Endoproteolysis of the β-amyloid precursor protein (APP) by β- and γ-secretases generates the toxic amyloid β-peptide (Aβ), which accumulates in the brain of Alzheimer’s disease (AD) patients. Here, we established a novel approach to regulate production of Aβ based on intracellular expression of single chain antibodies (intrabodies) raised to an epitope adjacent to the β-secretase cleavage site of human APP. The intrabodies rapidly associated, within the endoplasmic reticulum (ER), with newly synthesized APP. One intrabody remained associated during APP transport along the secretory line, shielded the β-secretase cleavage site and facilitated the alternative, innocuous cleavage operated by α-secretase. Another killer intrabody with an ER retention sequence triggered APP disposal from the ER. The first intrabody drastically inhibited and the second almost abolished generation of Aβ. Intrabodies association with specific substrates rather than with enzymes, may modulate intracellular processes linked to disease with highest specificity and may become instrumental to investigate molecular mechanisms of cellular events.

Introduction

Aging is a major risk factor for Alzheimer’s disease (AD) and the number of AD patients will increase in the near future. For this reason, therapeutic treatments against this devastating disease are urgently sought for (Hardy and Selkoe, 2002; Dodel et al., 2003; Cummings, 2004; Mattson, 2004; Tanzi et al., 2004). The amyloid hypothesis holds that generation and deposition of amyloid β-peptide (Aβ) are key events driving neurodegeneration in AD (Glenner and Wong, 1984). Immuno-therapy involving injection of synthetic Aβ aggregates to elicit neutralizing and aggregate-breaking antibodies and passive Aβ immunization showed promising results in delaying cognitive decline (Youkin, 2001; Haass, 2002), but also underscored the risk of side effects (Pfeifer et al., 2002; Nicoll et al., 2003). Other approaches aim at reducing Aβ generation by inhibiting the secretase activities. γ-Secretases cleave several substrates and their inactivation appears to interfere with physiologically important signaling pathways (Haass, 2004), but β-secretase remains an obvious therapeutic target because its activity can fully be removed in mice by knocking out BACE (β-site APP cleaving enzyme) without any obvious toxicity (Luo et al., 2001; Ohno et al., 2004). Inhibitors of BACE are under active study, but the development of specific, cell-permeable drugs that penetrate into the brain remains a challenging task (Kahle and De Strooper, 2003).

Here, we propose a novel approach to control Aβ production in vivo. The approach is based on intracellular expression of single chain antibodies (intrabodies; Biocca et al., 1990; Bird et al., 1988; Huston et al., 1988; Marasco and Dana Jones, 1998; Lobato and Rabbitts, 2004; Stocks, 2004) that interfere with pathologic endoproteolysis by binding close to the β-secretase cleavage site of huAPP (Fig. 1). One intrabody associated within the ER with newly synthesized β-amyloid precursor protein (APP). Association persisted during APP transport along the secretary line, protected APP from β-secretase cleavage and favored the alternative cleavage by α-secretase. This resulted in decreased production of the toxic Aβ peptide and increased production of P3. Another intrabody carrying a carboxy-terminal ER retention sequence caused quantitative ER retention and slow disposal of APP, thereby virtually abolishing Aβ production.

Results and discussion

The monoclonal antibody β1 (Paganetti et al., 1996) specifically binds to the EFRH tetrapeptide adjacent to the β-secretase cleavage site of huAPP (Fig. 1, at position Aβ3–6). β1 was used....

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Abbreviations used in this paper: Aβ, amyloid β-peptide; AD, Alzheimer’s disease; APP, β-amyloid precursor protein; BACE, β-site APP cleaving enzyme; HEK, human embryonic kidney 293; PS, P56 and permeabilized with 0.05% saponin.

The online version of this article contains supplemental material.
as template for preparation of two intrabodies named sFvβ1 and sFvβ1-KDEL. sFvβ1 consists of the light and heavy chain variable regions of β1 (132 and 120 residues, respectively) covalently linked by a GGGGS pentapeptide repeated three times. sFvβ1-KDEL is a variant of the same intrabody carrying the SEKDEL carboxy-terminal residues of BiP/GRP78 to confer ER retention (Munro and Pelham, 1987). The native signal sequence of the light chain was maintained to target the intrabodies to the ER lumen. Liquid chromatography mass spectrometry of secreted sFvβ1 expressed in human embryonic kidney 293 (HEK) cells revealed that the signal peptide was removed at the consensus site similar to the original β1 antibody (unpublished data).

We first determined if sFvβ1 maintained the capacity of the β1 template to associate with huAPP when expressed intracellularly. HEK cells were transfected for expression of the Swedish variant of huAPP (Mullan et al., 1992) with or without sFvβ1. 1 d after transfection, cells were metabolically labeled with 35S-methionine and cysteine, chased for 10 min or 2 h, and detergent solubilized. Labeled huAPP was immunoprecipitated from cell lysates with specific antibodies. In mock-transfected cells, huAPP was the only labeled protein isolated (Fig. 2 A, lanes 1 and 2). After a 10-min chase APP is immature (APP\textsubscript{i}; M\textsubscript{i} = 120 kD) in the ER as shown by EndoH sensitivity of its single N-linked glycan (Fig. 2 B). After a 2-h chase most of huAPP was released from the ER and the N-glycan became EndoH resistant (Fig. 2 B). Maturation of huAPP (APP\textsubscript{m}; M\textsubscript{m} = 130 kD) also involves tyrosine-sulfation and O-glycosylation resulting in higher M\textsubscript{r} (Weidemann et al., 1989). In cells also expressing sFvβ1, the intrabody (M\textsubscript{r} = 26 kD) coprecipitated with APP, after a 10-min chase (Fig. 2 A, lane 3) and association persisted through the chase (lane 4). Monitoring kinetics of association revealed that the half-time for formation of the intracellular sFvβ1-huAPP complex was 11 min (Fig. 2 C) and that association between sFvβ1 and huAPP did not prevent huAPP maturation (Fig. 2 A, lane 4 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200410047/DC1). The specificity of sFvβ1 for huAPP was confirmed by the substantial reduction in the amount of sFvβ1 coprecipitated with a variant of APP characterized by a carboxy-terminal APP antibody. When present, sFvβ1 associates and coprecipitates with huAPP but not with RGAPP. APP\textsubscript{i} denotes the immature and APP\textsubscript{m} denotes the mature form of APP. (B) Analysis on 8% SDS PAGE better visualizes APP maturation and EndoH sensitivity. At 10 min labeled APP, is still EndoH sensitive; but after 2 h APP is released from the ER and APP\textsubscript{m} becomes EndoH resistant and shows increased M\textsubscript{r} upon N-glycan modification, tyrosine-sulfation, and addition of O-glycans. (C) Kinetics of APP:sFvβ1 association were determined by communoprecipitations and plotted as a function of the maximal amount of sFvβ1 coprecipitated with APP. The position of M, markers of 200, 116, 97, 66, 45, and 32 kD is shown with thin lines.

Next, we coexpressed huAPP with sFvβ1-KDEL to determine first if this intrabody maintained the capacity to associate with APP, and second to establish if appending an ER-retention sequence to an APP-targeted intrabody also caused retention of huAPP. Cells were metabolically labeled and chased for 10 min and 3 h. sFvβ1-KDEL had slower electrophoretic mobility than sFvβ1 (Fig. 3 A). It rapidly associated with newly synthesized huAPP as shown by communoprecipitation after a 10-min chase (Fig. 3 A, lane 3) and association persisted through the chase (Fig. 3 A, lane 4). Unlike sFvβ1, however, association of sFvβ1-KDEL with huAPP prevented export of the latter from the ER, as shown by EndoH sensitivity of its carboxy-terminal glycans (Fig. 3 B). The time half for formation of the intracellular sFvβ1-KDEL-huAPP complex was 11 min (Fig. 3 C) and that association between sFvβ1-KDEL and huAPP was confirmed by the substantial reduction in the amount of sFvβ1-KDEL coprecipitated with a variant of APP characterized by an EGFH versus EFRIH mutation in the β1 epitope (Fig. 3 A, RGAPP). Also the parental antibody β1 has strongly decreased affinity for this epitope present in mouse APP.

Figure 1. **Scheme of APP processing by the secretases.** APP is a type I transmembrane protein with a single hydrophobic domain for membrane retention. The amyloidogenic processing of APP produces the β-amyloid peptide ([Aβ]) through sequential cleavages by BACE at the β-site and by γ-secretase. Shedding of the APP ectodomain occurs through redundant proteolytic events at the cell surface ([s-s] or in endosomes ([β-s]) by the secretases. The Swedish mutation at the consensus site similar to the original APP\textsubscript{i} through sequential cleavages by BACE at the β-site strongly favors BACE cleavage of APP on route to the cell surface. The 40 aa sequence of Aβ is also depicted (bold letters) as well as the 3 aa exchanged in murine Aβ\textsubscript{i}/H9252. The 40 aa sequence of the light chain was maintained to target the intrabodies to the ER lumen. Liquid chromatography mass spectrometry of secreted sFvβ1 expressed in human embryonic kidney 293 (HEK) cells revealed that the signal peptide was removed at the consensus site similar to the original β1 antibody (unpublished data).

Figure 2. **Specific binding of sFvβ1 to human APP in cells.** (A) HEK cells were transfected for expression of huAPP (lanes 1 and 2), sFvβ1 and huAPP (lanes 3 and 4), or sFvβ1 and RGAPP (lanes 5 and 6). After metabolic labeling with 35S-amino acids and chasing with unlabeled amino acids, huAPP and RGAPP were immunoprecipitated from cell extracts with a carboxy-terminal APP antibody. When present, sFvβ1 associates and coprecipitates with huAPP but not with RGAPP. APP\textsubscript{i} denotes the immature and APP\textsubscript{m} denotes the mature form of APP. (B) Analysis on 8% SDS PAGE better visualizes APP maturation and EndoH sensitivity. At 10 min labeled APP, is still EndoH sensitive; but after 2 h APP is released from the ER and APP\textsubscript{m} becomes EndoH resistant and shows increased M\textsubscript{r} upon N-glycan modification, tyrosine-sulfation, and addition of O-glycans. (C) Kinetics of APP:sFvβ1 association were determined by comminoprecipitations and plotted as a function of the maximal amount of sFvβ1 coprecipitated with APP. The position of M, markers of 200, 116, 97, 66, 45, and 32 kD is shown with thin lines.
the ER. In fact, the molecular weight of APP did not increase with progression of the chase (Fig. 3 A, lanes 3 and 4) and the protein failed to acquire EndoH resistance or other posttranslational modifications even after 3 h (Fig. 3 A, EndoH).

Cytochemical analysis by indirect immunofluorescence revealed that part of huAPP colocalized with the ER marker calnexin (Cnx) and part of it stained clustered regions free of Cnx (Fig. 3 B, squares, panels 1 and 2) but stained with an antibody to the Golgi marker Giantin (Fig. 3 B, panels 3 and 4). Note that all cells are labeled with the markers but only transfected cells are positive for anti-APP. ER and Golgi localization is expected for APP, a secretory protein synthesized in the ER and transported for maturation along the secretory pathway (Weidemann et al., 1989). Expression of sFvβ1 did not affect the intracellular localization of huAPP, which colocalized in part with Cnx (Fig. 3 B, panels 5 and 6) but was also in perinuclear clusters stained with Giantin (Fig. 3 B, panels 5, 7, and 8).

On the other hand, expression of sFvβ1-KDEL caused retention of huAPP in the ER as shown by exclusion of APP from Giantin-containing structures (Fig. 3 B, panels 9–12). Thus, we generated one intrabody (sFvβ1) that associated with APP in the ER lumen and remained associated with it during transport along the secretory line. A second intrabody (sFvβ1-KDEL) was equally efficient and fast to associate with APP, but prevented exit of the target protein from the ER. sFvβ1-KDEL actually acted as a killer intrabody because it triggered slow disposal of newly synthesized APP in a process that was delayed by MG132, an inhibitor of the cytosolic proteasome (Fig. 3 C).

We next determined if intracellular association of sFvβ1 and of sFvβ1-KDEL with APP affected secretase-mediated endoproteolysis resulting in the shedding of the ectodomain of this type I membrane protein. Cells coexpressing huAPP and sFvβ1 or sFvβ1-KDEL were metabolically labeled and chased for 10 min or 2 h. To analyze protein secretion, conditioned media were harvested, boiled in sample buffer and analyzed by SDS PAGE. Because of CMV-driven expression, labeled sAPP (and sFvβ1) are the major secretory products of transfected HEK cells. After 10 min, no labeled ectodomain was secreted (Fig. 4 A, lanes 1, 3, and 5; Fig. S1) as the labeled proteins are still folding in the ER. After a 2-h chase, ectodomain shedding of labeled huAPP had occurred in mock-treated cells (Fig. 4 A, lane 2; Fig. S1). Coexpression of sFvβ1 significantly reduced (Fig. 4 A, lane 4; Fig. S1), and coexpression of sFvβ1-KDEL virtually abolished secretase-mediated release of huAPP from cells (Fig. 4 A, lane 6). APP with the mutated β1 epitope served again as specificity control because coexpression of sFvβ1 did not reduce release of the RGAPP ectodomain in the extracellular media compared with controls (Fig. 4 C).
ies targeted to Aβ (Dodart et al., 2002). To establish if in situ release of intrabody immunization may rapidly reverse behavioral deficits in mice (Frenkel et al., 1998; Pfeifer et al., 2002). Moreover, passive prevention formation or to disassemble preexisting Aβ plaques (Dodart et al., 2002) is expected for a polypeptide carrying an ER retention signal. Part of the secreted sFv1 was associated and coprecipitated with the secreted huAPP (Fig. 4 B, lane 4) but not with the control protein RGAPP (Fig. 4 C, lane 8). Thus, the complex between the intrabody and huAPP was maintained during secretion and processing and release of the APP ectodomain (lanes 2 and 4, cond. media; lanes 2 and 4, anti-APP) and sFv1 does not associate with RGAPP as shown by lack of coprecipitation (lane 4, anti-APP). (D) sAPP secretion was quantified in a series of five independent experiments. Error bar represents SD.

Labeled sFv1 was secreted in the conditioned medium (Fig. 4, A and C, lane 4), whereas virtually no labeled sFv1-KDEL was detected extracellularly (Fig. 4 A, lane 6), as expected for a polypeptide carrying an ER retention signal. Part of the secreted sFv1 was associated and coprecipitated with the secreted huAPP (Fig. 4 B, lane 4) but not with the control protein RGAPP (Fig. 4 C, lane 8). Thus, the complex between the intrabody and huAPP was maintained during secretion and after shedding. From the data described above, we concluded that uncomplexed sFv1 was also released from cells. This is of interest and might have beneficial consequences in case of a therapeutic application of our approach because recognition of the EFRH epitope proved essential for antibodies able to prevent formation or to disassemble preexisting Aβ plaques (Frenkel et al., 1998; Pfeifer et al., 2002). Moreover, passive immunization may rapidly reverse behavioral deficits in mice (Dodart et al., 2002). To establish if in situ release of intrabodies targeted to Aβ exerts protective and/or therapeutic activity awaits further experimentation in an animal model for the disease. Thus, in a series of several independent experiments summarized in Fig. 4 D, we proved that by associating close to the β-secretase cleavage site, sFv1 inhibited by >60% the processing and release of the Swedish variant of APP. Addition of a SEKDEL-retention signal led to production of a killer intrabody that retained newly synthesized APP in the ER preventing secretase processing and eventually leading to slow degradation of APP.

The endoproteolysis of APPSwedish consists mainly in sequential cleavages by β- and γ-secretase releasing the soluble APP ectodomain and the toxic Aβ peptide. A minor alternative endoproteolysis initiated by α-secretase releases the soluble APP ectodomain and the peptide P3 (Fig. 1). Therefore, we next determined how coexpression of sFvβ1 or sFvβ1-KDEL affected production of the APP metabolites Aβ and P3. APP-derived peptides produced by HEK cells were identified independently by matrix-assisted laser desorption ionization time of flight mass spectrometry (Wang et al., 1996; unpublished data). Here, we determined the peptides by combining immunoprecipitation and immunoblotting with two well-characterized antibodies to the carboxy terminus of Aβ (Paganetti et al., 1996) and electrophoretic comigration with synthetic peptides (Fig. 5).

As demonstrated above using metabolic labeling, steady-state analysis of cell lysates by Western blot confirmed that sFv1 did not interfere with APP maturation (Fig. 5 A, lanes 1 and 2). In contrast, sFvβ1-KDEL strongly impairs APP maturation (Fig. 5 A, lane 3). Overexpression of APPSwedish produced abundant Aβ as expected for this substrate efficiently processed by β-secretase (Fig. 5 B, lane 1). Consistent with the...
effects observed for the ectodomain shedding. Aβ generation was lowered by coexpression of sFvβ1 (Fig. 5 B, lane 2) and virtually prevented by sFvβ1-KDEL (Fig. 5 B, lane 3). In contrast, sFvβ1 did not affect cleavage of the RGAPP carrying the point mutation in the sFvβ1 epitope (Fig. 5, A and B, lane 5) and sFvβ1-KDEL slightly affected RGAPP maturation (Fig. 5 A, lane 6) and did not affect significantly the production of Aβ (Fig. 5 B, lane 6).

Analysis of the ratio Aβ (M, = 4 kD) versus P3 (M, = 3 kD) produced in mock-transfected cells and in cells expressing sFvβ1 (Fig. 5 B, longer exposure, lanes 2 and 3) revealed that sFvβ1 augmented to a certain extent P3, i.e., shifted APP cleavage from the β- to the α-site. Thus, association of intrabodies to a sequence adjacent to the β-secretase–mediated cleavage site interfered with the amyloidogenic processing of huAPP by the β-secretase as determined by reduction of release from cells of the APP ectodomain and Aβ. In contrast, the innocuous cleavage by α-secretase was slightly favored as shown by increased production of P3.

In summary, we engaged the cellular protein factory, the ER, to produce therapeutic agents inhibiting production of Aβ. By rapidly associating with newly synthesized APP and by escorting it during intracellular transport, sFvβ1 shielded APP from the pathologic intervention on the enzyme, liabilities for side effects that may derive from the pathologic intervention in an enzymatic process leading to a human disease. By avoiding direct intervention with primary antibody diluted in PS for 45 min, washed 15 min with PS, then incubated with conjugated secondary antibody diluted in PS for 50 min. Cells were rinsed with PS and water and mounted in Mowiol. Microscopic images were collected at RT using a microscope (model E-800; Nikon) equipped with a 60×/1.4 Plan Apo objective, filter cubes for CFP and YFP fluorescence, and a camera (QImaging), controlled by Openlab 3.5 software. Image cropping and adjustment were accomplished using Photoshop (Adobe).

Online supplemental material
Fig. S1 shows kinetics of APP maturation and disappearance from cells (Intracellular) and of secretion of soluble APP (sAPP, extracellular). Longer persistence of the mature form of APP [APP*, top gel, panel on the right] in cells expressing sFvβ1 is a further indication that intrabody coexpression interferes with shedding of the APP ectodomain. This is proved by the lower amount of sAPP in the extracellular media (bottom panel compare labeled sAPP in lanes 4 vs. 5). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200410047/DC1.

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