Phosphorylation of Amyloid-β Peptide at Serine 8 Attenuates Its Clearance via Insulin-degrading and Angiotensin-converting Enzymes

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Background: Amyloid-β peptide (Aβ) is degraded by different proteases. We recently demonstrated phosphorylation of Aβ.

Results: Phosphorylation of Aβ decreases its clearance by microglial BV-2 cells and selectively inhibits the cleavage by insulin-degrading and angiotensin-converting enzymes.

Conclusion: Phosphorylation at Ser-8 negatively regulates Aβ degradation.

Significance: Phosphorylation could play a dual role in Aβ metabolism. It decreases the clearance by microglial cells and also promotes Aβ aggregation.

Alzheimer disease (AD)3 is characterized by the progressive deposition of amyloid-β peptides (Aβ) in the brain (1, 2). Aβ derives from proteolytic processing of the amyloid precursor protein involving sequential cleavages by enzymes called β- and γ-secretases (3, 4). A critical role of Aβ in the pathogenesis of AD is strongly supported by several mutations within the genes encoding the amyloid precursor protein itself or the two presenilins that represent the proteolytically active components of the γ-secretase complex. All of these mutations affect the production and/or aggregation of Aβ and cause early-onset forms of familial AD (5–7). Although early-onset familial AD appears to be commonly associated with an elevated production of aggregation-prone Aβ variants, it remains unclear whether increased Aβ generation also contributes to the much more common form of late-onset AD. Recent evidence rather indicated a decreased clearance rate of Aβ in AD compared with control brains (8–10).

Several mechanisms for Aβ clearance have been identified, including drainage via the blood-brain barrier (11, 12), internalization of Aβ by phagocytosis and pinocytosis (13–15), and degradation by cell surface-localized or secreted peptidases (16, 17). Major proteases in the degradation of extracellular Aβ are the insulin-degrading enzyme (IDE) and neprilysin (NEP) (18–20), but other proteases, including the angiotensin-converting enzyme (ACE), endothelin-converting enzymes, and plasmin, could also contribute to efficient clearance of Aβ in the brain (21–23).

IDE is localized principally in the cytosol but is also released from cells and found in extracellular fluids and conditioned media of cultured cells (24, 25). However, IDE lacks canonical signal sequences that target the enzyme to the conventional secretory pathway (26). Recent data demonstrated that IDE is secreted via a nonconventional pathway in association with exosomes (27, 28). This nonconventional secretion of IDE is dependent on a hexapeptide amino acid motif in its C-terminal converting enzyme; pAβ, phosphorylated Aβ; npAβ, non-phosphorylated Aβ; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethy]glycine.
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domain (29). The pathophysiological relevance of IDE is demonstrated by the deletion of IDE in mice that showed decreased Aβ degradation and increased cerebral Aβ accumulation (30–32). Conversely, enhancement of IDE activity in neurons effectively reduced Aβ accumulation in AD mouse models (30, 33). Recent data demonstrated that extracellular Aβ could undergo phosphorylation by secreted variants of protein kinase A and that the phosphorylation of Aβ at Ser-8 strongly promoted its aggregation into oligomeric and fibrillar assemblies (34).

Here, we sought to assess the effect of phosphorylation on the clearance of Aβ by microglial BV-2 cells. Our data demonstrate that phosphorylated Aβ (pAβ) has increased stability against microglial degradation compared with non-phosphorylated Aβ (npAβ). Interestingly, phosphorylation significantly decreases its proteolytic degradation by secreted IDE.

EXPERIMENTAL PROCEDURES

Reagents—Synthetic npAβ(1–40) (npAβ) and pAβ(1–40) (pAβ) peptides were purchased from Peptide Specialty Laboratories. Recombinant human IDE, ACE, NEP, and purified human plasmin were procured from R&D Systems. Acetonitrile and α-cyano-4-hydroxycinnamic acid were from Sigma. The SilverQuest silver staining kit and precast 4–12% NuPAGE BisTris minigels were from Invitrogen. Precast 16% Tricine gels were from Anamed. The ZipTip (C18) pipette tips used for mass spectrometric analysis were from Millipore. Primary and secondary antibodies were obtained from the indicated suppliers: anti-Aβ primary antibody 82E1 (IBL Corp.), anti-IDE primary antibody (Abcam), and anti-mouse and anti-rabbit secondary antibodies (Sigma).

Aβ Degradation Assays with BV-2 Cells or Recombinant Enzymes—Synthetic npAβ and pAβ were solubilized in 10 mM NaOH at a concentration of 1 mg/ml (230 μM), sonicated for 5 min, and stored at −80 °C until used. Aliquots were thawed and diluted in the appropriate buffer.

For the cellular degradation assays, BV-2 cells were cultured in Dulbecco’s modified Eagle’s medium with GlutaMAX (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen) in a 24-well plate to 70% confluency. Media were replaced with serum-free media and incubated with 22.5 nM siRNA (target sequence GCCTGTGTGCA-GAAGCTCA) using HiPerFect transfection reagent (Qiagen) according to the supplier’s instructions. Knockdown of IDE was analyzed after 24 h in cell supernatants and cell lysates by Western immunoblotting. For Aβ degradation experiments, cells were then incubated for 12 h in serum-free medium. Synthetic npAβ and pAβ variants were added to conditioned media, and aliquots were taken at the indicated time points. Aβ was then detected by Western immunoblotting.

RESULTS

Recent data demonstrated phosphorylation of extracellular Aβ at Ser-8 by secreted or cell surface-localized variants of PKA (Fig. 1A) (34, 35). To test whether this phosphorylation affects the clearance of Aβ by microglia, mouse microglial BV-2 cells were incubated with synthetic pAβ or npAβ, and stability was assessed by detection of the peptides after different time periods of incubation by Western blotting (Fig. 1B). Consistent with previous data (28, 36), extracellular monomeric Aβ was efficiently cleared in the conditioned media of BV-2 cells. About 80% of npAβ was cleared within the first hour of incubation. In contrast, only ~5% of pAβ was cleared after the first hour. Even after 6 h, ~50% of pAβ was detected in the cell supernatant, a time point at which only residual amounts (~10%) of npAβ were left (Fig. 1C). The calculated half-life times of npAβ and pAβ in these experiments were 45 min and 6 h, respectively. These data demonstrate that phosphorylation of Aβ strongly decreases the clearance of extracellular Aβ by microglial cells.

As shown previously (24, 28, 36), microglial BV-2 cells efficiently degrade extracellular monomeric Aβ by secreted IDE. To specifically analyze the effect of Aβ phosphorylation on IDE-mediated cleavage, we next performed in vitro experiments using recombinant IDE. Synthetic pAβ or npAβ was incubated with IDE, and time-dependent cleavage was examined by SDS-PAGE and subsequent silver staining or by MALDI-TOF-MS (Fig. 2, A–C). Although the amount of npAβ gradually decreased during the incubation, pAβ appeared to be very stable under these conditions (Fig. 2, A and B). MALDI-TOF-MS analysis demonstrated that, at the start of incubation, only full-length Aβ variants were detected (Fig. 2C). Already after 5 and 15 min, an additional peak was detected in the samples with npAβ, demonstrating cleavage of Aβ by IDE. The m/z ratio of this peak corresponds to Aβ(1–14), indicating cleavage.
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**FIGURE 1.** Decreased clearance of pAβ by microglial BV-2 cells. A, primary amino acid sequence of human Aβ40/42 with the phosphorylation site at Ser-8, indicated in boldface. B, BV-2 cells were incubated with synthetic npAβ or pAβ (1 μM), and aliquots of the conditioned media were taken at the indicated time points. Samples were separated by SDS-PAGE, and Aβ was detected by Western immunoblotting. C, quantification of Western blots in B was performed by densitometric analysis. Aβ levels at time 0 were set as 100%. Values represent means ± S.D. of three independent experiments (n = 3). ***,, p < 0.001 (t test).

between amino acids 14 and 15. This peak increases over time and was the predominant peak detected after 30 and 60 min of incubation. However, some additional peptides were detected that represent minor degradation products of less efficient cleavages between amino acids 13 and 14, 15 and 16, 18 and 19, 19 and 20, 20 and 21, and 28 and 29, corresponding to Aβ cleavages between amino acids 13 and 14, 15 and 16, 18 and 19, 19 and 20, 20 and 21, and 28 and 29, respectively. These data indicate that phosphorylation of Aβ does not completely block IDE-mediated degradation. However, it is important to note that the efficiency of cleavage by IDE is reduced in the presence of phosphorylated residues.

DISCUSSION

This study revealed a novel mechanism in the regulation of Aβ metabolism. The phosphorylation at Ser-8 inhibits the degradation by IDE, a major Aβ-degrading enzyme secreted by microglial BV-2 cells.

Increased concentrations of Aβ favor its aggregation and deposition in the form of β-amyloid plaques in the human brain. This is well supported by rare mutations in amyloid precursor protein and the presenilin proteins that cause autosomal dominant early-onset AD and commonly

**FIGURE 2.** Decreased clearance of pAβ by microglial BV-2 cells. A, primary amino acid sequence of human Aβ40/42 with the phosphorylation site at Ser-8, indicated in boldface. B, BV-2 cells were incubated with synthetic npAβ or pAβ (1 μM), and aliquots of the conditioned media were taken at the indicated time points. Samples were separated by SDS-PAGE, and Aβ was detected by Western immunoblotting. C, quantification of Western blots in B was performed by densitometric analysis. Aβ levels at time 0 were set as 100%. Values represent means ± S.D. of three independent experiments (n = 3). ***,, p < 0.001 (t test).

In addition, we also carried out similar experiments with human cerebrospinal fluid. At similar concentrations of IDE, the degradation of Aβ was lower in cerebrospinal fluid samples compared with the IDE assay buffer. These data indicate that cerebrospinal fluid contains proteins or other factors that negatively affect the degradation of Aβ. However, the inhibitory effect of phosphorylation on Aβ degradation was still evident (supplemental Fig. 1).

Recently, we demonstrated that IDE is the main protease contributing to Aβ degradation in BV-2 cells (28). Consistent with these results, siRNA-mediated knockdown of IDE strongly reduced the expression of cellular as well as secreted IDE in control cells. After knockdown, IDE levels were strongly decreased in the conditioned medium and cell lysates by ~75% (Fig. 4, A and B). Importantly, siRNA-mediated knockdown of IDE in these cells decreased the degradation of Aβ also by ~75% (Fig. 4, C and D). Consistent with the previous results, the degradation of pAβ was also lower compared with npAβ upon siRNA-mediated knockdown of IDE. Together, these data strongly indicate that secreted IDE is the major protease in the degradation of extracellular Aβ in this cell system.

Because other proteases also can contribute to the degradation of Aβ in other cell systems or in vivo, we further tested the effect of phosphorylation on Aβ degradation by known Aβ-degrading enzymes, including ACE, NEP, and plasmin. Although NEP showed comparably low Aβ-degrading activity under the experimental conditions and even at higher enzyme concentrations (supplemental Fig. 2), plasmin and ACE efficiently degraded the synthetic Aβ variants. The main cleavage products generated by plasmin had m/z ratios of ~3265 and ~1958, representing Aβ(1–28), and Aβ(1–16), respectively (Fig. 5, A–C, and Table 1). However, phosphorylation had little (if any) inhibitory effect on plasmin-mediated degradation of Aβ, which was also indicated by the similar appearance of phosphorylated degradation products (Fig. 5C). In contrast, the degradation of pAβ by ACE was strongly decreased compared with that of npAβ (Fig. 6, A–C). Interestingly, cleavage of Aβ by ACE occurred directly N- or C-terminal of Ser-8, either between Asp-7 and Ser-8 or between Ser-8 and Gly-9, as indicated by the m/z ratios of C-terminal cleavage products of ~3451 (Aβ(8–40)) and ~3370 (Aβ(9–40)).
increase the production of Aβ and/or its aggregation (37). However, mechanisms that alter the metabolism of Aβ in the pathogenesis of the much more common late-onset form of AD are largely unclear. Interestingly, recent data indicated a decreased rate of Aβ clearance in the AD brain rather than increased production (8, 9). The half-life time of Aβ is 8–12 h in human cerebrospinal fluid and 3–4 h in the interstitial fluid of mouse brain, indicating efficient clearance mechanisms that counteract the production of Aβ (9, 10, 38). The clearance of Aβ from the brain involves internalization via pinocytosis or receptor-mediated endocytosis/phagocytosis (13–15, 39) and subsequent degradation in the endosomal/lysosomal compartments (40), transcytosis and drainage via the blood-brain barrier to the vasculature (12, 41), and proteolytic degradation of extracellular Aβ by cell surface-localized and secreted proteases (21, 28, 36).

Microglial cells could contribute to the clearance of Aβ, as they are the main phagocytes in the brain and also express several proteases at the cell surface that could degrade extracellular Aβ (27). Recent evidence demonstrated that microglia secrete substantial amounts of IDE, thereby allowing efficient degradation of monomeric Aβ variants also at some distance from the microglial cell (19, 42). IDE is a zinc metalloprotease with broad substrate specificity that is involved in the degradation of several peptides, including insulin, glucagon, transforming growth factor, and...
other peptide hormones (43). The different peptide substrates have little (if any) sequence homology, but many of the substrates share a propensity to form a \( \beta \)-sheet-rich conformation under certain conditions (e.g., insulin, glucagon, amylin, atrial natriuretic factor, and calcitonin) (44–47). Accordingly, substrate selection of IDE might be determined mainly by the size and secondary and tertiary conformation of the peptides (46). Co-crystallization indicated that, after being engulfed into the catalytic center of IDE, \( \beta \) could undergo conformational changes that might be important for the subsequent hydrolysis reaction (48). We recently showed that the phosphorylation of \( \beta \) affects its conformation (34). Thus, the conformational change induced by phosphorylation might decrease the efficiency of hydrolysis by IDE. The introduction of a negatively charged phosphoryl group could also directly impair enzyme-substrate interaction. Similar effects of phosphorylation have been shown previously for caspase-mediated processing of

### TABLE 1
MALDI-TOF-MS analysis of cleavage products of np\( \beta \) and p\( \beta \)

Shown are the various peptide fragments generated upon cleavage of np\( \beta \) and p\( \beta \) variants by different proteases and their calculated mass (Da) and observed mass (Da) by MALDI-TOF. The double-ionized full-length peptide \((m/z)\) and the double-ionized full-length peptide \((1–40^\circ)\) of both peptide variants are the mean values \((n = 10)\). The difference between the observed mass and the calculated mass is \( \pm 1–5\) Da. The proteolytic products with a mass below 1000 kDa and C-terminal fragments of \( \beta \) generated after the proteolytic cleavage were barely detectable even at spectrum recordings at low mass range (between 100 and 1000 kDa). ND, peaks that were not detected by MALDI-TOF-MS.

| Peptide fragment | np\( \beta \) Calculated mass | np\( \beta \) Observed mass | p\( \beta \) Calculated mass | p\( \beta \) Observed mass |
|------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|
| 1–7              | 888.4                        | ND                          | 889.4                       | ND                          |
| 1–8              | 975.4                        | ND                          | 1055.4                      | ND                          |
| 1–12             | 1423.6                       | ND                          | 1503.6                      | ND                          |
| 1–13             | 1560.7                       | 1560.5                      | 1640.7                      | 1644.3                      |
| 1–14             | 1697.7                       | 1697.1                      | 1777.7                      | 1782.5                      |
| 1–15             | 1825.8                       | 1826.3                      | 1905.8                      | 1910.7                      |
| 1–16             | 1953.9                       | 1957.7                      | 2033.9                      | 2038.2                      |
| 1–17             | 2067.0                       | 2069.4                      | 2147.8                      | ND                          |
| 1–18             | 2166.0                       | 2164.6                      | 2256.0                      | 2251.8                      |
| 1–19             | 2313.1                       | 2316.3                      | 2393.1                      | 2399.4                      |
| 1–20             | 2460.2                       | 2460.2                      | 2540.2                      | 2545.8                      |
| 1–28             | 3260.5                       | 3261.2                      | 3340.5                      | 3340.3                      |
| 1–29             | 3317.6                       | 3321.8                      | 3397.6                      | ND                          |
| 8–40             | 3456.8                       | 3451.2                      | 3536.8                      | ND                          |
| 9–40             | 3369.8                       | 3367.7                      | 3369.8                      | ND                          |
| 15–28            | 1580.8                       | 1584.7                      | 1580.8                      | 1584.7                      |
| 15–40            | 2647.4                       | 2646.8                      | 2647.4                      | ND                          |
| 16–40            | 2519.4                       | ND                          | 2519.4                      | ND                          |
| 19–40            | 2179.1                       | ND                          | 2179.1                      | ND                          |
| 20–40            | 2032.1                       | ND                          | 2032.1                      | ND                          |
| 30–40            | 1028.3                       | ND                          | 1028.3                      | ND                          |
| 1–40             | 4163.6                       | 2164.5                      | 2203.6                      | 2204.3                      |

**FIGURE 3.** Similar cleavage products of np\( \beta \) and p\( \beta \) by recombinant IDE. A and B, synthetic np\( \beta \) and p\( \beta \) were incubated with a higher concentration of recombinant IDE \((0.9\, \text{ng/\mu l})\) for 120 min at 37 °C. Samples obtained before \((0)\) and after \((120)\) the incubation period were analyzed by SDS-PAGE and silver staining (A) and by MALDI-TOF-MS (B). Degradation products from both peptide variants are clearly evident after 120 min of incubation. Notably, the resulting pattern of degradation products of p\( \beta \) is very similar to that of np\( \beta \) except for the increased masses due to the phosphoryl group.
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FIGURE 4. siRNA-mediated knockdown of endogenous IDE decreases degradation of Aβ in BV-2 cells. A and B, BV-2 cells were transfected with IDE-targeting siRNA and incubated for 24 h. After 24 h, expression of secreted and cellular IDEs was analyzed by Western immunoblotting. Cellular actin was detected as a loading control. IDE levels were strongly decreased in the conditioned media and cell lysates in IDE siRNA-transfected cells compared with non-transfected controls. Quantification by densitometric analysis of Western blots indicated the percentage of reduction of secreted and cellular IDE expression in siRNA-transfected cells (IDE si-RNA) compared with the control. Values represent means ± S.D. (n = 4). *** p < 0.001 (t test). The values are normalized to actin. C and D, conditioned media collected from control and IDE siRNA-transfected cells were incubated with npAβ or pAβ (1 μM) for 6 h. Aβ levels at the beginning (0 h) and end (6 h) of the incubation period were analyzed by Western immunoblotting (C). Densitometric analysis of the Western blots indicated that an ~75% reduction in degradation activity was observed after siRNA-mediated knockdown of IDE in BV-2 cells for both npAβ and pAβ (D). Values represent means ± S.D. (n = 4). **, p < 0.01; *, p < 0.01 (t test).

several other AD-related proteins, including presenilin-1, presenilin-2, and Tau (49–51). Thus, it would be interesting to co-crystallize IDE with pAβ or npAβ to further elucidate the structural basis of the inhibitory effect of phosphorylation on Aβ degradation.

In addition to IDE, several other proteases involved in Aβ degradation that contribute to the regulation of Aβ levels in the human brain have been identified (52). In particular, NEP, ACE, and plasmin are considered to be physiologically and pathologically relevant in sporadic AD. NEP is a membrane-bound zinc metallopeptidase localized at the cell surface and in cytoplasmic vesicles preferentially hydrolyzing extracellular oligopeptides on the amino side of hydrophobic residues (53) and has been shown to be capable of degrading extracellular oligopeptides on the amino side of hydrophobic

is unlikely. However, it will be interesting to further investigate the effect of Aβ phosphorylation on NEP-dependent degradation.

Plasmin, a serine protease released after cleavage of its zymogen plasminogen, can also modulate the clearance of Aβ (59). In the nervous system, plasmin/plasminogen is expressed in neurons, whereas plasminogen activator is synthesized by neurons and microglial cells (60). The plasmin system is involved in many neural functions, such as neuronal plasticity, learning, and memory (61). The plasmin-dependent degradation of Aβ decreases its neurotoxicity in rat cortical cultures (62, 63). Our mass spectrometric analysis revealed main cleavage sites between Lys-16 and Leu-17 and between Arg-28 and Gly-29. However, the phosphorylation of Aβ had a negligible (if any) inhibitory effect on plasmin-mediated degradation.

ACE, also known as dipeptidyl carboxypeptidase (EC 3.4.15.1), is a membrane-bound zinc metalloprotease (64) and is widely expressed in peripheral tissues and the brain. The involvement of ACE in the pathogenesis of AD was suggested by the genetic association of polymorphisms in the ACE-1 gene (DCPI) with an increased risk of AD (65–67). However, recent large genome-wide association studies did not identify ACE as a significant genetic risk factor (68–70). However, recombinant ACE is capable of cleaving both Aβ40 and Aβ42 in vitro and thereby decreases Aβ aggregation and toxicity (71–73). We also observed efficient cleav-
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This cleavage occurs directly before or after Ser-8. Interestingly, the phosphorylation at Ser-8 strongly inhibited ACE-mediated degradation of Aβ.

In this study, we have demonstrated that phosphorylation at Ser-8 could selectively affect the degradation of Aβ by different proteases. Although the degradation of Aβ by plasmin was independent of phosphorylation, the cleavage of Aβ by IDE and ACE was strongly decreased upon phosphorylation at Ser-8. These data could have important implications for the accumulation of Aβ observed during the pathogenesis of AD. The decreased degradation of phosphorylated Aβ by IDE and ACE would eventually result in increased concentrations of this peptide in the brain. As shown recently, ~20–30% of the total extractable pool of Aβ was phosphorylated at Ser-8 and found in plaques and oligomeric aggregates (34). Thus, phosphorylation not only decreases the clearance of Aβ but also increases its propensity to aggregate (35). This modification would promote the formation of neurotoxic variants in a dual way. As the phosphorylation of Aβ at Ser-26 has also been described (74, 75), it will be interesting to also assess the effect of phosphorylation at this site on Aβ metabolism. The targeting of extracellular Aβ phosphorylation by inhibition of extracellular kinases or stimulation of Aβ dephosphorylation could be explored to facilitate Aβ clearance in the brain for prevention or therapy of AD.

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**FIGURE 6. Phosphorylation of Aβ decreases degradation by recombinant ACE.** A–C, synthetic npAβ and pAβ were incubated with recombinant ACE at 37 °C for various time intervals (0, 5, 15, 30, and 60 min). Sample aliquots of the reaction mixture collected at different time intervals were analyzed by SDS-PAGE and silver staining (A and B) or by MALDI-TOF-MS (C). The rate of degradation of pAβ was significantly reduced (A and B). The peaks representing cleavage products Aβ(8–40) and Aβ(9–40) were detected only with npAβ, whereas no peaks were detected with pAβ (C). The corresponding masses of the cleavage products are provided in Table 1. Values represent means ± S.D. (n = 3). ***, p < 0.001 (t test).
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