Expression cloning screen for modifiers of amyloid precursor protein shedding

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A large number of membrane proteins are proteolytically converted to their soluble counterparts. This process is referred to as ectodomain shedding and is an important way of regulating the biological activity of membrane proteins (Blobel, 2005; Huovila et al., 2005). This process has been described for type I, type II and GPI-anchored membrane proteins (reviewed in Blobel, 2005; Hooper et al., 1997; Huovila et al., 2005) and is found in many multicellular organisms. Ectodomain shedding is required in various biological processes, such as contact-mediated axon repulsion (Hattori et al., 2000), during embryonic development (Hartmann et al., 2002; Peschon et al., 1998) and in the immune system (for an overview see Schlondorff and Blobel, 1999), but also in the pathogenesis of Alzheimer's disease (AD). In AD, shedding of the amyloid precursor protein (APP) is an essential regulatory step in the formation of the amyloid β peptide (Aβ). Generation and subsequent deposition of Aβ are assumed to be the first events in the pathogenesis of AD (for a review see Selkoe and Schenk, 2003). The shedding of APP may occur through two different protease activities termed α- and β-secretase, which cleave APP within its ectodomain close to its transmembrane domain (Fig. 1A). The β-secretase is the aspartyl protease BACE1 and cleaves APP at the N-terminus of the Aβ peptide domain, thus catalyzing the first step in Aβ peptide generation (reviewed in Citron, 2004). After the initial cleavage of APP by BACE1, the remaining C-terminal APP-fragment is cleaved by γ-secretase within its transmembrane domain at the C-terminus of the Aβ domain, leading to the secretion of the Aβ-peptide (for an overview see Haass, 2004). In contrast to β-secretase, α-secretase cleaves within the Aβ-sequence and thereby precludes Aβ peptide generation. The α-secretase is a member of the ADAM-family of proteases and may be ADAM10, ADAM17 (TACE) or ADAM9 (Allinson et al., 2003). The α-secretase cleavage of APP is assumed to take place at or

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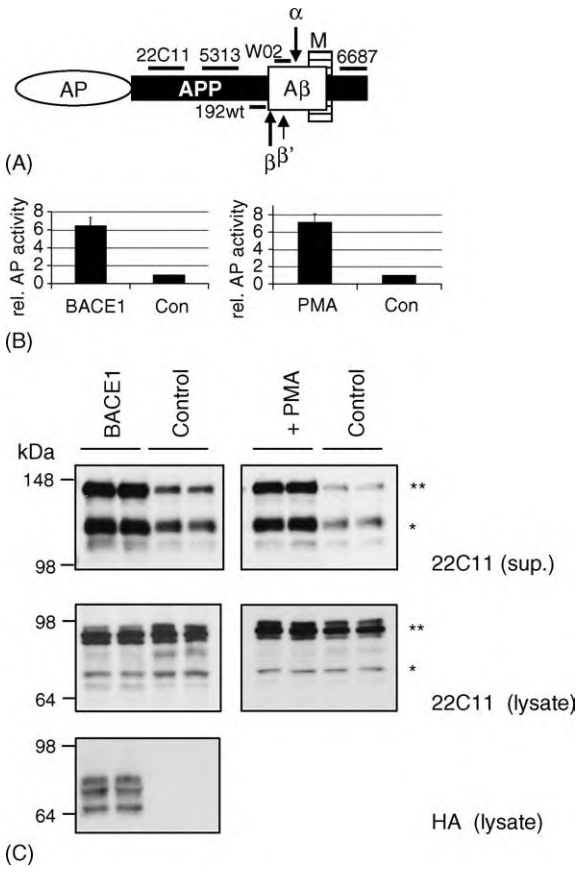

Fig. 1. Alkaline phosphatase reporter assay for the ectodomain shedding of APP. (A) Schematic drawing of APP695 fused to the ectodomain of the reporter enzyme alkaline phosphatase (AP), resulting in the AP-APP fusion protein. Horizontal bars show the epitopes recognized by the indicated antibodies. Arrows point to the proteolytic α-, β- and β′-cleavage sites within the APP ectodomain. The β′-cleavage site is an additional cleavage site of the β-secretase BACE1. M: membrane. (B) Human embryonic kidney 293 cells stably expressing AP-APP (clone SABC70) were transiently transfected with a BACE1-encoding plasmid or control vector (left panel) or treated with the phorbol ester PMA (1 μM, right panel). PMA and the overexpression of BACE1 stimulate the secretion of AP-APP as determined by the increased AP activity in the conditioned medium relative to control cells. The AP activity represents the mean and standard deviation of two independent experiments, each one carried out in duplicate. (C) Aliquots of the experiments in (B) were separated by electrophoresis and analyzed by immunoblot for secreted APP (upper panels, using antibody 22C11), for cellular APP (middle panels, using antibody 22C11) and for BACE1 expression (lower panel, detected with an antibody against the C-terminal HA epitope tag of BACE1). *Endogenous APP; **AP-APP; sup.: supernatant.

close to the cell surface, whereas β-secretase cleavage takes place after endocytosis of APP into endosomes.

α-Secretase and β-secretase compete for the ectodomain cleavage of APP (Skovronsky et al., 2000), but have opposite effects on Aβ generation. Additionally, α- but not β-secretase cleavage generates a secreted form of APP (APPsα), which has neurotrophic and neuroprotective properties (Furukawa et al., 1996; Meziane et al., 1998; Stein et al., 2004). Thus, shifting APP shedding away from β- towards α-secretase cleavage may be therapeutically beneficial for AD. In order to do so, it is essential to understand the cellular pathways that regulate the activity of both proteases. However, to date little is known about such cellular pathways (for reviews see Huovila et al., 2005; Lichtenthaler and Haass, 2004). To explore the contributory pathways systematically, we employed sib-selection expression cloning, which is a powerful genetic screening method to identify cDNAs by their function. A human brain cDNA library was used to obtain seven cDNAs that stimulate the ectodomain shedding of APP. To control for specificity, the influence of the identified cDNAs was analyzed on the shedding of the two unrelated membrane proteins TNF receptor 2 (TNFR2) and P-selectin glycoprotein ligand-1 (PSGL-1). Like APP, both proteins are subject to ectodomain shedding by ADAM proteases. Two of the proteins that strongly and specifically activated shedding of APP are members of the endophilin family of endocytic and signal transducing proteins (for reviews see Huttner and Schmidt, 2002; Reutens and Glenn Begley, 2002).

1. Experimental procedures

1.1. Reagents

The following antibodies were used: anti-HA epitope antibody HA.11 (Covance), HRP-conjugated goat anti-mouse secondary antibody (DAKO), HRP-coupled goat anti-mouse (DAKO). Antibodies 5313 (against APP ectodomain) and 6687 (against APP C-terminus) were described previously (Steiner et al., 2000) and provided by Christian Haass. Antibody 192 wt specific for the C-terminus of APPsβ (Seubert et al., 1993) was provided by Dale Schenk. Antibodies 22C11 (anti APP ectodomain) and W02 (against amino acids 5–8 of Aβ) (Ida et al., 1996) were provided by Konrad Beyreuther. Antibody sc-10880 against endophilin A3 was from Santa Cruz Biotechnology. Alexa555-coupled secondary anti-rabbit antibody was from Molecular Probes. PMA was obtained from Sigma. The metalloprotease inhibitor TAPI was purchased from Peptides International.

1.2. Plasmid construction

Generation of vector peak12 expressing BACE1 and ADAM10 has been described (Lichtenthaler et al., 2003). The following cDNAs in the peak8 expression vector were obtained from a human brain cDNA library (Edgebio): endophilin A1 and A3, catalytic α-subunit of PKA, Numb-like, MEKK2 (lacking 707 nucleotides at the 5′ end of the coding sequence), PPT1 (lacking the first four nucleotides of the coding sequence and having an insertion of one adenosine into the stretch of seven adenosines within the 5′ half of the coding sequence), mGluR3. The cDNAs of endophilin A1, A2 and A3 tagged with N- or C-terminal epitope tags (HA, GFP) were cloned into the expression vector peak12. The identity of all constructs obtained by PCR was confirmed by DNA sequencing. Peak12 vectors encoding the alkaline phosphatase (AP) fusion proteins AP-APP, AP-PSGL-1 and AP-TNFR2 as well as the vector encoding Bcl-X_L, CrmA and RIP were described previously (Lichtenthaler et al., 2003; Pimentel-Muinos and Seed, 1999). AP-APP consists of the ectodomain of AP fused to full-length APP695 lacking its signal peptide.

1.3. Cell culture, transfections, Western blot

293-EBNA cells were cultured in DMEM (Gibco) containing 10% FBS (Hyclone). Clonal 293 cells expressing AP-APP and Bcl-X_L/CrmA (clone SABC70) were generated and cultured as described (Lichtenthaler et al., 2003). Transfections were carried out using Lipofectamine 2000 (Invitrogen). One day after transfection, the medium was replaced with fresh medium. After over-night incubation conditioned medium and cell lysate (in 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40) were collected. AP activity was measured as described previously (Lichtenthaler et al., 2003). Aliquots of the conditioned medium were treated for 30 min at 65 °C to heat-inactivate the endogenous alkaline phosphatase activity.

To analyze the effect of PMA and TAPI on shedding, cells were treated as described previously (Lichtenthaler et al., 2003). For the detection of secreted and cellular APP by immunoblot, the protein concentration in the cell lysate was measured, and corresponding aliquots of lysate or conditioned medium were directly loaded onto an electrophoresis gel. Immunoblot detection was carried out using the indicated antibodies.

1.4. Expression cloning

Individual bacterial clones from a human adult brain cDNA library (Edgebio) were arrayed into 384-well plates using the Qbot (Genetix, UK). Subsequently, 96 clones were pooled using the Biomek FX Workstation (Beckman Coulter). Miniprep DNA of the pools was generated with the Biorobot 9600 (Qiagen) and transfected into the AP-APP reporter cell line using lipofectamine 2000 (Invitrogen). One day after transfection the medium was replaced with fresh medium. Two days after transfection, supernatants were collected and assayed for AP activity. In cDNA pools leading to an increased AP activity, the individual cDNA was isolated with two more rounds of screening: first, the initial 96 clones were arrayed into pools of 8 clones, which were tested as above for an increased AP activity. In the positive pool, all eight individual clones were tested. The identified cDNAs were sequenced from both ends for several hundred base pairs.

1.5. APP antibody uptake assay

COS cells plated on coverslips were cotransfected with wild-type APP695 and C-terminally GFP-tagged endophilin A3 or GFP as a control. APP endocytosis was determined as described previously (Kaether et al., 2002). In brief, transfected COS cells were washed with ice cold PCM (PBS supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), and incubated on ice in a 1:200 dilution of antibody 5313 in PCM. After 20 min, cells were washed in PCM on ice and then PCM was replaced by prewarmed culture medium and cells were placed for the indicated time points (see Fig. 4A) in a 37 °C incubator. Afterwards, coverslips were transferred to 4% paraformaldehyde, 4% sucrose in PBS, fixed for 20 min and processed for standard immunofluorescence using Alexa555-coupled secondary anti-rabbit antibodies. Fixed cells were analyzed on a Zeiss Axioskop2 plus microscope equipped with a 63×/1.25 objective and standard FITC and TRITC fluorescence filter sets. Images were obtained using a Spot Camera (Zeiss AxioCam HRm) and the MetaView Imaging software (Universal Imaging Corp.) Experiments were performed at least three times, and around 100 cells were scored per cell line and time point.

1.6. Transferrin uptake assay

COS cells plated on coverslips were transfected with C-terminally GFP-tagged endophilin A3 or GFP as a control. Transferrin receptor endocytosis was performed as described previously (Leprince et al., 2003) under slightly modified conditions. The seeded COS cells were preincubated for 1 h in serum-free DMEM containing 20 mM Hepes pH 7.5 at 37 °C. Endocytosis of AlexaFluor555 conjugated transferrin was performed at 37 °C for different time points (see Fig. 4B) in endocytosis medium (DMEM, 20 mM Hepes pH 7.5, 1 mg/ml BSA) containing 50 μg/ml Alexa555-conjugated transferrin (Molecular Probes). After the incubation with the conjugate, cells were rapidly cooled at 4 °C, washed twice with ice cold PBS and fixed with 4% paraformaldehyde, 4% sucrose in PBS for 20 min at 4 °C. Cells were then processed for standard immunofluorescence as above.

2. Results

2.1. Reporter cell line for the analysis of APP ectodomain shedding

For the expression cloning screen a reporter cell line was generated that allows a rapid detection of APP shedding in a high-throughput format. To this aim human embryonic kidney 293 cells were used, which stably express a fusion protein (AP-APP) consisting of secretory alkaline phosphatase (AP) upstream of full-length APP (Fig. 1A). To protect the cells against possible cell death induced by proapoptotic genes represented in the cDNA pools, the 293 cells were additionally transfected with a plasmid encoding the cDNA of two antiapoptotic proteins, Bcl-X_L and CrmA. Clonal cell lines were established and analyzed for protection against apoptosis as well as for the secretion of AP-APP. Clone SABC70 was resistant to apoptosis induced by overexpression of the proapoptotic kinase RIP (receptor-interacting protein; not shown) and was used for further analysis throughout this study (referred to as AP-APP cells). As a positive control, the AP-APP cells were transiently transfected with control vector or with the cDNA of the β-secretase BACE1, which controls the shedding of APP. Transfection of BACE1 strongly increased AP-APP shedding, as determined by AP activity measurement in the conditioned medium (Fig. 1B). The increase in AP-APP shedding was also observed by immunoblot analysis, where both AP-APP and the endogenous APP were detected (Fig. 1C). As an additional control, the AP-APP cells were treated with the phorbol ester PMA, a known inducer of the ADAM protease mediated ectodomain shedding of APP. PMA increased AP-APP shedding as measured in the AP activity assay (Fig. 1B) and by immunoblot analysis (Fig. 1C). The PMA and BACE1-induced increase in secreted AP-APP (as determined by immunoblot analysis) correlated well with the increase of AP activity in the conditioned medium (Fig. 1B and C), showing that the measurement of AP activity is a reliable means of detecting the amount of the secreted AP-APP fusion protein regardless of whether the cleavage occurred through an ADAM protease or BACE1.

2.2. Expression cloning of shedding-activating cDNAs

For the sib-selection expression cloning we used an unamplified human adult brain cDNA library, which was arrayed into pools of 96 cDNAs each. Screening of >100,000 cDNAs yielded seven cDNAs that increased secretion of AP-APP. The strongest shedding-inducing activity was observed for the proteins endophilin A1 and A3, which increased the secretion at least four-fold (Fig. 2A). A 1.5–3-fold increase was observed for five additional proteins: the metabotropic glutamate receptor 3 (mGluR3), Numb-like (Nbl), which is a modulator of Notch signal transduction and binds to the APP cytoplasmic domain (Roncarati et al., 2002), the catalytic α-subunit of protein kinase A (PKA), palmitoyl-protein thioesterase 1 (PPT1; two individual clones of this cDNA were obtained) and a N-terminally truncated form of the kinase MEKK2 (Fig. 2B). The identification of PKA is in agreement with previous publications showing that an activation of PKA by forskolin in rat pheochromocytoma PC12 cells (Xu et al., 1996) and in human embryonic kidney 293 cells (Marambaud et al., 1998) increased the shedding of APP. This validates the screening approach as it shows that physiologically relevant cDNAs can be obtained.

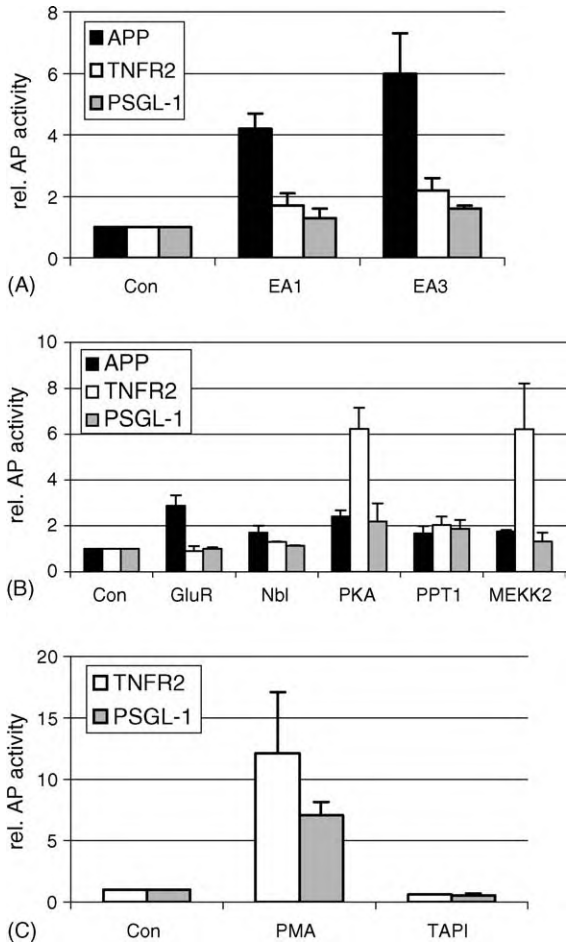

Fig. 2. Stimulatory effect of the identified cDNAs on the shedding of APP, TNFR2 and PSGL-1. (A and B) Kidney 293 cells stably expressing AP fusion proteins of APP, TNFR2 or PSGL-1 were transiently transfected with plasmids encoding the cDNAs indicated below the graphs. AP activity was measured in the conditioned medium and represents the mean and standard deviation of two to three independent experiments, each one carried out in duplicate. Con: control, EA1: endophilin A1; EA3: endophilin A3; GluR: mGluR3; Nbl: Numb-like. (C) Kidney 293 cells stably expressing AP-TNFR2 or AP-PSGL-1 were treated for 3 h with PMA (1 μM) or with the metalloprotease inhibitor TAPI (25 μM). AP activity was measured as above.

2.3. Shedding-inducing cDNAs stimulate the shedding of different membrane proteins

Several membrane proteins besides APP undergo ectodomain shedding. Therefore, we studied whether the identified seven cDNAs specifically influenced the shedding of APP or also stimulated the shedding of other type I membrane proteins and thus may be part of a cellular machinery regulating ectodomain shedding in general. To this aim, the TNF receptor 2 and the PSGL-1, which mediates leukocyte adhesion in inflammatory reactions (McEver and Cummings, 1997), were expressed as AP-fusion proteins in 293 cells. The AP-fusion proteins show the same regulation of their shedding as reported previously for the wild-type proteins (Davenpeck et al., 2000; Peschon et al., 1998), since the phorbol ester PMA stimulated the shedding of both AP-TNFR2 and AP-PSGL-1 (Fig. 2C). Moreover, the metalloprotease inhibitor TAPI inhibited the shedding of both fusion proteins by ∼40% (Fig. 2C), as expected for the cleavage by a metalloprotease of the ADAM-family.

Next, the seven cDNAs identified in the screen were transiently transfected into the AP-PSGL-1 and AP-TNFR2 cells in addition to the AP-APP cells. Measurement of AP activity revealed that they had differential effects on the shedding of TNFR2 and PSGL-1 (Fig. 2A and B). Endophilins A1 and A3 as well as mGluR3 clearly activated APP shedding, but had no or only little effect on the shedding of TNFR2 and PSGL-1. Likewise, Nbl weakly activated shedding of APP but not of TNFR2 and PSGL-1 (Fig. 2B). In contrast, PKA and MEKK2 strongly activated TNFR2 shedding, but only had a mild effect on the shedding of APP or PSGL-1. PPT1 mildly activated the cleavage of all three proteins. Taken together, the analysis of the shedding of the three fusion proteins shows that they fall into three different classes. Class I comprises endophilins A1, A3, mGluR3 and the weak activator Nbl, which activated APP shedding in a relatively specific manner. Class II consists of the kinases PKA and MEKK2 that are strong activators of TNFR2 shedding, but only modestly affected shedding of APP and PSGL-1. PPT1 belongs to the third class and seems to be a general stimulator of ectodomain shedding. Because endophilin A3 was the strongest and a relatively specific activator of APP cleavage, we focused on this protein to determine the mechanism underlying its shedding enhancing activity.

2.4. Endophilin A3 stimulates the shedding of endogenous APP

First, we tested whether endophilin A3 stimulated the shedding of APP by α- or by β-secretase. To this goal, human embryonic kidney 293 cells expressing the endogenous, wild-type APP were transiently transfected with endophilin A3 carrying a C-terminal HA epitope tag or with control vector. Soluble APP generated by α-secretase (APPsα) or β-secretase (APPsβ) was detected in the conditioned medium using cleavage site-specific antibodies. Antibody 192 wt specifically detects APPsβ (Seubert et al., 1993), whereas antibody W02 binds to an epitope between the β- and the α-secretase cleavage sites (Ida et al., 1996) (Fig. 1A). Thus, W02 does not detect APPsβ, but instead APPsα. Additionally, W02 detects APPsβ′, which starts at amino acid Glu11 of the Aβ sequence and represents a minor secondary cleavage site of BACE1. Compared to control cells W02 detected a strong increase in soluble APP in the endophilin A3 expressing cells (Fig. 3A). No increase in soluble APPsβ was detected with antibody 192 wt. In some experiments a slight reduction in APPsβ was observed (data not shown). This reveals that endophilin A3 mainly stimulated the α-secretase cleavage and only had a minor effect on the β-secretase cleavage of APP. Expression of endophilin A3 very slightly increased the amount of mature APP in the cell lysate, as determined using an antibody against the C-terminus of APP (Fig. 3A). Endophilin A3 was clearly expressed in the cell lysate with an apparent molecular weight of around 50 kDa (Fig. 3A).

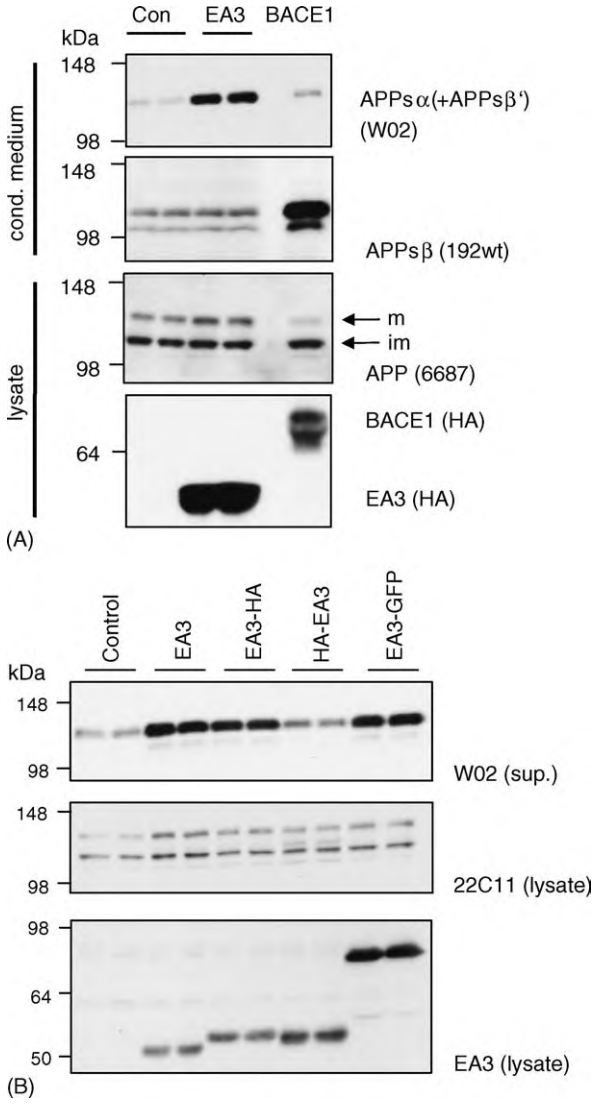

Fig. 3. Endophilin A3 increases α-secretase cleavage of endogenous APP. (A) 293 cells were transiently transfected with empty control vector (Con), endophilin A3 (EA3) or BACE1 as a control. Aliquots of the conditioned medium and the cell lysate were separated by electrophoresis. Soluble APP in the conditioned medium was detected by immunoblot analysis using antibody W02 (detecting soluble APP cleaved at the α- or β′-site) and antibody 192wt (against the β-cleaved soluble APP, APPsβ). The total amount of APP in the cell lysate was detected using antibody 6687, which binds to the C-terminus of APP. Expression of endophilin A3 and BACE1 in the cell lysate is shown using a HA-antibody against their C-terminal epitope tag. Shown are representative blots of at least three independent experiments. m: mature APP; im: immature APP. (B) To test whether an epitope tag or a GFP fusion might alter the shedding stimulatory function of endophilin A3 (EA3), the indicated constructs were transiently transfected in 293 cells. Soluble APP in the conditioned medium was detected by immunoblot analysis using antibody W02 (detecting soluble APP cleaved at the α- or β′-site). APP in the cell lysate was detected with antibody 22C11 against the ectodomain of APP. Expression of endophilin constructs was shown using an antibody against endophilin A3. Experiments are shown in duplicate. sup.: supernatant; EA3-HA: EA3 with a C-terminal HA-tag; HA-EA3: EA3 with an N-terminal HA-tag; EA3-GFP: EA3 with a C-terminal fusion to GFP.

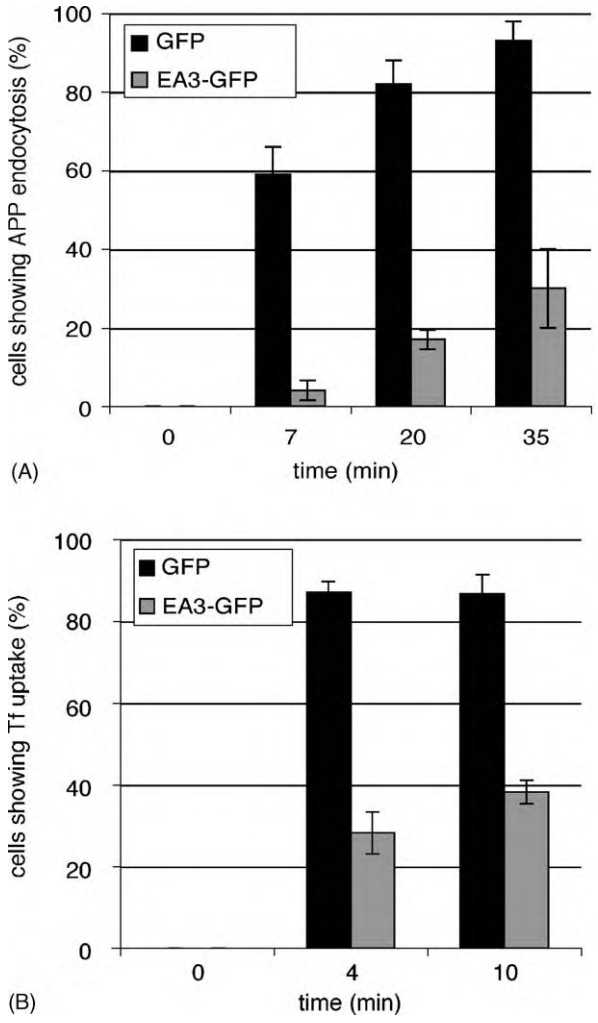

Fig. 4. Endophilin A3 reduces APP endocytosis and transferrin uptake. (A) COS cells were transiently cotransfected with APP695 and C-terminally GFP-tagged endophilin A3 (EA3-GFP) or GFP as a control. On ice, cells were incubated with antibody 5313 against the ectodomain of APP. Cells were returned to 37 °C for the indicated time points to allow endocytosis to occur. Cells were fixed, stained with a secondary antibody against the primary APP antibody and analyzed by fluorescence microscopy. Around 100 cells expressing GFP or EA3-GFP were analyzed at every time point and scored as showing or not showing endocytosis. Given are the mean and the standard deviation of two independent experiments. (B) COS cells were transiently transfected with EA3-GFP or GFP as a control. Cells were incubated with fluorescently labeled transferrin (Tf) for the indicated time periods as described in the methods section. Shown are the mean and the standard deviation of two independent experiments. Around 100 cells were analyzed at every time point and scored as showing or not showing endocytosis.

As a control, the kidney 293 cells were transiently transfected with the β-secretase BACE1. As expected, this led to a strong increase in APPsβ secretion, as determined by immunoblot analysis using antibody 192 wt (Fig. 3A). Additionally, a slight increase in APP secretion was detected with antibody W02, presumably resulting from the increase in APP β′-cleavage (alternative β-secretase cleavage site as described above).

In an additional control experiment we verified that the C-terminal HA-epitope tag used for the above transfections did not alter the APP shedding-stimulatory function of endophilin A3. To this aim, kidney 293 cells were transiently transfected with control vector, with endophilin A3 without an epitope tag or with endophilin A3 carrying a C-terminal HA-tag (EA3-HA) or a C-terminal fusion to GFP (EA3-GFP). Both EA3-HA and

EA3-GFP stimulated APP shedding in a similar manner as untagged wild-type endophilin A3 (Fig. 3B), as detected by immunoblot analysis using antibody W02. Similar results were obtained when the tagged endophilin A3 proteins were expressed in the AP-APP reporter cell line (results not shown). In contrast, a N-terminal HA epitope tag (HA-EA3) completely abolished the shedding enhancing effect of endophilin A3 (Fig. 3B). This construct was not further used in this study.

2.5. Endophilin A3 inhibits APP endocytosis and transferrin uptake

APP α-cleavage occurs at or close to the plasma membrane (Sisodia, 1992), whereas β-cleavage of wild-type APP mainly occurs after endocytosis in the endosomes. Given that endophilin A3 increased the α- but not the β-cleavage of APP, we tested for a possible influence of endophilin A3 on APP endocytosis using an APP antibody uptake assay as described previously (Kaether et al., 2002). APP together with C-terminally GFP-tagged endophilin A3 or with GFP (as a control) was transiently cotransfected into COS cells. Surface APP was labeled on ice with an antibody against the APP ectodomain. Returning the cells to 37 °C allowed APP to be endocytosed together with the prebound antibody. After different time periods the cells were fixed, permeabilized and stained with a secondary antibody against the prebound anti-APP antibody. Using immunofluorescence analysis the transfected cells were identified by their green fluorescence (GFP or endophilin A3-GFP). Individual cells were scored as showing endocytosis, if the APP-staining revealed endocytic vesicles. In contrast, they were scored as not showing endocytosis if patchy cell surface APP staining was visible. At every time point 100 cells were analyzed. Compared to control transfected cells endophilin A3 strongly reduced the rate of APP endocytosis (Fig. 4A). In contrast to the specific effect of endophilin A3 on APP shedding (Fig. 2A), it did not only act on APP endocytosis, but it also reduced the rate of transferrin uptake (Fig. 4B), which is in agreement with a previous study (Sugiura et al., 2004).

3. Discussion

The ectodomain shedding of APP can be stimulated by several different proteins, including kinases and cytosolic adapter proteins, as well as by extracellular stimuli, such as phorbol esters, growth factors and estrogen (for a review see Allinson et al., 2003). Many of these stimuli activate the α-secretase cleavage of APP. However, the molecular and cellular processes, which are activated by these stimuli and which finally execute the increased cleavage of APP, remain largely unknown. To explore the contributory pathways systematically, we have undertaken a sib-selection expression cloning study to identify genes that might regulate the shedding of APP. The screening approach is validated by our identification of PKA, which is a known regulator of the shedding of APP (Marambaud et al., 1998; Xu et al., 1996). The other six proteins have not previously been reported to be involved in APP shedding. Because the ectodomain shedding of APP by β-secretase, but not by α-secretase is tightly linked to the pathogenesis of Alzheimer's disease (Selkoe and Schenk, 2003), proteins that regulate this process may be useful targets for prophylaxis or treatment of the disease and might potentially allow to shift APP cleavage from the β- towards to the α-secretase. Moreover, a comparative study of the shedding of unrelated proteins – as carried out in this study using PSGL-1 and TNFR2 – may lead to the discovery of cDNAs that strongly affect the shedding of APP but only have a minor effect on the shedding of other proteins. In this regard, mGluR3 may be particularly important, as it is one of the cDNAs showing a specific effect on the shedding of APP. Two members of the mGluR family, mGluR1 and mGluR5, have previously been shown to stimulate the secretion of APP (Lee et al., 1995). Both mGluRs activate phospholipase D. In contrast, the identified mGluR3 negatively regulates adenylate cyclase and thus, points to a role of this separate pathway in the control of APP shedding.

The strongest activators of APP shedding were the endocytic proteins endophilin A1 and A3.

Together with endophilin A2 they form a family of evolutionarily conserved proteins that can associate with the cytoplasmic surface of membranes. Additionally, two distantly related family members, endophilin B1 and B2, have been described. Of the three endophilins A (A1, A2 and A3), the neuron-specific endophilin A1 has been best studied (for reviews see Huttner and Schmidt, 2002; Reutens and Glenn Begley, 2002). It is a cytoplasmic protein essential for the budding and fission of synaptic vesicles from the plasma membrane. Through its C-terminal SH3 domain endophilin A1 interacts with proline-rich domains of other endocytic proteins, such as synaptojanin and dynamin. The N-terminal domain of endophilin A1 possesses lysophosphatidic acid acyl transferase activity, which is implicated in the generation of membrane curvature during the endocytic process. Endophilins A2 and A3 are highly homologous to endophilin A1 and share the same domain structure. In contrast to the neuron-specific endophilin A1, endophilin A2 is ubiquitously expressed, whereas endophilin A3 is expressed in brain, thymus and testis. The endophilins are also linked to signal transduction of the tyrosine kinase receptors for hepatocyte and epidermal growth factor (Petrelli et al., 2002; Soubeyran et al., 2002) and of c-Jun N-terminal kinase (Ramjaun et al., 2001). Interestingly, endophilin A3 has been shown to bind to the Huntington's disease protein huntingtin and to promote the formation of polyglutamine containing aggregates (Sittler et al., 1998).

In this study we show that endophilin A3 strongly activates APP α-cleavage, but does not have a major effect on APP β-cleavage. Concerning the molecular mechanism underlying this shedding-stimulatory effect, we found that transfection of endophilin A3 inhibits APP endocytosis. As a result, more APP may be available for an increased α-secretase cleavage, which is assumed to occur at or close to the cell surface (Sisodia, 1992). This fits with previous studies showing that an inhibition of dynamin-dependent endocytosis resulted in more APP at the cell surface and in an increased APP shedding, mainly by α-secretase (Carey et al., 2005; Chyung and Selkoe, 2003; Koo

and Squazzo, 1994). Conversely, a reduction of APP presence at the cell surface lowers APP shedding, such as in Munc13-1 deficient neurons (Rossner et al., 2004) and upon retention of APP in early compartments of the secretory pathway (Suga et al., 2005).

Endophilin A3 also inhibited transferrin endocytosis, which is in agreement with a recent study (Sugiura et al., 2004). Taken together, our data suggest that transfection of endophilin A3 negatively regulates endocytosis – including the endocytosis of APP – and thereby increases the access of APP to α-secretase, resulting in more APP α-secretase cleavage. Despite their more general role in endocytosis, the endophilins acted relatively specifically on APP shedding and not on the shedding of TNFR2 or PSGL-1. This finding suggests that APP stands out among other shedding substrates, in that its shedding is particularly sensitive to changes in the rate of endocytosis.

Besides the inhibition of endocytosis an additional mechanism might contribute to endophilin A3's stimulatory effect on APP shedding. Endophilin A3 and its homologs A1 and A2 bind to the metalloproteases ADAM9 and ADAM15 (Howard et al., 1999). The endophilins have been suggested to be involved in the intracellular trafficking and maturation of these ADAM proteases (Howard et al., 1999), but it remains unknown whether this interaction affects the ADAM protease activity. Since ADAM9 is one of the candidate α-secretases for APP (Koike et al., 1999), it is tempting to speculate that endophilin A3 may not only increase APP shedding by inhibiting APP endocytosis but additionally by increasing ADAM protease activity.

Currently it is unclear how Nbl, PPT1 and MEKK2 influence the shedding of APP. However, some clues may be inferred from what is already known about them. For example, Nbl is a modulator of Notch signaling and may play a role in Notch endocytosis (Santolini et al., 2000). Moreover, Nbl has recently been shown to bind to APP, but the effect of this interaction on APP shedding has not been explored (Roncarati et al., 2002). Nbl binds to the NPXY amino acid motif in the cytoplasmic domain of APP. Other cytoplasmic proteins, such as FE65 and X11 bind to the same site and are known to affect APP shedding, potentially by altering APP trafficking. Thus, Nbl may compete with such proteins for binding to APP and thereby influence APP shedding.

Activation of the Erk- and p38-MAP-kinase pathways increases the shedding of different membrane proteins (Fan and Derynck, 1999). Since MEKK2 is one of the kinases activating the MAP-kinase pathway, it is possible that MEKK2 exerts its shedding-stimulatory function through an activation of the MAP-kinase pathway. A similar mechanism may hold for PPT1, which is a thioesterase that removes palmitoyl modifications from proteins in the lysosome. However, the two PPT1 cDNAs identified in this study differ from the published sequence of wild-type PPT1: both lack the start codon and contain a one-nucleotide insertion, giving rise to a mutant PPT1 protein without a signal peptide. Because the library used for arraying was unamplified, these are likely to be independent isolates of a relevant in vivo species. The predicted protein is not expected to localize to the lysosomes, but instead may act as a cytosolic thioesterase and mimic the function of the cytosolic palmitoyl-protein thioesterase APT1 (Resh, 1999). One possible mechanism by which the variant PPT1 may exert its shedding-inducing effect might be through H-Ras, which upon depalmitoylation moves from lipid rafts to the bulk plasma membrane and can activate the Map-kinase pathway (Prior et al., 2001), which in turn can activate the shedding of various proteins (Fan and Derynck, 1999). This proposal is consistent with the finding that the variant PPT1 also stimulates the shedding of TNFR2 and PSGL-1.

In summary, we have shown that it is possible to identify proteins that are able to regulate ectodomain shedding. Besides the proteolytic processing of APP, ectodomain shedding regulates a variety of diverse biological processes, among them embryonic development (Peschon et al., 1998) and axonal pathfinding (Hattori et al., 2000). To date the pathways that regulate these processes have not been explored in much detail. Our study suggests that a relatively well-conserved set of proteins with connections to central signaling pathways, such as PKA and MEKK2, may be involved in the shedding of many, if not all shedding substrates, whereas other proteins, such as the endophilins or mGluR3, may preferentially control the shedding of a small number of membrane proteins.

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