Distinct transcriptional regulation and function of the human *BACE2* and *BACE1* genes

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ABSTRACT **Amyloid β protein (Aβ) is the principal component of neuritic plaques in Alzheimer's disease (AD). Aβ is derived from β amyloid precursor protein (APP) by β- and γ-secretases. Beta-site APP cleaving enzyme 1 (BACE1) has been identified as the major β-secretase. BACE2 is the homolog of BACE1. The *BACE2* gene is on chromosome 21 and has been implicated in the pathogenesis of AD. However, the function of *BACE2* in Aβ generation is controversial. Some studies have shown that *BACE2* cleaved APP at the β-site whereas other studies showed it cleaved around the α-secretase site. To elucidate the involvement of *BACE2* in AD pathogenesis, we compared *BACE2* and *BACE1* gene regulation and their functions in Aβ generation. We cloned and functionally characterized the human *BACE2* promoter. The *BACE2* gene is controlled by a TATA-less promoter. Though Sp1 can regulate both *BACE1* and *BACE2* genes, comparative sequence analysis and transcription factor prediction showed little similarity between the two promoters. BACE1 increased APP cleavage at the β-site and Aβ production whereas *BACE2* did not. Overexpression of BACE2 significantly increased sAPP levels in conditioned media but markedly reduced Aβ production. Knockdown of BACE2 resulted in increased APP C83. Our data indicate that despite being homologous in amino acid sequence, BACE2 and BACE1 have distinct functions and transcriptional regulation. BACE2 is not a β-secretase, but processes APP within the Aβ domain at a site downstream of the α-secretase cleavage site. Our data argue against *BACE2* being involved in the formation of neuritic plaques in AD.—Sun, X., Wang, Y., Qing, H., Christensen, M. A., Liu, Y., Zhou, W., Tong, Y., Xiao, C., Huang, Y., Zhang, S., Liu, X., Song, W. Distinct transcriptional regulation and function of the human *BACE2* and *BACE1* genes. *FASEB J.* 19, 739–749 (2005)**

Key Words: secretase · amyloid β · transcription · promoter · Alzheimer's disease

Aβ IS THE MAJOR COMPONENT of the neuritic plaque, a pathological hallmark of AD. Aβ is generated from APP by sequential cleavages by β- and γ-secretase. However, the majority of APP is cleaved by α-secretase and γ-secretase, which will preclude the production of Aβ (1). BACE1 has been identified as the major β-secretase in vivo (2–7). BACE1 is a type 1 membrane-associated aspartyl protease of 501 amino acids. BACE1 mediates cleavage of APP at the major Asp+1 site and a minor Glu+11 of Aβ (4). *BACE2* was identified in the Down syndrome critical region as a homolog of BACE1 (5, 8–11). *BACE2* is expressed in a wide variety of organs and tissues, with several transcripts resulting from alternative splicing and the use of two polyadenylation signals. *BACE2* is a single transmembrane aspartyl protease of 518 amino acids. It contains a 20-residue signal peptide and two putative N-glycosylation sites (10). The coding sequences of *BACE2* and *BACE1* are 45% identical (5, 11). Despite extensive studies about the function of BACE1, the function of *BACE2* remains unknown. Several studies showed that *BACE2* can cleave APP at the β-secretase cleavage site in vitro (12, 13). BACE2, but not BACE1, was reported to be responsible for the production of Aβ in the Flemish mutant APP transfected cells (12). However, other studies showed that *BACE2* cleaves APP at the Phe^{+19} and Phe^{+20} sites, which are adjacent to the α-secretase cleavage site; *BACE2* functions as an alternative α-secretase and as an antagonist of BACE1 (14, 15). Furthermore, there is no compensatory up-regulation of *BACE2* in *BACE1* knockout mice, which have a deficiency in generating Aβ (16). The *BACE1* gene is localized on chromosome 11q23.3. There have been no mutations in the *BACE1* gene coding sequence genetically associated with AD (17). However, the *BACE2* gene is located on chromosome 21q22.3, a critical region of Down syndrome (DS). Nearly all DS patients will develop AD neuropathology, including neuritic plaques and neurofibrillary tangles, in their brains after middle age. Although a

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genotyping study has shown no association between AD and the intronic polymorphism in *BACE2* (18), immunoreactivity for *BACE2* was detected in neurofibrillary tangle-bearing neurons from elderly DS brains with Alzheimer-type neuropathology, but not in DS brains without Alzheimer-type neuropathology or in control brains of any age (19). The extra copy of *BACE2* in DS suggests it might be involved in the early onset of AD in DS. Therefore, it is important to elucidate the function of BACE2 and the regulation of *BACE2* gene expression.

BACE1 mRNA has the highest expression in the pancreas and has high levels in the brain (5). In situ hybridization analysis showed that BACE1 was highly expressed in neurons. The BACE1 protein is abundant in both normal and AD brains (4, 20). However, *BACE2* mRNA was nearly undetectable in human adult or fetal brain (8). The level of *BACE2* mRNA is very low in most peripheral tissues, but high in kidney, prostate, placenta, colon, and pancreas. Our lab cloned the *BACE1* promoter and demonstrated that *BACE1* gene transcription is tightly regulated and that SP1 plays an important role in *BACE1* gene expression (21). There are few studies of the mechanism of *BACE2* gene transcription. Understanding the regulation of gene transcription can provide additional information on BACE2's function in cells under physiological or pathological conditions. Gene expression is often coordinately regulated to achieve an efficient process (22–26).

To investigate the roles of BACE2 and BACE1 in the pathogenesis of AD, we compared the transcriptional regulation of these two genes and their effects on the production of Aβ. We cloned and functionally characterized the *BACE2* promoter. Like the *BACE1* promoter, the *BACE2* promoter is TATA-less. Despite high similarity in the coding sequence region, the sequence analysis showed no similarity in the *BACE1* and *BACE2* promoter regions or 3′UTR. The *BACE2* promoter has a higher activity in peripheral cells whereas the *BACE1* promoter has higher activity in neuronal cells. The functional comparison of BACE1 and BACE2 in the cleavage of APP showed that BACE1 increased Aβ production, whereas BACE2 had no effect on β-secretase cleavage of APP and decreased Aβ production. Our data showed BACE2 and BACE1 are distinct in gene transcriptional regulation and function.

MATERIALS AND METHODS

Plasmids and primers

Primers were designed to include restriction enzymes sites so that the resulting PCR-amplified fragments could be easily cloned into the multiple cloning site of vector pGL3-Basic (Promega, Madison, WI, USA). Various fragments of *BACE2* from –1583 to +442 bp relative to the transcription start site (+1, adenine) were amplified by PCR and inserted in front of the luciferase reporter gene (Luc) in the pGL3-Basic expression vector. Primers used to generate different promoter deletion plasmids include: forward, –446Nhe I (5′-ATAGCTAGCGCAGCCAGACCCGGCGACTG-3′), –371Nhe I (5′-ATAGCTAGCTAGTTCAGGCCCTCGCTGC-3′),-200NheI (5′-ATAGCTAGCGTATCAGATGAGCCTCGTC-3′), –54Nhe I (5′-ATAGCTAGCGAGGAAATTCGGGACTCG-3′), and reverse, +278Hind III (5′-AGAAGCTTCGCGCCCAGCCTAGCCGG-3′), and +442 Hind III (5′-ATAAGCTTCGGGGTGGGGCGCAACTAC-3′). Primers –1582Kpn I (5′-ATGGATCCCTGCATCGGTCACCATGGT-3′) and +442Mlu I (5′-ATACGCGTCGGGGTGGGCGCAACTAC-3′) were used to amplify the –1582 to +442 region, and this fragment was cloned into pGL3-Basic at the *Kpn* I and *Mlu* I sites. The *BACE2* promoter region and inserts of the promoter-luciferase plasmids were sequenced by an automatic fluorescence-based DNA sequencer (ABI PRISM DNA analyzer; Applied Biosystems, Foster City, CA, USA). Computer-aided sequence analysis was performed with the GCG Wisconsin Package (Genetics Computer Group, Inc., Madison, WI, USA). Human BACE2 cDNA was cut from pcDNA3.1-BACE2 (12) and the fragment was cloned into pcDNA4mycHis (Invitrogen, San Diego, CA, USA) to generate pZ-BACE2mycHis with BACE2 tagged with mycHis epitope. The plasmid was confirmed by restriction digestion and sequencing. BACE2 antisense oligo 5′GUACUUCACUGUGACGUCAAAGCCC3′ was synthesized; the sequence corresponds to the coding region 531-555 bp of the human BACE2 cDNA. pCGN-Sp1 expression plasmid contains Sp1 cDNA under the control of the CMV promoter (27).

Cell culture

Neuro-2a (N2a) cells, a murine neuroblastoma cell line, and HEK293 cells, a transformed human embryonic kidney cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1 mM sodium pyruvate, 2 mM *L*-glutamine and 50 units of penicillin and 50 μg of streptomycin. Sp1 wild-type (Sp1-WT) and Sp1knockout (Sp1-KO) embryonic cells were cultured in DMEM supplemented with 10% fetal bovine serum (21). All cells were maintained at 37°C in an incubator containing 5% CO_2.

Generation of stably transfected cell lines

20E2 is a Swedish mutant APP stably transfected HEK cell line (28). Human BACE1 and BACE2 cDNA were cloned into pcDNA4mycHis vector (Invitrogen), which contains the Zeocin resistance gene. A 2EB2 cell line stably expressing BACE1 and Swedish APP695 was established by transfecting plasmid pZ-BACE1mycHis into 20E2 cells under selection of Zeocin (28). To generate a BACE2 stably transfected cell line 4EB2, plasmid pZ-BACE2mycHis was transfected into 20E2 cells and selected with 1 mg/mL Zeocin. 4EB2 cells stably express Swedish APP695 and mycHis-tagged BACE2 proteins. Stable cell lines were maintained with 100 μg/mL Geneticin and/or Zeocin.

Transfection and luciferase assay

Cells were seeded 1 day before transfection and grown to ~70% confluence at the time of transfection. Cells were transfected with 1 μg of plasmid DNA in a well of a 24-well plate with Lipofectamine 2000 (Invitrogen). The *Renilla* (sea pansy) luciferase vector pCMV-Rluc (Promega) was cotransfected to normalize the transfection efficiency. Cells were washed with PBS 48 h after transfection and lysed in 100 μL of 1× passive lysis buffer for the Dual Luciferase Assay (Promega). Lysates (2 μL) were mixed with the firefly luciferase assay reagent II and the luminescent signal was mea-

sured using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Stop & Glo® Reagent (10 μL) was added to the same tube and the luminescent signal from the *Renilla* luciferase was measured by the same luminometer. The firefly luciferase activity was normalized according to *Renilla* luciferase activity and expressed as relative luciferase units (RLU) to reflect the promoter activity.

Mithramycin A inhibition assay

Sp1 binding inhibitor Mithramycin A (21) was used to treat *BACE2* promoter transfected cells. HEK293 cells were transfected with 2 μg of pB2Luc-A and 0.01 μg of pCMV-Rluc in a 35 mm plate with Lipofectamine 2000 Reagent (Invitrogen). Cells were then treated with Mithramycin A (Sigma, St. Louis, MO, USA) at different doses and times. Cells were harvested at 48 h in 1× Reporter lysis buffer, and dual cell luciferase activities were measured as described above.

Primer extension assay

A primer extension assay was performed to determine the transcription initiation site. Total RNA was extracted from HEK293 cells with TRI Reagent following the manufacturer's protocol (Sigma). A reverse primer, corresponding to +20 to +39 bp of the 5′ untranslated region (UTR) of *BACE2* gene, 5′-GCAAGTTCTTCTCCGCTGCC-3′, was synthesized and radioactively labeled with [γ-^{32}P]ATP (6,000 Ci/mmol, Amersham Biosciences, Arlington Heights, IL, USA) by T4 polynucleotide kinase (Promega). Eighty micrograms of RNA and 20 μL of ^{32}P-labeled primer (10 pmol) were precipitated and hybridized in 30 μL of hybridization buffer (Promega) at 30°C overnight. The hybridized RNA primer samples were precipitated and incubated in 20 μL of 2× reverse transcriptase buffer (10 μL of avian myeloblastosis virus [AMV] primer extension buffer, 1.4 μL of 40 mM sodium pyrophosphate, 6.6 μL of nuclease-free water, 1.0 μL of 1U/μL AMV reverse transcriptase, 1.0 μL of RNase Inhibitor) at 42°C for 40 min. The same radiolabeled primer was used for DNA sequencing with DNA Sequencing Kit (USB, Cleveland, OH, USA). The primer extension assay samples were analyzed on 6% denaturing polyacrylamide gels; the DNA sequencing sample with the same primer was loaded in the same gel and used as the sequence marker.

Immunoblotting

Cell lysates were resolved by 12% SDS-PAGE and immunoblotting was performed as described (28). Full-length APP and its C-terminal fragments C99 and C83 were detected by the antibody C20 against APP C terminus. Monoclonal antibody 9E10 was used to detect myc-tagged BACE1 and BACE2 proteins. A rabbit polyclonal antibody 208 against BACE1 C terminus was used to detect BACE1 proteins (28). To detect BACE2 proteins, a polyclonal antibody 210 was raised in a rabbit against a BACE2 C-terminal amino acid sequence PRDPEVVNDESSLVRH. 22C11 antibody, a mouse monoclonal antibody against amino acids 66-81 of the APP N terminus (Chemicon, El Segundo, CA, USA), was used to analyze secreted APP in the conditioned media. β-Actin expression was detected as an internal control using monoclonal anti-β-actin antibody AC-15 (Sigma).

Aβ40/42 sandwich ELISA assay

Conditioned media were collected from cells and an ELISA assay was performed as described (28). Briefly, protease inhibitor cocktail (Roche, Nutley, NJ, USA) and AEBSF (Sigma) were added to prevent degradation of Aβ. The concentration of Aβ40/42 was determined by β-amyloid 1-40 or 1-42 Colorimetric ELISA kit (Biosource International, Inc., Camarillo, CA, USA) according to the manufacturer's instructions.

RESULTS

Cloning the human *BACE2* gene promoter and mapping the transcription start site

The 5′ upstream region of *BACE2* gene was amplified from a human genomic library. We cloned and sequenced a 2025 bp 5′ flanking region of *BACE2* gene and the N terminus of the coding region (**Fig. 1*A***). The sequence was deposited to GenBank™ under accession number AY769996. The primer extension assay was performed to map the transcription start site of the human *BACE2* gene. An antisense primer (5′-GCAAGTTCTTCTCCGCTGCC) located 262 bp upstream of the translation start codon ATG was used to hybridize with RNA isolated from HEK293 cells. The primer extension assay yielded a ~38 bp major cDNA product. DNA sequencing gel analysis indicates that the major transcription start site of the human *BACE2* gene is located at 301 bp upstream of the translation start site. This transcription start site begins with adenine and was designated as +1 (Fig.1*B*). Computer-based transcription factor binding site search revealed that this 2.0 kb 5′ flanking region contains several putative regulatory elements, such as NF1, SP1, AP1, AP2, GATA, OCT1, and USF (Fig 1*A*). The human *BACE2* gene promoter lacks typical TATA and CAAT boxes and has a very high GC content in its proximal region. The GC content of the 600 bp region surrounding the transcription start site is 78%.

Identification of *BACE2* promoter and its transcriptional activity

To determine whether the 5′UTR fragment of the *BACE2* gene contained the promoter of the *BACE2* gene, we subcloned 5′ flanking fragments of the *BACE2* gene into the promoterless vector pGL3-Basic. The pGL3-Basic vector plasmid lacks a eukaryotic promoter or enhancer sequence upstream of the reporter firefly (*Photinus pyralis*) luciferase gene. Expression of luciferase indicated by luciferase activity therefore depends on the promoter inserted upstream of the reporter gene. A series of deletion plasmids containing various fragments of the 5′ upstream region of *BACE2* gene was generated. The plasmid constructs were checked by agarose gel electrophoresis after restriction enzyme digestion and confirmed by DNA sequencing (**Fig. 2*B***). The pB2Luc-A was generated to contain the 1858 bp 5′UTR from –1580 bp to +278 bp of the *BACE2* gene upstream of the luciferase reporter gene. Compared with the empty pGL3-Basic control, the pB2Luc-A transfected cells had a significantly higher luciferase activity

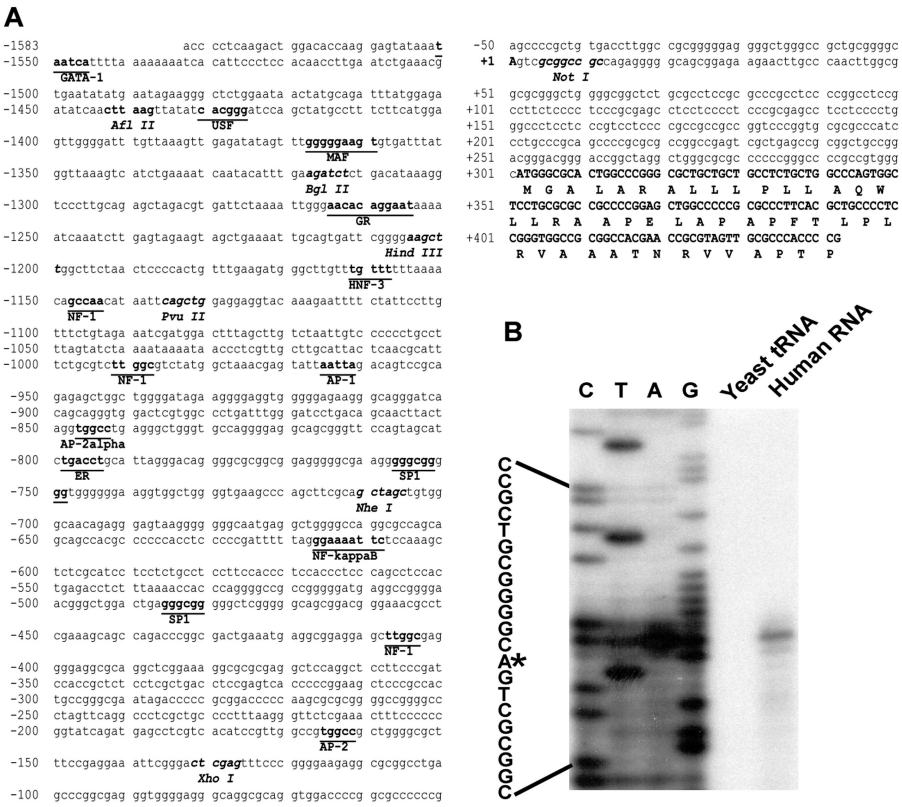

Figure 1. The human *BACE2* gene promoter sequence. *A)* Nucleotide sequence of the human *BACE2* gene promoter. A 1583 bp fragment of the 5′ flanking region and the first exon of the human *BACE2* gene was isolated from a human genomic library and sequenced by the primer walking strategy. Adenine +1 represents the major transcription start site. Positions of some of the unique and common restriction enzymes are indicated in italics. Putative transcription factor binding sites are underlined in boldface. The codon of the first exon is indicated. Genbank™ accession number is AY769996. *B)* Primer extension assay. HEK293 RNA was extracted by TRI-Reagent and yeast tRNA was used as a control. A ^{32}P-labeled reverse primer complementary to +20 to +39 was used for primer extension and sequencing reaction. Plasmid pB2Luc-B was used as sequencing template. Samples were analyzed by 6% denaturing PAGE gel electrophoresis. *Major transcription start site.

(52.52±1.97 RLU vs. 5.06±0.37, $P<0.0001$), indicating that the 1858 bp 5′UTR from –1580 bp to +278 bp contains a functional promoter of the human *BACE2* gene (Fig. 2*C, D*).

To investigate the transcriptional regulation of the *BACE2* gene in different cell types, the deletion plasmids were transfected into a murine neuroblastoma cell line (N2a) and a human embryonic kidney cell line, HEK293. Plasmids pB2Luc-A, pB2Luc-B, pB2Luc-C, pB2Luc-D, and pB2Luc-E contained the *BACE2* promoter region from –1580, –446, –371, –200, and –54 to +278 bp, respectively. These plasmids were transfected into cells and luciferase activity was measured 48 h after transfection. In HEK293 cells, pB2Luc-D had the highest promoter activity (79.06±3.72 RLU) and pB2Luc-A possessed the lowest promoter activity (52.52±1.97 RLU) ($P<0.001$). Deletions from –446 to –54 bp region had no significant effect on *BACE2* promoter

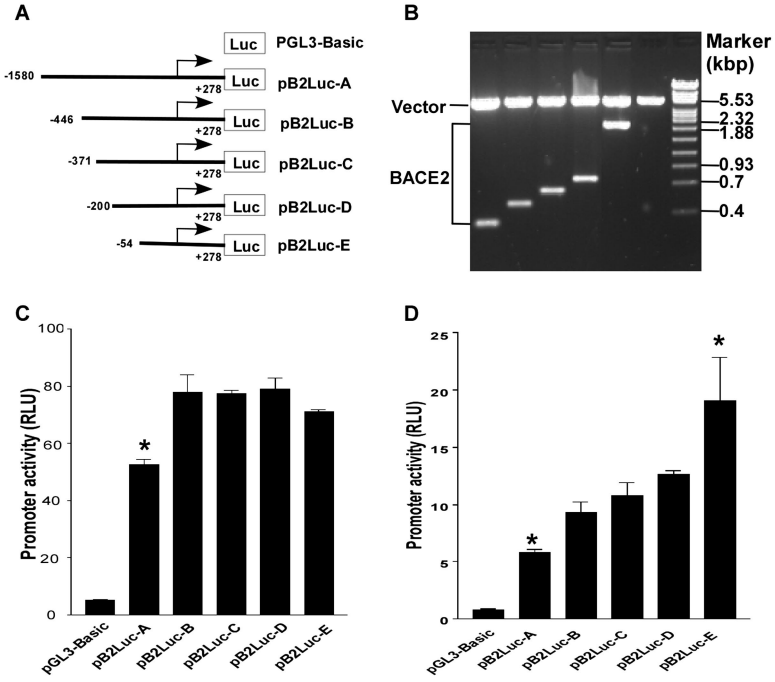

Figure 2. Functional analysis of the *BACE2* gene promoter. *A)* Schematic diagrams of the *BACE2* promoter deletion constructs containing various fragments of the 5′ flanking region of *BACE2* gene in the promoter-less vector plasmid pGL3-Basic. Firefly (*Photinus pyralis*) luciferase gene (Luc) was used as a reporter gene. Arrows indicate the direction of transcription. Numbers represent the end points of the *BACE2* insert. *B)* The deletion plasmids were confirmed by sequencing and restriction enzyme digestion, and the digested samples were analyzed on a 1% agarose gel. Vector size is 4.7 kb, and the *BACE2* gene 5′ flanking fragment insert sizes range from 0.3 to 2.0 kb. Deletion plasmids were cotransfected with pCMV-Rluc into HEK293 cells (*C*) and N2a cells (*D*). Luciferase activity was measured at 48 h by a luminometer. *Renilla* luciferase activity was used to normalize transfection efficiency. Values represent means ± SE ($n=4$). *$P < 0.001$ by ANOVA with the post hoc Newmann-Keuls test.

activity in HEK cells. Promoter activity of plasmids pB2Luc-C, and pB2Luc-E were 77.37 ± 1.07 and 71.13 ± 0.65 RLU, respectively, similar to pB2Luc-B, 77.78 ± 6.11 RLU ($P>0.05$) (Fig. 2*C*). However, *BACE2* promoter activity in the neuroblastoma N2a cells was much lower. pB2Luc-A had the lowest promoter activity of 5.86 ± 0.26 RLU; pB2Luc-B, pB2Luc-C, pB2Luc-D, and pB2Luc-E had promoter activity of 9.32 ± 0.89, 10.74 ± 1.14, 12.62 ± 0.33, and 19.04 ± 3.78 RLU, respectively. The fragment from –54 to +273 bp region had the highest promoter activity in neuronal cells ($P<0.001$ relative to others) (Fig. 2*D*). These data indicate that the –54 bp fragment is essential for *BACE2* transcriptional activation in neuronal and non-neuronal cells and that the *BACE2* gene is preferentially transcribed in non-neuronal cells.

Sp1 regulates *BACE2* gene expression

Previously we demonstrated that Sp1 regulates *BACE1* gene transcription (21). Though we detected no sequence similarity in promoters of *BACE1* and *BACE2*, the transcription factor data mining program suggested that the human *BACE2* promoter had putative Sp1 binding elements. To see whether Sp1 could regulate *BACE2* gene expression, we transfected the Sp1 expression plasmid pCGN-Sp1 and the *BACE2* promoter plasmid pB2Luc-A or pB2Luc-B into HEK293 cells. Sp1 overexpression significantly up-regulated the *BACE2* promoter activity in pB2Luc-A transfected cells (149.83±4.51%, $P<0.001$ relative to control) and had no significant effect on the pB2Luc-B transfected cells ($P>0.05$) or control vector transfected cells (**Fig. 3*A***). These data indicate that the putative Sp1 binding site at –755 bp was functional to regulate *BACE2* transcription. To confirm these data, we transfected pB2Luc-A plasmid into $Sp1^{+/+}$ (Sp1-WT) and $Sp1^{-/-}$ (Sp1-KO) embryonic cells (21). pB2Luc-A had significantly lower promoter activity in Sp1-KO cells (20.56±1.82 RLU) than in Sp1-WT cells (55.92±4.64 RLU) ($P<0.005$) (Fig. 3*B*). This indicates that Sp1 is required for adequate transcription of the *BACE2* gene. When pB2Luc-A transfected HEK293 cells were treated with a Sp1 binding inhibitor, Mithramycin A (21), *BACE2* promoter activity was significantly inhibited in a dose- and time-dependent manner. Addition of 25 nM, 75 nM, 125 nM, and 250 nM of Mithramycin A for 48 h decreased the promoter activity from 55.06 ± 4.41 RLU in control to 27.77 ± 1.68, 9.11 ± 0.32, 7.10 ± 0.21, and 4.67 ± 0.10 RLU, respectively ($P<0.001$ by ANOVA) (Fig. 3*C*). Treatment with 125 nM Mithramycin A for 24 and 48 h inhibited the promoter activity from 55.06 ± 4.41 RLU in control to 34.71 ± 2.06 and 5.58 ± 0.22 RLU, respectively ($P<0.001$ by ANOVA) (Fig. 3*D*). Taken together, these data clearly demonstrate that Sp1 regulates the transcription of the human *BACE2* gene.

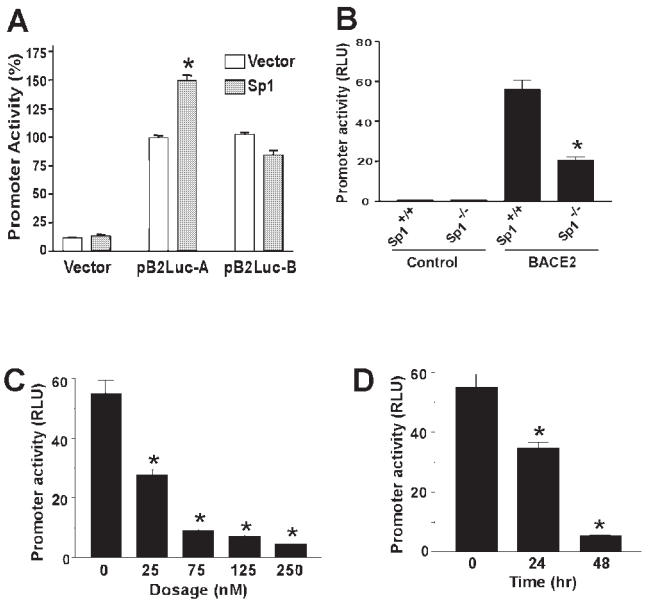

Figure 3. Potentiation of *BACE2* promoter activity by SP1. *A)* SP1 expression plasmid pCGN-Sp1 was cotransfected with pGL3-Basic, *BACE2* promoter pB2Luc-A, or pB2Luc-B plasmids into HEK293 cells. Plasmid pCMV-Rluc was used to normalize the transfection efficiency. Sp1 overexpression significantly increased the *BACE2* promoter activity in pB2Luc-A transfected cells but not in pB2Luc-B transfected cells. Values represent % of normalized luciferase activity (means±SE) ($n=3$ to 6). *$P < 0.001$ by ANOVA with the post hoc Newmann-Keuls test. *B) BACE2* transcriptional activation was markedly reduced in Sp1-KO cells ($Sp1^{-/-}$). Sp1-WT ($Sp1^{+/+}$) and Sp1-KO cells were transfected with the *BACE2* promoter plasmid pB2Luc-A or pGL3-Basic and pCMV-Rluc. *C, D)* Sp1 binding inhibitor Mithramycin A reduced the *BACE2* promoter activity. HEK293 cells were transfected with the *BACE2* promoter plasmid pB2Luc-A and treated with vehicle solution control or Mithramycin A for 48 h at 25, 75, 125, or 250 nM (*C*) or Mithramycin A at 125 nM for 24 or 48 h (*D*). Cells were harvested at the same transfection end point and luciferase activity was measured and expressed as mean ± SE. RLU of control promoter activity. *$P < 0.001$ relative to control by ANOVA with post-hoc Newmann-Keuls test.

Comparative sequence analysis of *BACE2* and *BACE1* genes

BACE2 is the homolog of BACE1, but it has been reported that the *BACE2* gene might function differently from *BACE1.* To investigate whether *BACE2* and *BACE1* gene expression were distinctly regulated, we first examined the sequence differences between *BACE2* and *BACE1* genes. Sequence alignment analysis showed that the amino acid sequences of the human *BACE1* and *BACE2* gene coding regions are 45% identical and 75% homologous (**Fig. 4*A***). Both gene promoters contain many putative transcription factor binding sites (**Table 1**). Comparative sequence analysis has evolved as an essential technique to identify functional coding and noncoding elements conserved throughout evolution. To compare transcriptional regulation of the *BACE1* and *BACE2* gene, we aligned sequences of the 5′ promoter regions and 3′UTR of human *BACE1* and

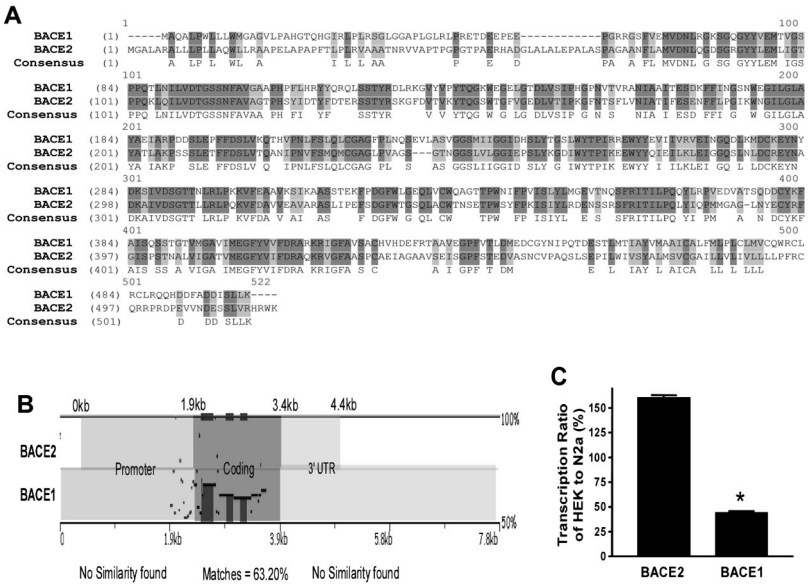

Figure 4. Comparative sequence and promoter activity analysis of the human *BACE1* and *BACE2* gene. *A)* The amino acid sequences of BACE1 and BACE2 were aligned by multiAlign tool. The dark shaded area indicates identical amino acid sequences and light gray represents similar sequences between two proteins. *B)* Sequences of the *BACE1* and *BACE2* genes including the promoter region, the coding sequence, and the 3′UTR were aligned with the zPicture alignment tool and submitted to rVista for analysis of the conservative sequence and transcription factor binding sites. The promoter, coding, and 3′UTR are indicated. The scores of similarity were plotted between 50% and 100%. Location of the black dots or lines represents the similarity score between *BACE2* and *BACE1* gene in the region. Black bars indicate the evolutionarily conserved region (ECR). *C)* *BACE1* and *BACE2* promoter activity in the neuronal and non-neuronal cell. pGL3-Basic, the *BACE2* promoter construct pB2luc-A, and the *BACE1* promoter construct pB1P-G (21) were transfected into HEK293 and N2a cells. The pCMV-Rluc was cotransfected to normalize the transfection efficiency. Cells were harvested 48 h after transfection and dual luciferase assay was performed to measure luciferase activity. Values represent means ± SE. (n=4). *$P < 0.001$ by Student's t test.

BACE2 genes using a zPicture alignment tool. The homology between the *BACE1* and *BACE2* genes was analyzed with the rVista program, which is used to identify evolutionarily conserved regions (ECR), including transcription factor binding sites, and combines transcription factor binding site search with comparative sequence analysis to reduce false positive predictions by the normal database search (29–31). ECR has been used to discover novel genes, identify distant gene regulatory elements, and predict transcription factor binding sites. As homologous genes are likely due to a gene duplication, the promoter region of the two homologous genes might have an evolutionary conservation. The alignment by zPicture identified three evolutionarily conserved regions (ECR) in the coding region with an ECR length of at least 100 bp and an ECR similarity of at least 70%. The analysis showed there is no similarity in the promoter region or the 3′UTR of these two genes. The similarity localized only in the coding region of the human *BACE1* and *BACE2* genes with a matching score of 63.20% (Fig. 4*B*). We analyzed the promoters of *BACE2* and *BACE1* by other

TABLE 1. *Putative transcription factor binding sites in the human BACE1 and BACE2 genes*[a]

BACE1 binding sites*		Transcription factors	*BACE2* binding sites*		Transcription factors
−1891	−1881	HSF-1	−1551	−1446	GATA-1
−1771	−1764	HSF-1	−1431	−1426	USF
−1703	−1698	PU-Box	−1368	−1360	MAF
−1634	−1628	AP-1	−1265	−1255	GR
−1618	−1613	PU-Box	−1162	−1558	HNF-3
−1424	−1418	AP-1	−1448	−1444	NF-1
−1119	−1111	AP-2	−992	−987	NF-1
−987	−982	GATA-1	−966	−962	AP-1
−911	−906	SP-1	−847	−843	AP-2
−877	−870	AP-2	−799	−794	ER
−796	−789	AP-1	−758	−749	SP1
−738	−733	CREB	−617	−609	NF-κB
−612	−605	AP-3	−408	−404	NF-1
−309	−304	C-myc	−262	−255	WT-1
−252	−247	PU-Box	−166	−162	AP-2
−208	−201	C/EBP	+61	+66	SP-1
−141	−135	c-fos-SRE			
−97	−88	Lymphokine			
+82	+87	AP-2			

[a] Numbers represent the bp location of the putative core sequence of the transcription factor binding site with +1 as the major transcription start site. − indicates the location upstream of the major transcription start site; + indicates the location downstream of the major transcription start site.

alignment tools; no significant similarity was found. The results indicate that despite the homology between BACE2 and BACE1 protein sequences, the regulatory nucleic acid sequences that control these two genes are significantly different.

Distinct transcriptional activation of *BACE1* and *BACE2* genes in neuronal and non-neuronal cell lines

Earlier studies showed that BACE1 and BACE2 expression is different in the nervous system and peripheral tissues. BACE1 is highly expressed in neurons whereas BACE2 is highly expressed in non-neuronal tissues. To investigate the role of the promoter in regulating the cell-specific expression of these two genes, we transfected the *BACE1* or *BACE2* promoter plasmids into HEK293 or N2a cells. The result showed that the *BACE2* promoter has higher activity in HEK293 cells (160.11±2.55%) than in N2a cells ($P<0.05$), whereas the *BACE1* promoter activity is higher in the neuronal N2a cells than in HEK293 cells (44.05±1.64%) ($P<0.05$) (Fig. 4*C*).

BACE2 and BACE1 distinctly regulate APP processing and Aβ production

Aβ is generated from APP by β-secretase and γ-secretase. Previous studies showed that BACE1 is the major β-secretase in vivo (2–7). However, the function of BACE2 protein was not fully defined. We generated stably transfected cell lines 2EB2 and 4EB2. pBACE1-mycHis plasmid containing mycHis-tagged BACE1 cDNA was stably transfected into the Swedish mutant APP695 cells 20E2 to generate an APP-BACE1 double stable cell line 2EB2 (28). The 4EB2 cell line was established by stably transfecting pZ-BACE2mycHis into 20E2 cells so that the cells stably express Swedish mutant APP695 and human *BACE2* genes. To detect BACE2 protein, a synthetic peptide B2CT with sequence PRDPEVVNDESSLVRH corresponding to the C terminus of human BACE2 protein was used to immunize a rabbit, and a polyclonal antibody 210 was raised against BACE2 protein. To characterize the BACE2 antibody, HEK293 cells were transfected with empty vector, pBACE1-mycHis, or pZ-BACE2mycHis plasmids. **Figure 5*A*** showed that our antibody 210 detected BACE2 protein but not BACE1 protein in the transfected cells. Overexpressed BACE2 protein could not be detected with preimmunization sera Pre210. Preincubation of 210 with excess B2CT peptides resulted in clearance of BACE2-specific antibody and the precleared antibody could not detect BACE2 protein. These data clearly demonstrated that our 210 antibody is specific to detect BACE2 protein. Western blot analysis showed that 2EB2 cells had robust expression of BACE1 as detected by anti-myc antibody 9E10 and anti-C-terminal BACE1 antibody 208; 4EB2 cells had robust expression of BACE2 detected by 9E10 and 210 (Fig. 5*B*).

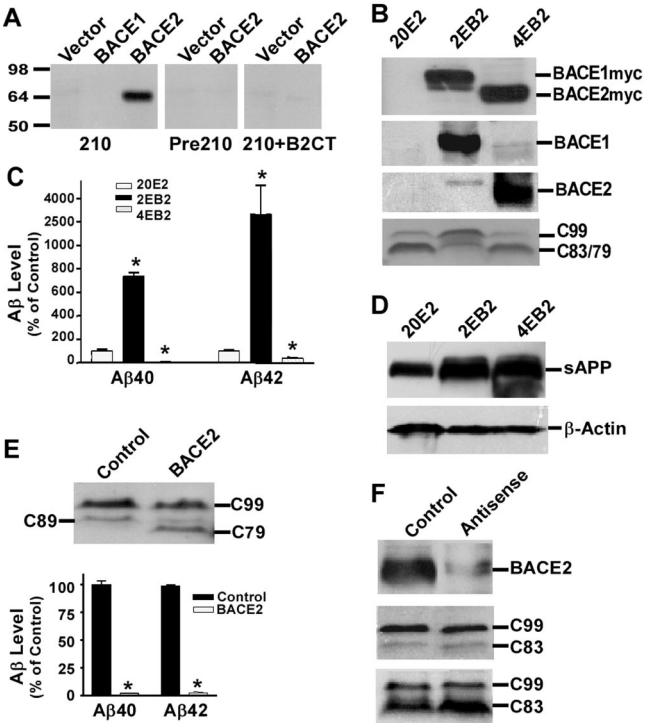

Figure 5. Regulation of APP processing and Aβ production by BACE1 and BACE2. *A)* Characterization of BACE2 antibody 210. pBACE1-mycHis, pZ-BACE2mycHis, or pcDNA4mycHis were transfected into HEK 293 cells. Cell lysates were separated by 12% SDS-glycine gel and immunoblotted with specific BACE2 C-terminal antibody 210, rabbit serum drawn before immunization with BACE2 peptide B2CT, or 210 antibody preincubated with B2CT peptides. *B)* Lysate samples from 20E2, 2EB2, and 4EB2 cells were subjected to Western blot analysis with anti-myc antibody 9E10, anti-BACE1 antibody 208, anti-BACE2 antibody 210, or APP C-terminal antibody C20. *C)* Aβ40 and Aβ42 levels in conditioned media from 20E2, 2EB2, and 4EB2 cells were measured by colorimetric ELISA assay. $n = 4$. $^*P < 0.001$ by ANOVA. Details described in Materials and Methods. *D)* sAPP in the conditioned media was analyzed by 12% Tris-glycine gel with an N-terminal APP antibody 22C11. β-Actin was detected by AC-15 antibody (Sigma). *E)* The BACE1 stable cells 2EB2 were transfected with empty vector as control or pZ-BACE2mycHis. APP CTFs were analyzed on 16% Tris-Tricine gel with C20 antibody. Conditioned media were used for Aβ measurement by ELISA. $n = 3$. $^*P < 0.001$ by Student's t test. *F)* 2EB2 and 4EB2 cells were transfected with BACE2 antisense oligos. The BACE2 level in 4EB2 was detected by antibody 210. APP CTFs in 2EB2 cells (middle gel) and in 4EB2 cells (bottom gel) were separated by 16% Tris-Tricine gel and blotted with C20.

To examine the role of BACE2 and BACE1 in APP processing, cell lysates from stable cells 4EB2 and 2EB2 were subjected to Western immunoblot analysis with C20 antibody to detect APP C-terminal fragments (CTFs) (Fig. 5*B*). In 20E2 cells the major APP CTF was C83; in 2EB2 cells the majority of CTFs were APP C99 fragments, indicating that overexpression of the *BACE1* gene significantly increased APP processing at the β-secretase site, resulting in markedly increased generation of APP C99 fragments. In contrast, the majority of the CTFs in 4EB2 cells were C79 fragments; the level of

APP C99 in 4EB2 cells was slightly decreased relative to that in 20E2 cells, suggesting that overexpression of the human *BACE2* gene has no effect on the β-secretase cleavage of APP.

To define the role of BACE2 and BACE1 in regulating APP processing and Aβ generation, the levels of Aβ production in 20E2, 2EB2, and 4EB2 cells were determined. Conditioned media from the cells were collected and Aβ colorimetric sandwich ELISA assay was performed to measure Aβ levels. Aβ40 and Aβ42 levels were significantly increased in 2EB2 cells by 734 ± 33% and 3791 ± 746% ($P<0.001$), respectively, but drastically decreased in 4EB2 cells to 6.57 ± 1.55% and 39.06 ± 5.58% ($P<0.001$), respectively, relative to control cells 20E2 (Fig. 5*C*). 22C11, an APP N-terminal antibody, was used to check the secreted forms of APP (sAPP) in these cells. sAPP species include α-secretase-generated sAPPα, β-secretase-generated sAPPβ and BACE2-generated sAPP. Conditioned media from the cells were subjected to 12% Tris-glycine PAGE analysis. Overexpression of BACE1 significantly increased the secretion of sAPPβ into the conditioned media and overexpression of BACE2 markedly increased production of BACE2-generated sAPP, resulting in an overall elevation of total sAPP levels in the conditioned media relative to control 20E2 cells (Fig. 5*D*). The significant increase in sAPP together with a reduction in Aβ generation indicates that BACE2 functions not as a β-secretase, but instead cleaves APP within the Aβ domain to preclude Aβ generation.

To confirm the distinct role of BACE2 in APP processing, BACE1 stable cells 2EB2 were transfected with BACE2 cDNA. Due to the significant increase in β-secretase activity in 2EB2 cells, the majority of the APP CTFs were the major β-secretase cleavage product C99 and the minor product C89 in 2EB2 cell lysates. However, overexpression of BACE2 significantly changed the APP processing pattern in BACE1 stable cells, resulting in a decrease in C99 and C83 generation but a significant increase in C79 production (Fig. 5*E*). This suggests that BACE2 cleaves APP at a site downstream of the β-secretase sites. Consistent with this observation, Aβ production was inhibited by overexpression of BACE2 in BACE1 stable cells, 2EB2. Levels of Aβ40 and 42 were markedly reduced to 1.99 ± 0.45% and 2.40 ± 0.87% in the BACE2 transfected cells relative to controls ($P<0.001$) (Fig. 5*E*). Transfection of BACE2 antisense oligos in BACE2 stable cell 4EB2 cells resulted in significant reduction in the level of BACE2 expression (Fig. 5*F*). Such knockdown of BACE2 expression markedly increased the levels of C83 fragment in 4EB2 cells but had no significant effect on C99 generation in BACE1 stable cell 2EB2 (Fig. 5*F*). These data clearly demonstrate that BACE2 processes APP not at the β-secretase sites, as BACE1 does, but rather cleaves APP at a site downstream of α-secretase site. This cleavage of APP within the Aβ domain significantly reduced Aβ generation.

DISCUSSION

Neuritic plaques and neurofibrillary tangles are the two pathological hallmarks of AD. The neuritic plaques consist of the fibrillary form of Aβ. The production of Aβ, especially Aβ42, is elevated in familial AD bearing dominant mutations in APP or presenilin genes. Levels of Aβ and APP C99 are elevated in the brains of DS patients who have three copies of chromosome 21 (32, 33). The elevation of APP C99 and Aβ in DS is partially due to an extra copy of the APP gene in chromosome 21. However, the overexpression of APP cannot fully explain the AD pathogenesis in DS patients (34–36). Thus, several other genes on chromosome 21, including *BACE2*, a homolog of *BACE1*, have been implicated in AD pathogenesis.

BACE1 is the major β-secretase in vivo. siRNA suppression of BACE1 reduced CTFβ and Aβ production in neurons derived from wild-type and Swedish APP mutant transgenic mice (37). Disruption of the presenilin genes inhibits γ-secretase activity and abolishes Aβ production. Presenilin deficiency inhibits Notch signaling and embryonic development (38–42). In contrast, *BACE1*-KO mice have abolished Aβ generation, but exhibit a normal phenotype without any observed developmental deficits (6, 7, 43). Disruption of the *BACE1* gene rescues memory deficits and cholinergic dysfunction in Tg2576 Swedish APP mutant transgenic mice (44). These results suggest that inhibition of BACE1 is a valid therapeutic target for AD. BACE2 and BACE1 share many similarities, including 63% identity in amino acids and two aspartic protease active site motifs, six conserved lumenal cysteine residues, a C-terminal transmembrane domain, N-linked glycosylation sites, as well as other structural features. Like BACE1, BACE2 undergoes a complex set of post-translational modifications, including prodomain processing between Leu^{62} and Ala^{63}. Prodomain processing of BACE2 is autocatalytic (45). The high degree of similarity between BACE2 and BACE1 protein sequences suggested that BACE2 might function as a β-secretase. It was reported that BACE2, but not BACE1, was responsible for the production of Aβ in Flemish mutant APP transfected cells (12). BACE2 cleaves at the β site and more efficiently at another site within Aβ. The familial AD-associated Flemish mutant APP is adjacent to this latter site. BACE1 and BACE2 respond identically to conservative β-site mutations, and alteration of a common active site Arg inhibits β-site cleavage but not cleavage within Aβ by both enzymes (12). However, other studies showed that BACE2 mainly cleaved in the middle of the Aβ sequence, after the 19th and 20th residues that are adjacent to the α-secretase cleavage site (46, 47), leading to accumulation of the N-terminal truncated product C79 in BACE2-expressing cells (46). BACE2 functions as an alternative α-secretase and as an antagonist of BACE1 (14, 15). While in situ hybridization showed the coexpression of APP, BACE1, and ADAM10, expression of BACE2 and ADAM17 only partially overlapped

with that of APP, suggesting that ADAM10 and BACE1 are authentic α- and β-secretases (20). BACE1 knockout mice do not acquire compensatory gene expression changes or develop neural lesions over time. In particular, BACE2 expression is not up-regulated (16). Selective inactivation of BACE2 by RNAi results in increased β-cleaved secreted APP and Aβ secretion from cells (15). These studies suggest that BACE2 might have distinct functions from BACE1.

Regulation of gene transcription is closely related to the function of a particular gene. Some genes involved in common processes are often coordinately regulated to achieve efficiency. To define the molecular mechanism by which *BACE2* gene expression is regulated at the transcriptional level, we cloned the 2.0 kb 5′ flanking region of the human *BACE2* gene and identified the gene promoter. Sequence analysis showed that the *BACE2* promoter, unlike most type II eukaryotic gene promoters, does not contain a typical TATA and CATA box and has a high GC content. This TATA-less and high GC feature of the *BACE2* gene is similar to that of the *BACE1* gene and is common in many housekeeping genes (21, 48). The 5′ flanking region has various possible transcription factor binding elements such as NF1, SP1, AP1, AP2, GATA, and OCT1. The region of –54 to +278 bp had similar promoter activity to –446 to +278 in HEK cells and the highest promoter activity in N2a cells, suggesting this proximal region of –54 to the transcriptional start site was important in regulating the *BACE2* gene. Our deletion assay showed that the region from –1580 to –446 contained negative regulatory element(s); removing this region significantly increased the BACE2 promoter activity in neuronal and non-neuronal cells. Deletions from –652 bp to –54 bp resulted in a gradual increase in the promoter activity in N2a cells but not in HEK293 cells. Deletion of the 146 bp fragment from the –200 to –54 bp region significantly increased BACE2 promoter activity. These data indicate there might be a neuronal specific repressive element in this region and that *BACE2* gene expression is tightly regulated at the transcription level in neuronal and non-neuronal cells. Computer analysis for putative transcription factor binding sites showed several NF1 sites in this region. NF1 is believed to interfere with the binding of TBP to its recognition site, thus inhibiting transcription (49–51). NF1 is also important in tissue-specific and developmentally specific gene expression. In the *BACE2* promoter, NF1 might interact with other regulators to achieve repression in neuronal cells. Future studies could be directed to identify the transcription factors in this region of the *BACE2* promoter.

To examine the function of BACE2 and its relationship to BACE1, the transcriptional regulation of *BACE2* and *BACE1* genes was analyzed. We previously cloned and characterized the human *BACE1* promoter (21). Analysis of the BACE2 and BACE1 sequences showed that despite 75% homology in the coding sequence, there is no similarity in the promoter regions. There are different putative transcription factor binding sites in these two promoters. BACE1 and BACE2 have distinct expression patterns. *BACE1* mRNA is highly expressed in neurons of most brain regions. Northern analysis reveals that *BACE2* mRNA is expressed at low levels in most human peripheral tissues and at higher levels in colon, kidney, pancreas, placenta, prostate, stomach, and trachea. Human adult and fetal whole brain and most adult brain subregions express low or undetectable levels of *BACE2* mRNA (8). Our study shows that the *BACE2* promoter has higher activity in HEK293 cells whereas the *BACE1* promoter has higher activity in neuronal cells. Our study provides a mechanism underlying the distinct distribution of BACE1 and BACE2 in nervous and peripheral tissues.

We earlier reported that transcription factor Sp1 plays an important role in *BACE1* gene expression and APP processing (21). Sp1 and TAFII 130 transcriptional activity are disrupted in early Huntington's disease (52–55). In this study we discovered that Sp1 regulates human *BACE2* gene transcription. BACE2 gene transcriptional activation was markedly reduced in the Sp1-KO cells relative to the Sp1-WT cells. Treatment with Sp1 binding inhibitor Mithramycin A resulted in inhibition of the *BACE2* promoter activity in a time- and dose-dependent manner. Although sequence analysis revealed that the BACE2 gene promoter contained two Sp1 binding sites at –755 and +63 bp, Sp1 overexpression significantly up-regulated *BACE2* promoter activity only in pB2Luc-A transfected cells and had no significant effect on the pB2Luc-B transfected cells. These data indicate that the putative Sp1 binding site at –755 bp is functional in regulating human *BACE2* transcription. Our study demonstrates that Sp1 regulates *BACE2* and *BACE1* genes. Sp1 has been shown to play an important role in regulating the expression of many genes. Its C-terminal domain interacts with other transcription factors in a synergistic manner that controls gene expression in time and space (56). Our data suggest that Sp1 might work with other transcription factors to regulate different tissue expression patterns of the *BACE2* and *BACE1* genes.

The BACE2 function, either as a β-secretase or α–secretase, has not been fully defined. To examine this, a stable cell line expressing BACE2 and Swedish mutant APP695 was established. Our results did not show that BACE2 had an effect similar to that of BACE1 on production of C99 and Aβ, both of which were elevated in the BACE1 stable cell line 2EB2. In contrast, overexpression of BACE2 significantly increased sAPP secretion but decreased Aβ generation. These data clearly show that BACE2 is not a β-secretase in the APP processing pathway. BACE2 inhibits Aβ generation by BACE1, and knockdown of BACE2 expression increased the level of the α-secretase product C83. These data indicate that BACE2 processes APP within the Aβ domain at a site downstream of α-secretase cleavage, consistent with a report that the major BACE2 cleavage site is located at the 19th and 20th residues of Aβ domain (46, 47). Our results at the transcriptional level and functional level argue against the involvement of

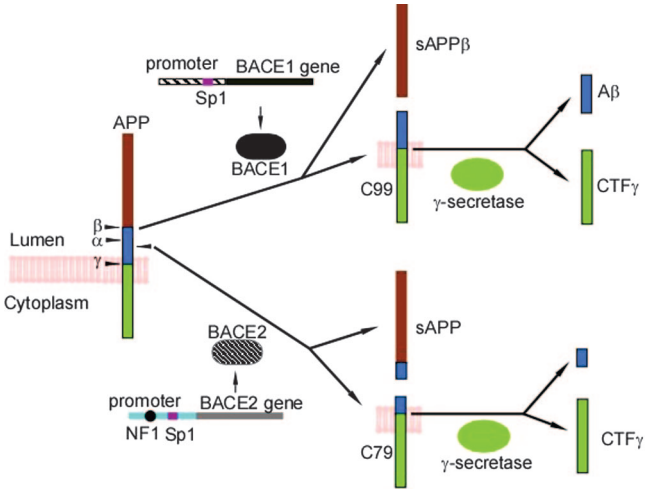

Figure 6. Schematic diagram of transcriptional regulation and function of the *BACE2* and *BACE1* gene. *BACE2* and *BACE1* genes are distinctly regulated at a transcriptional level. Though Sp1 can regulate both of the *BACE1* and *BACE2* genes, comparative sequence analysis and transcription factor prediction showed little similarity between the two promoters. Despite being homologous in amino acid sequence, BACE2 and BACE1 have distinct function in processing APP. BACE1 is the β-secretase to cleave APP at β-secretase site to generate Aβ. However, BACE2 is not a β-secretase but processes APP within the Aβ domain at a site downstream of α-secretase cleavage site to preclude Aβ generation.

BACE2 in the production of Aβ, and thus the pathogenesis of AD (**Fig. 6**). Since BACE2 is almost undetectable in brain tissues and BACE2 antagonized the action of BACE1 on APP processing, future study may be needed to examine whether up-regulation of BACE2 in neuronal cells can be one potential strategy in AD therapy. FJ

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