The PCR products of hXB51α and hXB51β were thus 397 and 295 bp, respectively. The PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced. Total RNAs of healthy human cerebrum, mouse cerebrum, SH-SY 5Y cells and HEK-293 cells were also prepared with an acid guanidinium/phenol/chloroform procedure and analysed for XB51 expression. Glyceraldehyde-3-phosphate dehydrogenase was analysed as a ubiquitously expressed protein using the forward 5′-ATGGTGAAGGTCGGTGTGAA-3′ and reverse 5′-AGCCCTTCCACAATGCCAAAGTTGTC-3′ primers.

XB51 protein expression in cultured cells

African green monkey kidney COS-7 cells and human embryonic kidney HEK-293 cells that stably express human APP695 (HEK-293APPwt cell line) were cultured as described in [14]. These cells were induced to express the various hXB51 proteins by transiently transfecting 5×10^5–1×10^6 COS-7 cells or (2–4) $\times 10^5$ HEK-293APPwt cells with the indicated amounts of each plasmid in Lipofectamine or Lipofectamine 2000 (Invitrogen). The transfected cells were then cultured for 24–48 h, after which they were harvested and lysed for 0.5 h at 4 °C in Hepes-buffered saline with Triton X-100 [HBST; 50 mM Hepes, pH 7.6, 0.5% (v/v) Triton X-100, 150 mM NaCl, 5 μg/ml chymostatin, 5 μg/ml leupeptin and 5 μg/ml pepstatin A]. The lysed cells were then centrifuged at 12000 ***g*** for 15 min at 4 °C, and the supernatant was used for analysis.

Antibodies and co-immunoprecipitation assay

Anti-FLAG (M2; Sigma), anti-HA (12CA5; Roche Diagnostics) and anti-Myc (Invitrogen) monoclonal antibodies were purchased. Specificity of rabbit polyclonal anti-APP antibody G369 was described in [15]. The anti-X11L monoclonal antibody #347-5 was raised against a peptide, Cys plus 15 amino acids of the hX11L C-terminal end. The specificity of this antibody was confirmed by Western-blot analysis using mouse brain lysate. The #347-5 antibody specifically detected X11L protein, but not other proteins, including X11 (results not shown).

The lysate of transfected cells was incubated with antibody at 4 °C for 1 h. The resulting immunocomplexes recovered with Protein G–Sepharose beads (Amersham Biosciences) were analysed by Western blot with the antibody indicated.

Aβ quantification

HEK-293APPwt cells were transiently transfected with the indicated amounts of plasmids and then cultured for 28 or 48 h. Aβ40 and Aβ42 secreted into the medium were quantified by sandwich ELISA as described previously [14]. Intracellular Aβ40 and Aβ42 were prepared [16] and quantified by sandwich ELISA. Briefly, the cells were lysed by sonication in 40 μl of PBS (10 mM sodium phosphate, pH 7.4/154 mM NaCl) containing 6 M guanidinium chloride and centrifuged at 20000 ***g*** for 15 min at 4 °C. The resulting supernatant was diluted up to 12-fold by adding PBS and quantified with Aβ40 and Aβ42 end-specific monoclonal antibodies [14].

RESULTS

Structure and expression of human XB51 isoforms

We previously isolated, using yeast two-hybrid screening, human and mouse cDNAs encoding the X11L-binding protein XB51.

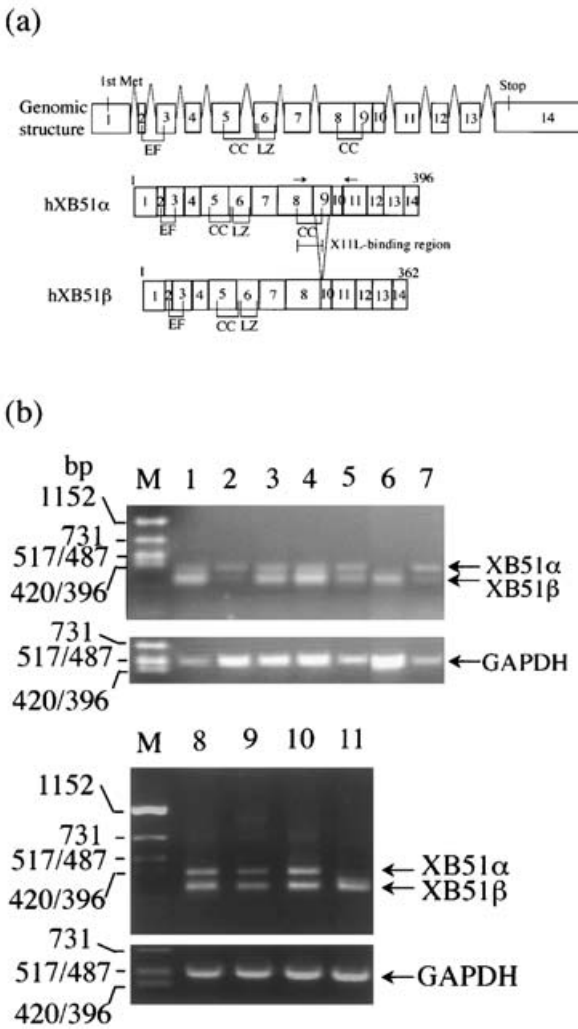

Figure 1 Structure and expression of the human *XB51* gene

(**a**) Genomic structure of *hXB51* (upper), and protein structures of hXB51α (middle) and hXB51β (lower). Numbered boxes indicate the exons. EF, EF hand motif; CC, coiled-coil structure; LZ, leucine zipper structure. The positions of the first methionine (1st Met) and the stop codon (Stop) are indicated. Arrows indicate the position of the primers used in RT-PCR. X11L-binding region of XB51 and amino acid numbers are indicated. (**b**) Expression profiles of XB51α and XB51β in human and mouse tissues and cells. Total RNA was analysed with RT-PCR using the primers indicated in (**a**) and the products derived from XB51α and XB51β are indicated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the product derived from the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene. Lane 1, human adrenal gland; lane 2, human bone marrow; lane 3, human cerebellum; lane 4, human total brain; lane 5, human heart; lane 6, human skeletal muscle; lane 7, human spleen; lane 8, human cerebrum; lane 9, SH-SY 5Y cells; lane 10, HEK-293 cells; lane 11, total mouse brain. Numbers indicate the sizes (bp) of the DNA markers (lane M).

The *hXB51* encoded 273 amino acids, and mXB51 (mouse XB51) lacked the middle 34 amino acids with 75 % homology with human protein [12]. In this latter paper [12], we designated these proteins hXB51αshort and mXB51βshort. In the present study, we discovered a longer cDNA species that contains within it the hXB51αshort cDNA sequence and encodes a protein of 396 amino acids (Figure 1a). Analysis of the *hXB51* gene structure showed that this species is encoded by all 14 exons of the *hXB51* gene and that hXB51αshort lacks 123 amino acids encoded by exons 1–4. Recent research also showed that a product of *hXB51* gene, hNECAB3, was a 362-amino-acid protein [17]. This information suggests that hXB51αshort, mXB51βshort [12] and hNECAB3 are truncated proteins or variants with exons spliced out from the *hXB51* gene. A previous paper has shown that exon 9 can be removed by RNA splicing of a short intron that is inserted into the intronless transcript [13], and analysis of the mXB51 protein [12] shows that it lacks the sequence encoded by exon 9. We consequently speculated whether the *XB51* gene in humans encodes two isoforms, one that is unspliced and one that lacks the 34 amino acids encoded by exon 9 (Figure 1a). We denoted the larger species as XB51α, while the spliced isoform is denoted as XB51β, which is identical with hNECAB3 [17].

We assessed with RT-PCR whether the XB51 isoforms including or excluding exon 9 occur endogenously in various human tissues and cell lines and in mice (Figure 1b). We found that, in humans, both isoforms are expressed, although their expression profiles varied depending on the tissue and cell line examined. In the mouse, however, the isoform excluding exon 9 was expressed only in the brain (Figure 1b, lane 11) while another isoform including exon 9 was not found in any of the tissues examined (results not shown). These observations are consistent with our previously reported Northern-blot analysis of *mXB51* and *hXB51* gene expression [12]. Thus in humans, the *XB51* gene, at least, expresses two variants: one including exon 9 composed of 396 amino acids, XB51α protein, and another excluding exon 9 composed of 362 amino acids, XB51β protein.

Interaction of hXB51 isoforms with X11L

Given that hXB51αshort associates with X11L, which in turn is known to be involved in APP metabolism [12], we assessed the ability of the two isoforms, hXB51α and hXB51β, to bind X11L. COS-7 cells were transiently transfected with FLAG-hXB51α or FLAG-hXB51β together with untagged X11L and APP and then subjected to Western blotting with various antibodies (Figure 2a). Anti-FLAG antibody immunoprecipitated X11L together with FLAG-hXB51α or FLAG-hXB51β (Figure 2a, lanes 3 and 4). APP is also recovered with the complex composed of X11L and FLAG-hXB51α (Figure 2a, lanes 5). However, in the presence of APP, hXB51β co-immunoprecipitated less X11L and resulted in no recovery of APP (Figure 2a, lanes 6). Interaction of X11L with XB51β may be weaker than that with XB51α in the presence of APP, or the interaction of APP with X11L may inhibit the ability of X11L to associate with XB51β but not XB51α. Alternatively, XB51β may compete with APP for X11L binding. This issue was resolved when hXB51α-FLAG and hXB51β-FLAG were both immunoprecipitated together with X11L antibody (Figure 2b, lanes 6 and 7). APP was partially recovered with anti-X11L antibody in the presence of hXB51α-FLAG (Figure 2b, lanes 8), but not in the presence of hXB51β-FLAG (Figure 2b, lanes 9). The interaction of hXB51β with X11L thus seems to inhibit the association of X11L to APP. This finding is not in agreement with the observation shown in lanes 6 of Figure 2(a), because recovery of X11L by immunoprecipitation with anti-FLAG antibody was moderate. Therefore, to analyse further the interaction among the three proteins, APP was immunoprecipitated (Figure 2c). HA-hXB51α and X11L were both co-immunoprecipitated with APP (lanes 5 in Figure 2c) but HA-hXB51β and X11L were not (Figure 2c, lanes 6). Thus XB51α forms a tripartite complex together with X11L and APP.

In conclusion, the interaction of X11L with APP is not influenced by the association of XB51α with X11L, and the interaction of X11L with XB51α is also not influenced by the association of X11L and APP. On the contrary, the association of XB51β with X11L interferes with the interaction of X11L with APP (Figure 2c). This result coincides with the observation in Figure 2(b). The hXB51 isoforms have different properties

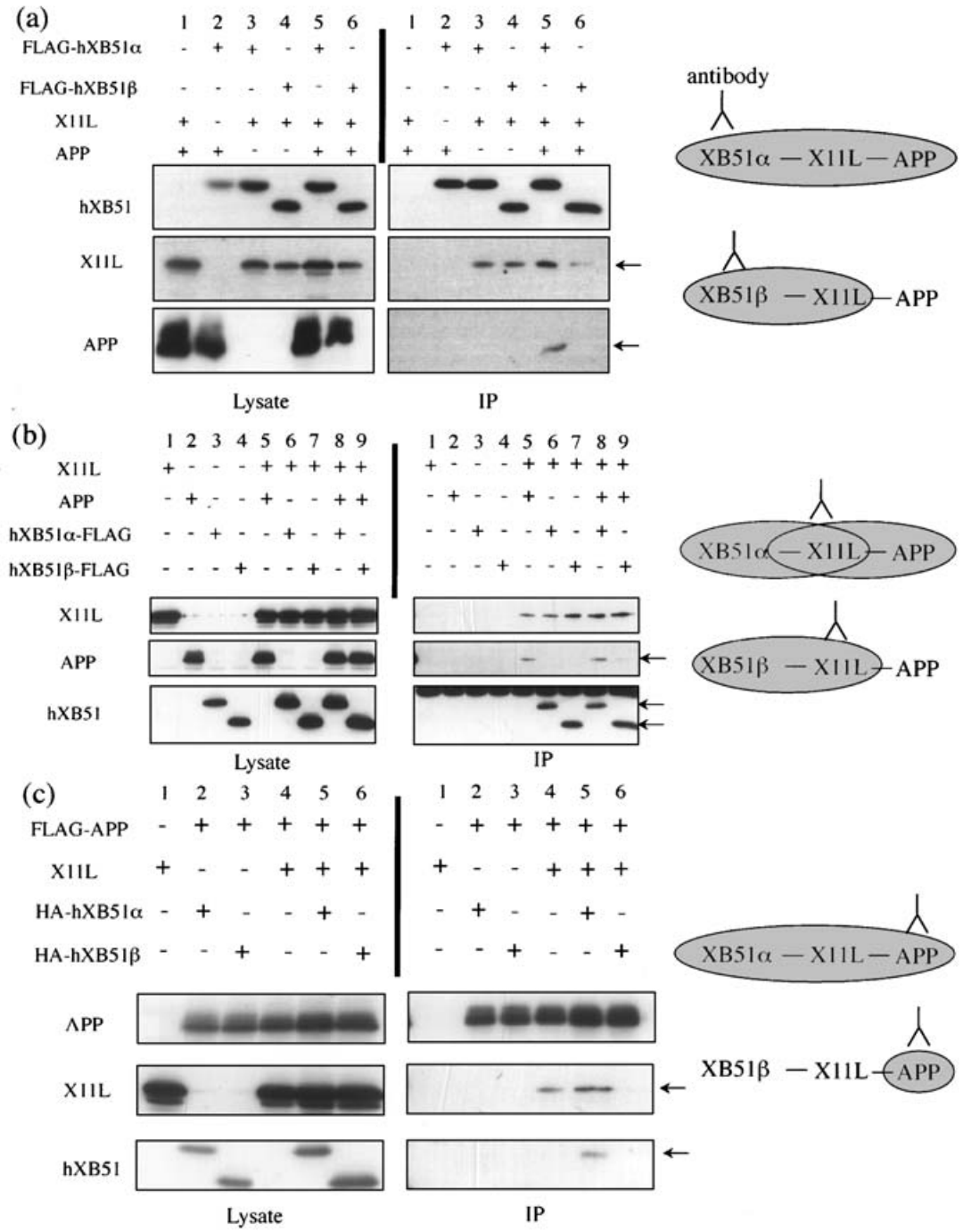

Figure 2 Interaction of hXB51 with X11L and its effect on the intracellular association of X11L with APP

(**a**) Co-immunoprecipitation of X11L and APP with anti-FLAG antibody. COS-7 cells were transiently transfected with pcDNA3.1-FLAG-hXB51α[no-sp] (2 μg) or pcDNA3.1-FLAG-hXB51β (2 μg) together with pcDNA3-hX11L (0.5 μg) and pcDNA3APP695 (1 μg). To standardize the plasmid concentration, adequate amounts of pcDNA3 or pcDNA3.1 vector (−) were added (up to 3.5 μg total). Cells were then lysed with HBST and FLAG-hXB51α or FLAG-hXB51β was recovered by immunoprecipitation with anti-FLAG antibody M2 (3 μg of IgG). Crude lysate (Lysate) and immunoprecipitates (IP) were analysed by Western blotting with anti-FLAG (upper panels), X11L (middle panels) and APP (lower panels) antibodies. Arrows indicate co-precipitated X11L and APP. (**b**) Co-immunoprecipitation of XB51-FLAG and APP with anti-X11L antibody. COS-7 cells were transiently transfected with pcDNA3-hX11L (1 μg) and pcDNA3APP695 (1 μg) together with pcDNA3.1-hXB51α[no-sp]-FLAG (1 μg) or pcDNA3.1-hXB51β-FLAG (1 μg). To standardize the plasmid concentration, adequate amounts of pcDNA3 or pcDNA3.1 vector (−) were added (up to 3 μg total). Cells were lysed with HBST and X11L was recovered by immunoprecipitation with anti-X11L antibody #347-5 (3 μg of IgG). Crude lysate (Lysate) and immunoprecipitates (IP) were analysed by Western blotting with anti-X11L (upper panels), APP (middle panels) and FLAG (lower panels) antibodies. Arrows indicate co-precipitated APP and hXB51α-FLAG or hXB51β-FLAG. (**c**) Co-immunoprecipitation of X11L and HA-hXB51 with FLAG-APP. COS-7 cells were transiently transfected with pcDNA3-FLAG-APP695 (2 μg) and pcDNA3-hX11L (0.5 μg) together with pcDNA3.1-HA-hXB51α[no-sp] (1 μg) or pcDNA3.1-HA-hXB51β (1 μg). To standardize the plasmid concentration, adequate amounts of pcDNA3 or pcDNA3.1 vector (−) were added (up to 3.5 μg total). Cells were lysed with HBST and APP was recovered by immunoprecipitation with anti-FLAG antibody M2 (3 μg of IgG). Crude lysate (Lysate) and immunoprecipitates (IP) were analysed by Western blotting with anti-FLAG (upper panels), X11L (middle panels) and HA (lower panels) antibodies. Arrows indicate co-precipitated X11L and HA-hXB51α. The results, derived from studies expressing XB51α or XB51β, X11L and APP, are shown schematically on the right-hand side. Proteins in the shaded circles were co-immunoprecipitated.

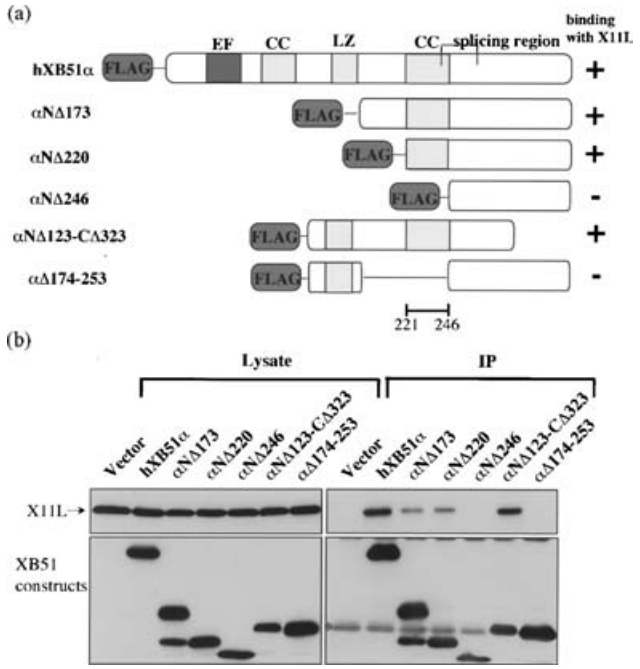

Figure 3 Determination of the X11L-binding site on hXB51α

(**a**) FLAG-tagged hXB51α protein constructs expressed in COS-7 cells are indicated schematically. The ability of the constructs to bind to X11L is indicated as positive (+) or negative (−). (**b**) COS-7 cells were transiently transfected with pcDNA3-hX11L (1 μg) together with 1 μg of pcDNA3.1 (vector), pcDNA3.1-FLAG-hXB51α[no-sp], pcDNA3.1-FLAG-hXB51α [no-sp]NΔ173, pcDNA3.1-FLAG-hXB51α[no-sp]NΔ220, pcDNA3.1-FLAG-hXB51αNΔ246, pcDNA3.1-FLAG-hXB51α[no-sp]NΔ123–CΔ323 or pcDNA3.1-FLAG-hXB51$\alpha\Delta$174–253. The cells were lysed with HBST and the FLAG-hXB51α proteins were recovered by immunoprecipitation with anti-FLAG antibody (M2; 8 μg of IgG). Crude lysate (Lysate) and immunoprecipitates (IP) were analysed by Western blotting with antibodies against X11L (upper panels) or FLAG (lower panels). The asterisk indicates the IgG light chain.

regarding the binding to X11L and the regulation of X11L binding to APP, regardless of small difference between two isoforms.

X11L-binding site on XB51

hXB51α contains an EF-hand motif and three coiled-coil domains, the second of which has a leucine zipper-like structure. The third coiled-coil domain is composed of exons 8 and 9, and thus the splicing of exon 9 in hXB51β is expected to influence the structure of the third coiled-coil domain (Figure 1a). Several domain-deleted FLAG-hXB51 proteins were constructed and examined for their ability to bind to X11L intracellularly by transiently expressing them with X11L co-immunoprecipitating with anti-FLAG antibody (Figure 3). The XB51 constructs lacking the third coiled-coil domain (αNΔ246 and $\alpha\Delta$174–253) were unable to associate with X11L. The construct lacking N-terminal 173 (αNΔ173) and 220 (αNΔ220) amino acids bound to X11L weakly. We can estimate that the X11L-binding site locates between amino acid positions 221 and 246 of XB51α. Thus the third coiled-coil domain contains the binding site of X11L, and deletion of N-terminal region towards the third coiled-coil domain affects the binding ability of the third coiled-coil domain to X11L.

XB51-binding site on X11L and different binding abilities to X11L of XB51 isoforms

We first determined the XB51α-binding site on X11L. Human X11L consists of an N-terminal region (amino acid positions

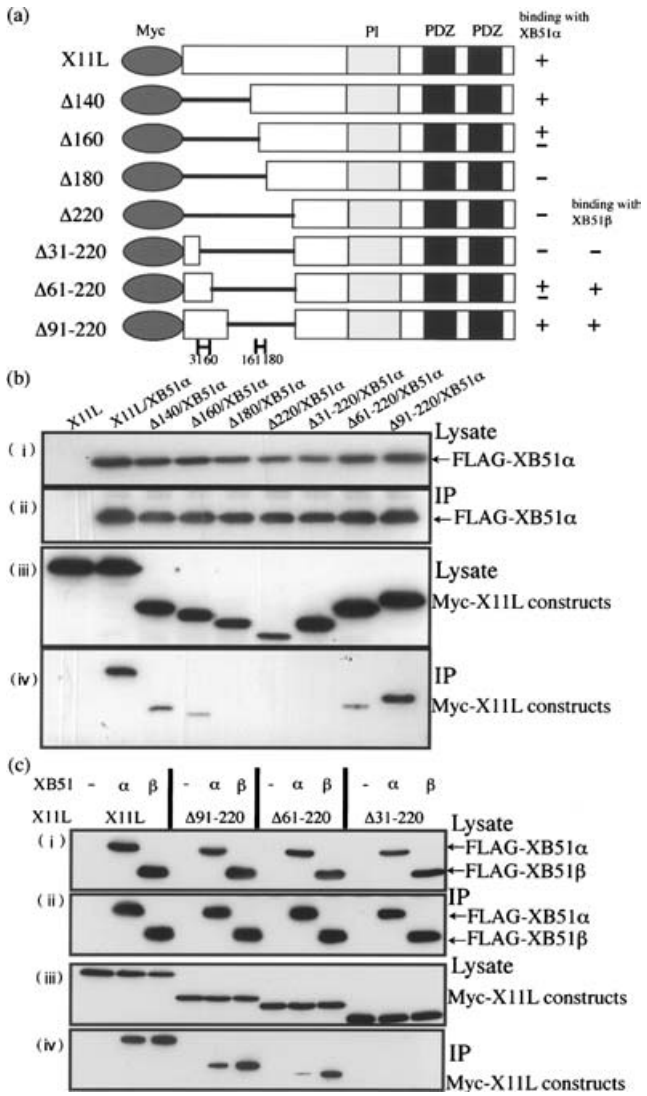

Figure 4 Identification of the hXB51-binding site on X11L

(**a**) The Myc-tagged protein constructs of X11L expressed in COS-7 cells are indicated schematically. The ability of the constructs to bind to hXB51 is indicated as positive (+), weakly positive ($\pm$) or negative (−). The regions of hX11L necessary for interacting with hXB51 (amino acids 31–60 and 161–180) are indicated. PI, PI domain; PDZ, PDZ domain. (**b**) COS-7 cells were transiently transfected with 1 μg of pcDNA3.1-Myc-hX11L (X11L), pcDNA3.1-Myc-hX11LNΔ140 (Δ140), pcDNA3.1-Myc-hX11LNΔ160 (Δ160), pcDNA3.1-Myc-hX11LNΔ180 (Δ180), pcDNA3.1-Myc-hX11LNΔ220 (Δ220), pcDNA3.1-Myc-hX11LΔ31–220 (Δ31–220), pcDNA3.1-Myc-hX11LΔ61–220 (Δ61–220) or pcDNA3.1-Myc-hX11LΔ91–220 (Δ91–220) together with pcDNA3.1-FLAG-hXB51α[no-sp] (1 μg). (**c**) In a separate experiment, the cells are transiently trasfected with 1 μg of pcDNA3.1-Myc-hX11L and pcDNA3.1-Myc-hX11LΔs together with 1 μg of pcDNA3.1-FLAG-hXB51α[no-sp] and pcDNA3.1-FLAG-hXB51β. The cells were lysed with HBST and FLAG-hXB51 proteins were recovered by immunoprecipitation with anti-FLAG antibody M2 (3 μg of IgG). Crude lysate (Lysate, panels i and iii) and immunoprecipitates (IP, panels ii and iv) were analysed by Western blotting with anti-FLAG (panels i and ii) and anti-Myc (panels iii and iv) antibodies.

1–365), a central PI domain (365–555) and a C-terminal region (556–749) containing two PDZ domains [6]. Our previous study revealed that hXB51αshort interacts with the N-terminal region of X11L, but a detailed analysis of this interaction was not performed [12]. Therefore, we prepared various hX11L constructs with deleted N-terminal regions and examined their ability to interact with XB51α (Figures 4a and 4b). The X11L proteins lacking the N-terminal 140 (Δ140) and 160 (Δ160) amino acids

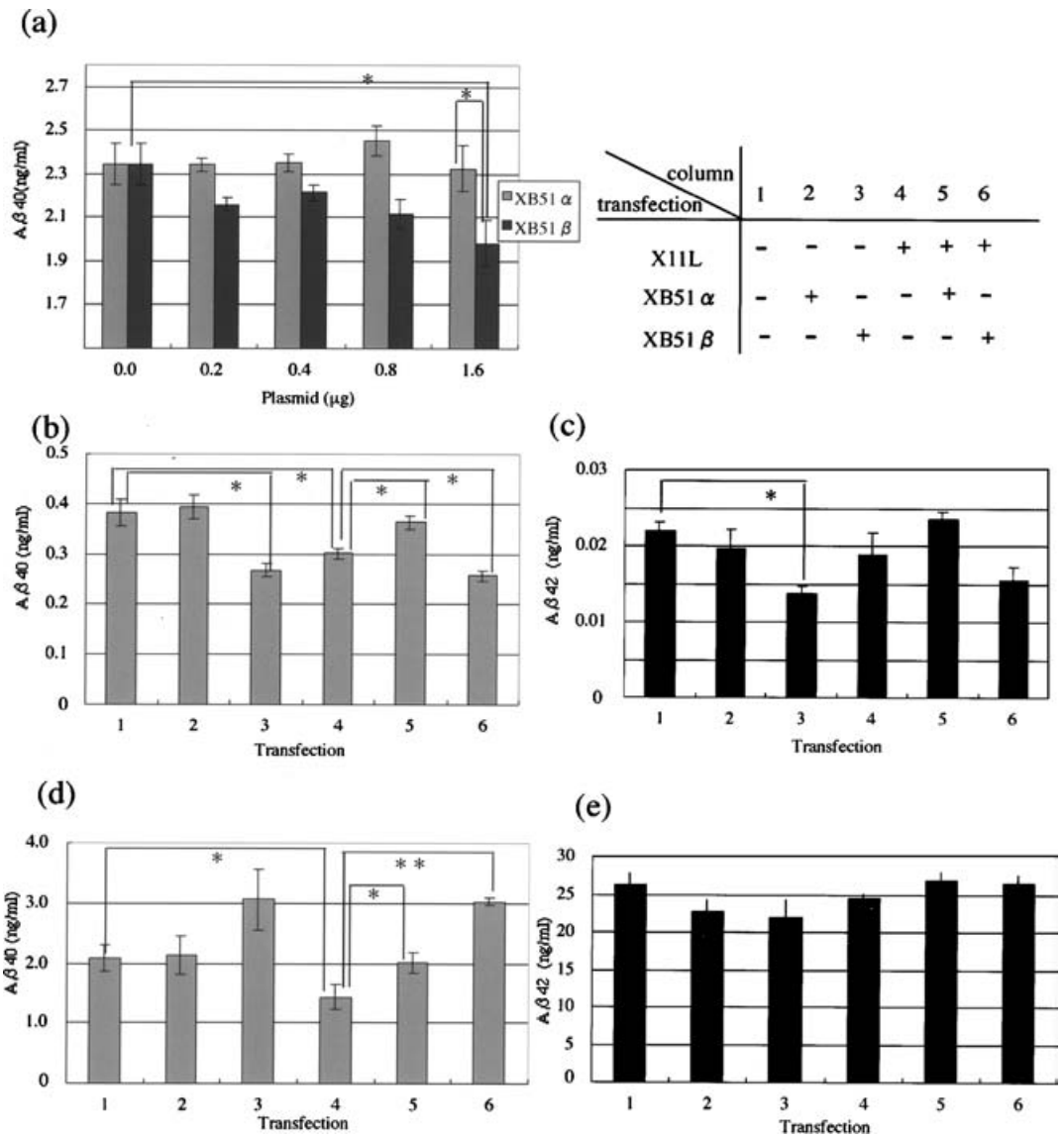

Figure 5 Effect of XB51 isoforms on Aβ generation in the presence or absence of X11L expression

(**a**) HEK-293APPwt cells were transiently transfected with the indicated amounts of pcDNA3.1-hXB51α[no-sp] (XB51α) or pcDNA3.1-hXB51β (XB51β) in the absence of X11L expression. The culture medium was collected 48 h after transfection, assayed for Aβ40, and indicated as ng/ml of medium. (**b** and **c**) HEK-293APPwt cells were transiently transfected with pcDNA3.1-hXB51α[no-sp] or pcDNA3.1-hXB51β (0.8 μg) together with pcDNA3-hX11L (0.4 μg). The culture medium was collected 24 h after transfection and assayed for Aβ40 (**b**) or Aβ42 (**c**) and indicated as ng/ml of medium. (**d** and **e**) The Aβ levels in the cell lysates were also assayed for Aβ40 (**d**) and Aβ42 (**e**) and indicated as ng/ml of lysate. Bars indicate means $\pm$ S.D. ($n = 6$ for **a**–**c** and 4 for **d** and **e**); $^{*}P < 0.05$; $^{**}P < 0.005$. The transfection combinations used (**b**–**e**) are shown in the upper-right-hand panel.

associated with XB51α, but were weaker when compared with the full-length X11L protein. However, the Δ180 and Δ220 proteins, which lacked the N-terminal 180 and 220 amino acids respectively, were completely unable to bind to XB51α. The simple explanation of these results is that hX11L binds to XB51α via a sequence located between amino acids 161 and 180. However, we also found that the constructs lacking amino acids 91–220 (Δ91–220) could bind to XB51α as competently as X11L. Construct lacking amino acids 61–220 (Δ61–220) also showed a weaker binding ability to XB51α, but construct lacking amino acids 31–220 (Δ31–220) had completely lost this ability. These results suggest that another site that contributes to XB51α binding is located between amino acids 31 and 60. It is possible that X11L possesses two co-operative sites located in amino acids 31–60 and 161–180 with which it interacts with XB51α.

We confirmed whether XB51β binds to the identical sites on X11L (Figure 4c). XB51β strongly binds to the Δ91–220 and Δ61–220 proteins when compared with XB51α, but both isoforms lost their binding ability to the Δ31–220. The results indicate that XB51α and XB51β recognize the identical sites on X11L, but XB51β is more tightly coupled to X11L when compared with XB51α.

Effect of XB51 isoforms on Aβ generation

We examined whether the XB51 isoforms affect this Aβ-generating process. HEK-293wt cells, which express APP, were transiently transfected with the cDNAs encoding XB51α or XB51β in the presence or absence of X11L. Secreted and intracellular Aβ levels were quantified (Figure 5). Expression of

XB51α in the absence of X11L did not alter the level of Aβ40 secretion (Figure 5a), but, when X11L alone was expressed, Aβ40 secretion was suppressed (column 4 in Figure 5b) [6]. This suppression was abolished by the expression of XB51α (column 5 in Figure 5b). X11L did not markedly suppress Aβ42 secretion whether XB51α was present or absent (columns 4 and 5 in Figure 5c). With regard to intracellular Aβ generation, X11L expression suppressed Aβ40 but not Aβ42 levels in the cell lysates (columns 4 in Figures 5d and 5e) whereas XB51α abolished this suppression (column 5 in Figure 5d).

In contrast with XB51α, when XB51β was expressed, the secretion of both Aβ40 and Aβ42 decreased in a dose-dependent manner (Figure 5a, and column 3 in Figures 5b and 5c). This occurred whether X11L was present or not (column 6 in Figures 5b and 5c). As the association of XB51β with X11L tends to abolish the binding of X11L to APP (Figure 2), it was expected that the expression of XB51β would abolish the suppression of Aβ40 secretion by X11L. However, we could not detect this X11L-dependent regulation by XB51β (column 6 in Figures 5b and 5c). That XB51β can suppress Aβ secretion in the absence of X11L (column 3 in Figures 5b and 5c) indicates that there is another mechanism by which this protein can regulate APP metabolism (Figure 5a). The expression of XB51β tended to increase the intracellular Aβ40 generation (columns 3 and 6 in Figure 5d), which was independent of X11L.

DISCUSSION

We and others previously reported that the two neuron-specific adaptor proteins X11 and X11L regulate APP metabolism, including the generation of Aβ [6–8,18]. The PI domain of X11 and X11L associates with the ^{681}GYENPTY^{687} sequence in the cytoplasmic domain of APP (human APP695 isoform numbering), and this interaction stabilizes the intracellular APP metabolism. The details of this mechanism are still being investigated. It has been reported that X11s can also interact with various other proteins, including Munc 18 [19], nuclear factor κB [18] and KIF17 (kinesin superfamily motor protein 17) [20]. Some of these are expected to regulate X11s' function, including its role in regulating APP metabolism.

In our previous work, we isolated a novel protein, XB51, that binds to the N-terminal region of the PI domain of X11L [12]. This protein is 273 amino acids long in humans and was designated hXB51αshort in that report. We reported that the interaction of hXB51αshort with X11L inhibits the association of X11L with APP and abolished its ability to suppress Aβ secretion. However, we know now that hXB51αshort may be a truncated form or one of several spliced products of the *hXB51* gene and that a larger polypeptide may act as the major transcript. In the present paper, we identified two other *hXB51* gene products, namely the unspliced 396-amino-acid XB51α protein, and XB51β, which lacks the sequence encoded by exon 9 of *hXB51*.

Here we demonstrate that XB51α and XB51β associate with X11L using a similar recognition site, but with different stabilities. The two isoforms seemed to affect the binding of APP to X11L differently. The association of XB51α with X11L did not inhibit the interaction of X11L with APP, resulting in the formation of tripartite complex composed of XB51α, X11L and APP. In contrast, the association of XB51β with X11L tended to block the binding of X11L to APP, and hXB51β did not form a tripartite complex with X11L and APP. We showed here that the X11L-binding site of XB51 is the third coiled-coil domain of XB51, which is encoded by exons 8 and 9. The lack of exon 9-encoded sequences in XB51β may thus influence the conformation of

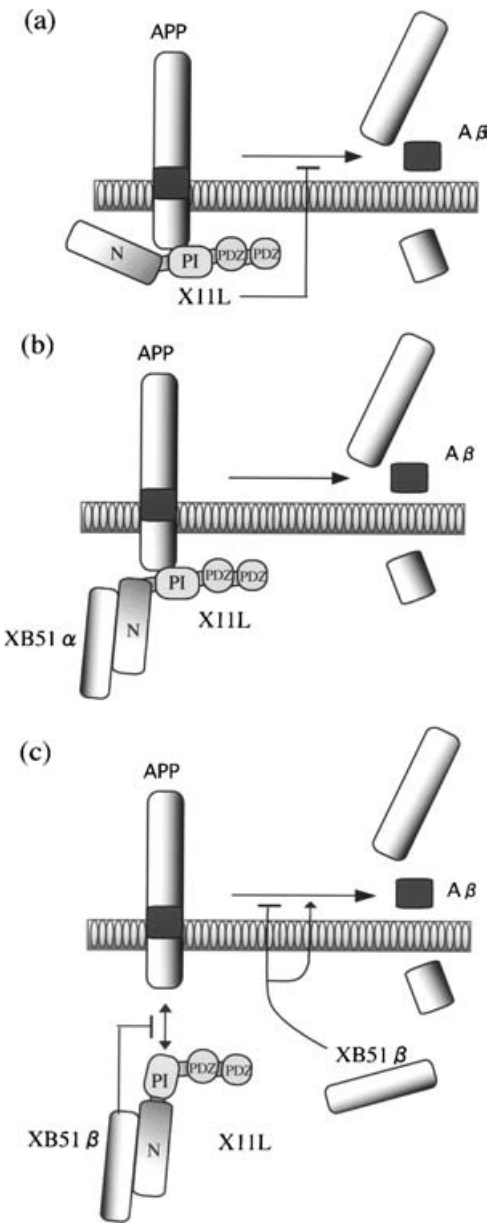

Figure 6 Schematic diagram showing the ability of XB51 isoforms to regulate Aβ generation and X11L association with APP

(**a**) The association of X11L with APP suppresses Aβ generation [6]. (**b**) The binding of XB51α to X11L may induce a conformational change in X11L. The altered X11L still associates with APP but it loses its ability to suppress Aβ generation. (**c**) The association of XB51β with X11L may inhibit the interaction of X11L with APP. The XB51β may also suppress Aβ secretion and increase intracellular Aβ levels by an unknown mechanism that is independent of its association with X11L. N, N-terminal region of X11L; PI, PI domain; PDZ, PDZ domain.

XB51β, resulting in altered binding stability to X11L, which itself affects the interaction of X11L with APP. A similar possibility may also explain why the XB51αshort isoform blocks complex formation of X11L and APP [12]. The latter XB51αshort lacks sequences encoded by the first four exons of *hXB51* and this may alter the conformation of the protein in some way, which affects the ability of X11L to bind APP.

Interestingly, the two XB51 isoforms affect Aβ generation differently and via a different mechanism. The XB51α isoform, through forming the tripartite complex, reverses the block imposed by X11L on Aβ generation from APP. One would expect

that XB51β would also enhance Aβ generation because, like XB51αshort [12], it also abrogates APP binding to X11L. However, the presence of XB51β in a cell actually decreases Aβ secretion and increases intracellular Aβ level. The mechanism by which this occurs is unclear, but it does not involve X11L. Thus the various XB51 protein isoforms can either enhance Aβ production by down-regulating the suppressive effect of X11L or they can regulate Aβ generation and/or secretion by an X11L-indpendent mechanism (Figure 6).

We found that two regions of X11L were involved, namely, amino acids 31–60 and 161–180, in binding to XB51. Although the three-dimensional structure of X11L is unknown, it is possible that the 31–60 and 161–180 sequences co-operate to form a conformation necessary for XB51 binding.

The intracellular system regulating APP metabolism, including Aβ generation, is likely to be very complex. We and others have reported multiple APP-binding proteins, such as X11s [6–9], Fe65 [5,21] and JIP1 (c-Jun N-terminal kinase-interacting protein 1) [22–24]. The binding of these proteins to APP is itself subject to various regulatory processes, including the requirements for APP phosphorylation [5,23,25] or the interaction of other proteins [12,18,26]. Identifying the components that regulate the activities of these APP-binding proteins will help to clarify the mechanisms that regulate Aβ generation. This in turn will aid our understanding of AD pathogenesis and lead to novel strategic approaches for the development of anti-AD drugs [27].

We thank Dr T. Nakaya and Dr H. Taru (Hokkaido University) for helpful discussions and useful comments. We also thank Professor Paul Greengard (Rockefeller University) and Professor Samuel Gandy (Thomas Jefferson University) for the anti-APP antibody G369. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (C)-Advanced Brain Science Project (TS 14017001 and TS 15016002) and a Grant in-Aid for Scientific Research (TS 15659012) from the Ministry of Education, Science, Culture, Sports, and Science and Technology, Japan, and The Mitsubishi Foundation and the TAKEDA Science Foundation (to T. S.) and the McCusker Foundation for Alzheimer's Disease Research (to R. N. M.).

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Received 31 March 2003/22 May 2003; accepted 3 June 2003
Published as BJ Immediate Publication 3 June 2003, DOI 10.1042/BJ20030489