γ-SECRETASE SUBSTRATE SELECTIVITY CAN BE MODULATED DIRECTLY VIA INTERACTION WITH A NUCLEOTIDE BINDING SITE.

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γ-Secretase is an unusual protease with an intramembranous catalytic site that cleaves many type I membrane proteins, including the amyloid β-protein (Aβ) precursor (APP) and the Notch receptor. Genetic and biochemical studies have identified four membrane proteins as components of γ-secretase: heterodimeric Presenilin (PS) composed of its N- and C-terminal fragments (NTF/CTF), Nicastrin (NCT), Aph-1 and Pen-2. Here, we demonstrate that certain compounds, including protein kinase inhibitors and their derivatives, act directly on purified γ-secretase to selectively block cleavage of APP- but not Notch-based substrates. Moreover, ATP activated the generation of the APP intracellular domain (AICD) and Aβ, but not the generation of the Notch intracellular domain (NICD) by the purified protease complex and was a direct competitor of the APP-selective inhibitors, as were other nucleotides. In accord, purified γ-secretase bound specifically to an ATP-linked resin. Finally, a photoactivatable ATP analog specifically labeled the PS1-CTF in purified γ-secretase preparations: the labeling was blocked by ATP itself and APP-selective γ-secretase inhibitors. We conclude that a nucleotide-binding site exists within γ-secretase, and certain compounds that bind to this site can specifically modulate the generation of Aβ while sparing Notch. Drugs targeting the γ-secretase nucleotide-binding site represent an attractive strategy for safely treating Alzheimer’s disease.

Alzheimer’s disease (AD) is characterized by the progressive accumulation of amyloid β-protein (Aβ) in brain regions subserving memory and cognition (1). Sequential proteolytic cleavages of the amyloid β-protein precursor (APP) by the β- and γ-secretases generate the amyloid β-protein (Aβ) (1). β-Secretase (BACE) is a single membrane-spanning aspartyl protease expressed at high levels in neurons (2). γ-Secretase is also an aspartyl protease but with an unprecedented intramembranous catalytic site (3,4) that is required for the cleavage of a wide range of type I membrane proteins that include APP and the Notch receptors (for a review see (5)). We recently reported a specific and reproducible procedure for the high-grade purification of active human γ-secretase and characterized various factors that affect its activity in vitro (6). In further investigating the properties of the purified enzyme, we have observed that ATP can activate purified γ-secretase in vitro by up to two fold. This observation is in agreement with the recent report of Netzer and colleagues that γ-secretase-mediated generation of Aβ in a mouse N2a neuroblastoma cell-free system is ATP dependent (7). These authors also found that imatinib mesylate (Gleevec or STI571), a selective Abl kinase inhibitor approved to treat chronic myelogenous leukemia (CML) (8-10), inhibited γ-secretase cleavage of APP without affecting Notch processing in a N2a cell-free system, in intact N2a cells expressing human APP and in rat primary neurons (7). Another compound with a pyrido-(2,3-d)pyrimidine structure (called Inhibitor 2 (7) or PD173955 (11)) showed a similar effect (7). Both Gleevec and Inhibitor 2 are known to inhibit Abl
kinase by targeting its ATP-binding site (12-16) but cells deficient in this enzyme were still sensitive to Gleevec with respect to lowering Aβ production (7). The mechanism by which these two compounds affect γ-secretase cleavage of APP is unknown. Because Gleevec and Inhibitor 2 target several tyrosine protein kinases besides Abl, Netzer et al. suggested that platelet-derived growth factor receptor (PDGFR), Src kinase or c-kit might be involved (7). Another proposed mechanism involves an effect on the localization of γ-secretase or APP in a way that prevents interaction of enzyme with substrate.

A central concern about inhibiting γ-secretase to lower Aβ production in AD is that this should also interfere with Notch processing, and lead to severe toxicity due to interference with cell differentiation. Indeed, significant adverse effects of γ-secretase inhibitors caused by blocking Notch signaling have been described in preclinical animal studies (17,18,19,20). Because Netzer et al. showed that Gleevec and Inhibitor 2 inhibited APP but not Notch cleavage in their systems (7), we investigated the effects of selected protein tyrosine kinase inhibitors on Aβ production and on Notch substrate cleavage using isolated, purified γ-secretase.

MATERIALS AND METHODS

Cell Lines and Cultures - HeLa S3 cells, the Chinese hamster ovary (CHO) γ-30 cell line (co-expressing human PS1, Flag-Pen-2, Aph1α2-HA), and the S-1 CHO cell line (co-expressing human PS1, Flag-Pen-2, Aph1α2-HA and NCT-GST) were cultured as previously described (6,21,22). Purification of γ-secretase and in vitro γ-secretase assays - The multistep procedure for the high-grade purification of human γ-secretase from the S-1 cells was performed as previously described (6). In vitro γ-secretase assays using the recombinant APP-based substrate C100Flag and the recombinant Notch-based substrate N100Flag were performed as previously reported (4,21). Basically, the proteolytic reaction mixtures contained C100Flag and N100Flag substrate at a concentration of 1 µM, purified γ-secretase solubilized in 0.2% CHAPSO-HEPES, pH 7.5, at 10-fold dilution from stock (stock = the M2 anti-Flag-eluted fraction in the purification protocol from S-1 cells (6)), 0.025% phosphatidylethanolamine (PE) and 0.10% phosphatidylcholine (PC). All the reactions were stopped by adding 0.5% SDS, and the samples were assayed for Aβ40 and Aβ42 by ELISA as described (23). The capture antibodies were 2G3 (to Aβ residues 33-40) for the Aβ40 species and 21F12 (to Aβ residues 33-42) for the Aβ42 species. Inhibitors - Powder containing Gleevec (Novartis) was dissolved from capsules or tablets in a mixture composed of ethyl acetate and aqueous saturated sodium bicarbonate solution. The organic layer was washed several times with brine, dried on sodium sulfate and evaporated under vacuum. Gleevec was purified and analyzed by RP-HPLC using a Vydac C18 preparative column (10 µM, 2.2 cm x 25 cm) and a C18 analytical column (5 µM, 0.46 cm x 25 cm), respectively. Chromatographic separations were performed at a flow of 1.5 ml/min, with a gradient of 0-100% MeOH in water over 30 min. This isolated material is referred to as “Gleevec extract”. Purified Gleevec was from Sequoia Research Products, UK. Final purity and characterization of the two Gleevec extracts (from capsules and tablets, respectively) and the purified Gleevec (Sequoia Research Products, UK) was performed by MALDI-TOF mass spectroscopy (Applied Biosystems Voyager System 4036). Gleevec was detected with a m/z of (M+H)+ = 494 g/mol (expected m/z of (M+H)+ = 493 g/mol). The well-characterized γ-secretase inhibitor III-31C was prepared as described previously (4). Bryostatin-1 was purchased from BIOMOL Research laboratories. All compounds listed in Fig. 4 were purchased from TOCRIS-UK; the structure and action of these compounds are described in Table 1 (modified from TOCRIS-UK in Supplementary Data). All inhibitors were added to the reaction mixtures from a DMSO stock (final DMSO concentration of 1%, which alone did not affect γ-secretase activity).

Western Blotting and Antibodies - For Western analysis of PS1-NTF, PS1-CTF, Aph1α2-HA, Flag-Pen-2 and NCT-GST, the samples were run on 4-20% Tris-glycine PAGE gels, transferred to polyvinylidene difluoride, and probed with Ab14 (for PS1-NTF, 1:2000, a gift of S. Gandy), 13A11 (for PS1-CTF, 5 µg/ml, a gift of Eli Lilly), 3F10 (for Aph1α2-HA, 50 ng/ml, Roche), anti-Flag M2 (for Flag-Pen-2, 1:1000, Sigma), or αGST antibodies (for NCT-GST, 1:3000, Sigma). Samples from the γ-secretase activity assays (above) were run on 4-20% Tris-glycine gels and transferred to PVDF membranes to detect AICD-Flag with anti-Flag M2 antibodies.
Purified γ-secretase and binding to ATP-immobilized resins - The purified γ-secretase was diluted 10-fold from stock (6) in 50 mM HEPES buffer, pH 7.0, containing 0.2% or 1% CHAPSO, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and incubated overnight, in the presence or absence of 50 mM ATP (Sigma), with ATP-agarose (ATP attached to agarose through the ribose hydroxyls, Sigma Prod. No. 71438-3). Each resin was washed 3 times with 0.2% or 1% CHAPSO-HEPES buffer, the bound proteins collected in 2X Laemmli sample buffer. For the photoaffinity labeling of the purified γ-secretase, the enzyme was diluted 10-fold from stock (6) in 50 mM HEPES buffer, pH 7.0, containing 0.2% or 1% CHAPSO-HEPES buffer, the bound proteins collected in 2X Laemmli sample buffer and resolved on 4-20% Tris-glycine gels and transferred to PVDF membranes to detect NICD-Flag, PS1-NTF, Aph1-HA, PS1-CTF and Flag-Pen2 as described above.

Photoaffinity Labeling Experiments - [γ³²P]-8-azidoATP (18 Ci/mmol) was purchased from Affinity Labeling Technology (ALT, Lexington, KY). For the photoaffinity labeling of the purified γ-secretase, the enzyme was diluted 10-fold from stock (6) in 50 mM HEPES buffer, pH 7.0, containing 0.2% or 1% CHAPSO, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 0.025% PE and 0.10% PC. The samples were exposed to UV light for 5 min (hand-held UV lamp at 254 nm; UVP Model UVGL-25) on ice, and the reaction was quenched with 1 mM DTT. The proteins were diluted in 0.5% CHAPSO-HEPES buffer and incubated overnight for affinity-precipitation with GSH-resin as described previously (6,22). The unbound nucleotides were removed by washing the membranes 3 times, then the washed membranes were resuspended for 1 hour in 0.5 ml of 1% CHAPSO-HEPES, pH 7.4. The solubilized proteins were diluted 1:2 in HEPES buffer (final CHAPSO conc. = 0.5%) and incubated overnight with X81 antibody for immunoprecipitation, as described previously (6,22). Samples were electrophoresed on 4-20% Tris-glycine gels and autoradiographed (BioMax MS films used with BioMax Transcreen HE (KODAK)).

ATPase assays - [α³²P]-ATP (11.9 Ci/mmol) was purchased from Affinity Labeling Technology (ALT, Lexington, KY). The purified γ-secretase was prepared as described for the photoaffinity labeling experiments; 5 μCi of [α³²P]-ATP was added, the reactions incubated at 37°C, and at the indicated time points aliquots were removed and reactions stopped by addition to 10% SDS. A total of 2 μL of each stopped reaction was analyzed by thin-layer chromatography (TLC) on polyethyleneimine cellulose plastic sheets (Baker-Flex, Germany) with 0.75 M KH₂PO₄ (pH 3.5) as the running buffer to separate ATP from ADP. To identify hydrolysis products, a reaction of [α³²P]-ATP incubated in the presence of 0.005U canine kidney phosphatase (Sigma) was loaded. Samples were autoradiographed as described above.

RESULTS

Non-hydrolyzed ATP can activate the generation of AICD and Aβ₄₂ but not the generation of NICD by purified γ-secretase - We took advantage of our highly purified γ-secretase complexes to investigate factors that might affect their cleavage of APP, using a C100Flag substrate consisting of the β-CTF (C99) portion of APP (amino acids 596-695) plus a Met at the N-terminus and a Flag tag at the C-terminus (4,24). We observed that increased concentrations of ATP promoted a dose-dependent rise in the production of both AICD-Flag (Fig. 1A, compare lanes 2-6 with lane 1) and Aβ (both Aβ40 and Aβ42), reaching a ~1.75-fold increase at 1-5 mM as estimated by Aβ ELISA (Fig. 1B). Densitometry of the AICD-Flag bands (Fig. 1A) showed that the fold increases were similar to those seen for Aβ by ELISA in Fig. 1B. These findings are in good agreement with the
reported data of Netzer \textit{et al.}, which showed that the addition of 1-3 mM ATP in a cell-free membrane preparation (derived from mouse N2a cells expressing human APP-695) results in a ~2-fold increase in Aβ production (7). Next, we addressed whether ATP can stimulate the cleavage of a Notch-based substrate (N100Flag) in a similar manner and under the same conditions as for C100Flag. N100Flag is an analog of a Notch-based substrate corresponding to residues Val1711 to Glu1809 from the mouse Notch-1 receptor (plus a Met and a Flag tag at the N- and C-termini, respectively, just like C100) (21). We found that increased concentrations of ATP did not alter the generation of NICD-Flag (Fig. 1A, compare lanes 2-6 with lane 1), and this was confirmed by densitometry.

ATP stores energy in the form of a chemical bond and releases it in the process of hydrolysis, providing a readily-available energy supply for many enzymatic reactions. Thus, we intended to determine whether the peptidase activity of γ-secretase is associated with ATP hydrolysis. As shown in Figure 1C, the purified γ-secretase incubated in the presence (lanes 21-25) or absence (lanes 6-10) of C100Flag substrate did not increase the hydrolysis of [α\(^{32}\)P]ATP into [α\(^{32}\)P]ADP when compared to the hydrolysis occurring in the reaction buffer alone (lanes 11-15). This result indicates that ATP hydrolysis is not required for the peptidase activity of γ-secretase. We further found that 1 mM levels of the nonhydrolyzable ATP analogue, ATP-γS, also resulted in a ~1.35 increase in the generation of Aβ40 and Aβ42 from C100Flag substrate by purified γ-secretase (data not shown), supporting our observation that ATP hydrolysis is not required for the effect.

\textit{Gleevec itself is not a direct γ-secretase inhibitor; however, a Gleevec extract inhibits the generation of Aβ by purified γ-secretase without affecting the cleavage of a Notch-based recombinant substrate.} Because ATP activated the purified γ-secretase complex (Figs. 1A & B), we used this preparation to examine the effects of a Gleevec extract (prepared from capsules and characterized as described in detail under Materials and Methods) on the cleavage efficiency of C100Flag substrate. We first confirmed that III-31C (4) and DAPT (25), two well-characterized inhibitors of γ-secretase, inhibited both C100Flag and N100Flag cleavage by our purified γ-secretase with similar potencies (III-31C : IC\(_{50}\) of ~50 nM for cleavage of C100Flag, as estimated by ELISA and densitometry, and ~100 nM for cleavage of N100Flag, as estimated by densitometry; DAPT : IC\(_{50}\) of ~100 nM for cleavage of C100Flag, as estimated by ELISA and densitometry, and ~100 nM for cleavage of N100Flag, as estimated by densitometry) (Fig. 2A, and ELISA data not shown). We then probed the effects of our Gleevec extract (extracted from capsules and isolated by HPLC as described in Materials and Methods) on Aβ production by the purified γ-secretase. The cleavage products, Met-Aβ40 and Met-Aβ42, (which we designate Aβ40 and Aβ42 for simplicity) were quantified by ELISA (Fig. 2B) and also detected by blotting with the 6E10 anti-Aβ antibody (Fig. 2C). The other proteolytic product, Flag-tagged AICD, was detected with anti-Flag M2 antibodies (Fig. 2C). Our Gleevec extract inhibited the generation of Aβ40, Aβ42 and AICD in a concentration-dependent fashion and with a similar potency, yielding an approximate IC\(_{50}\) value (estimated by Aβ ELISA) of ~75μM (Figs. 2B & 2C). Next, we examined the effects of our Gleevec extract on the cleavage of N100Flag by the purified protease. The Gleevec extract did not inhibit the generation of NICD-Flag, even at concentrations ~10-fold the IC\(_{50}\) value for the generation of Aβ40 and Aβ42 from C100Flag substrate (i.e., at 1 mM) (Fig. 2C, lane 7). Similarly, the Gleevec extract was found to inhibit the generation of Aβ by endogenous γ-secretase solubilized from HeLa cell membranes without affecting the cleavage of N100Flag substrate (Fig. 2D). Because pH is an important factor modulating the activity of the purified γ-secretase (6), the pH of all the above reactions was checked and found to be consistently at pH 7.4, even at high concentrations (1 mM) of the Gleevec extract. Because several other compounds (impurities or degradation products probably generated during the extraction and purification procedures - m/z of 200.1, 247.6, 268.2, 277.1, 286.6, 308.9, 332.9, 350.0, 380.1, 395.1, 516.2) were detected in the Gleevec preparation (Fig. 2E, left panel and data not shown), we decided to examine the effect on the purified γ-secretase of two additional Gleevec samples prepared from two different sources: Gleevec extracted from tablets as described in detail under Materials and Methods and purified Gleevec (Sequoia Research Products, UK). Final purity of the two Gleevec samples described above was addressed by MALDI-
TOF mass spectroscopy (Fig. 2E, middle and right panels, respectively). We found that these two Gleevec samples did not inhibit the generation of AICD, Aβ40 and Aβ42 by the purified γ-secretase (Fig. 2E, middle and right panels, respectively and ELISA data not shown). Because Gleevec (m/z of (M+H)+ = 494) was found in the three different Gleevec samples, our data strongly suggest that Gleevec is not the active compound found in the Gleevec preparation extracted from capsules. Interestingly, the analyses by mass spectroscopy of the three Gleevec samples revealed several compounds (m/z of 200.1, 308.9, 332.9, 350.0, 516.2) that were only present in the active Gleevec preparation (Fig. 2E, the compounds identified specifically in the active Gleevec preparation are labeled with arrow heads), leaving open the possibility that one or more of those compounds are active towards γ-secretase. Also, a very minor peak at 286.6 in the inactive extract is a major peak in the active extract (Fig. 2E, asterisk). Although purification and characterization of each of the compounds found in our active Gleevec extract will now be necessary for the identification of the active compound, our data show that (1) Gleevec itself is not a direct γ-secretase inhibitor and (2) the generation of Aβ from γ-secretase can be inhibited directly and selectively without affecting Notch proteolysis.

Next, we analyzed extracellular Aβ40 and Aβ42 levels after treatment of CHO cells stably expressing human APP with purified Gleevec (Sequoia Research Products, UK). Consistent with the previous report of Netzer and colleagues, treatment of cells with 10 µM of purified Gleevec reduced secreted Aβ40 and Aβ42 by ~50% (data not shown). Based on this observation, an attractive hypothesis is that Gleevec breaks down in cells to form the active compound found in the Gleevec preparation extracted from capsules.

Nucleotides are direct competitors of the active compound in the Gleevec extract on γ-secretase - Because ATP activated the cleavage of an APP- but not a Notch- based substrate and because Gleevec binds to the ATP-binding site of protein kinases (26,27), we compared ATP and other nucleotides (ADP, AMP, GTP, CTP and TTP) as potential competitors with respect to the active Gleevec extract, reasoning that the active Gleevec derivative may interact with a nucleotide binding site on γ-secretase. Again, all nucleotide stock solutions were neutralized to pH 7.4 before use. We found that the active Gleevec extract at 100 µM inhibited ~70% of the generation of both Aβ40 and Aβ42 and accordingly decreased AICD-Flag generation, when compared with levels observed in the absence of compound (Fig. 2B; Fig. 3A-compare lanes 1 & 2 (top panel); and Fig. 3B & C). In the presence of the active Gleevec extract at 100 µM, increasing concentrations of ATP (from 0.1 to 5 mM) restored γ-secretase activity in a concentration-dependent fashion to ~100% of that observed without the Gleevec extract (Fig. 3A, compare lanes 2-6 with lane 1 in the top panel; also, Figs. 3B & C). These highly consistent results suggest that ATP is a direct competitor of the active compound present in the Gleevec extract on purified γ-secretase. Interestingly, ADP and AMP also prevented the inhibitory effect of the Gleevec extract, but with slightly less potency than ATP. For example, in the presence of 1 mM ATP, ADP or AMP, the Gleevec Extract at 100 µM inhibited ~25%, 35%, and 50% of the generation of Aβ40, respectively, and ~25%, 42%, and 62% of the generation of Aβ42, respectively (Figs. 3B & C). Next, we assessed whether GTP, CTP and TTP can act as direct competitors with respect to the Gleevec extract. We found that both GTP and TTP were potent Gleevec extract competitors, whereas CTP was not, even at 5 mM (data not shown). Taken together, our results yield the following order of potency of competition against Gleevec extract: ATP = GTP > ADP = TTP > AMP > CTP. Moreover, they suggest a common binding site on γ-secretase for the active compound in the Gleevec extract and these nucleotides.

ZM 39923 (1367), a potent Janus kinase 3 inhibitor, preferentially blocks APP cleavage by purified γ-secretase - On the basis of the results described above, the Gleevec extract contains a direct competitive inhibitor of γ-secretase with respect to the cleavage of APP to Aβ that does not noticeably affect Notch cleavage. Because the active compound is apparently an impurity or degradation product of Gleevec (a potent tyrosine kinase inhibitor known to compete with ATP-binding sites (26,27)) and because ATP is a direct competitor of the active compound in the Gleevec extract, we decided to screen ~50 well-characterized protein kinase inhibitors (most of them
For the ability to inhibit C100Flag cleavage by purified \( \gamma \)-secretase. All of the reactions contained 1 \( \mu M \) C100Flag substrate, purified \( \gamma \)-secretase solubilized in 0.20\% CHAPSO-HEPES (pH 7.5) at 10-fold dilution from stock (6), 0.025\% phosphatidylethanolamine (PE), 0.10\% phosphatidylcholine (PC), and 100 \( \mu M \) of the respective compound. The concentration of 100 \( \mu M \) for each compound was chosen to attempt to identify inhibitors more potent than the active Gleevec extract (IC\(_{50} \) value for the cleavage of C100Flag is \( \sim 75 \mu M \)). III-31C (10 \( \mu M \)) or the Gleevec extract (100 \( \mu M \)) were used as positive controls to inhibit \( \gamma \)-secretase; Bryostatin 1 (100 \( \mu M \)), a protein kinase C activator which has been reported to increase APP processing by the \( \alpha \)-secretase pathway (28), was used as a negative control for the inhibition of \( \gamma \)-secretase. After 4 hr of incubation at 37\°C, the reactions were probed for the generation of AICD-Flag as described above. We found one compound (1367) that by western blotting had a potency at least equal to that of the Gleevec extract (Fig. 4, compare lanes 25, 26 and 27). Several other compounds (0433, 0577, 1321, 0744, 1037, 1405 and 1407) also inhibited purified \( \gamma \)-secretase (Fig. 4A, compare lanes 8, 13 and 24 with lanes 4-6). Interestingly, purified \( \gamma \)-secretase bound to the resin that had ATP attached to agarose through the ribose hydroxyls, or else ATP attached to acrylamide (ATP attached to acrylamide through the ribose hydroxyls (Fig. 5, lanes 1-12), suggesting a stimulatory effect on NICD-Flag production in the 30-100 \( \mu M \) range (Fig. 4B, left panel, compare lanes 5-6 and 8, and Fig. 4B, right panel, compare lanes 5-7 and 9). Still another compound, the sulfonamide 0433, behaved differently from the active Gleevec extract, 1367 and 1366, inhibiting the cleavage of C100Flag and N100Flag with a similar potency (Fig. 4C and ELISA data not shown).

**Purified \( \gamma \)-secretase binds to an ATP-immobilized resin** - In view of all of the above results, we examined the ability of \( \gamma \)-secretase to bind ATP. Purified \( \gamma \)-secretase (prepared as described in Methods and diluted in 0.2\% CHAPSO-HEPES) was incubated overnight with ATP-immobilized resins (ATP attached to agarose through the ribose hydroxyls, or else ATP attached to acrylamide through the \( \gamma \)-phosphate) in the absence or presence of excess (50 \( mM \)) ATP. We observed that purified \( \gamma \)-secretase bound to the resin that had ATP attached to acrylamide through the \( \gamma \)-phosphate (Fig. 5, lanes 1-3), and this binding was specific in that 50 \( mM \) ATP fully prevented the complex from binding (Fig. 5, lanes 4-6). Interestingly, purified \( \gamma \)-secretase diluted in 0.2\% CHAPSO-HEPES bound poorly to the resin in which ATP was attached to agarose through the ribose hydroxyls (Fig. 5, lanes 10-12), suggesting a lesser accessibility of the nucleotide to the binding pocket. We also found that \( \gamma \)-secretase present in crude whole lysates of \( \gamma \)-30 cells binds very poorly to the resin with ATP attached to acrylamide through the \( \gamma \)-phosphate, in contrast to the efficient binding of the

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**Known to compete with the ATP-binding site on their cognate targets** for their ability to inhibit C100Flag cleavage by purified \( \gamma \)-secretase.
purified enzyme (Fig. 5, compare lanes 7-9 to 1-3). Two hypothesis could explain this observation: a) the whole cell lysate contains ATP, which acts as a competitor for the γ-secretase nucleotide-binding site, consistent with the fact that the intracellular concentration of ATP can exceed 5 mM (30); and b) the lysate is enriched in ATP-binding proteins that act as competitors for the ATP bound to the resin. Yet, we cannot exclude that purified γ-secretase adopts subtle conformational changes that differ from γ-secretase present in crude whole lysates, thus promoting the binding to the ATP-resin. In any event, these observations suggest that such a resin would not be useful as an early step for the purification of endogenous γ-secretase.

**Photoaffinity labeling of the γ-secretase component PS1-CTF with the ATP analog 8-azido-ATP[γ32P]** - In an effort to identify the component(s) of the purified complex that mediates the interaction with the Gleevec extract, ZM39923 (1367) and ATP, we used the ATP analog 8-azido-ATP[γ32P] for attempted site-specific photolabeling of γ-secretase. First, we observed that like ATP itself, unlabeled 8-azido-ATP (5 mM) was able to rescue the inhibition of purified γ-secretase activity by 100 μM Gleevec extract (data not shown), indicating that azido-modified-ATP still binds to the apparent nucleotide binding domain. Next, purified γ-secretase was incubated with 8-azido-ATP[γ32P] (Fig. 6A, lane 4) in the presence of 10 mM ATP (Fig. 6Aa, lane 5) or 1 mM Gleevec extract (Fig. 6A, lane 6) and subjected to blue native (BN)–PAGE analysis after UV irradiation. The 32P labeling assessed by autoradiography revealed a high molecular weight complex (HMWC, Fig. 6A, lane 4) comigrating with the well-characterized active γ-secretase complex (31) that is detected by BN-PAGE analysis and western blotting (Fig. 6A, lane 1). The intensity of this band was lost in the presence of excess cold ATP or Gleevec extract (Fig. 6A, compare lanes 5 and 6 with lane 4), indicating that purified and active γ-secretase binds specifically 8-azido-ATP[γ32P]. Next, purified γ-secretase was incubated with 8-azido-ATP[γ32P] in the absence or presence of 10 mM ATP, 1 mM Gleevec extract, 1 μM III-31C, 1 μM C100Flag, 1 μM N100Flag, or 300 μM 1367; as a control for the specificity of photolabeling, purified γ-secretase was also incubated with ATP[γ32P] (without the 8-azido group). After UV irradiation, γ-secretase was affinity-precipitated with GSH-resin (which binds Nicastrin-GST in the complex), and its 32P labeling was assessed by SDS-PAGE and autoradiography. A prominent ~18 kDa band consistent with the known position of PS1-CTF was found to be photoaffinity-labeled in the purified γ-secretase (Fig. 6B, lanes 1 and 2). Next, the sample loaded in lane 1 (Fig. 6B) was probed simultaneously for PS1-CTF (13A11) and Aph1-HA (3F10); the photoaffinity-labeled ~18 kDa was found to comigrate with PS1-CTF but not with Aph1-HA (Fig. 6B, compare lane 10 with lanes 1 and 2), strongly suggesting that the ~18 kDa protein is PS1-CTF. The intensity of this band decreased markedly or was lost in the presence of excess cold ATP, Gleevec extract, or 1367 (Fig. 6B, compare lanes 1 & 2 with lanes 3, 4 and 9), indicating that azido-ATP[γ32P] bound to this protein specifically. In contrast, the presence of 1 μM III-31C, a transition-state analog inhibitor, did not affect the labeling of this protein (Fig. 6B, lane 5), suggesting that azidoATP[γ32P] does not bind to the active site of γ-secretase. The presence of 1 μM C100Flag or 1 μM N100Flag also did not affect the labeling of this protein (Fig. 6B, lanes 7 and 8, respectively), suggesting that azido-ATP[γ32P] does not bind to a putative common substrate docking site of γ-secretase (4,32). Interestingly, no labeling was observed with purified γ-secretase incubated with ATP[γ32P] (without the 8-azido group) (Fig. 6B, lane 6), supporting the specific photoaffinity labeling of PS1-CTF. Moreover, this observation indicates that the γ32P does not transfer from ATP[γ32P] to PS1-CTF. Together with our observation that ATP is not hydrolyzed when incubated with purified γ-secretase (Fig. 1B), these findings indicate that intact ATP[γ32P] binds directly to PS1-CTF. To confirm the interaction of ATP with this component of the γ-secretase complex when it is located within membranes, we incubated membranes from HeLa cells (expressing endogenous γ-secretase) with azido-ATP[γ32P]. After removing unbound nucleotide by washing the membranes with excess buffer and then co-immunoprecipitating solubilized γ-secretase with antibodies directed against PS1-NTF, we observed a specifically photoaffinity-labeled ~18 kDa protein migrating at the position of PS1-CTF (Fig. 6C, lane 1). This labeling was abolished in the presence of excess cold ATP (Fig. 6C, lane 2).
DISCUSSION

Here we demonstrate that ATP can activate the generation of AICD and Aβ by purified γ-secretase from an APP-based substrate without affecting the generation of NICD from a Notch-based substrate. We also demonstrate that Gleevec itself is not a direct γ-secretase inhibitor but a Gleevec extract from capsules contains a direct γ-secretase inhibitor, that is competitive with ATP and that has no effect on the cleavage of a Notch-based substrate. We further identify ZM39923 (1367) and ZM449829 (1366), two tyrosine kinase inhibitors known to bind to the ATP-binding site of Janus kinase 3 (29), as direct γ-secretase inhibitors that block preferentially the cleavage of the APP-based substrate compared to the Notch-based substrate. ZM39923, ZM449829 and Gleevec are tyrosine kinase inhibitors that bind to the ATP-binding sites of their corresponding targets. Accordingly, ATP is able to rescue the γ-secretase activity inhibited by the Gleevec extract, suggesting a potential nucleotide-binding domain on γ-secretase. In support of this hypothesis, we found that purified γ-secretase binds to a resin having ATP attached to acrylamide through the γ-phosphate. Moreover, γ-secretase and the PS1-CTF are specifically photolabeled with the ATP analog 8-azido-ATP[γ-32P] in reactions containing either purified γ-secretase from cells overexpressing all components of the protease or endogenous γ-secretase in membranes from untransfected HeLa cells. Finally, photolabeling of γ-secretase and PS1-CTF with the ATP analog 8-azido-ATP[γ-32P] was lost in the presence of excess cold ATP, Gleevec extract or ZM39923. Taken together, our data strongly suggest (1) that γ-secretase cleavage of an APP-based substrate (C100Flag), but not a Notch-based substrate (N100Flag), can be regulated in vitro by direct binding of nucleotide to the enzyme, and (2) that binding to this site by certain compounds including kinase inhibitors and/or their derivatives results in the selective inhibition of γ-secretase cleavage of APP vis-à-vis Notch. Intriguingly, binding to this site by ZM39923 or ZM449829 can even stimulate N100Flag cleavage, suggesting that this site allows tunable conformational changes for allosteric control of substrate selectivity.

ATP hydrolysis is likely not to be required for γ-secretase activity, suggesting that this enzyme does not belong to the Walker family of ATPases, a vast and functionally diverse collection of enzymes that includes ATP-dependent proteases (33,34). Supporting this assumption, no conserved Walker consensus sequence motif A (GXXGXXGKT/S) associated with ATPase activity (33,35) is present in PS, NCT, Aph-1 or Pen-2. One hypothetical model to explain the direct ATP effects on purified γ-secretase involves nucleotide binding to a hydrophobic pocket shaped by an integral protein component of the complex, namely PS1-CTF, in a way that effects a conformational change of the enzyme, perhaps promoting the opening of a “trap door” into the catalytic site that allows the substrate to enter and be processed. Other models could be proposed, and precisely how ATP acts on the purified γ-secretase requires further work. Nevertheless, we speculate that nucleotides and the inhibitors have different conformational effects upon binding to this site on the protease complex, in a manner analogous to the differential effects of agonists and antagonists on receptors.

Accumulating data have demonstrated that presenilin/γ-secretase is involved in many crucial physiological functions in virtually all cells. For example, genetic deletion has revealed that presenilin/γ-secretase is essential for regulation of the immune system, for memory and for synaptic plasticity (for a review, see (36)). To chronically prevent the accumulation of Aβ deposits by inhibiting γ-secretase without adversely affecting its other physiological functions, it has been a major goal to identify γ-secretase inhibitors that discriminate between APP and the processing of Notch and other substrates. Weggen et al (37) reported that certain non-steroidal anti-inflammatory drugs (NSAIDs) can subtly alter the γ-secretase-mediated generation of Aβ, selectively lowering the production of Aβ42 over that of Aβ40 without significantly decreasing Notch cleavage. More recently, Netzer et al. reported that Gleevec, an approved Abl kinase inhibitor used to treat chronic myelogenous leukemia, inhibits γ-secretase cleavage of APP without affecting Notch processing (7). Our work shows that Gleevec itself is not a direct γ-secretase inhibitor but supports this key finding in that certain kinase inhibitors (1367 and 1366) can act directly on γ-secretase via a mechanism.
that allows the protease to distinguish between the cleavages of APP and Notch. Gleevec may be metabolized or degraded under physiological conditions, thus leading to the generation of the active compound(s) found in our active Gleevec extract. Purification and characterization of each of the compounds found in our active Gleevec extract will now be necessary to address this question.

Intriguingly, while our manuscript was in preparation, PS2 and PS1 missense mutations causing cases of familial AD that are located within TM domain 5 of PS were reported to reduce production of the AICD fragment of APP without affecting NICD levels (38). This observation suggests that TM5 of both PS1 and PS2 is more important for γ-secretase cleavage of APP than of Notch. Interestingly, the apparently selective effect of these PS mutations on APP processing is mimicked pharmacologically by the Gleevec extract, ZM39923 (1367) and ZM449829 (1366), apparently via direct interaction with the protease complex. One plausible hypothesis is that these FAD mutations affect the conformation (either directly or allosterically) of a domain involved in the regulation of APP but not Notch cleavage. The binding to this domain by the tyrosine kinase inhibitors described in this study could result in a similar modulation of the protease, with selective inhibition of γ-secretase cleavage of APP vis-à-vis Notch. Another plausible but speculative explanation is that these kinase inhibitors may bind to a site on PS that interacts with APP substrates but not with Notch substrates; the binding of these compounds to this site would compete selectively with the binding of C83/C99 and result in the inhibition of γ-secretase cleavage of APP vis-à-vis Notch.

The physiological relevance of nucleotide binding and regulation of γ-secretase activity remains unknown. Nevertheless, whatever the precise biochemical mechanism, our findings are pharmacologically relevant and could have major therapeutic implications. Apparently, the γ-secretase complex can be directly affected in a way that allows selective inhibition of Aβ production without interfering with Notch proteolysis. Thus, the disturbing specter of Notch-associated toxicities previously reported upon in vivo administration of γ-secretase inhibitors may be avoidable, making this protease a much more attractive therapeutic target for the prevention and/or treatment of AD.

REFERENCES


**FOOTNOTES**

* We thank S. Gandy for PS1 antibody Ab14. We are also grateful to J. Sears, J. Strahle, and W. Xia for ELISA analysis, to F. Bihel, A. Kuppanna and E. Spooner for the preparation and analysis of Gleevec extracts and to A. Saci for helpful discussions about protein kinase/phosphatase assays.

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1 Aβ, amyloid β-protein; AD, Alzheimer’s disease; APP, amyloid β-protein precursor; AICD, APP intracellular domain; CHO, Chinese hamster ovary; CTF, C-terminal fragment; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FAD, familial Alzheimer’s disease; GST, glutathione-S-transferase; GSH, glutathione; HMW, high molecular weight; IP, immunoprecipitation; kDa, kiloDalton; NCT, Nicastrin; NTF, N-terminal fragment; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, presenilin; WT, wild-type.

**FIGURE LEGENDS**

**Fig. 1.** Non-hydrolyzed ATP can activate the generation *in vitro* of Aβ40 and Aβ42. *A*, Effect of ATP on purified γ-secretase activity. γ-Secretase diluted in 0.2% CHAPSO-HEPES, pH 7.5, was incubated at 37°C for 4 hr in the presence of 0.1% PC, 0.025% PE, the indicated concentrations of ATP, and 1 µM C100Flag (an APP-based substrate) or 1 µM N100Flag (a Notch-based substrate); both substrates were adjusted to 0.5% SDS prior to addition to the reactions, (see ref. 6). The reactions were Western blotted for AICD-Flag (with M2 anti-Flag antibody) and for NICD-Flag (with Notch Ab1744 antibody). Levels of Aph1-HA serve as equal-loading controls. Aβ40 and Aβ42 were measured by ELISA (B, means +/- SD; n = 3). Levels of AICD-Flag and NICD-Flag were estimated by densitometry (values are single determinations from the blot shown). Asterisks indicate significant differences in Aβ40 (*, p < 0.01) and Aβ42 (**, p < 0.01) production compared to samples without ATP. *C*, ATPase assays on purified γ-secretase. ATP[α32P] was incubated at 37°C for the indicated times in the reaction buffer (0.2% CHAPSO-HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, 0.025% PE and 0.10% PC) alone (lanes 11-15), or in the presence of purified γ-secretase (lanes 6-10), C100Flag substrate (lanes 16-20), or both purified γ-secretase and C100Flag substrate (lanes 21-25). Two µL of each reaction were then analyzed by TLC to separate ATP from ADP. As a positive control to show ATP hydrolysis products, [α32P]-ATP was incubated at the indicated times in the presence of canine kidney phosphatase (lanes 1-5).

**Fig. 2.** Gleevec itself is not a direct γ-secretase inhibitor but a Gleevec extract inhibits the generation of Aβ by purified γ-secretase without affecting the cleavage of a Notch-based substrate. *A*, Effect of III-31C and DAPT on the cleavage by purified γ-secretase of C100Flag and N100Flag. γ-Secretase diluted in 0.2% CHAPSO-HEPES,
pH 7.5, was incubated at 37°C for 4 hr with 1 μM C100Flag or N100Flag substrate, 0.1% PC, 0.025% PE and the indicated concentrations of III-31C and DAPT. The reactions were Western blotted for AICD-Flag (M2 antibody) and NICD-Flag (Ab1744 antibody). Levels of Aph1-HA serve as equal-loading controls. Levels of NCT serve as loading controls.

**Fig. 3.** Nucleotides prevent the inhibitory effect of Gleevec extract on purified γ-secretase. A, ATP, ADP and AMP are direct competitors with respect to the Gleevec extract. γ-Secretase diluted in 0.2% CHAPSO-HEPES, pH 7.5, was incubated at 37°C for 16 hr in the absence (lane 1) or presence (lanes 2-6) of 100 μM Gleevec extract and the indicated concentrations of ATP, ADP or AMP. The generation of AICD-Flag was probed by Western blotting with M2 anti-Flag. Levels of Aph1-HA served as equal-loading controls. Note that the lane 1 control without ATP is the same control for the absence of ADP or AMP. B & C. The effects of increasing concentrations of ATP, ADP and AMP on Aβ40 (B) and Aβ42 (C) generation by purified γ-secretase in the presence of 100 μM Gleevec extract (reactions performed at 37°C for 4 hr) were quantified by ELISA (n = 3).

**Fig. 4.** ZM39923 (1367), a potent Janus kinase 3 inhibitor, preferentially blocks the generation of Aβ by purified γ-secretase. A, Effect of a large number of protein kinase/phosphatase inhibitors or activators on purified γ-secretase activity. γ-Secretase diluted in 0.2% CHAPSO-HEPES, pH 7.5, was incubated at 37°C for 16 hr in the presence of 1 μM C100Flag, 0.1% PC, 0.025% PE, and 100 μM of the indicated compounds, except for III-31C (10 μM). The generation of AICD-Flag was probed by Western blotting with M2 anti-Flag antibody. B, Effect of ZM39923 (1367) and its break-down product ZM449829 (1366) (structures shown) on the cleavage by purified γ-secretase of C100Flag and N100Flag. Activity assays were performed as described above in the presence of the indicated concentrations of 1367 and 1366, and the generation of AICD-Flag and NICD-Flag were probed by Western blotting with M2 and Notch Ab1744 antibody, respectively. In all figures, levels of Aph1-HA are shown as equal-loading controls. C, Similarly, the effect of SC-9 (0433) (structure shown) on the cleavage by purified γ-secretase of C100Flag and N100Flag was probed.

**Fig. 5.** Purified γ-secretase binds specifically to an ATP resin. Purified γ-secretase (St. for starting material) was incubated overnight in the presence or absence of 50 mM ATP with two different ATP-immobilized resins: ATP attached to acrylamide through the γ-phosphate (lanes 1-9) or ATP attached to agarose through the ribose hydroxyls (lanes 10-12). The unbound fractions (Unb.) were recovered, and the resins washed three times in 0.2% CHAPSO-HEPES and resuspended in Laemmli sample buffer to recover the bound proteins (P. for Precipitate). All samples were electrophoresed on 4-20% Tris-glycine gels and transferred to polyvinylidene
difluoride membranes to detect NCT-GST (aGST), PS1-NTF (Ab14), Aph1-HA (3F10), PS1-CTF (13A1), and Flag-Pen2 (M2-anti Flag). Note that purified γ-secretase binds specifically to the resin in which ATP is attached to acrylamide through the γ-phosphate (lanes 1-6), whereas γ-secretase from a crude lysate (of γ-30 cells) is unable to bind to the same resin (lanes 7-9). Starting Material (St.) and unbound (Unb.) lanes were each loaded with the equivalent of 25% of the material that was bound to the resins (P.), so that the unbound and bound protein levels can be compared directly.

Fig. 6. Specific labeling of the γ-secretase component PS1-CTF with [γ32P]-8-azidoATP, a photoaffinity ATP analog. A, Photolabeling of γ-secretase with [γ32P]-8-azidoATP as revealed by non-denaturing BN-PAGE. Purified γ-secretase solubilized in 0.1% Digitonin-TBS was incubated with 22.5 μM of [γ32P]-8-azidoATP (10 μCi per reaction) in the presence of 10 mM ATP (lanes 2 & 5) or 1 mM Gleevec extract (Gleevec E., lanes 3 & 6), exposed to UV light for 5 min and subjected to BN–PAGE analysis as described (31). 32P labeling was assessed by autoradiography (lanes 4-6) (BioMax MS films used with BioMax Transcreen HE, KODAK). As a control for the migration of γ-secretase, the purified complex solubilized in 0.1% Digitonin-TBS (lane 1), in the presence of 10 mM ATP (lane 2) or 1 mM Gleevec extract (lane 3), was exposed to UV light for 5 min, subjected to BN–PAGE analysis on the same gel as above, and probed for the γ-secretase complex using 3F10 antibody to the Aph1-HA component (lanes 1-3). The asterisk denotes non-specific aggregates containing Aph1-HA (P. Fraering et al., unpublished data). HMWC, the high molecular weight γ-secretase complex (see ref. 31).

B, Photolabeling of γ-secretase with [γ32P]-8-azidoATP as revealed by denaturing SDS-PAGE. Purified γ-secretase was incubated with 22.5 μM of [γ32P]-8-azidoATP (10 μCi per reaction) in the absence (lane 1) or presence (lane 2) of PC and PE (lipids), or in the presence of 10 mM ATP (lane 3), 1 mM Gleevec extract (lane 4), 1 μM III-31C (lane 5), 1 μM C100Flag (lane 7), 1 μM N100Flag (lane 8), or 300 μM 1367 (lane 9). As a control for the specificity of photolabeling, purified γ-secretase was also incubated with ATP[γ32P] (without the 8-azido group) (lane 6). All samples were exposed to UV light for 5 min, and the reactions quenched with 1 mM DTT. The samples were diluted and were incubated overnight at 4°C with GSH resin for the affinity precipitation of the NCT-GST component and its associated proteins (see ref. 6). The resins were then washed three times, all precipitated proteins were electrophoresed on 4-20% Tris-glycine gels and the photolabeled proteins autoradiographed as in A. As a control for the mobility of the photolabeled proteins, the same sample as shown in lane 1 was electrophoresed on a 4-20% Tris-glycine gel and Western blotted simultaneously for PS1-CTF and Aph1-HA with 13A11 and 3F10 antibodies, respectively (lane 10). Molecular weight markers are in lane 11. The levels of the Aph1-HA on the gel in B are shown to demonstrate equal protein loading.

C, Photolabeling of endogenous γ-secretase with [γ32P]-8-azidoATP. Membranes from untransfected HeLa cells were incubated with [γ32P]-8-azidoATP (10 μCi per reaction) in the absence (lane 1) or presence (lane 2) of 10 mM ATP and exposed to UV light as described above. The membranes were washed, the proteins solubilized in 1% CHAPSO-HEPES, and γ-secretase co-IPed with anti-PS1-NTF antibodies. The photolabeled proteins were detected as described above and levels of PS1-NTF (Western blotted with MAB1563 antibody) serve as co-IP controls.
FIGURE 1

A

ATP (mM): 0 0.2 0.5 1 5 10

Densitometry:

1.0 1.05 1.3 1.57 1.7 1.98

C100Flag
AlCD-Flag

Aph1-HA

NiCD-Flag

Purified γ-secretase

B

γ-secretase activity (% control)

[ATP] (mM)

0 0.2 0.5 1 5 10

C

γ-secretase: – + – – – +

C100Flag: – – – + +

Phosphatase: + – –

Time (min): 0 5 10 30 60 120 240

AMP

ADP

ATP
FIGURE 2

A

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Purified γ-secretase

B

Graph showing the relative Ap levels (% of control) versus [Gleevec Extract] (µM) for Ap40 and Ap42.
FIGURE 2

C

Gleevec Extract (μM) 0 0 30 100 300 1000

III-31C

C100Flag

AICD-Flag

Aβ

Aph1-HA

NICD-Flag

NICD-Flag (short exp.)

Aph1-HA

Purified γ-secretase

D

Gleevec Extract (μM) 0 0 30 100 300 1000

III-31C

-C100Flag

-AICD-Flag

Aβ

NCT

NICD-Flag

NICD-Flag (short exp.)

NCT

HeLa membrane lysate
FIGURE 2
FIGURE 3

A

Nucleotide (mM): 0 0 0.1 0.5 5
Gleevec Extract (100 μM): - + + + +

ATP | C100-Flag
ADP | AICD-Flag
AMP | AICD-Flag

ATP | Aph1-HA
ADP | Aph1-HA
AMP | Aph1-HA

1 2 3 4 5 6

B

Gleevec Extract (100 μM)

C

Gleevec Extract (100 μM)
FIGURE 6

A

![Image of gel electrophoresis results showing bands labeled with different conditions: ATP, Gleevec E, and [32P]8N3 ATP. Bands are marked with 'Aph1-HA' and 'HMWC'.]

B

![Image of gel electrophoresis results showing bands labeled with different conditions: ATP, Gleevec E, Ill-3TC, C100C, N100C, and 1367. Bands are marked with 'Aph1-HA', 'PS1-CTF', and 'PS1-NTF'.]

C

![Image of gel electrophoresis results showing bands labeled with different conditions: ATP, [32P]8N3 ATP, and [32P]ATP. Bands are marked with 'PS1-CTF'.]
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