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A surface plasmon resonance-based biosensor with full-length BACE1 in a reconstituted membrane

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ABSTRACT

A surface plasmon resonance (SPR) biosensor-based assay for membrane-embedded full-length BACE1 (β-site amyloid precursor protein cleaving enzyme 1), a drug target for Alzheimer's disease, has been developed. It allows the analysis of interactions with the protein in its natural lipid membrane environment. The enzyme was captured via an antibody recognizing a C-terminal His6 tag, after which a lipid membrane was reconstituted on the chip using a brain lipid extract. The interaction between the enzyme and several inhibitors confirmed that the surface was functional. It had slightly different interaction characteristics as compared with a reference surface with immobilized ectodomain BACE1 but had the same inhibitor characteristic pH effect. The possibility of studying interactions with BACE1 under more physiological conditions than assays using truncated enzyme or conditions dictated by high enzyme activity is expected to increase our understanding of the role of BACE1 in Alzheimer's disease and contribute to the discovery of clinically efficient BACE1 inhibitors. The strategy exploited in the current study can be adapted to other membrane-bound drug targets by selecting suitable capture antibodies and lipid mixtures for membrane reconstitution.

Membrane proteins are key regulators of cellular function, and more than 60% of approved drug targets are thought to be membrane bound [1]. Despite the importance of this protein class, methods to study them in their natural environment are limited. Standard biochemical methods are not generally applicable due to the often complex structure of the proteins and the hydrophobicity of their transmembrane regions. The proteins are typically removed from the membrane during purification, and the lipid core is replaced by detergents. This may have a drastic impact on their structure and function and may even induce denaturation of the proteins. In the case of membrane-bound enzymes, the catalytic site is often located in a soluble ectodomain, protruding from the membrane. By using a truncated form of the protein consisting only of the ectodomain, many common problems can be avoided and standard enzymological techniques can often be used. But there may be fundamental differences in the functional characteristics of the ectodomain and the full-length protein. In this study, we have taken an approach where a full-length membrane protein is studied in a membrane resembling its natural environment, avoiding the drawbacks of the commonly used approaches.

The studied protein is the β-site amyloid precursor protein (APP)[1] cleaving enzyme 1 (BACE1, EC 3.4.23.46, also known as β-secretase or memapsin-2) [2,3]. This membrane-bound aspartic-type protease performs the initial cleavage of APP, which is also membrane bound. Subsequent cleavage of the remaining membrane-attached stub (C99) by the γ-secretase complex gives rise to Aβ peptides (e.g., 1–40, 1–42). These are the main constituents of amyloid plaques and the hallmarks of Alzheimer's disease. The enzyme is consequently an attractive target for drugs against this disease [4].

Because the catalytic site of membrane-bound BACE1 is localized in an ectodomain, structurally similar to non-membrane-bound aspartic proteases, biochemical studies of BACE1 have primarily been limited to a truncated form corresponding to the

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[1] *Abbreviations used:* APP, amyloid precursor protein; BACE1, β-site amyloid precursor protein cleaving enzyme 1; C99, C-terminal 99 amino acids of APP; SPR, surface plasmon resonance; cDNA, complementary DNA; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; RU, resonance units; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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ectodomain. It is believed to be a relevant in vitro system enabling the development of BACE1 inhibitors because it cleaves APP-based substrate peptides at the correct cleavage site [5]. An advantage of using ectodomain BACE1 is that it allows the same standard enzymological techniques already established for other aspartic proteases (e.g., HIV protease) to be used.

However, despite significant efforts and design of BACE1 inhibitors showing very good potency in enzyme activity and cell assays, very few compounds have reached the clinic. This lack of success is often attributed to poor pharmacokinetics. However, it can be speculated that current assays for lead identification and optimization are suboptimal and that our understanding of the functional characteristics of BACE1 as a drug target is insufficient. We have consequently been interested in developing alternative assays for BACE1 that both increase our knowledge of the target and are better at guiding the drug discovery process. In an earlier study, we revealed the importance of using neutral pH for inhibition assays [6]. Here we focused on developing an assay that allows studies of full-length BACE1 in its natural environment attached to a lipid membrane.

The approach exploited a surface plasmon resonance (SPR)-based biosensor, a technology of increasing importance for drug discovery [7]. We have previously used it for numerous applications, including inhibitor interaction studies with ectodomain BACE1 [6]. The method enables the direct and time-resolved monitoring of interactions between two molecules without labeling the interaction partners. There are only a few examples of SPR biosensor-based assays with membrane proteins so far [8–11], despite the large interest in identifying generally applicable methods for designing assays for membrane proteins. Still, the difficulties in developing such assays can primarily be attributed to problems in producing and handling the proteins rather than to the technology itself. Therefore, our strategy involved the expression of BACE1 in Sf9 cells and a direct capture of the protein from the cell lysate to the sensor chip by an antibody specific for a His6 tag (Fig. 1). A lipid membrane was reconstituted around the captured enzyme, stabilizing the transmembrane region and providing a physiologically relevant environment.

The surfaces were validated with a series of BACE1 inhibitors selected on the basis of their different degrees of potency in enzyme assays and cell-based assays (Fig. 2) and whose interactions we previously studied with the biosensor assay developed for ectodomain BACE1 [6], used here as a reference system. This enabled a comparison of the interaction characteristics of the inhibitors with the two forms of BACE1. In addition, the influences of pH and Ca^{2+} concentration on the interactions were investigated. The former was inspired by our recent study with immobilized ectodomain BACE1, where we showed that pH can have a significant effect on inhibitor interactions and that neutral pH appears to represent a physiologically relevant environment for the binding of inhibitors [6]. Our interest in calcium was based on the obvious importance of this ion for regulation of physiological processes as well as a recent report that calcium binding to BACE1 influences both the structure and proteolytic activity of the enzyme [12].

Materials and methods

Construction of baculovirus clones for full-length BACE1

Complementary DNA (cDNA) encoding full-length BACE1 (aa 1–501) with a C-terminal His6 tag was generated by polymerase chain reaction (PCR) using a plasmid containing the gene for full-length human BACE1 as template (previously amplified by PCR from human brain cDNA and cloned into pCR2.1 TOPO). Oligonucleotides were obtained from MWG (Ebersberg, Germany). A primer combination with the forward primer Baceforw (5′-TAT AGG ATC CAT GGC CCA AGC CCT GCC CTG G-3′) and a reverse primer Bacerevhis (5′-ATA TCT CGA GTC AGT GAT GGT GAT GGT GAT GCT TCA GCA GGG AGA TGT CAT C-3′) was used (where the recognition sequences for restriction enzymes *Bam*HI and *Xho*I are underlined). The PCR contained 0.4 μM of each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 5 μl of 10× buffer supplied with the polymerase, 0.5 μl of 10× diluted plasmid miniprep template (sequence as in NCBI ref. no. NM_012104.3), 2 μl of dimethyl sulfoxide (DMSO), 1 μl of *Pfu* Turbo polymerase (Stratagene, La Jolla, CA, USA), and water to a final volume of 50 μl. The PCR program started with a denaturation step at 95 °C for 5 min, followed by 26 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min and ended at 72 °C for 10 min.

The PCR product was analyzed by agarose gel electrophoresis, excised, and gel purified using a QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany). After purification, the plasmid was subsequently digested with *Bam*HI and *Xho*I. Plasmid pFastBac1 (Invitrogen, Carlsbad, CA, USA) was digested with the same restriction enzymes; gel purified, and dephosphorylated using calf intestinal phosphatase (Roche, Basel, Switzerland). After ligation and transformation, minipreps of recombinant plasmids were prepared using the QIAprep Spin Miniprep Kit and further used for restriction analysis and DNA sequencing (MWG). Competent DH10Bac *Escherichia coli* cells (Invitrogen) were transformed with the pFastBac/BACE1 constructs to generate recombinant bacmids. Positive bacmid clones were identified by blue/white selection, followed

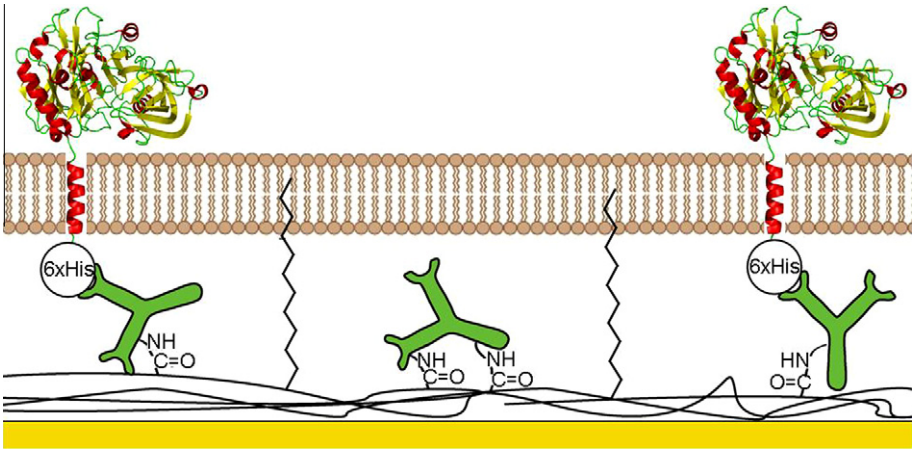

Fig.1. Schematic illustration of the design of the biosensor surface with immobilized full-length BACE1 embedded into a lipid membrane.

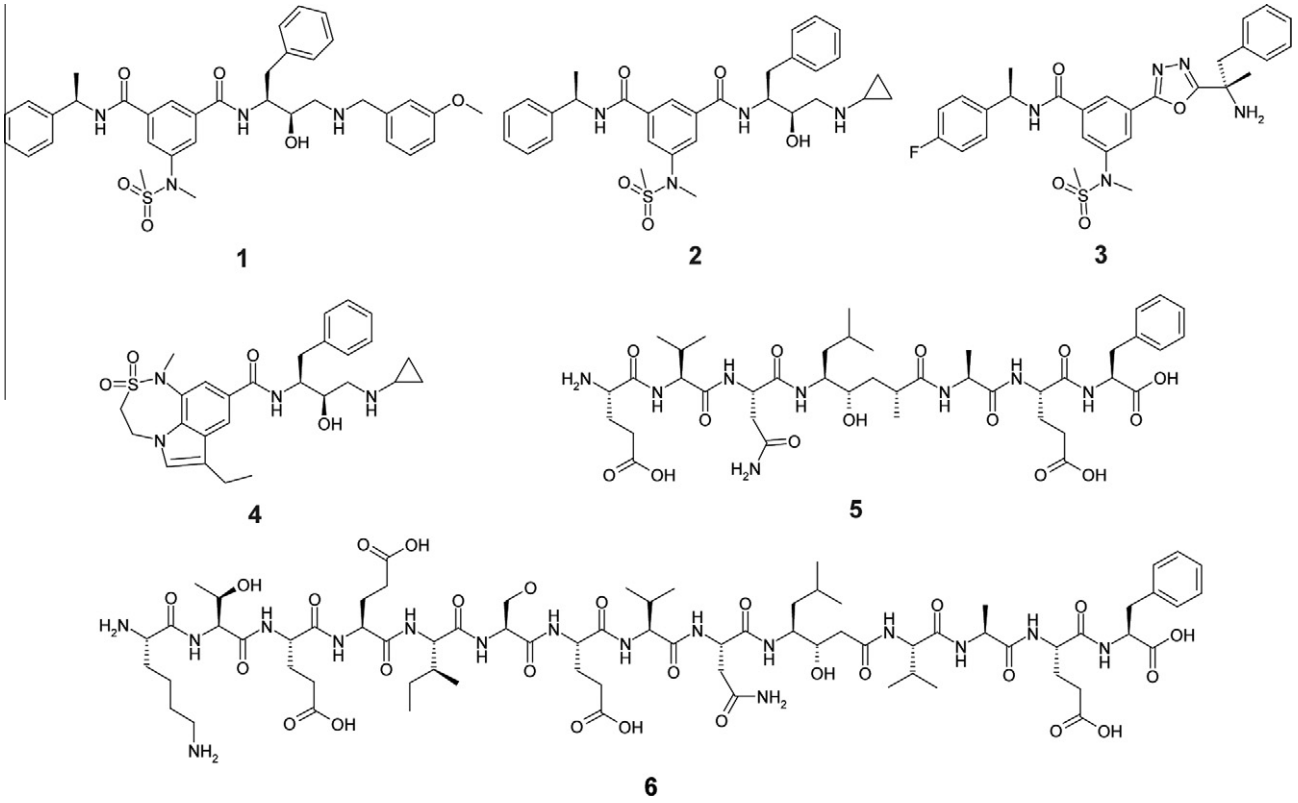

Fig.2. Structures of BACE1 inhibitors used for the validation of the biosensor assay.

by PCR to verify the insertion of cDNA encoding BACE1 into the viral genome, according to procedures described by the manufacturer (Invitrogen). Large-scale bacmid preparations were performed with a Plasmid MidiPrep Kit from Qiagen.

Transfection of Sf9 insect cells and production of a viral stock for full-length BACE1

Sf9 cells were grown continuously in solution in Insect-XPRESS medium (Cambrex, East Rutherford, NJ, USA) supplemented with PEST (50 U penicillin/50 μg/ml streptomycin) and L-glutamine. Cells were split twice a week to a cell density of 0.9×10^6 cells/ml. Cultivated Sf9 cells were diluted to 0.5×10^6 cells/ml with Insect-XPRESS growth medium supplemented with L-glutamine. Various amounts of bacmid DNA and transfection agent FuGENE6 (Roche) were added to cells in 6-well plates and left at 27 °C for 72 h. After visual inspection, cells were harvested by centrifugation at 500*g* for 10 min. The supernatants were stored as the first viral stocks at 4 °C and subsequently used for further amplifications of virus by infection of larger volumes of insect cells. After three rounds of amplification, the viral titers were estimated using a plaque assay.

Expression of full-length BACE1

Small-scale (25 ml) test expressions were performed using a fixed number of cells and a different number of viruses. Virus/cell ratios ranged from 0.01 to 30. Cultures were grown for 72 h, after which the cells were harvested. The expression level was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot according to standard procedures using a NuPAGE Precast Gel System (Invitrogen), anti-BACE1 antibodies (D-16, cat. no. sc-10053, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and chemiluminescence for detection. The conditions that generated the highest expression level were used for larger scale expression of BACE1.

Full-scale expression of full-length BACE1 was performed using 550 ml of cell culture ($\sim 2.5 \times 10^6$ cells/ml) infected with recombinant virus at a multiplicity of infection of approximately 3. The culture was grown for 72 h at 27 °C in a shaking incubator at 90 rpm. Cells were harvested by centrifugation at 2800 rpm for 10 min. The cells were washed with cold phosphate-buffered saline (PBS), pelleted again, and frozen at −80 °C for future use.

For the detection of dimeric full-length BACE1 by SDS–PAGE and Western blot, the samples were prepared in standard Laemmli buffer (4% SDS, 20% glycerol, 125 mM Tris–HCl [pH 6.8], and 0.004% bromophenol blue). Several variations to this buffer were tested, including changes in the SDS concentration and additions of 20% β-mercaptoethanol or 0.1% Triton X-100. The samples were boiled at 100 °C for 10 min before some experiments.

Enzymatic assay

A TruPoint β-Secretase Assay Kit (PerkinElmer, Waltham, MA, USA), based on fluorescence resonance energy transfer (FRET), was used to measure the catalytic activity of BACE1. All measurements were carried out with a Fluoroskan Ascent (Thermo Labsystems, Philadelphia, PA, USA) and in 96-well plates. The reaction was monitored at 320 nm (λ_{ex}) and 612 nm (λ_{em}).

The activity of BACE1 in the crude cell lysate was determined in a 10-μl sample of an Sf9 cell pellet. It was resuspended in 250 μl of PBS with 2% Triton X-100 and then lysed by pipetting the sample through a tip 10 times. The lysate was stirred for 30 min and then cleared by centrifugation for 30 min at 40,000*g*. The activity was determined by preincubating the FRET substrate in the reaction buffer at a final concentration of 200 nM for 15 min at 25 °C and then adding the sample of cell lysate, after which the fluorescence was measured every 15 min over a 2-h period. An identical sample containing 50 μM compound **2** was used as a control.

Biosensor analysis

All interaction studies were performed at 25 °C with a Biacore 2000 instrument (GE Healthcare, Uppsala, Sweden). The interaction was measured in resonance units (RU) and is presented graphically as overlaid time series data (sensorgrams) from a set of experiments at different inhibitor concentrations.

Preparation of biosensor surfaces

BACE1 was immobilized via antibody capture directly from Sf9 cell lysates, and a lipid membrane was subsequently reconstituted on the sensor chip. A monoclonal anti-polyhistidine antibody (R&D Systems, Minneapolis, MN, USA) was first immobilized on an L1 sensor chip by amine coupling according to standard procedures (GE Healthcare). Prior to immobilization, the surface of the L1 chip was washed three times for 30 s with 0.02 M Chapso (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate). The antibody buffer was changed to 10 mM Na-acetate (pH 4.75), and the concentration was adjusted to 100 μg/ml. The running buffer during immobilization was 0.01 M Hepes (pH 7.4) and 0.15 M NaCl.

BACE1 was captured directly from cell lysates prepared from Sf9 cells as described for the activity assay above. The clarified cell lysate was injected over the surface with the immobilized antibody for 15 min. The captured enzyme was embedded into a lipid membrane, essentially as described previously [11] but with a lipid mixture prepared according to Karlsson and Löfås [9], starting out with 2 mg of a total brain lipid extract or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in chloroform (Avanti Polar Lipids, Alabaster, AL, USA) that was first dried for several minutes in an N_2 stream and then afterward in vacuum overnight. The dried lipids were dissolved again in 800 μl of 50 mM Hepes (pH 7.4), 0.15 M NaCl, and 0.03 M *n*-octyl-β-D-glucopyranoside, and the mixture was shaken for several hours. The lipid membrane was created by an injection of lipid mixture for 10 min employing the COINJECT function of the Biacore 2000. The amount of immobilized protein was determined after the interaction studies by removing the lipids with three 30-s injections of 0.02 M Chapso. The signal after lipid removal was interpreted to correspond to the amount of immobilized protein.

All steps were performed at a flow rate of 5 μl/min. The reference surface was prepared in the same way but with a cell lysate of nontransfected Sf9 cells.

Inhibitor interaction studies

Compounds **1** (GRL-8234) [13], **2** [14], **3** [15], and **4** [16] were synthesized according to published procedures. Compounds **5** (OM99-2) and **6** (P_{10}–P_4statV) were purchased from Sigma–Aldrich. For the interaction studies with substrate, a peptide (SEVNLDAEFR) derived from the sequence of APP with the Swedish mutation was used (Anaspec, Fremont, CA, USA). All interaction studies were performed at a flow rate of 30 μl/min and in a running buffer composed of 50 mM Hepes (pH 7.4), 0.15 M NaCl, and 3% DMSO or 10 mM Na-acetate (pH 4.5), 0.15 M NaCl, and 3% DMSO. For the interaction studies in the presence of calcium, 50 μM $CaCl_2$ was included in the running buffer. Control experiments in which up to 1 mM $CaCl_2$ was injected as analyte were also performed. The inhibitors were diluted in running buffer and injected into two independent concentration series for 180 s. The dissociation was recorded until the inhibitor had cleared the surface, but for at least 600 s. A blank, consisting of running buffer, was injected before and after each concentration series.

Data analysis

BIAevalution 4.1 software (GE Healthcare) was used to analyze the data. The reference and an average of the blanks were subtracted from the data before determining the association and dissociation constants and the affinity using global nonlinear regression analysis and a 1:1 binding model with mass transfer. This results in independent determinations for the rate constants and the affinity for each experiment. The reported standard deviations are based on the means for the replicates. To compensate for bulk changes between the association and dissociation phases, an additional correction term was included in the analysis model. The sensorgrams were normalized to the amount of immobilized protein and the molecular weight of the compound. As a reference, kinetic data for the interactions with truncated BACE1 were also extracted, as described above, from experiments performed in a previous study [6]. The maximal signal that could theoretically be expected at saturating concentration of the analyte (i.e., concentrations >> K_D) was based on the relationship $R_{max} = R_{protein} \cdot (Mw_{analyte}/Mw_{protein}) \cdot BC$, where $R_{protein}$ is the response of immobilized protein alone, BC is the apparent binding capacity, and $Mw_{analyte}$ and $Mw_{protein}$ are the molecular weights of the analyte and protein, respectively.

Results

Enzyme production and activity

Full-length BACE1 was successfully expressed in Sf9 cells. The presence of a protein with an approximate molecular weight of 62 kDa was confirmed by Western blot of the total Sf9 cell lysate using anti-BACE1 antibodies (Fig. 3A). Another diffuse band of approximately 55 kDa was also detected by the antibody, suggesting that the lysate contained BACE1 with different glycosylation patterns. There was no sign of a BACE1 dimer under any conditions tested. The expressed BACE1 was active in the lysate, as illustrated by the hydrolysis of a FRET-based peptide substrate (Fig. 3B). The enzyme was not purified for the interaction experiments because it was considered an advantage to keep it in as natural an environment as possible until preparation of the sensor surfaces. Moreover, the specific capture strategy used when preparing sensor surfaces serves as a purification step.

Creation of BACE1 sensor surfaces

BACE1 sensor surfaces were prepared by antibody capture and lipid membrane reconstitution, as illustrated schematically in Fig. 1. The enzyme was captured directly from the Sf9 cell lysate

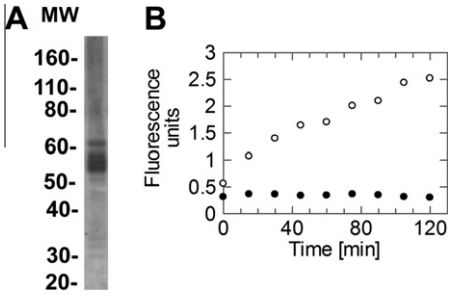

Fig.3. (A) Verification of the presence of full-length BACE1 in Sf9 cell lysate via Western blot analysis using an anti-BACE1 antibody. (B) Enzyme activity monitored as hydrolysis of a FRET peptide with a sequence corresponding to APP with the Swedish mutation (○). The addition of 50 μM compound **2** was included as a control (●).

via a monoclonal anti-polyhistidine antibody previously immobilized to the sensor chip by amine coupling to a level of 8000 RU. A total brain lipid extract was subsequently injected to stabilize the captured protein. Sensorgrams for the capture and reconstitution steps are shown in Appendix 1 in the Supplementary material.

This method for immobilization resulted in a level of BACE1 together with lipids between 3500 and 4500 RU (Appendix 1), whereas the preparation of a reference surface with Sf9 cells not transfected with full-length BACE1 resulted in a signal of approximately 2300 RU. The amount of immobilized protein, determined by removing the lipids after the experiments, was between 1700 and 2200 RU.

The reconstitution of a lipid membrane was crucial because the enzyme alone could be immobilized only to a level of 2000 RU, and the surface showed a strong baseline drift and a rapidly decreasing binding capacity. Reconstitution using only POPC resulted in unstable surfaces unable to bind any inhibitor after 12 h. In contrast, by using a total brain lipid extract, a surface with a stable baseline and binding capacity for more than 24 h was produced (Appendix 1). The binding capacity of the surface was typically around 45%, and the sensitivity of the surface was in a range that small molecules ($M_r \sim 500$ Da) produced a maximum signal of approximately 8 RU.

Interaction between inhibitors and BACE1 and effect of pH

The characteristics of the BACE1 sensor surface was evaluated by analyzing its interactions with different BACE1 inhibitors (Fig. 2). The interactions were analyzed at pHs 4.5 and 7.4 because pH has recently been shown to have a major influence on the interaction characteristics of BACE1 inhibitors [6]. Fig. 4 shows representative examples of the sensorgrams for the interactions and the theoretical sensorgrams (overlaid) obtained by global regression analysis using a 1:1 interaction model. Due to the rapid kinetics for some of the inhibitors, a term accounting for limited mass transfer was included. The kinetic rate constants and the affinities obtained for a series of replicate experiments are summarized in Table 1. The table also contains the corresponding data for interaction experiments previously performed with truncated BACE1, for which only the affinities were published [6].

There were significant differences in the sensorgrams for the different compounds and between the two pH conditions (Fig. 4). Compound **1** appeared to bind slowly but essentially irreversibly at both pH values, whereas compounds **2**–**4** interacted reversibly with both enzyme forms at both pH values. The interactions of compounds **5** and **6** were also characterized by slow association and dissociation rates. Due to the slow dissociation rates, the kinetics or affinities could not be determined for compound **1** at either pH value or for Compound **5** at pH 4.5. For these interactions, the K_D values were defined to be below 10 nM for the sake of the quantitative analysis. Similarly, the interactions of compound **6** showed no interaction up to 1 μM at pH 7.4. Because the estimated R_{max} for a compound with this molecular weight is 20 RU with these sensor surfaces, the K_D value was concluded to be much higher than 1 μM.

The slow interactions of compounds **5** and **6** were accompanied by some type of mechanistic complexity because the 1:1 interaction model did not describe the data perfectly. Therefore, the observed kinetic rate constants for these compounds (Table 1) are approximations of the association and dissociation rate constants. Because there were no signs of solubility problems for any of the compounds within the concentration range used, the cause of the complexity can be assumed to reflect true complexities in the interaction.

The effect of pH on the interactions is visualized in Fig. 5, revealing that the primary effect of pH on compound **3** is on the rate of dissociation, whereas for compounds **2** and **4** there is also an effect

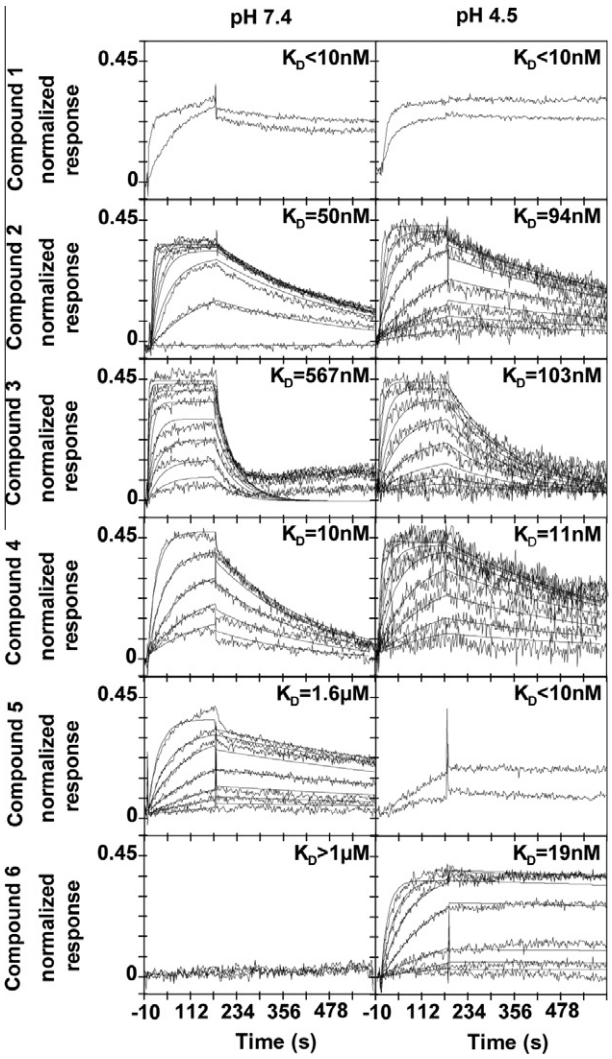

Fig.4. Interaction between inhibitors and full-length BACE1. The sensorgrams were normalized to the theoretical maximum signal estimated from the mass of the inhibitor and the amount of immobilized protein. A 1:1 binding model with mass transfer was fitted to the data (shown as solid lines). The corresponding K_D values are given in each graph. For interactions where the affinities could not be estimated, the upper or lower limit is given.

on association. Because kinetic constants could not be determined for both pH values for compounds **5** and **6**, the graph simply illustrates the change in affinity and the magnitude of the effect on association or rate constants cannot be distinguished.

Effect of Ca^{2+} on inhibitor interactions

The influence of 50 μM Ca^{2+} on the interaction between the different compounds and full-length BACE1 was explored. The concentration was chosen so that a recently reported Ca^{2+} binding site with a K_D of 2 μM [12] would be saturated. However, the presence of Ca^{2+} did not result in any significant differences in K_D values or interaction kinetics at either pH 4.5 or pH 7.4 (Table 2). Injections of up to 1 mM Ca^{2+} as analyte did not show evidence of binding of the ion to BACE1 under these conditions.

Comparison of kinetics for inhibitor interactions with full-length and truncated BACE1

The kinetic constants for the interaction with full-length and truncated BACE1 were determined under the same experimental

Table 1
Kinetic constants for inhibitors interacting with full-length and truncated BACE1 at pHs 7.4 and 4.5.

Compound	pH	Full-length BACE1			Truncated BACE1[a]		
		k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
1	7.4			<10			<10
	4.5			<10			<10
2	7.4	$8\ (\pm1)\times10^{4}$	$3\ (\pm1)\times10^{-3}$	47 ± 17	$4\ (\pm2.5)\times10^{5}$	$7\ (\pm5)\times10^{-3}$	16 ± 3
	4.5	$2.1\ (\pm0.1)\times10^{4}$	$2.0\ (\pm0.1)\times10^{-3}$	94 ± 6	$5.03\ (\pm0.01)\times10^{5}$	$4.7\ (\pm0.1)\times10^{-3}$	93 ± 17
3	7.4	$3.9\ (\pm0.3)\times10^{4}$	$2.2\ (\pm0.5)\times10^{-2}$	567 ± 72	$4.6\ (\pm0.5)\times10^{5}$	$1.9\ (\pm0.5)\times10^{-2}$	42 ± 20
	4.5	$3.7\ (\pm0.1)\times10^{4}$	$3\ (\pm1.5)\times10^{-3}$	103 ± 45	$1.4\ (\pm0.1)\times10^{5}$	$1.4\ (\pm0.5)\times10^{-2}$	105 ± 34
4	7.4	$6\ (\pm5)\times10^{5}$	$5\ (\pm1.5)\times10^{-3}$	10 ± 5	$8\ (\pm1.5)\times10^{5}$	$4.2\ (\pm0.1)\times10^{-3}$	5 ± 0.8
	4.5	$1.5\ (\pm0.3)\times10^{5}$	$1.5\ (\pm0.5)\times10^{-3}$	11 ± 7	$4.9\ (\pm0.5)\times10^{5}$	$1.86\ (\pm0.02)\times10^{-3}$	4 ± 0.3
5	7.4	$6\ (\pm2)\times10^{2}$	$9\ (\pm1.5)\times10^{-4}$	1630 ± 60	$1.19\ (\pm0.01)\times10^{3}$	$7.3\ (\pm0.5)\times10^{-4}$	619 ± 2.8
	4.5			<10			<10
6	7.4			>1000			
	4.5	$6\ (\pm1)\times10^{3}$	$1.1\ (\pm0.5)\times10^{-4}$	17 ± 2			

Note: The sample standard deviations are based on three replicates.
[a] The values for truncated BACE1 were extracted from previously published experiments [6] as described in "Materials and methods".

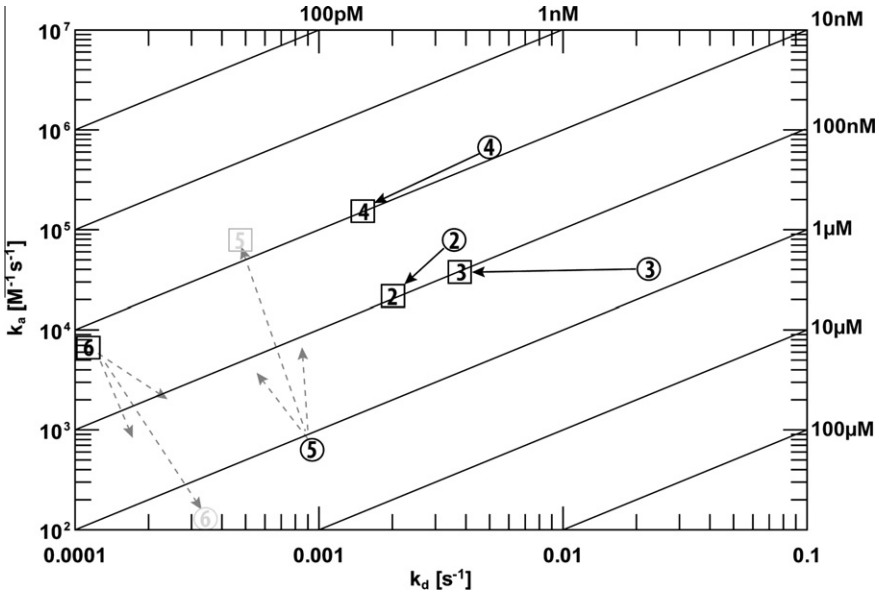

Fig.5. Interaction kinetic plot of compounds **2–6** at pH 4.5 (squares) and 7.4 (circles). The gray dashed arrows indicate the direction of the change for the compounds that have only one point in the graph, and the symbols indicate approximate positions with respect to affinity.

Table 2
Kinetic rate constants and affinities for interactions of selected inhibitors with full-length BACE1 in the presence of 50 μM $CaCl_2$.

Compound	pH	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
2	7.4	4.67×10^{4}	2.00×10^{-3}	43
	4.5	1.94×10^{4}	2.16×10^{-3}	111
3	7.4	2.33×10^{4}	1.58×10^{-2}	677
	4.5	4.47×10^{4}	5.80×10^{-3}	130
4	7.4	4.35×10^{5}	2.75×10^{-3}	6
	4.5	1.84×10^{5}	1.73×10^{-3}	9

Note: The data were otherwise obtained under the same conditions as the corresponding data in Table 1.

conditions. Differences in the assays, such as immobilization from a cell lysate and the presence of a lipid membrane, were accounted for by using appropriate reference surfaces. In Fig. 6, the kinetic rate constants for the interactions between the inhibitors with full-length and truncated BACE1 are compared. The correlation graphs show that association rates (Fig. 6A) were generally slightly faster for truncated BACE1 as compared with the full-length enzyme. No significant differences were seen for most of the dissociation rates (Fig. 6B).

Interaction between full-length BACE1 and substrate

The interaction between BACE1 with a substrate peptide based on APP with the Swedish mutation was also investigated. But despite using up to 1 μM, the substrate was not seen to interact with BACE1 at either of the two pH values used or in the presence of 50 μM Ca^{2+} (not shown). The same result was obtained with a surface immobilized with ectodomain BACE1, demonstrating that this is an inherent characteristic of BACE1 rather than the full-length construct and the currently used immobilization procedure. These results indicate that the K_D is above 1 μM under these conditions.

Discussion

The current study involved the development of a new biosensor-based interaction assay for analysis of interactions between BACE1 and inhibitors. The assay was based on an experimental design where the full-length form of the enzyme was immobilized to a sensor chip to which lipids were attached, enabling the subsequent reconstitution of a lipid membrane on the surface. Previously published biosensor-based assays for BACE1 have been in

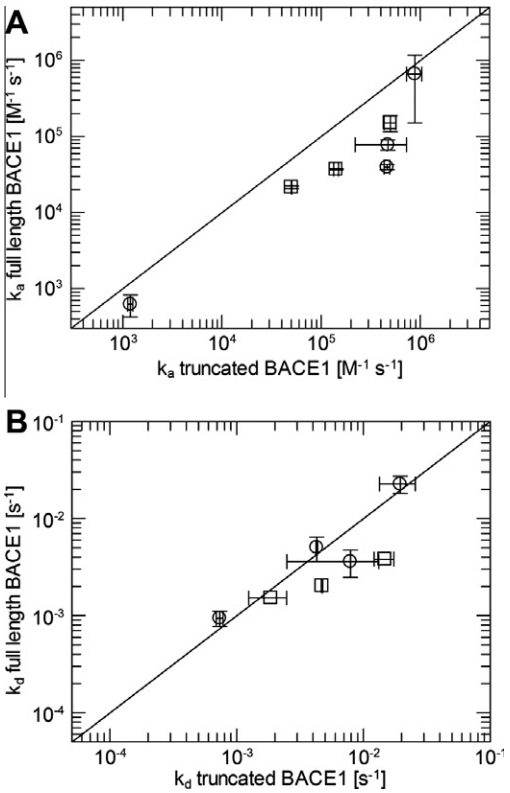

Fig.6. Comparison of association rate constants (A) and dissociation rate constants (B) (k_a and k_d, respectively) for the interaction between truncated and full-length BACE1 with the different compounds. The values for full-length BACE1 were taken from Table 1, and the values for truncated BACE1 were taken from a previous study [6]. The sample standard deviations are based on at least two independent experiments.

the form of a competition assay where ectodomain BACE1 was injected as analyte together with an inhibitor [17] or the direct immobilization of truncated BACE1 [6,18]. The new assay is expected to be more physiologically relevant and has enabled a comparison of inhibitor interactions with the full-length and ectodomain forms of BACE1. Furthermore, the sensitivity of the assay allowed the study of interactions with small molecules, making the assay useful for drug discovery.

To avoid purification and unnecessary handling of the membrane-bound enzyme, Sf9 cells expressing BACE1 were simply lysed and the enzyme was captured to the sensor surface via an antibody. A weakness of using a lysate is that the properties of the protein are not as well defined as when using purified protein. Therefore, some basic controls were performed to define the characteristics of the enzyme in the lysate to verify that it was suitable for the intended studies. SDS–PAGE and Western blot experiments showed that the expressed protein appeared to have undergone some posttranslational modification (e.g., glycosylation, lipidation) as the antibody detected two bands: one with the theoretical molecular weight of BACE1 and a second larger band. This has previously been described for the enzyme [2,19,20] and is not expected to have any practical consequence for the current study.

More significant, due to the conflicting data in the literature, a rigorous analysis of the quaternary structure of the enzyme in the lysate was performed. Because BACE1 has been reported to occur both as SDS-resistant BACE1 dimers and in monomeric form [20–23], the quaternary structure of numerous samples prepared under various conditions was analyzed. A variety of different conditions for sample preparation were explored because the exact procedures for sample preparation in previous studies have not been clearly described in the literature. Only monomeric BACE1 was detected despite these efforts. Still, it is not possible to unequivocally exclude the presence of dimers under certain conditions or conclude that it is a result of aggregation during sample preparation, which is not unusual for membrane proteins [24]. But it can be assumed that if dimerization occurs, it must involve a specific mechanism or require very special conditions. So, it is still unresolved if the reported differences in quaternary structure are physiologically relevant. Considering the catalytic mechanism of BACE1 and the fact that a monomer consists of a complete active site, there is no obvious reason for dimerization—at least from a catalytic perspective.

An important control was to verify that the enzyme was active in the lysate (i.e., before immobilization). After immobilization, the functionality was confirmed by the observation that the enzyme interacted with inhibitors (see below). Therefore, it was rather unexpected that it was not possible to detect the binding of a peptide derived from APP with the Swedish mutation even when tested up to a concentration of 1 μM. Because the same lack of substrate binding was also observed with ectodomain BACE1, it is interpreted as an effect of the very low affinity of BACE1 for its substrate. As illustrated for compound **6**, a peptide of similar features and molecular weight as the substrate could also not be detected to bind at pH 7.4. For both the substrate and compound **6**, the affinity was calculated to be more than 1 μM. Such a high K_D for the substrate is reasonable considering the micromolar K_m values reported for peptides derived from APP [25–28]. To overcome the low affinity for the substrate, BACE1 activity assays are designed so that they measure the hydrolysis of substrate over a much longer time frame (hours) than is typically used for other enzymes (e.g., HIV protease) [28–30] and these biosensor studies (minutes). Nevertheless, it is curious that the enzyme appears to be so sluggish, especially given that it must compete with α-secretase for APP cleavage in vivo. Therefore, it has been speculated that other proteases may be involved in Aβ production and that the low affinity for APP is compensated by concentrating APP and BACE1 in special areas of the lipid membrane such as lipid rafts [31]. Another explanation for the observed poor activity could be that the peptide substrate does not correspond to the natural substrate because it has a higher conformational flexibility compared with APP and that only a very small fraction of the substrate is in fact in a conformation that can bind the enzyme. This is perhaps reflected for compound **6**, which is also a "long" peptide whose conformational flexibility may restrict efficient binding under certain conditions. However, the experimental design developed in the current study can be further extended for studies of the interaction between full-length BACE1 and full-length APP, potentially allowing these aspects to be studied further.

By capturing BACE1 via an antibody directed against the His tag, the enzyme could be immobilized without any purification steps. Moreover, the orientation of the protein on the chip was suitable for reconstitution of a membrane, leaving the extracellular domain accessible for binding of analyte. Because membrane proteins can have a strong lipid core associated with their transmembrane regions [32], it can be assumed that BACE1 has such a lipid core and that it stays intact during the short period between cell lysis and rebuilding of the lipid membrane. However, it was necessary to stabilize the immobilized protein by a lipid membrane as it exhibited a strong baseline and capacity drift when only a detergent was used to keep it solubilized. The lipid composition was important because POPC did not result in a stable surface, whereas the surface was stable and showed a good binding capacity for several days when using a brain lipid mixture. This is reasonable because it has been shown that different transmembrane regions require interactions with special lipid chains or head groups and that just a hydrophobic environment is not sufficient [33]. It

indicates that the mixture of added lipids interacted specifically with the captured BACE1, providing a stable environment for the protein and acting as an additional anchor for the protein (Fig. 1). Still, small differences in the final immobilization levels between experiments suggest that there may be minor differences in the amount of enzyme in the cell lysate and perhaps also in the brain lipid extracts used.

A drawback of capturing a protein from a lysate is that the amount of functional protein immobilized can be too low for a sensitive surface. Due to the multistep immobilization procedure and the possibility that other compounds than the intended protein were nonspecifically captured, it was not possible to exactly quantify the binding capacity of the surface, although it appeared to be approximately 45%. Apparent binding capacities down to 20% are rather typical and not a problem unless the signals become too low, which was not the case here.

The interactions between BACE1 and compounds **2**–**4** were well described by a 1:1 interaction model, and the association and dissociation rate constants could be determined. For some of the compounds, the standard deviations for k_a and k_d were much higher than those for K_D. This is often observed for fast interactions, which are limited by mass transfer effects that influence the rate constants but not the affinity estimates (personal communication with Olof Karlsson, Biacore, GE Healthcare). Compounds **1**, **5**, and **6** appeared to interact by a more complex mechanism. The mechanism for compound **1** could not be elucidated from the current data due to the stability of the formed complexes. The complexity observed for compounds **5** and **6** can be speculated to be due to effects on the conformation or aggregation state of the inhibitors in solution because the slow association suggests that a significant fraction of the inhibitors are not in a binding competent form and that the equilibrium between two forms of the uncomplexed inhibitor is rate limiting for association. The compounds for which association is slow are the longest compounds, for which the conformational flexibility is expected to be the largest.

In a previous study, we investigated the interaction between truncated BACE1, without a membrane-spanning domain, and compounds **1**–**5** [6]. Minor differences were seen for most of the dissociation rates, supporting earlier data that the transmembrane region has no influence on the interaction with inhibitors [25]. However, previous studies have been performed in an acidic environment, where BACE1 shows the highest activity. Because we and Stachel and coworkers recently showed that interactions at a neutral pH are also critical for the efficacy of BACE inhibitors [6,34], it was important to elucidate the influence of the transmembrane region also at neutral pH. Our results confirm the previous results from acidic pH but also show that the transmembrane region has only a slight influence on the interaction at neutral pH. This suggests that truncated BACE1 is a good model system to study the interaction with inhibitors and that the transmembrane region has no major effects on the structure of the BACE1 active site at either pH 4.5 or 7.4.

It is not clear why these studies could not substantiate the recent report that full-length BACE1 has a calcium binding site in the intracellular region and that calcium binding modifies both the structure of BACE1 and its proteolytic activity [12]. The fact that none of the inhibitors or the substrate showed differences in their interactions with the enzyme in the presence of calcium indicates that the calcium binding has no influence on the structure of the inhibitor binding site, as we previously demonstrated for C-reactive protein by similar experiments [35]. Still, it cannot be excluded that calcium could perhaps have an effect on the function of BACE1 by influencing, for example, the location within the cell membrane or its activity in a different context than what has been tested here.

Conclusions

The current study illustrates that direct capture of a membrane protein from a cell lysate and the subsequent reconstitution of a membrane around the protein, using a complex lipid extract, is an efficient strategy for preparing biosensor surfaces suitable for SPR biosensor analysis of the enzyme in a physiologically relevant environment. Nevertheless, it revealed that ectodomain BACE1 is a good model system to study the interaction with inhibitors, although the characteristics of full-length BACE1 are not completely reflected. This experimental design will now be extended for studies of other membrane proteins, and its potential as a standard method to immobilize membrane proteins will be explored.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.02.041.

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