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BACE1- and BACE2-expressing Human Cells

CHARACTERIZATION OF β-AMYLOID PRECURSOR PROTEIN-DERIVED CATABOLITES, DESIGN OF A NOVEL FLUORIMETRIC ASSAY, AND IDENTIFICATION OF NEW *IN VITRO* INHIBITORS*

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We have set up stably transfected HEK293 cells overexpressing the β-secretases BACE1 and BACE2 either alone or in combination with wild-type β-amyloid precursor protein (βAPP). The characterization of the βAPP-derived catabolites indicates that cells expressing BACEs produce less genuine Aβ1–40/42 but higher amounts of secreted sAPPβ and N-terminal-truncated Aβ species. This was accompanied by a concomitant modulation of the C-terminal counterpart products C89 and C79 for BACE1 and BACE2, respectively. These cells were used to set up a novel BACE assay based on two quenched fluorimetric substrates mimicking the wild-type (JMV2235) and Swedish-mutated (JMV2236) βAPP sequences targeted by BACE activities. We show that BACEs activities are enhanced by the Swedish mutation and maximal at pH 4.5. The specificity of this double assay for genuine β-secretase activity was demonstrated by means of cathepsin D, a "false positive" BACE candidate. Thus, cathepsin D was unable to cleave preferentially the JMV2236-mutated substrate. The selectivity of the assay was also emphasized by the lack of JMV cleavage triggered by other "secretases" candidates such as ADAM10 (A disintegrin and metalloprotease 10), tumor necrosis α-converting enzyme, and presenilins 1 and 2. Finally, the assay was used to screen for putative *in vitro* BACE inhibitors. We identified a series of statine-derived sequences that dose-dependently inhibited BACE1 and BACE2 activities with IC_{50} in the micromolar range, some of which displaying selectivity for either BACE1 or BACE2.

Alzheimer's disease is characterized by the cortical and subcortical accumulation of proteinaceous deposits called senile plaques (1). The main constituent of these aggregates is referred to as amyloid β peptide (Aβ).[1] Aβ is generated from the β amyloid precursor protein (βAPP) by the subsequent attacks by β- and γ-secretases, which liberate the N- and C-terminal moieties, respectively (2). It is now clear that under this generic terminology, "Aβ" gathers a series of distinct "Aβ-related species," some of them truncated at their β-secretase-derived N terminus (3). Although the nature of the γ-secretase is still discussed (4, 5), the identity of the β-secretase candidate is more consensual.

BACE1 (β-site APP-cleaving enzyme) is an aspartyl protease recently identified by several groups (6–9), which displays all the properties expected from a genuine β-secretase candidate (for review, see Refs. 10 and 11). Thus, BACE1 is an acidic protease mainly localized in the Golgi apparatus and in endosomal compartments and exhibits a lumenal active site fitting well with the lumenal cleavage of βAPP occurring in these acidic compartments where Aβ had been detected. The overexpression of BACE1 leads to increased recovery of Aβ-related fragments, mainly 1–40 and 11–40 that are also observed when recombinant BACE is incubated with βAPP (7, 9). The opposite phenotype is observed when BACE is down-regulated by an antisense approach (7, 9). BACE2 is a parent protease (12) that seems to contribute poorly to neuronal Aβ production. However, BACE2 could be important in Down syndrome pathology because the enzyme is encoded by chromosome 21 (13, 14) and elevated BACE2 expression is observed in trisomic brains (15).

BACE1 not only behaves as a β-secretase, *in vitro*, but likely corresponds to the main contributor of the β-secretase pathway, *in vivo*. Thus, it was evidenced that the invalidation of the *BACE* gene led to abolishment of Aβ production in knockout mice (16) and neurons (17). That this deletion appeared totally innocuous (18) makes BACE likely the most interesting target of an Aβ-directed AD therapy. Here we fully characterize the βAPP-derived catabolites generated by BACE1 and BACE2-

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[1] The abbreviations used are: Aβ, amyloid β peptide; βAPP, β-amyloid precursor protein; Abz, *p*-aminobenzoic acid; tBu, *tertio*-butyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPLC, high pressure liquid chromatography; Boc, *tert*-butyloxycarbonyl; *Sta*, statine; *Norsta*, norstatine; *AHPPA*, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenyl-pentanoic acid; *ACHPA*, (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxy-pentanoic acid; *Qui*, quinolein-2-carboxylic acid; TACE, tumor necrosis α-converting enzyme; CS, commercial substrate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Bz, benzoyl.

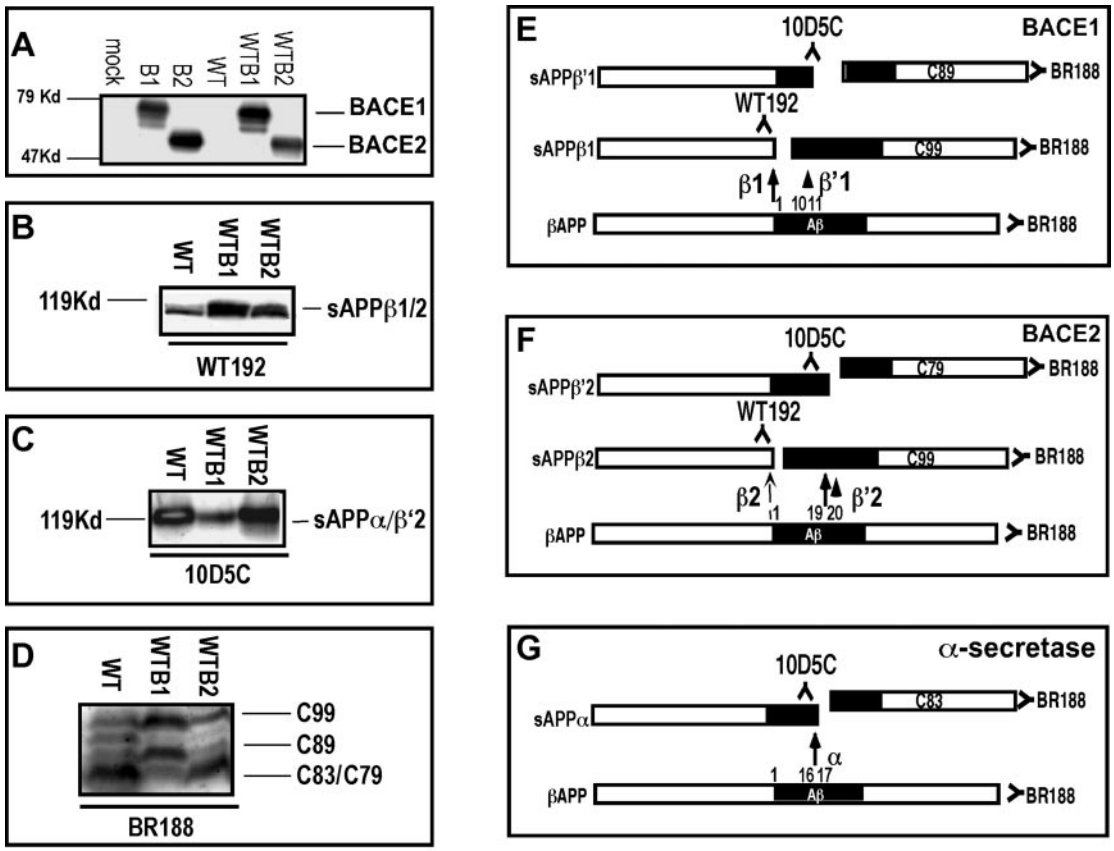


FIG. 1. **Characterization of secreted βAPP-derived products and their C-terminal counterparts generated by BACE1- and BACE2-expressing cells.** HEK293 cells overexpressing 1D4-tagged BACE1 or BACE2 alone (*B1* and *B2*, respectively) or in combination with wild-type βAPP (*WTB1* and *WTB2*) have been obtained as described under "Experimental Procedures" and monitored by means of anti-1D4 antibodies (*panel A*). sAPPβ1/2 derived from BACE1 (*panel E*) and BACE2 (*panel F*) are identical and are monitored by means of WT192 (*panel B*) as described under "Experimental Procedures." sAPPα and sAPPβ′2 generated by α-secretase (*panel G*) and BACE2 (*panel F*), respectively, were monitored by means of 10D5C (*panel C*) as described under "Experimental Procedures." Intracellular C-terminal counterparts derived from the above cleavages were monitored in the indicated cell lysates with BR188 (*panel D*) as described under "Experimental Procedures."

expressing cells. Furthermore, we describe a novel fluorimetric assay and new *in vitro* inhibitors of potential interest to further characterize BACE1 and BACE2 properties.

EXPERIMENTAL PROCEDURES

Chemical Reagents—Fmoc-amino acids, HBTU, and the resin were purchased from Senn Chemicals (Gentilly, France). The reagents and solvents for solid-phase peptide synthesis were obtained from Acros (Noisy-le-Grand, France) or from SDS (Peypin, France). All other chemicals were of the purest grade available.

Synthesis of Peptides and Inhibitors—The intramolecularly quenched fluorogenic peptidic substrates Abz-Val-Lys-Met-Asp-Ala-Glu-EDDnp (JMV2235) and Abz-Val-Asn-Leu-Asp-Ala-Glu-EDDnp (JMV2236) contain the *ortho*-aminobenzoyl (Abz)/dinitrophenyl groups as the donor/acceptor pair (19). They were synthesized essentially by solid phase methods (20), starting from the C-terminal residue Glu-EDDnp. The latter was first assembled in solution by coupling Fmoc-Glu(γ-tBu)-OH to EDDnp using HBTU as the coupling agent, in the presence of diisopropylethylamine. The resulting Fmoc-Glu(γ-tBu)-EDDnp was treated by trifluoroacetic acid to free the γ carboxylate group that was subsequently attached to the linker of a Wang resin by the symmetric anhydride method. After manual assembling of the substrates using the solid phase Fmoc strategy and HBTU as the coupling agent, cleavage of the peptides from the resin and simultaneous deprotection of side chains were carried out by treatment with a solution containing trifluoroacetic acid, thioanisole, water, phenol (8.5/0.5/0.5/0.5) for 2 h at room temperature. The final deprotected peptides were purified by reverse-phase chromatography on a C18 column (Deltapack Waters, 40 × 100 mm) by means of a linear gradient of 20–35% acetonitrile in 0.1% aqueous trifluoroacetic acid over 30 min (flow rate 50 ml/min), and their purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry (Abz-VKMDAE-EDDnp: experimental mass 1018.7 ± 0.2, calculated mass 1018.4; Abz-VNLDAE-EDDnp: experimental mass 986.6 ± 0.2, calculated mass 986.4).

All peptidic inhibitors were amidated at their C-terminal end and blocked at their N terminus, either by an acetyl or by a Boc group (excepted the Qui-containing compounds), to protect them from exopeptidasic attacks. The peptide backbone of the compounds was stepwise assembled by classical methods, using Boc as the α-amino protecting group and benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate as the coupling reagent, either in homogeneous phase or on solid phase with methylbenzhydrylamine resin, necessitating a final HF cleavage procedure. Published protocols were followed for the formation of the peptide bond isostere moieties, reduced amide bond (Leu-Ψ(*CH$_s$NH*)-Asp in JMV963) (21, 22), norstatine (*Norsta*-containing compounds) (23, 24), and statine and analogs (*Sta*-, *AHPPA*-, *ACHPA*-containing compounds) (25, 26). All synthetic inhibitors were purified on C18 reverse-phase HPLC, and their purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry.

HEK293 Culture and Stable Transfection—HEK293 and stably transfected HEK293 cells overexpressing wtβAPP751 (WT) and swβAPP751 (SW) (27) were stably transfected with DAC30 (according to the manufacturer's instruction Eurogentec) containing 2 μg of pcDNA3 vector encoding either 1D4-tagged BACE1 (WTB1) or BACE2 (WTB2) and zeocin resistance. 1D4 is a 10-amino acid C-terminal tag derived from bovine rhodopsin (28). Medium was replaced 48 h after transfection with selective medium containing neomycin and/or zeonin (1 g/liter). Transfectants were screened by 10% SDS-PAGE gels analysis and Western blotting (see below). Positive clones overexpress 75- and 50-kDa immunoreactive proteins, respectively, corresponding to BACE1 and BACE2 (28). Tumor necrosis α-converting enzyme (TACE), A disintegrin and metalloprotease 10 (ADAM10), and presenilin 1 and presenilin 2-expressing cells were previously described (29–31).

Western Blot Analyses—HEK293 cells were scraped and lysed in

RIPA 1× buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.1% deoxycholate, and 1% Nonidet P-40). Equal quantity of proteins were separated on a 10% SDS-PAGE gel for the detection of BACE1 and BACE2 proteins and transferred to Hybond-C (Amersham Biosciences) membranes. After transfer, membranes were blocked with 5% nonfat milk and incubated overnight with the primary antibody anti-1D4 (1:1000) (mouse monoclonal antibody donated by Scott Waniger at the National Cell Culture Center, National Cell Culture Center, Minneapolis, MN). Immunological complexes were revealed with an anti-mouse peroxidase (Amersham Biosciences) antibody followed by enhanced chemiluminescence (Amersham Biosciences). All protein concentrations were determined by the Bradford (32) procedure as described.

Measurements of Total Aβ—Stably transfected WT, WTB1, WTB2, and SW HEK293 cells (see above) were incubated for 7 h in the presence of phosphoramidon (1 μM) (Sigma). Media were collected, diluted in 1/10 RIPA 10× buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.1% SDS, and 1% Nonidet P-40), and incubated overnight with a 200-fold dilution of FCA18 (total Aβ) as described (33). After further incubation for 3 h with protein A-Sepharose (Zymed Laboratories Inc.) and centrifugation, pelleted proteins were submitted to 16.5% Tris/Tricine gels and then Western blotted on Hybond C membranes (Amersham Biosciences) for 45 min. Nitrocellulose sheets were heated in boiling phosphate-buffered saline for 5 min and capped with 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20 for 1 h. Membranes were then incubated overnight with WO2 antibody (ABETA GmbH, Heidelberg, Germany) specific for the 5–8 N-terminal region of Aβ (34)) and detected by enhanced chemiluminescence.

Measurements of sAPPβ, sAPPα, and C-terminal Products—WT, WTB1, and WTB2 cells were allowed to secrete for 7 h as above and then 15 (sAPPα/β′2) or 50 μl (sAPPβ1/2) of secretate were loaded on 8% SDS-PAGE and Western blotted with 10D5C or WT192 (kind gift from Dr. D. Schenk, Elan Pharmaceuticals), respectively. Immunological complexes were revealed with the adequate IgG coupled to peroxidase. C-terminal products were analyzed from the same cell lysates and were separated on large Tris/Tricine 16.5% gels, Western blotted, and probed with BR188 as described (35).

Fluorimetric Assay—Different cell types were lysed, in 10 mM Tris, pH 7, and then various amounts of total homogenate proteins were preincubated for 10 min with a commercial β-secretase inhibitor (KTEEISEVN-(*Sta*)-VAEF-OH, Enzyme System Products, Livermore, CA) or with distinct JMV inhibitors (10 μM) in 96-well plates. The JMV2235, JMV2236, or a β-secretase commercial substrate (Mca-SEVNLDAEFRK-(dinitrophenyl)-RR-NH_2, R & D Systems) were then added (10 μM) and incubated for various times at 37 °C. At the end of the incubation, fluorescence was recorded at 320 and 420 nm as excitation and emission wavelengths, respectively. When the effect of pH was monitored, lysed cells were pelleted, then resuspended in 25 mM Na^+-acetate/MES/Tris/TES adjusted at pH values ranging from 2 to 10 and then incubations were performed as above. Controls include membrane proteins or substrate alone and background fluorescence was subtracted to recorded BACE activities. This fluorescence was very low and did not change with the time of incubation at 37 °C. Cathepsin D activity (Sigma, 0.5 μg) was assayed as above with JMV2235, JMV2336, or with the commercial substrate.

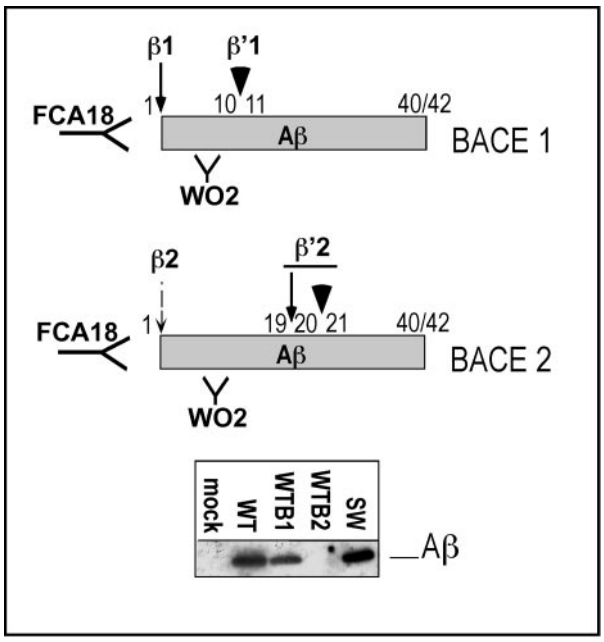


FIG. 2. **Characterization of genuine Aβ secreted by BACE1- and BACE2-expressing cells.** Mock-transfected HEK293 cells or cells expressing either Swedish-mutated βAPP (*SW*), wild-type βAPP alone (*WT*), or in combination with BACE1 or BACE2 (*WTB1* and *WTB2*, respectively) were allowed to secrete Aβ for 7 h at 37 °C and then genuine Aβ was immunoprecipitated with FCA18 (which recognizes only the Asp-1 residue liberated by β1 and β2 cleavages). Aβ was then monitored after Tris/Tricine gel analysis and Western blot with W02 as described under "Experimental Procedures."

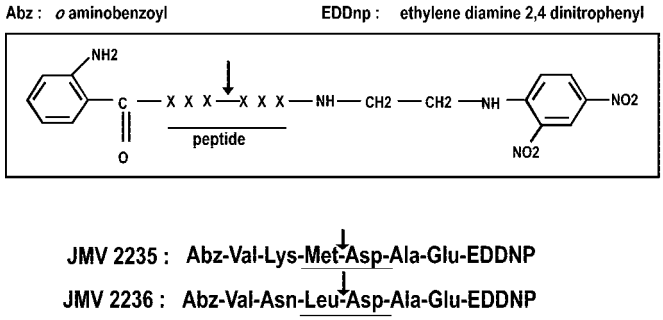


FIG. 3. **Structure of the JMV2235 and JVMV2236 quenched fluorimetric substrates.**

RESULTS

Characterization of Secreted and Intracellular βAPP-derived Products Generated by BACE1 and BACE2-expressing Human Cells—We have set up stably transfected HEK293 human cells overexpressing 1D4-tagged BACE1 and BACE2 with expected molecular weights (Fig. 1*A*), either alone (called B1 and B2) or in combination with wild-type βAPP (referred to as WTB1 and WTB2). We have used WT192 monoclonal antibodies that recognize the two last amino acids of the secreted products derived from the canonical β-secretase cleavages taking place at the N terminus of the Asp-1 residue of Aβ (see β1 and β2 sites in Fig. 1, *E* and *F*). sAPPβ1 and sAPPβ2 generated by BACE1 and BACE2 were identical (Fig. 1, *E* and *F*) and theoretically both were recognized by WT192. As expected, secreted WT192-immunoreactive products were recovered in higher amounts in both BACE1- and BACE2-expressing cells than in WT cells (Fig. 1*B*). Additional β′1- and β′2-derived cleavages targeted by BACE1 and BACE2, respectively, occur inside the Aβ sequence and theoretically liberate sAPPβ′1- and sAPPβ′2-secreted fragments (see Fig. 1, *E* and *F*). sAPPβ′2, as well as sAPPα, the physiological α-secretase-derived APP fragment, retain a 10D5C epitope that was disrupted by β′1 cleavage (see Fig. 1, *E* and *F*). As expected, 10D5C-positive fragments secreted by WT and WTB2 cells (corresponding to sAPPα/β′2) were recovered in much higher amounts than with WTB1 cells (Fig. 1*C*).

The C-terminal counterparts of the above products were probed in cell lysates using BR188, a polyclonal antibody that interacts with the C terminus of all fragments (Fig. 1, *E–G*). As expected, β1- and β2-derived cleavages (Fig. 1, *E* and *F*) increase C99 in WTB1 and WTB2 (Fig. 1*D*), in agreement with higher production of their N-terminal counterpart sAPPβ1/2 (Fig. 1*B*). The nature of C99 was confirmed by means of FCA18 (36), a polyclonal antibody that specifically interacts with the free Asp-1 residue of Aβ and C99 (not shown). A fragment of lower molecular weight was specifically recovered with WTB1 cells (Fig. 1*D*). Although not definitively identified, this product likely corresponds to C89, the β′1-derived specific product generated by WTB1 cells (Fig. 1, *D* and *E*). Finally, a low molecular

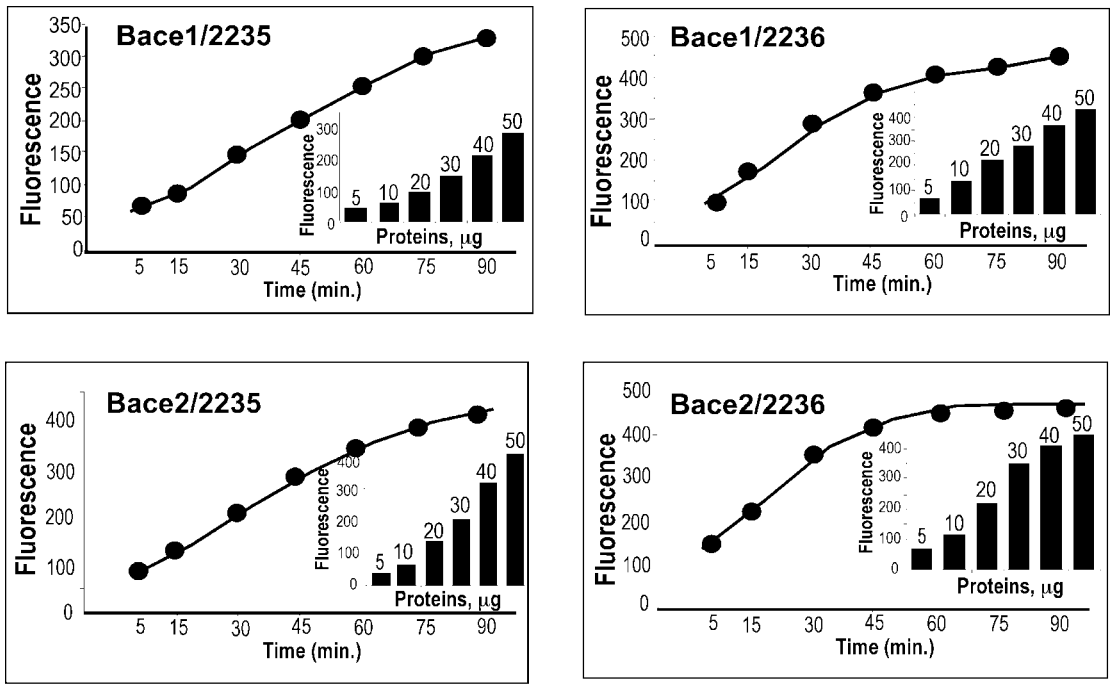


FIG. 4. **Kinetic analyses of JMV2235 and JMV2236 hydrolysis by BACE1- and BACE2-expressing cells.** BACE1- (*upper panels*) and BACE2- (*lower panels*) expressing cells (30 μg of proteins) were assayed for their JMV2235- (*left panels*) or JMV2236- (*right panels*) hydrolyzing activities for the times indicated at pH 4.5 as described under "Experimental Procedures." *Insets*, activities were measured for 30 min at pH 4.5 with the indicated varying amounts of proteins. Fluorescence was recorded at 320 and 420 nm as excitation and emission wavelengths, respectively, as described under "Experimental Procedures."

weight product present in BACE2 but not BACE1 cells could derive from the β′2 cleavage (Fig. 1*F*). This product tentatively ascribed to C79 co-migrates with the C83 major product generated by α-secretase in WT cells (Fig. 1, *D* and *G*). C89- and C79-like products were FCA18-negative (not shown), confirming that the fragments lack the intact N terminus present in C99.

Characterization of Genuine Aβ in BACE1- and BACE2-expressing HEK293 Cells—The use of FCA18 was proved useful to monitor secreted genuine Aβ. Thus, this antibody only recognizes free Asp-1 residue of Aβ or C99 (Fig. 2) because acetylation or removal of this aspartyl residue abolishes recognition by FCA18 (36). Therefore, FCA18 does not label N-terminal truncated Aβ-related species. As expected, the overexpression of wild-type βAPP increases Aβ1–40/42 (compare mock and WT in Fig. 2). Interestingly, as previously described (27, 37–39) the introduction of the Swedish mutation triggers increased Aβ secretion (compare WT and SW). Both WTB1 and WTB2 cells secrete less Aβ than WT cells (Fig. 2), indicating that the β′1 and β′2 cleavages are likely more efficient than the β1 and β2 breakdowns elicited by BACE1 and BACE2 (see Fig. 2), respectively. It is noteworthy that while "complete" Aβ is still detectable in BACE1-expressing cells, it was not recovered in WTB2. The fact that sAPPβ was augmented in BACE2 (Fig. 1*B*) suggests that the β2 cleavage before Asp-1 of Aβ sequence indeed occurs. However, the relatively faint augmentation of the C-terminal counterpart C99 and the accumulation of C79 (Fig. 1*D*) suggests that BACE2-elicited β′2 cleavage was particularly efficient and could take place using C99 as a substrate, thereby explaining the low recovery of "full-length Aβ" observed with these cells (Fig. 2).

Design of a Novel BACE Fluorimetric Assay—Several studies have indicated that the Swedish mutation responsible for a familial form of Alzheimer's disease leads to increased production of Aβ via an exacerbation of the β-secretase-derived cleavage (37–39). We have set up a new assay based on the cleavage of quenched fluorimetric substrates mimicking the wild-type (JMV2235) and Swedish-mutated (JMV2236) sequences (Fig. 3) targeted by β-secretase (s) with the assumption that a good assay should be reflected by a favored hydrolysis of mutated JMV2236.

At acidic pH, B1 and B2 cell extracts (see Fig. 1) hydrolyze both JMV2235 and JMV2236 in a time- and dose-dependent manner (Fig. 4). It should be noted that kinetic analyses indicate that activities recovered with JMV2236 plateaued at the same fluorescence value (Fig. 4). This could be because of either depletion of available substrate or, alternatively, to an inhibitory effect by one of the products of the reaction. The latter hypothesis was ruled out by the fact that the rates of hydrolyzes of JMV2235 by B1 and B2 cells were not affected by 100 μM Bz-VNL, Bz-VKL, Bz-VNL-NH_2, and Bz-VKL-NH_2. These N-terminal degradation products lack the N-terminal fluorescent moiety and were either free or amidated at the C terminus (to prevent it from putative carboxypeptidase attack (not shown). In agreement with a substrate extinction, standard fluorescence observed with synthetic Abz-VNL (10 μM) corresponds to the value observed at plateau when 10 μM substrate is used, indicating that this fluorescence indeed corresponds to 100% hydrolysis of the substrate (not shown).

We used a β-secretase commercial inhibitor to further validate our assay. Interestingly, mock-transfected cells exhibit faint JMV2235- and JMV2236-hydrolyzing activities that remained insensitive to the inhibitor (Fig. 5, *A* and *B*), indicating that endogenous β-secretase activity was low in HEK293 cells. The JMV2235- and JMV2236-hydrolyzing activities of B1 cells returned to the mock-transfected cell value in the presence of the inhibitor (Fig. 5, *A* and *B*), indicating that most of the fluorescence recorded was indeed because of BACE1 in B1 cells. This conclusion also stands for BACE2-expressing cells (Fig. 5*C*) although the extent of inhibition was slightly lower (69 *versus* 97% of inhibition for B2 and B1 cells, respectively, see Fig. 6*B*). Interestingly, in B1 cells, hydrolysis of JMV2236-

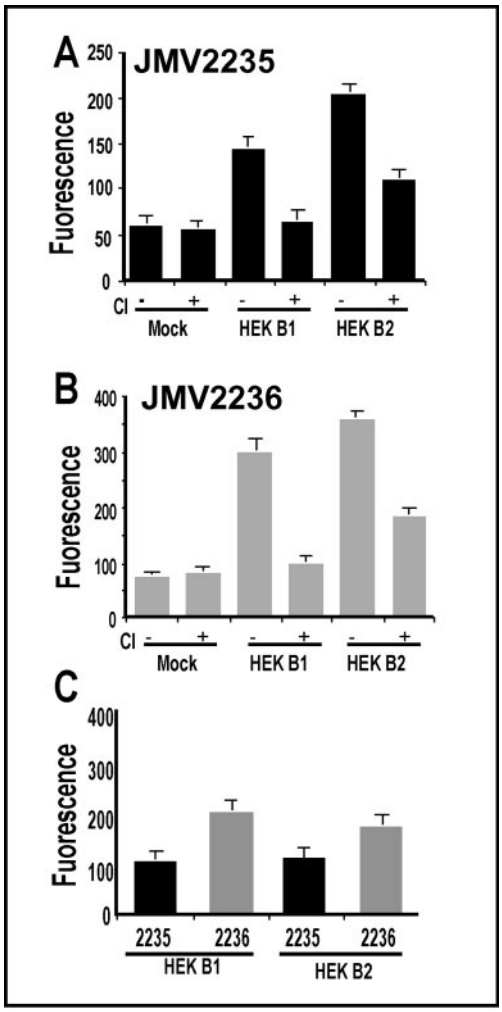
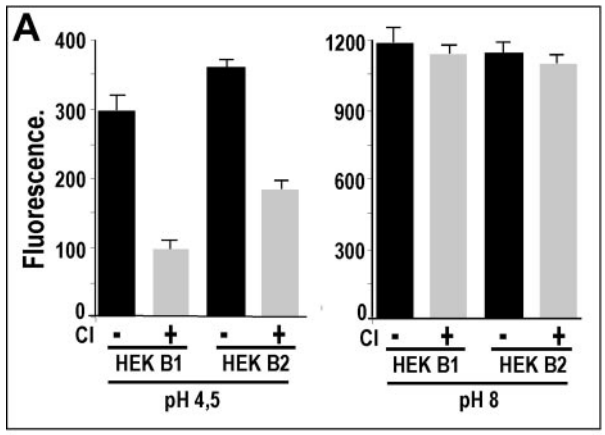


FIG. 5. **Comparative analysis of JMV2235 and JMV2236 hydrolysis by BACE1 and BACE2 activities: effect of a β-secretase commercial inhibitor.** Mock, BACE1- (*HEKB1*), or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with 10 μM JMV2235 (*A*) or JMV2236 (*B*) for 30 min at pH 4.5 in the absence (−) or in the presence (+) of a commercial β-secretase inhibitor (CI, 10 μM), and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations. *Panel C* compares the JMV2235- and JMV2236-hydrolyzing activities displayed by HEKB1 and HEKB2 cells.



B	HEK B1		HEK B2	
V (F/h/mg)	pH 4,5	pH 8	pH 4,5	pH 8
Control	19998	79992	24064	78867
+ Inhibitor	6599	76659	12332	75210
% Inh°	97	4	69	5

FIG. 6. **Inhibitor-sensitive JMV2236 hydrolysis by BACE1- and BACE2-expressing cells is pH-dependent.** BACE1- (*HEKB1*) or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with 10 μM JMV2236 (*A*) for 30 min at pH 4.5 or 8, in the absence (−) or presence (+) of a commercial β-secretase inhibitor (*CI*, 10 μM), then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations. Specific activities in *B* correspond to initial velocity measurements of the JMV2236-hydrolyzing activity obtained with or without inhibitor. The % of inhibition was calculated after subtraction of the CI-insensitive activity obtained in mock-transfected cells (see "Results"). A fluorescence of 700 corresponds to 1 nmol of Abz-VNL-OH liberated.

mutated substrate is twice as efficient as that observed for the non-mutated analog (Fig. 5*C*).

Another clue for stating that our novel assay was β-secretase-specific was the strong pH requirement observed. We have carried out the assay at pH ranging from 2 to 10 (in 25 mM Na^+-acetate/MES/Tris/TES to avoid any intrinsic influence other than the pH). The activity was sharply maximal at pH 4–4.5 for JMV2236- (Fig. 6*A*) and JMV2235- (not shown) hydrolyzing activities in both B1 and B2 cells. It was interesting to note that at pH 8, very high JMV2236-cleaving activities were displayed by B1 and B2 calls but that, unlike at pH 4.5, remains totally insensitive to the β-secretase commercial inhibitor (Fig. 6, *A* and *B*). This further indicates that our assay allows selective dosage of β-secretase only at pH relevant for BACE biological activity.

Comparison of JMV-based Assay with a Commercial β-Secretase Assay by Use of Cathepsin D—BACE1 and BACE2 hydrolyze another commercial fluorimetric substrate (CS) with identical pH (Fig. 7*A*) and inhibitor-sensitive (Fig. 7, *A* and *C*) manners. At acidic pH, CS appeared even better cleaved by B1 and B2 cells than JMV2235 and JMV2236 substrates (Fig. 7*B*). We took advantage of the description of cathepsin D as an *in vitro* β-secretase-like activity (40, 41) to further compare the usefulness and accuracy of these two assays. Interestingly, although purified cathepsin D potently hydrolyzed CS and JMV2235, this enzyme was unable to hydrolyze the mutated substrate JMV2236 (Fig. 8, *A* and *B*). This shows that on the basis of the hydrolysis of CS or JMV2235 only, cathepsin D would have behaved as a good β-secretase candidate. The dual screening with JMV2235 and -2236 makes it fall in the category of the β-secretase "false positive" candidates. These data indicate the drastic improvement brought by our new assay for the monitoring of putative unknown β-secretase-like candidates and also confirm the fact that cathepsin D is not β-secretase. The selectivity of the assay was further emphasized by the inability of ADAM10, TACE, and presenilins 1 and 2, the α- and γ-secretases candidates to cleave JMV2236 (Fig. 9).

Novel in Vitro Inhibitors of BACE1 and BACE2—The above data suggested the use of JMV2236 as a good probe to screen for putative *in vitro* β-secretase inhibitors. We have studied the putative inhibitory effect of 26 peptidic sequences modified at the β-secretase site and harboring various N- and C-terminal lengths (Table I). Most of the molecules are displaying a statine-derived group, a non-cleavable residue that mimics the tetrahedral intermediate of catalysis by aspartyl proteases. The statine group can be replaced by an AHPPA ((3*S*,4*S*)-4-amino-3-hydroxy-5-phenyl-pentanoic acid) moiety without influencing the inhibitory activity toward both BACE1 and BACE2 (compare JMV1197 and JMV1200 in Table I and Fig. 10). JMV1195 and JMV1197 block BACE1 (Fig. 10*A*) and

BACE2 (Fig. 10*B*) with IC_{50} values in the micromolar range (Fig. 10*C* and Table II), suggesting that shortening the N-terminal part of the inhibitor does not significantly alter its potency. The C-terminal length appears more important because shortening it by only one amino acid leads to an inactive inhibitor on BACE2 and less potent agent against BACE1 (compare JMV1196 and JMV1197; Fig. 10, *A* and *B*). It is noteworthy that this screening led to the identification of molecules that appear to discriminate between BACE1 and BACE2. This appears to be the case for JMV1321 which is more potent on BACE2 than on BACE1 whereas the contrary is true for JMV1196 (Fig. 10, *A* and *B*).

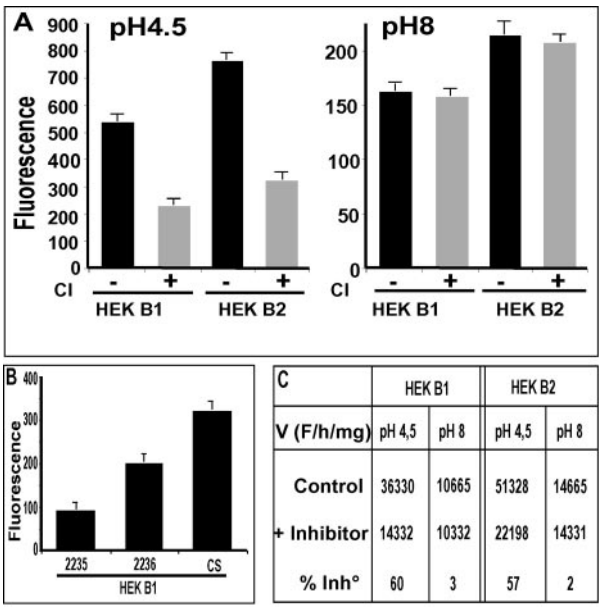
DISCUSSION

Although the etiology of Alzheimer's disease is not formally known, it is difficult to consider the overproduction of the amyloid β peptide (Aβ) as an innocuous and inert event in the progression of the disease. Causative or not, Aβ is the most obvious biochemical common denominator between sporadic and familial forms of this disease. Thus, the mutations triggering the genetic forms of Alzheimer all lead to an acceleration of the disease progression that, if not directly because of a modulation of Aβ production, appears at least linked to it (for reviews, see Ref. 42–45). This so called "amyloid cascade" hypothesis (46) implies that the secretases that generate Aβ could be considered as major targets of therapeutic strategies aimed at slowing down the onset and progression of the disease.

Aβ is generated from a transmembrane precursor, the β-amyloid precursor protein, by subsequent attacks by β- and γ-secretases, which liberate the N- and C-terminal moieties of Aβ, respectively (for reviews, see Refs. 2 and 47–49)). The nature of the γ-secretase is still discussed (4, 5, 50, 51) and awaits definitive identification whereas the β-secretase function is more consensually ascribed to BACE1 (β-site APP cleaving enzyme also called memapsin 2 or Asp2, (6–9)). Both β- and γ-secretase inhibition could be theoretically seen as a means to prevent Aβ production but several apparently uncircumventable problems lead to the conclusion that γ-secretase is not an adequate target. Thus, inhibitors of the γ-secretase activity not only prevent Aβ production but also alter the processing of various proteins involved in vital functions at adulthood (52–56). Furthermore, preventing βAPP processing at the γ-secretase site increases the recovery of the highly toxic C99 product (3) that accumulates in AD brains (57).

At first sight, β-secretase appears as a much better target. First, it is noteworthy that BACE expression and activity are elevated in sporadic Alzheimer's disease brains, particularly in the cortical and hippocampal areas affected in the disease (58–60). Second, the abrogation of the *BACE1* gene totally abolishes the formation of Aβ-related species and C99 product in knockout neurons (17) and BACE1-deficient mice brain (16). Of most interest, mice devoid of BACE1 develop normally and have an unaltered phenotype (16, 18). The latter indicates that even if BACE1 specificity for βAPP is not exclusive, the other targeted substrates do not share essential functions or that another enzyme can complement for BACE-mediated proteolysis. This contrasts with presenilins-dependent γ-secretase-like cleavages, which when abolished, trigger lethality at the embryonic stage (61–63) and severe alterations at adulthood, particularly in thymocyte development (52, 64).

We have set up stable transfectants overexpressing BACE1. When expressed together with βAPP, we observed that the production of sAPPβ and C99, the two β-secretase-derived complementary products were increased, but to a much lesser extent for the latter. This is likely because of the subsequent cleavage of C99 inside the Aβ domain, leading to an N-terminal-truncated fragment as previously described (7, 65, 66). This agreed well with our observation that genuine Aβ, *i.e.* Aβ starting at the canonical Asp-1 residue, was drastically reduced after overexpression of BACE1. These features also stand and were even accentuated when studying cells overexpressing BACE2, the BACE1 parent protein. In this case, full-length Aβ appeared barely detectable in agreement with studies indicating that BACE2 mainly cleaved in the middle of the Aβ sequence, after the 19th and 20th residues (28, 67, 68), thereby



C

V (F/h/mg)	HEK B1 pH 4,5	HEK B1 pH 8	HEK B2 pH 4,5	HEK B2 pH 8
Control	36330	10665	51328	14665
+ Inhibitor	14332	10332	22198	14331
% Inh°	60	3	57	2

FIG. 7. **BACE1 and BACE2-hydrolyzing activity toward a β-secretase commercial substrate.** BACE1- (*HEKB1*) or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with a commercial β-secretase substrate (10 μM) for 30 min at pH 4.5 or 8, in the absence (−) or presence (+) of a commercial β-secretase inhibitor (*CI*, 10 μM) and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of six (*C*) or eight (*A* and *B*) determinations.

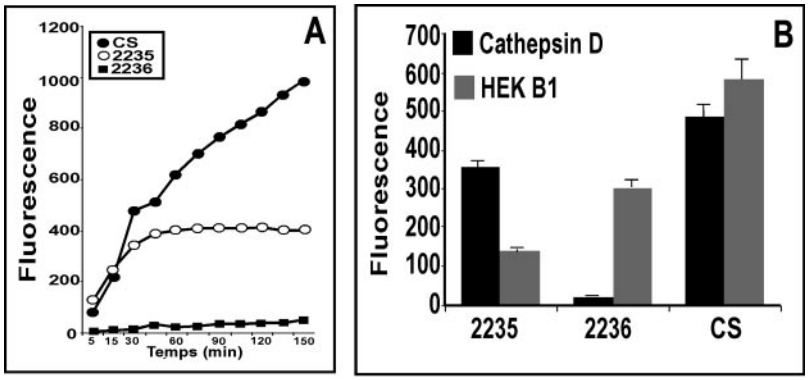


FIG. 8. **Hydrolysis of JMV2235, JMV2236, and the β-secretase commercial substrate by purified cathepsin D.** *A*, purified cathepsin D activity (0.5 μg) was assayed for the indicated time periods at pH 4.5 with 10 μM JMV2235, JMV2336, or commercial substrate (*CS*). In *B*, *bars* are the mean ± S.E. of six determinations and compare the fluorescence generated from the indicated substrates by cathepsin D and BACE1. A fluorescence of 700 corresponds to 1 nmol/30 min of Abz-VNL-OH liberated.

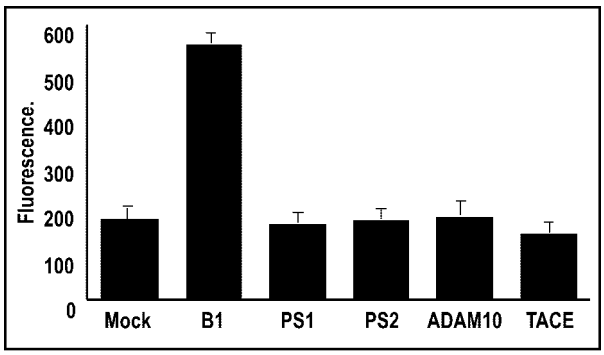


FIG. 9. **Hydrolysis of JMV2236 by several "secretase" candidates reveals selectivity for β-secretase.** Mock-transfected HEK293 cells or cells expressing BACE1 (*B1*), presenilin 1 (*PS1*), presenilin 2 (*PS2*), ADAM10 or TACE were obtained as described under "Experimental Procedures." Thirty μg of proteins were assayed for 30 min at pH 4.5 (B1 cells) or neutral pH (other cells) with 10 μM JMV2236 and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations.

TABLE I
Inhibitors nomenclature and structures

JMV	Inhibitors
963	Boc-Asn-Leu-ψ(*CH*$_2$*NH*)-Asp-Ala-NH$_2$
946	Boc-Asn- *Sta* -Asp-Ala-NH$_2$
945	Boc-Val-Asn- *Sta* -Ala-NH$_2$
931	Boc-Asn- *Sta* -Ala-NH$_2$
947	Boc-Asn- *Sta* -Ala-Glu-NH$_2$
1104	Ac-Glu-Val-Asn- *Sta* -Ala-Glu-Phe-NH$_2$
1200	Ac-Val-Asn- *Sta* -Ala-Glu-Phe-NH$_2$
1201	Ac-Asn- *Sta* -Ala-Glu-Phe-NH$_2$
1105	Ac-Val-Asn- *Sta* -Ala-Glu-NH$_2$
1242	Ac-Glu-Val-Lys- *Sta* -Ala-Glu-Phe-NH$_2$
1251	Qui-Val-Asn- *Sta* -Ala-NH$_2$
1250	Qui-Asn- *Sta* -Ala-NH$_2$
1318	Qui-Asn-*Norsta*-Asp-NH$_2$ (S,S)
1317	Qui-Asn-*Norsta*-Asp-NH$_2$ (S,R)
1319	Qui-Val-Asn-*Norsta*-Asp-NH$_2$ (S,S)
1320	Qui-Val-Asn-*Norsta*-Asp-NH$_2$ (S,R)
1244	Boc-Asn-*Norsta*-Ala-NH$_2$ (S,S)
1245	Boc-Asn-*Norsta*-Ala-NH$_2$ (S,R)
1322	Ac-Glu-Val-Asn-*Norsta*-Ala-Glu-Phe-NH$_2$ (S,S)
1321	Ac-Glu-Val-Asn-*Norsta*-Ala-Glu-Phe-NH$_2$ (S,R)
1195	Ac-Glu-Val-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$
1197	Ac-Val-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$
1202	Ac-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$
1196	Ac-Val-Asn-*AHPPA* -Ala-Glu-NH$_2$
1243	Ac-Glu-Val-Lys-*AHPPA* -Ala-Glu-Phe-NH$_2$
1300	Ac-Glu-Val-Asn-*ACHPA* -Ala-Glu-Phe-NH$_2$

leading to N-terminal truncated products that escaped immunological detection in our conditions. In line with these data, N-terminal truncated C89 and C79 accumulated in BACE1- and BACE2-expressing cells, respectively.

We have set up a new β-secretase assay based on the hydrolysis of two quenched fluorimetric substrates, one of which harboring the Swedish mutation is thought to enhance β-secretase cleavage (see Introduction). The assay allows to monitor a time- and dose-dependent fluorescence specifically increased by BACE1 and BACE2 overexpression. More important, we establish that this activity was maximal at acidic pH and enhanced by the Swedish mutation, in agreement with the reported properties of β-secretase activity (for reviews, see Refs. 10, 11, and 69). It should be noted that the effect of the mutation on BACE activity appears weaker with these fluorigenic substrates than with βAPP itself. This is likely because of the fact that BACEs act better as protease rather than peptidases. This semantic discrimination implies that these proteases hydrolyze preferentially proteins rather than small peptides because the former likely fit better with a relatively larger recognition/catalytic pocket (70).

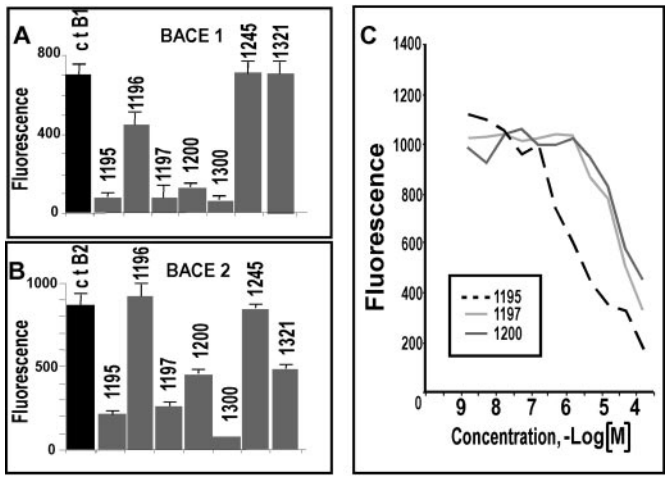


FIG. 10. **Effect of new inhibitors toward BACE1 and BACE2.** BACE1- (*A*) or BACE2- (*B*) expressing cell proteins (30 μg) were incubated with JMV2236 (10 μM) for 30 min at pH 4.5 in the presence of the indicated JMV inhibitors (10 μM) and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of four determinations. In *C*, a complete dose-response curve of the indicated JMV inhibitors obtained with BACE2-expressing cells is shown.

TABLE II
IC$_{50}$ values of inhibitors on BACE1 and BACE2 activities

Inhibitor	IC$_{50}$	
	B1 cells	B2 cells
Commercial inhibitor	1 μM	10 μM
JMV1195	3 μM	1 μM
JMV1197	3 μM	10 μM
JMV1200	3 μM	10 μM
JMV1300	1 μM	1 μM

It is interesting to note that the signature of a genuine β-secretase activity is indeed revealed by the mutation-induced differential fluorescence monitored by this dual assay. Thus, cathepsin D, a protease with *in vitro*-like β-secretase activity (40, 41), indeed cleaves efficiently a commercial substrate mimicking the β-secretase-targeted sequence. However, our assay demonstrated that this protease did not behave as a good β-secretase candidate because it did not hydrolyze JMV2236, the fluorimetric substrate bearing the Swedish mutation. This dual assay therefore proved useful to monitor genuine β-secretase activities. In this context, it is interesting to note that recently, splice variants of BACE1 have been identified in human brain and pancreas (71, 72). Our assay should allow the monitoring of other putative BACE-like activities and help reveal yet unknown functions of these activities.

The most potential interest of our assay would be to design highly potent, bioavailable and metabolically stable inhibitors of BACE1. This is a real challenge because until now, the inhibitors designed are mostly peptide-based (for reviews see Refs. 10, 11, and 73), and therefore poorly enter the blood-brain barrier and are susceptible to proteolysis. Our rapid, reproducible and sensitive assays should allow to screen for numerous inhibitor candidates. Our data allow to establish that the length of the C-terminal tails adjacent of a stabilizing statine group is a more drastic structural requirement to maintain full efficiency than length of the N-terminal moiety. Our data also showed the potential of designing fully specific BACE1 or BACE2 inhibitors. Thus, we found inhibitors of BACE1 or

BACE2 that appeared inactive on the parent protease. Although these compounds clearly did not fully discriminate between the two enzymes, they constitute the starting point of a rational design leading to the selection of fully selective blocking agents. This is not only a biochemical challenge but indeed an important issue. Thus, BACE2 cleaves mainly inside the Aβ sequence, at a nonamyloidogenic site and therefore likely contributes to the depletion of Aβ or BACE1-generated Aβ-related species. In this case that likely reflects most of sporadic AD, inhibition of BACE1 but not BACE2 is an important issue. On the other hand, BACE2 is encoded by chromosome 21 and therefore possibly contributes to the neuropathological AD-like stigmata that take place in trisomic brains. Thus it has been shown that brains affected with Down's syndrome display elevated BACE2 expression (15). BACE2 activity also appears increased by the Flemish AD mutation (28). In these cases, a BACE2-specific inhibitor would be likely very useful.

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