BACE1 Cytoplasmic Domain Interacts with the Copper Chaperone for Superoxide Dismutase-1 and Binds Copper*

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The amyloidogenic pathway leading to the production and deposition of Aβ peptides, major constituents of Alzheimer disease senile plaques, is linked to neuronal metal homeostasis. The amyloid precursor protein binds copper and zinc in its extracellular domain, and the Aβ peptides also bind copper, zinc, and iron. The first step in the generation of Aβ is cleavage of amyloid precursor protein by the aspartic protease BACE1. Here we show that BACE1 interacts with CCS (the copper chaperone for superoxide dismutase-1 (SOD1)) through domain I and the proteins co-immunoprecipitate from rat brain extracts. We have also been able to visualize the cotransport of membranous BACE1 and soluble CCS through axons. BACE1 expression reduces the activity of SOD1 in cells consistent with direct competition for available CCS as overexpression of CCS restores SOD1 activity. Finally, we demonstrate that the twenty-four residue C-terminal domain of BACE1 binds a single Cu(I) atom with high affinity through cysteine residues.

A major pathological hallmark of Alzheimer disease is the presence of senile plaques in the brain. The main protein components within the core of plaques are the 40–42-amino acid Aβ peptides derived from the amyloid precursor protein (APP).1 APP is a type-1 membrane-spanning protein, and release of Aβ involves consecutive proteolytic cleavage by β- and γ-secretases. β-Secretase (BACE1) cleaves at the N terminus, and γ-secretase cleaves at the C terminus of the Aβ sequence, respectively. BACE1 is an aspartic protease and, in common with APP, is a type-1 membrane-spanning protein. BACE1 may function as a dimer, whereas γ-secretase comprises a complex of presenilin-1/2, Aph1, Pen2, and Nicastrin (1, 2). Numerous studies have described the neurotoxic properties of Aβ, and increased production and accumulation of Aβ are believed to be significant, possibly major, contributing factors to Alzheimer disease (3). However, the mechanisms that regulate BACE1 and γ-secretase activities and Aβ deposition are not fully understood (3, 4).

In a screen for peptides that may interact with the short cytosolic domain of BACE1, we recovered CCS, the copper delivery protein for copper/zinc superoxide dismutase, SOD1 (5). Using multiple methods we have validated the interaction detected initially in yeast. It has been replicated in vitro using pull-down assays with purified proteins, confirmed to occur in extracts from rat brain via co-immunoprecipitation of endogenous proteins, and most graphically visualized within living neuronal axons via fluorescent tagging. CCS has three well-defined structural domains (6, 7). The N- and C-terminal domains bind copper, whereas the central domain shows homology to SOD1 and is involved in dimerization with SOD1 during the copper transfer reaction (6, 7). Here we also establish that it is the N-terminal copper-binding domain of CCS that interacts with BACE1. On the basis of the recent clarification of the mechanism by which CCS activates SOD1 and the fact that CCS is limiting in cells, we reasoned that binding to BACE1 would reduce the level of CCS available for SOD1 activation (8–10). We have found that overexpression of BACE1 does reduce SOD1 activity in cells consistent with our hypothesis, and moreover, increasing levels of CCS restore SOD1 activity in these cells.

Alterations in copper metabolism are strongly linked to Alzheimer disease, both APP and Aβ bind copper, and copper is present within amyloid plaques (11–16). Metal binding is important for a number of the known properties of Aβ peptides including aggregation and the ability of Aβ peptides to form pores in the membrane and to both generate and clear reactive oxygen species (17–20). Senile plaques are enriched in metals, particularly copper and zinc, and modulating copper availability can influence amyloid pathology in animal models of Alzheimer disease (21, 22). APP has many of the features of a copper transporter, and the Cu(II) binding site at residues 135–155 can promote the reduction of bound Cu(II) (23). Separate lines of transgenic mice overexpressing different mutant forms of human APP all show reduced copper levels in the brain (24–26). Conversely APP knock-out animals have increased copper.
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levels in the brain (27). We also show that the BACE1 cytoplasmic tail peptide binds a single Cu(I) atom with high affinity and specificity through conserved cysteine residues. A number of tissues, notably the pancreas but including the brain, express splice variants of BACE1 that are poorly active or completely inactive but have the same C-terminal domain (28–30). Our findings therefore indicate that BACE1 provides a link between metal homeostasis and oxidative stress in Alzheimer disease, relevant to both full-length BACE1 and the inactive isoforms.

MATERIALS AND METHODS

Construction of Two-hybrid Baits and Two-hybrid Screen—CCS was identified in a two-hybrid screen using a fragment of the C-terminal domain of BACE1 (Arg481–Lys501) as a bait to screen a library constructed with cDNA made from human adult brain in a vector derived from pG4.5 (Clontech). Longer bait sequences tended to autoactivate and were not used further. Screening was performed using standard protocols, and 9.2 × 10^6 colonies were screened, equivalent to approximately three times library coverage.

Cloning, Expression, and Purification of BACE1 Fusion Proteins—Two different C-terminal fragments of BACE1 were cloned in pGEX-4T-1 (Amersham Biosciences) and expressed as GST fusion proteins in BL21(DE3) (Invitrogen). One encoded the peptide Cys478–Lys501 corresponding to the proposed cytoplasmic domain of BACE1 (31). The other encoded the longer sequence Pro475–Lys501. The fusion proteins were expressed untagged or with tandem C-terminal myc-His tags and purified using glutathione-agarose.

Cloning, Expression, and Purification of Recombinant CCS-Fc Fusion Proteins in Mammalian Cells—Oligonucleotide primers were used to amplify the full-length human CCS cDNA and the separate domains I (amino acids 1–79), II (80–241), and III (amino acids 242–274). The PCR products were cloned into signal pGEx-6P-1 (Amersham Biosciences) and expressed as GST fusion proteins with the CD3 signal sequence. The constructs were sequenced and then used to transfect Cos-1 cells. The secreted fusion protein was purified on protein A-agarose (Invitrogen).

GST Pull-down and Immunoprecipitation Assays—GST or GST-BACE1 myc-His-tagged protein (500 ng) bound to 25 µl of packed GST-Sepharose resin were mixed with 10 µl of in vitro translated ^35S-labeled CCS in 160 µl of 10 mHEPES, pH 7.2, 150 mM NaCl, 0.5% Nonidet P-40 and incubated for 2 h at 4 °C with the following additions as indicated: 10 µM TTA final concentration, 2 mM; 25 µM CuCl2, 25 µM CuSO4, and 0.1 mM DTT. The complexes formed were detected by SDS-PAGE and immunoblotting. CCS was detected by its ability to complex with Cu(I) and not for its activity.

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Production of BACE1-GFP Constructs—Plasmid expressing CCS tagged at its N terminus with DsRed was created by cloning of the human CCS cDNA into plasmid pDsRed-C1 (Clontech) as an EcoRI-BglII fragment. To create C-terminal enhanced green fluorescent protein (EGFP)-tagged BACE1, human BACE1 coding sequences in which the stop codon had previously been removed and replaced with a unique HindIII site (32) were cloned as XhoI-HindIII fragments into vector pEGFP-N1 (Clontech).

CCS and BACE Transport in Neurons—Primary cortical neurons were obtained from 18-day-old rat embryos and cultured on glass coverslips coated with poly-β-lysine in six-well plates in neurobasal medium and B27 supplement (Invitrogen) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 5 mM HEPES buffer. The neurons were transfected 6 days after plating using Lipofectamine 2000 transfection reagent (Invitrogen). DNA, 2.5 µg in 60 µl of OpiMEM, was mixed with 2.5 µl of Lipofectamine 2000 in 60 µl of OptiMEM and incubated at room temperature for 20 min. The transfection mix was then added directly to neurons plated onto coverslips and incubated at 37 °C and 5% CO2. The neurons were observed 48 h post-transfection as described previously (33). Briefly, the cells grown on coverslips were viewed in normal medium (neurobasal medium with B27 supplement) stained with Axiovert S100 to assess nuclear morphology and lack of apoptosis. Data were only acquired from healthy cells.

SOD1 Activity Assays—SOD1, CCS, and BACE1 DNA (8 µg) were transfected in CHO cells as indicated; transfections receiving only one or two plasmids were balanced with empty vector such that all received the same total amount of DNA. 24 h after transfection, the plates were washed twice with phosphate-buffered saline, harvested by scraping into phosphate-buffered saline, and then pelleted by centrifugation at 2,000 × g for 5 min at 4 °C. The pelleted cells were resuspended and lysed in water by a freeze-thaw cycle (34) and centrifuged at 10,000 × g for 5 min, and the supernatant was collected. A sample of supernatant from each transfection was analyzed by SDS-PAGE and immunoblotting to determine the expression of SOD1, CCS, and BACE. The remainder was adjusted to 0.125 M Tris chloride, pH 6.8, 20% (v/v) glycerol, 0.025% bromophenol blue, and 0.001% sodium dodecyl sulfate, and then centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was assayed using nitro blue tetrazolium in-gel staining assays as described (35). SOD1 activity assay gels and immunoblots were further analyzed by pixel densitometry using a Bio-Rad GS-710 densitometer and Quantity One software to obtain the ratio of SOD1 activity to BACE1. For each expression condition, this ratio was set at 100% for the SOD1-only transfected cells, and the remaining activities were expressed relative to this. Data from three separate sets of transfections were analyzed together using an analysis of variance.

BACE1 Cytoplasmic Domain Peptides—A synthetic peptide corresponding to the C-terminal region of BACE1 (amino acids Cys475–Lys501) was obtained from California Peptide Research, Inc. (Napa, CA) and checked by mass spectrometry. Peptides in which cysteine residues were changed to alanine were synthesized by the Molecular Tools Group of GlaxoSmithKline and checked by mass spectrometry.

Spectroscopic Methods—A stock solution of CuCl was prepared in an anaerobic chamber by dissolving in concentrated HCl, 0.1 M NaCl, then diluted to 100 mM with 50 mM HEPES, pH 7.4, 150 mM NaCl, and stored in an anaerobic chamber. This was used for serial dilution with 50 mM HEPES, pH 7.4, 150 mM NaCl, immediately prior to each titration. BACE1 cytoplasmic domain peptides were resuspended in 10 mM HEPES, pH 7.4, 150 mM KCl in an anaerobic chamber and transferred to a stoppered quartz cuvette for UV-visible measurements. The concentration of peptides was 15–25 µM for precise values calculated using a theoretical εCu2+ = 5690 M^−1 cm^−1. Evidence of precipitation and base line lifting occurred at additions of Cu(I) above 1.75 molar equivalents. Analogous titrations were performed in the presence of equimolar concentrations of Zn(II) and also (separately) Co(II). Spectra for mutant peptides were obtained in the presence of 0.5 molar equivalents of Cu(I) and also in the presence of 1 molar equivalent of Co(II). To compare Cu(I) affinities with the chelator BCS, 22 µM...
Cu(I) was titrated with BCS in either the presence or absence of an equivalent concentration of peptide, with Cu(I) forming a complex with two molecules of BCS to generate a spectral feature at 483 nm.

**Gel Filtration of Peptide-Metal Complexes—**An aliquot of BACE1 was incubated with a 2-fold molar excess of Cu(I) and a 2-fold molar excess of Zn(II) under anaerobic conditions, and then bound and free metal was resolved by gel filtration on Sephadex G-25, again in the anaerobic N₂ chamber. Fractions (1 ml) were analyzed for peptide and also for metal by atomic absorption spectrophotometry.

**RESULTS**

The Cytoplasmic Domain of BACE1 Interacts with Domain I of Human CCS—We carried out a yeast-two-hybrid screen using the cytoplasmic domain of BACE1 as “bait” and identified an interaction with CCS, the copper chaperone for SOD1. In addition to the conventional selection screens, quantitative β-galactosidase assays (Fig. 1) indicate that this interaction is specific and unlikely to be mediated by a nonspecific metal-dependent interaction. Such an interaction could occur through the MXCXXC motif in CCS and cysteine residues in the BACE1 bait sequence. However, the interaction is relatively specific because HAH1 (Atox1 or Atx1), the copper chaperone that delivers copper to both the Wilson and Menkes disease protein, interacts poorly (Fig. 1) even though it contains a canonical copper-binding motif (M[^15]HENDC) that is very similar to that found in domain I of CCS ([^20]TCQSC). This weak interaction is almost indistinguishable from that found with the empty vector pJG4.5, the Saccharomyces cerevisiae CCS orthologue lys7, and two other unrelated proteins including cysteine-rich CRIPT ([^36]Fig. 1).

CCS has three distinct domains that are clearly defined both functionally and structurally (6–9, 37–39). We therefore used the two-hybrid system to provide an initial indication of which domain of CCS interacts with BACE1. Quantitative β-galactosidase assays show a clear interaction with domain I but not with either of the other domains (Fig. 1). We went on to confirm this interaction in vitro using a range of methods and most importantly, in vivo in a physiologically relevant tissue. In vitro we used a pull-down approach with a GST-BACE1 fusion protein to capture radiolabeled CCS produced by in vitro transcription-translation. One fusion protein carried BACE1 amino acids Cys[^478]–Lys[^501] corresponding to the proposed cytoplasmic domain of BACE1 ([^31]). We also used a fusion protein carrying BACE1 amino acids Pro[^572]–Lys[^501] (PLCLMVCQWRCLRQLQQHDDFADDISLLK) that includes an additional cysteine residue thought to lie in the transmembrane domain but that we have shown to be palmitoylated and hence potentially exposed in the cytoplasm (data not shown). Both fusion proteins gave the same results with clear binding of CCS to GST-BACE1 detected but not to GST alone (Fig. 2A). Under these conditions the addition of copper salts to the binding reaction may have a subtle effect on the amount of protein bound (Fig. 2A), but generally the binding observed was not affected by the presence of excess copper or zinc or the metal chelators EDTA, bathocuproine disulfonate, and clioquinol. This supports the interaction from the two-hybrid screen that the interaction is not mediated via metal binding. This was confirmed using a GST-BACE1 fusion protein in which the cysteine residues were changed to alanine. The binding of this fusion protein to CCS was indistinguishable from the binding of the unmodified GST-BACE1 (Fig. 2B).

We have used a different configuration of the binding assay to eliminate possible nonspecific binding through the carrier protein and to confirm the two-hybrid identification of the subdomain of CCS, which interacts with BACE1. Using an Fc-CCS fusion protein bound to protein A and soluble GST-BACE1, we detected clear binding of CCS to BACE1 (Fig. 2C). The three separate domains of CCS are defined structurally and functionally. Domain I is a copper-binding domain that transfers a bound copper atom to domain III. CCS docks with SOD1 by dimerization through domain II, and the bound copper atom is transferred from domain III of CCS to the bound SOD1 (6–9, 37–39). We expressed each CCS domain as individual Fc fusion proteins and evaluated binding of each of these proteins to GST-BACE1. The results (Fig. 2C) indicate a clear, specific interaction between domain I of CCS and GST-BACE1.
BACE1 and CCS Can Be Co-immunoprecipitated from Rat Brain—Although the data presented above show specific binding between CCS and the cytoplasmic domain of BACE1, it is essential to demonstrate that endogenous CCS and BACE1 interact in vivo. To test this we immunoprecipitated endogenous CCS from rat brain and probed for the presence of endogenous BACE1 by immunoblotting. We found that BACE1 was present in the immunoprecipitate and migrated as both monomer and dimer in SDS-PAGE (Fig. 2D). BACE1 has recently been reported to occur as a dimer in brain, and it has been suggested that this is the active form of the enzyme (40, 41).

BACE1 and CCS Are Co-transported through Axons—BACE1 is a type 1 integral membrane protein that is located in the endoplasmic reticulum, Golgi, and plasma membrane (1). CCS is a cytosolic protein found in cell bodies, dendrites, and axons in neurons (10). BACE1 is transported through axons to the synapse, and there is evidence that BACE1 cleavage of APP may occur during this axonal transport (42–44). Axonal transport of CCS has not been properly investigated, but SOD1 is known to undergo axonal transport (45). We therefore examined whether BACE1 and CCS are transported together by monitoring movement of GFP-tagged BACE1 and DsRed-tagged CCS in axons of co-transfected living rat cortical neurons. These studies revealed that a proportion of BACE1 and CCS are co-transported during axonal transport in these co-transfected neurons (Fig. 3 and supplemental material). It should be noted that signal overlap is not precise because there is a delay of almost 2 s in image collection from each channel because of the motorized switching of the filters for GFP and DsRed and image capture (Fig. 3). This can be seen clearly in the Quicktime movie (supplemental material) where DsRed-CCS moves first, followed by GFP-BACE1. Confocal images showing precise coincidence of the signals are presented in the supplemental material. At the 5–10-s time points the labeled proteins are moving at the maximal rate of ~1.6 microns/s. Hence the apparent separation of the images at this time point is greatest, and the signals appear as smears rather than discrete points. At the lower movement rates seen at the later time points, the separation is less. Moving GFP-BACE1 and DsRed-CCS particles were transported in both anterograde and retrograde directions. Pausing in the movement of GFP-BACE1 and DsRed-CCS was also observed. The net speeds of movement (excluding any pauses) in the anterograde direction were between 0.07 and 0.69 m/s (average, 0.2 m/s); movements in the retrograde direction were between 0.07 and 1.37 m/s (average, 0.22 m/s). These movement characteristics correspond to those generated by conventional kinesin and dynein motor protein families.

Overexpression of BACE1 Reduces SOD1 Activity, Which Can Be Restored by CCS—Two recent reports describe an essential role for CCS in the activation of SOD1 in response to increases in oxygen tension and in the formation of the intramolecular disulfide bond, which is required to generate the mature, active enzyme (8, 9). Because SOD1 is present in considerable excess over CCS in cells (10), we reasoned that as BACE1 binds CCS, it would compete with SOD1 for the limited pool of CCS, leading to reduced levels of active SOD1 enzyme. To test this we transfected the corresponding cDNA constructs into CHO cells, prepared cell lysates, and measured protein expression and SOD1 activity using the conventional native gel assay (34). We found clear down-regulation of SOD1 activity when BACE1 is overexpressed, and this reduction was fully restored by increasing the levels of CCS (Fig. 4).

A Cytoplasmic Domain BACE1 Peptide Binds a Single Cu(I) Atom with High Affinity—The cytoplasmic domain of BACE1 contains a number of sequence motifs including a di-leucine

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**Fig. 2.** BACE1 binds CCS in vitro and in vivo. A, SDS-PAGE analysis of purified GST and GST-BACE1 fusion proteins, 35S-labeled input CCS, and specific binding of 35S-labeled CCS to GST-BACE1 fusion protein. B, SDS-PAGE analysis of purified GST and a GST-BACE1 C-A fusion protein in which the cysteine residues have been changed to alanine and specific binding of 35S-labeled CCS to GST-BACE1 C-A fusion protein. C, binding of GST-BACE1 to full-length CCS-Fc fusion protein and CCS-Fc fusion proteins containing the separate domains of CCS. D, co-immunoprecipitation of BACE1 with CCS. CCS from rat brain was immunoprecipitated by using a rabbit polyclonal CCS antibody. The immunoprecipitates were analyzed by SDS-PAGE and probed for the presence of BACE1 and CCS using antibodies 9B21 and G19, respectively. + and − refer to absence or presence of antibody.
endosomal retrieval motif, an adjacent phosphorylation site, plus a cluster of cysteine residues that have been shown to be palmitoylated (46–48). We noted that this cysteine-rich sequence includes the putative metal-binding motif Cys-Xaa-Xaa-Cys, one additional Cys plus other potential metal-ligand residues. To establish whether this domain could bind copper, we used UV-visible absorption spectroscopy under anaerobic conditions to detect saturable Cu(I) binding to a synthetic 24-amino acid peptide corresponding to amino acids Cys478–Lys501 of BACE1. The synthetic 30-amino acid peptide corresponding to amino acids Pro472–Lys501 was insufficiently soluble to be used in these experiments.

A Cu(I) titration revealed Cu(I)-dependent increases in absorption below 400 nm extending into the UV with a pronounced shoulder around 265 nm and a less prominent feature around 292 nm (Fig. 5A). The shoulder at 265 nm is diagnostic for thiolate to Cu(I) (ligand-to-metal) charge transfer and clearly saturates at one molar equivalent of Cu(I). Linearity indicates an affinity too tight to measure with these volumes and protein concentrations; $k_{app}$ was tighter than $10^{-11}$ M (Fig. 5B). The shoulder at 292 nm continues to increase beyond one molar equivalent of Cu(I) with a slight discontinuity in the fit at around one equivalent (Fig. 5C), suggesting that further Cu(I) atom(s) may enter a chemically distinct site(s), but no more cysteinyl-thiols are recruited. This additional binding could involve conversion of terminal thiols into bridging thiols and/or intermolecular liganding.

Competition for copper between protein ligands and bathocuproine disulfonate has been used to determine copper binding affinities in a number of proteins. For example, an average dissociation constant of $10^{-19}$ M was estimated for Cu(I) binding to the cytosolic region of the copper importer Ctr1 (49). Our data suggest that BACE1 has a similar high affinity for Cu(I) (Fig. 5, D–F).

Identification of BACE1 Ligands for Cu(I)—More than one BACE1 cysteine residue coordinates Cu(I) because the molar absorptivity at 265 nm ($\varepsilon = 8.9 \times 10^3$ M$^{-1}$ cm$^{-1}$; Fig. 5A) exceeds all reported values for single thiolate-Cu(I) bonds (50, 51). To identify the cysteine residues involved in metal binding, we prepared a series of peptides in which the three cysteine residues, corresponding to amino acids 478, 482, and 485 in the primary sequence, were changed to alanine individually or in combination (Fig. 6A).

The difference spectra of single cysteine mutants at 0.5 equivalent of Cu(I) are consistent with a key role for the central cysteine (residue 482) in Cu(I) binding with a significant reduction in the feature at 265 nm in peptide CAC but relatively little change in this feature in the peptides ACC and CCA (Fig. 6B). In the peptides in which pairs of cysteines are changed to alanine (sequences AAC, ACA, and CAA), the reduction in absorption at 265 nm is similar to that seen in the CAC peptide consistent with a major role for cysteine 482. However, the ACA peptide alone shows an enhanced feature that is red-
BACE1 binds Cu(I) more tightly than Zn(II)—A Cu(I) titration of BACE1 in the presence or absence of Zn(II) gave similar intensities, implying that spectroscopically silent zine is not significantly inhibiting Cu(I) binding (Fig. 7A). Preferential binding of Cu(I) was confirmed by gel filtration after mixing BACE1 with equal concentrations of Cu(I) and Zn(II) under anaerobic conditions. The profile shows co-elution of peptide and Cu(I) with a stoichiometry of one tightly bound Cu(I) atom/BACE1 with no co-migrating Zn(II) (Fig. 7B). Unbound Cu(I) and Zn(II) migrate as free ions (Fig. 7B, peaks II and III). These data imply an affinity for Cu(I) that is at least an order of magnitude tighter than for Zn(II).

Co(II) is often used as a spectral probe for Zn(II)-binding sites. A Co(II)-difference spectrum shows two features (Fig. 7C). The first feature is absorbance below 400 nm, which is due to sulfur to metal charge transfer; isothermal titration (Fig. 7D) indicates that this feature at 370 nm is saturable with a maximal molar absorptivity of 1929 M\(^{-1}\) cm\(^{-1}\). This implies that at least one of the three Cys is used in Co(II) and probably Zn(II) binding. The second feature is a broad absorbance in the region of 650–750 nm caused by d-d transitions with an extinction coefficient of 401 M\(^{-1}\) cm\(^{-1}\) at 680 nm, suggesting a pseudotetrahedral site. For a tetrahedral or pseudotetrahedral (four ligand) site, the extinction coefficient should be in the region of 300–500 M\(^{-1}\) cm\(^{-1}\).

To resolve this from other components of the spectrum, two difference-difference spectra were generated. Subtraction of the spectrum for the ACC mutant from that of the wild type peptide reveals a theoretical contribution (Fig. 6D, spectrum I) of a single Cu(I)-thiolate bond. Further subtraction of this difference-difference spectrum I, from the spectrum obtained for the ACA mutant, reveals a residual putative non-thiol component (spectrum II). The origin of this feature is unknown, although “weak” absorbance in the region 260–360 nm can arise from metal-to-metal charge transfer in clusters, providing the distance between copper atoms is relatively short. Unexpectedly, the spectra show some evidence of copper binding to the triple cysteine mutant (AAA); although sulfur ligands are clearly lost, some absorbance at 292 nm is retained (Fig. 6C). Additional candidate ligands for this binding are histidine and aspartic acid residues. We conclude that the high affinity Cu(I) site in BACE1 involves Cys\(^{452}\) in conjunction with one of the two flanking cysteines and a third non-thiol ligand.

**BACE1 Binds Cu(I) More Tightly than Zn(II)**—A Cu(I) titration of BACE1 in the presence or absence of Zn(II) gave similar intensities, implying that spectroscopically silent zine is not significantly inhibiting Cu(I) binding (Fig. 7A). Preferential binding of Cu(I) was confirmed by gel filtration after mixing BACE1 with equal concentrations of Cu(I) and Zn(II) under anaerobic conditions. The profile shows co-elution of peptide and Cu(I) with a stoichiometry of one tightly bound Cu(I) atom/BACE1 with no co-migrating Zn(II) (Fig. 7B). Unbound Cu(I) and Zn(II) migrate as free ions (Fig. 7B, peaks II and III). These data imply an affinity for Cu(I) that is at least an order of magnitude tighter than for Zn(II).

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If the site were octahedral (six ligands), a much lower extinction coefficient would be predicted. For all three single amino acid changes, the Co(II) spectra (Fig. 7E) show a reduced shoulder around 370 nm, reflective of Co(II)-thiolate binding, implying a contribution from all three cysteines.

**FIG. 5.** BACE1 cytoplasmic domain peptide binds a single Cu(I) ion with high affinity through more than one thiolate-ligand. A, apo-subtracted difference UV-visible absorption spectra of BACE1 cytoplasmic domain peptide titrated with increasing amounts of Cu(I) under anaerobic conditions. B and C, Cu(I) binding isotherms generated from the optical spectra at 265 (B) and 292 nm (C). D, BCS titration. 22 μM Cu(I) was titrated against 44, 88, 132, and 176 μM BCS. Saturation occurs with the first addition of BCS. E, an identical titration to that in D but in the presence of 22 μM BACE1 peptide. BACE1 competes with BCS, implying affinities of similar orders of magnitude. Inset F compares the absorbance at 483 nm from two analogous titrations in the absence (closed circles) or presence (open circles) of BACE1.

**FIG. 6.** Identification of cysteine ligands for Cu(I) binding in BACE1 cytoplasmic domain peptide. A, sequences of peptides in which cysteine residues are changed to alanine. B, apo-subtracted difference spectra of BACE1 single residue mutant peptides in the presence of 0.5 molar equivalents of Cu(I). C, apo-subtracted difference spectra of BACE1 double and triple residue mutant peptides in the presence of 0.5 molar equivalents of Cu(I). D, difference-difference spectra subtracting the difference spectrum for peptide CAC from wild type (I) to obtain a “pure” Cu(I)-thiol spectrum and subtracting spectrum I from the difference spectrum for peptide ACA to obtain a pure non-thiol spectrum (II).
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DISCUSSION

Our results identify two novel features of BACE1, the rate-limiting enzyme in the production of Aβ. First, we show that the enzyme interacts with CCS, the interaction being mediated by the C-terminal cytoplasmic domain of BACE1 and domain I of CCS. This interaction is sufficiently stable to allow co-immunoprecipitation from rat brain and for the proteins to remain co-localized during axonal transport and hence apparently distinct from the transient interaction expected of a copper donation event (39). We have already directly measured one physiological consequence of this interaction by overexpressing BACE1 in cells and monitoring SOD1 activity. We have found that under these conditions SOD1 activity is reduced consistent with a model in which BACE1 competes with SOD1 for the limiting pool of CCS molecules. Second, we also show that a peptide encompassing the BACE1 cytoplasmic domain binds a single Cu(I) atom with high affinity, greater than that for Zn(II), by ligands that include the C-terminal cysteine residues.

The bulk of BACE1 protein is located in the trans-Golgi network, whereas a small proportion resides at the cell surface and in the endosomal compartments (1). The cytoplasmic domain of BACE1 has been shown to be important in trafficking in the cell and to interact with the VPS-27, Hrs, and domain of the GGA (Golgi-localized γ-adaptin-containing AFR binding) proteins (52, 53). The di-leucine motif has been shown clearly to act as an endosomal targeting signal, and its removal or mutation results in more of the enzyme residing at the endosomal compartments (26). Using a yeast model, it has recently been noted that clioquinol can facilitate cross-linking of the peptide and the generation of H2O2 and reactive hydroxyl radicals; these may then induce oxidative damage to proteins, lipids, and nucleic acids. Binding of copper to Aβ may therefore be a mediator of Aβ toxicity (11–16). Finally, the APP gene harbors a copper response element within its promoter such that depletion of copper leads to a marked decrease in APP expression (59).

Such findings have promoted investigations into the effects that modulating copper levels have on the deposition of Aβ within the brain. The copper chelator clioquinol inhibits Aβ accumulation and deposition in APP transgenic mice, and trace amounts of copper in drinking water induces Aβ deposition in a rabbit model of Alzheimer disease (21, 22). However, other studies have provided differing results on the effects of modulating copper availability. Dietary copper supplements reduce Aβ production in APP transgenic mice (24), and crossing of APP mice with mice bearing a mutant copper transporter that limits enzyme Cu,Zn-SOD1γ expression. The proposed mechanism of activation of SOD1 by CCS involves the docking of copper-bound CCS with a molecule of reduced SOD1 carrying a single bound zinc atom. In the presence of oxygen, this noncovalently linked dimer is converted into a disulfide-linked heterodimer (8, 9). It is proposed that this heterodimer undergoes disulfide exchange to form an intramolecular disulfide in SOD1 generating the active enzyme Cu,Zn-SOD1(8,8), and this provides oxygen-dependent post-translational control of SOD1 activation (8, 9). Cellular levels of SOD1 are ~15-fold higher than CCS levels, and in the human and rodent brains CCS is expressed in those regions where BACE1 is expressed (10). Hence it is possible that CCS binding to BACE1 might simply reduce the availability of CCS for SOD1 activation by direct competition. Alternately, binding of CCS by BACE1 could influence copper loading onto SOD1 and interfere directly with the activation mechanism. Indeed binding of the adaptor protein X11α to domain III of CCS likewise negatively regulates SOD1 activity (58). It is even possible that SOD1 and BACE1 compete for binding to CCS and that X11α regulates this process. Future studies will address these issues.

As summarized in the introduction, defective copper metabolism is implicated in Alzheimer disease; APP and Aβ both bind copper, and copper is present within amyloid plaques (11–14). There is also evidence that the binding of copper to Aβ promotes cross-linking of the peptide and the generation of H2O2 and reactive hydroxyl radicals; these may then induce oxidative damage to proteins, lipids, and nucleic acids. Binding of copper to Aβ may therefore be a mediator of Aβ toxicity (11–16). Finally, the APP gene harbors a copper response element within its promoter such that depletion of copper leads to a marked decrease in APP expression (59).

We have demonstrated that BACE1 overexpression in cells leads to a decrease in SOD1 activity in extracts made from those cells. Activity could be restored in these cells by increasing CCS expression. The proposed mechanism of activation of SOD1 by CCS involves the docking of copper-bound CCS with a molecule of reduced SOD1 carrying a single bound zinc atom. In the presence of oxygen, this noncovalently linked dimer is converted into a disulfide-linked heterodimer (8, 9). It is proposed that this heterodimer undergoes disulfide exchange to form an intramolecular disulfide in SOD1 generating the active enzyme Cu,Zn-SOD1(8,8), and this provides oxygen-dependent post-translational control of SOD1 activation (8, 9). Cellular levels of SOD1 are ~15-fold higher than CCS levels, and in the human and rodent brains CCS is expressed in those regions where BACE1 is expressed (10). Hence it is possible that CCS binding to BACE1 might simply reduce the availability of CCS for SOD1 activation by direct competition. Alternately, binding of CCS by BACE1 could influence copper loading onto SOD1 and interfere directly with the activation mechanism. Indeed binding of the adaptor protein X11α to domain III of CCS likewise negatively regulates SOD1 activity (58). It is even possible that SOD1 and BACE1 compete for binding to CCS and that X11α regulates this process. Future studies will address these issues.

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Our observations suggest mechanisms by which Cu(I) binding to the cytoplasmic domain of BACE1 could modulate APP processing and Aβ release. This could be brought about by copper-dependent alterations in BACE1 activity, dimerization, trafficking, or association with APP in the membrane. However, association with CCS and copper binding would also be properties of the inactive BACE1 isoforms. This suggests other, nonproteolytic roles for BACE1, linked to metal homeostasis and the clearance of oxygen radicals. Further studies are urgently required to investigate these novel aspects of BACE1 biochemistry.
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