Soluble amyloid-β precursor protein binds its cell surface receptor in a cooperative fashion with glypican and syndecan proteoglycans

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Summary

Proteolytic processing of amyloid-β precursor protein (APP) generates the amyloid-β peptide, which plays a central role in Alzheimer disease. The physiological function of APP and its proteolytic fragments, however, remains barely understood. Here we show that, on the basis of its binding characteristics, the secreted ectodomain of APP (sAPP) is a new member of the heparin-binding growth factor superfamily. Like other of its members, sAPP binds in a bivalent manner to the plasma membrane with two different subdomains. The N-terminal growth-factor-like domain (GFLD) is necessary and sufficient for protein-receptor binding, whereas the E2-domain mediates interaction with membrane-anchored heparan sulfate proteoglycans (HSPGs). The membrane-anchored HSPGs function as low-affinity co-receptors for sAPP and enhance the affinity to the sAPP receptor. Our findings provide a solid basis for the further identification of this receptor.

Key words: Amyloid precursor protein (APP), Alzheimer, Ectodomain of APP, sAPP, APP-receptor, Proteoglycan, Growth factor

Introduction

Despite advances in our understanding of amyloid-β precursor protein (APP) processing in Alzheimer disease, its normal physiological function has proven more difficult to elucidate (Turner et al., 2003; Anliker and Müller, 2006). Several membrane-anchored proteins interact with the extracellular domain of APP and modulate APP processing (Rice et al., 2013), such as APP family members themselves (Soba et al., 2005; Kaden et al., 2008), TAG-1 (Ma et al., 2008), Notch family members, the Nogo-66 receptor (Wolfe and Guénette, 2007), BRI2 (Fotinopoulou et al., 2005; Matsuda et al., 2005) and Pancortins (Rice et al., 2012). Another class of binding partners for APP comprises extracellular matrix components, such as collagen, laminin, fibulin-1 and heparan sulfates (Turner et al., 2003; Reinhard et al., 2005), and NgCAM and contactins (Osterfield et al., 2008). Both classes of protein were studied in the context of membrane-anchored APP. Recent data, however, suggest that the main biological function of APP is carried out by its secreted ectodomain sAPP. This ectodomain is necessary and sufficient to rescue prominent abnormalities of APP-KO mice such as impairment in spatial learning and long-term potentiation (Ring et al., 2007). Likewise, rescue of the apl-1 lethality in *C. elegans* is possible by expression of the extracellular domain of APL-1, the homolog of APP in *C. elegans*. (Hornsten et al., 2007). Thus, more focus on sAPP and its interaction with the cell surface is needed.

sAPP has a complex domain organization (Fig. 1A) see also (Reinhard et al., 2005). It has been associated with neurite outgrowth, synaptogenesis, neurogenesis, cell survival and cell proliferation (Turner et al., 2003). These cellular effects point to a function as growth factor and imply key differences in biological functions of sAPP and its membrane anchored precursor. Thus, sAPP-specific cell surface receptors should exist that are likely to be different from the proteins discussed above. The existing literature remains diffuse and even contradictory with regard to the domains of sAPP that are involved in receptor binding. Several heparin-binding sites in sAPP are postulated to bind also to heparan-sulfate proteoglycans (HSPGs) (Clarris et al., 1997). The growth-factor-like domain (GFLD) activates mitogen-activated protein kinase (Greenberg et al., 1995) and stimulates synapse formation (Morimoto et al., 1998). Finally, the penta-peptide RERMS in the E2-domain seems to bind to a cell surface receptor and to stimulate cell growth and neurite extension (Jin et al., 1994; Ninomiya et al., 1994). Thus, a more systematic analysis of sAPP-binding to the cell surface and the specific contribution of its subdomains is needed.

Results and Discussion

Expression of sAPP$_{695}$α-receptors on neurons and CHO cells

We cloned sAPP$_{695}$α (hereafter referred to as sAPP) and its subdomains GFLD and E2 into bacterial expression vectors carrying N-terminal fusion tags (Fig. 1B). As shown in Fig. 2A (middle panel), Xpress-tagged sAPP binds strongly to neurons, with a slight preference for the neurites. The observed signal is specific as it could be competed by adding a tenfold excess of S-tagged sAPP (Fig. 2A, right panel). Similar results were obtained

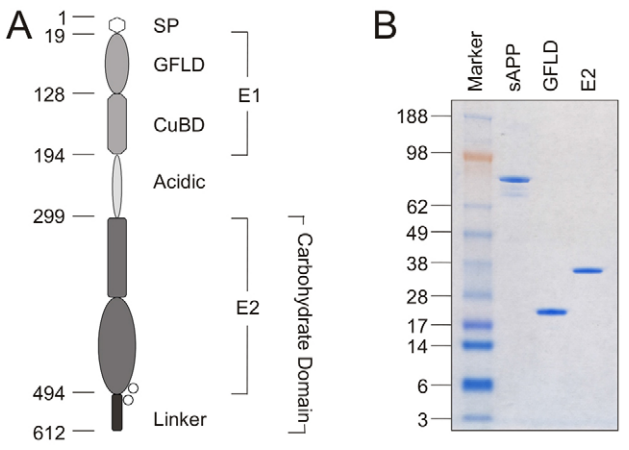

Fig. 1. Domains in secreted amyloid precursor protein ectodomain. (A) Domain organization of $sAPP_{695}\alpha$ (elsewhere in this article referred to as sAPP).The signal peptide sequence (SP) is followed by the E1-domain, which consists of the N-terminal growth factor-like domain (GFLD) and the copper-binding domain (CuBD). The E1-domain is linked through the acidic region to the carbohydrate domain, which contains two N-glycosylation sites (open spheres). The carbohydrate domain is subdivided into the E2-domain and the linker region. The numbers indicate the amino acid residues. (B) Coomassie blue staining of sAPP, GFLD and E2.

with CHO wild-type cells (CHO-WT, Fig. 2B). Under the conditions used in this study, binding of sAPP appears to be independent of membrane-anchored APP or APLP1 and/or APLP2 since the neuroblastoma cell line B103, which does not express any member of the APP-family, and APP and/or $APLP2^{-/-}$ mouse embryonic fibroblasts are still able to bind sAPP. Thus, APP-independent receptors exist and their binding-properties are further characterized in this study.

Binding of sAPP through membrane-anchored heparan-sulfate proteoglycans

To investigate binding of sAPP to glycosaminoglycans (GAGs) and to discriminate between GAG and protein-protein-mediated binding at the cell-surface, we compared binding of sAPP between wild-type Chinese hamster ovary (CHO-WT) cells and two CHO cell lines deficient in the biosynthesis of GAGs. The CHO-677 cell line lacks N-acetylglucosaminyltransferase and glucuronyltransferase and is, therefore, defective in HSPGs (Esko et al., 1988). The CHO-745 cell line lacks xylosyltransferase, which initiates GAG-formation by transferring xylose to serine residues of the core protein, and is therefore completely deficient in GAGs (Esko et al., 1985). We radiolabeled sAPP with ^{125}I (specific activity ~100 cpm/fmol) and incubated the cells with 100 nM [^{125}I]sAPP in the presence or absence of 20 μM non-labeled sAPP. Specific cell-surface binding of sAPP was calculated by subtraction of the non-specific from total sAPP binding. In line with the immunofluorescence data (Fig. 2B), CHO-WT cells strongly bound sAPP, whereas the lack of HSPGs in CHO-677 resulted in a tenfold reduction of sAPP-binding (Fig. 3A) supporting the hypothesis that HSPGs are main sAPP binding-sites. Most importantly, even in the complete absence of GAGs in the CHO-745 cells, we still observe substantial and specific binding of about 50 fmol/10^6 cells (Fig. 3A) indicative for the existence of a specific sAPP-receptor next to the proteoglycans (see below).

HSPGs are either components of the extracellular matrix or membrane-anchored molecules. The latter mainly consists of two gene families, namely type I transmembrane syndecans and glycosylphosphatidylinositol-anchored glypicans. Both families can function as co-receptors that modulate ligand-receptor interactions in cell proliferation, motility and differentiation (Park et al., 2000). Because of the heterogeneity of the heparan-sulfate (HS) chains, HSPG migrate in SDS-PAGE as smears and are difficult to detect. Treatment of intact cells with heparitinase I removes the HS chains, leaving behind the core proteins, which can be detected with monoclonal antibody 3G10 (David et al., 1992). CHO-WT were incubated with or without heparitinase I and solubilized, and proteins were analyzed by SDS-PAGE and western blotting (Fig. 3B, first two lanes). We detected bands that corresponded in their molecular sizes to those of glypicans and syndecans (Häcker et al., 2005). To confirm that, indeed, glypicans and syndecans are involved in sAPP-binding, we analysed sAPP-binding to CHO-WT cells, and to CHO cells that had been stably transfected with syndecan 2 (Syn2) or glypican 1 (Gpc1) (Fig. 3B, last two lanes). Binding assays performed with [^{125}I]sAPP revealed that higher concentrations of Syn2 or Gpc-1 at the plasma-membrane correlated substantially with increased sAPP-binding (Fig. 3C), confirming that these membrane-anchored HSPGs are mainly responsible for sAPP-binding.

sAPP binds to a protein-receptor at the plasma membrane through its growth-factor-like N-terminal domain

Although HSPGs play an important role in sAPP-binding, complete lack of GAGs in CHO-745 cells did not lead to a complete loss of sAPP-binding (Fig. 3A), implying the existence of a specific protein receptor. To determine the binding characteristics, we performed classic binding studies with sAPP. CHO-745 cells were incubated with increasing amounts of [^{125}I]sAPP in the absence (total binding) and presence of a 200-fold excess of non-labeled protein (non-specific binding). Non-linear, global curve fitting was used to determine total and non-specific binding. Total binding was defined as the sum of specific binding plus non-specific binding, assuming that non-specific binding was linear. As shown in Fig. 3D, non-specific binding of sAPP follows, indeed, a linear regression with $R^2=0.84$. Specific binding of sAPP to CHO-745 cells depicts in a hyperbolic saturation curve, best represented by a single-binding-site model [$Y=B_{Max}\times X/(K_D+X)$]. In four independent experiments, the K_D (81 nM±35 nM) and B_{Max} (50.000±8.400 molecules per cell) for sAPP were determined. Surprisingly, incubation of CHO-745 cells with concentrations of sAPP above 500 nM resulted in an additional increase in binding of sAPP (data not shown), which is indicative for a second binding site with very low affinity (≥5 μM) and high B_{Max}. This additional very-low-affinity binding site might consist of glycolipids, e.g. gangliosides that interact with the heparin-binding sites in sAPP.

We focused on the high-affinity binding site. sAPP is composed of several subdomains (Fig. 1); GFLD and the E2-domain, which contains the RERMS sequence, have been associated with the neurite outgrowth and neuronal-differentiation properties of APP. Thus, we used the purified domains (Fig. 1B) and performed competition studies using [^{125}I]sAPP. CHO-745 cells were incubated with 100 nM [^{125}I]sAPP in the absence (total binding) or presence of 20 μM sAPP, GFLD or the E2-domain. Competition with sAPP represents the maximum competition that could be achieved,

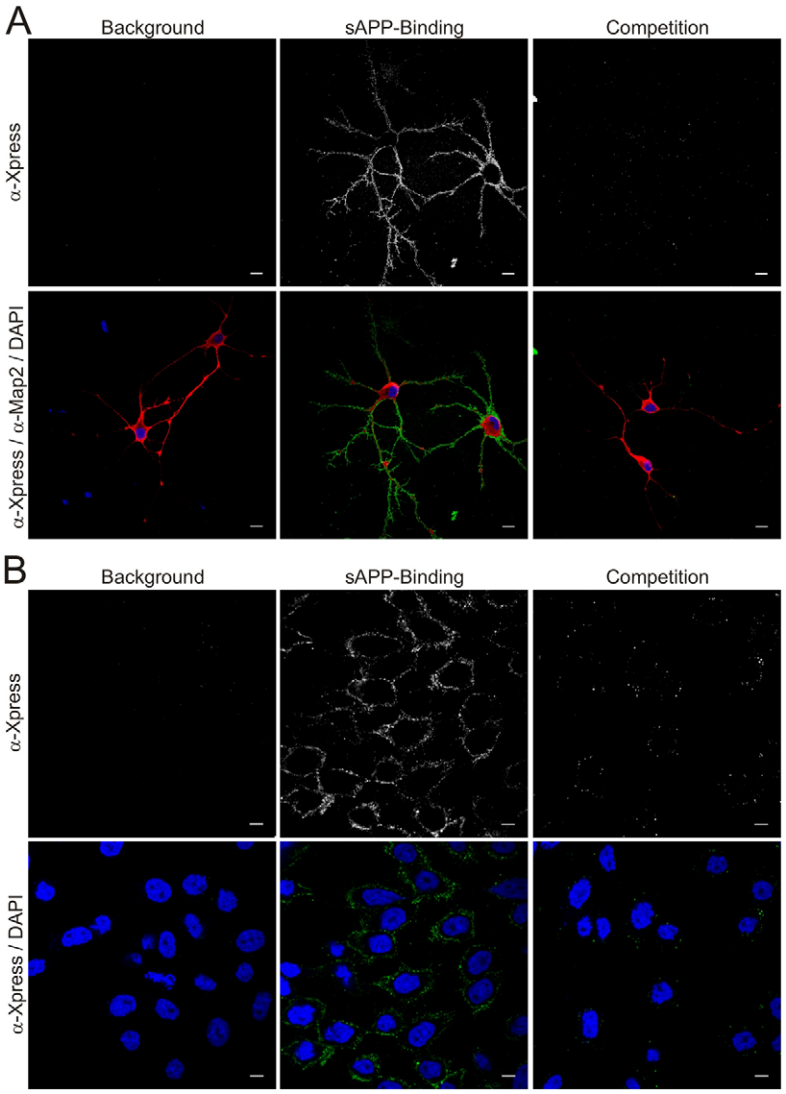

Fig. 2. sAPP-binding on hippocampal neurons and CHO cells. (A) Hippocampal neurons were incubated with recombinant sAPP at 4℃, fixed and processed using antibodies against sAPP (Xpress-epitope tag) and against Map2 as a neuronal marker, and Alexa-488- and Alexa-568-conjugated to secondary antibodies. Background, no addition of sAPP; sAPP-Binding, addition of 1 μM Xpress-tagged sAPP; Competition, addition of 1 μM Xpress-tagged and 10 μM S-tagged sAPP. Scale bars: 10 μM. (B) Similar binding studies on CHO-WT cells. Scale bars: 5 μM.

and corresponds with ~20% of total binding (Fig. 3E, non-specific binding). The GFLD significantly reduced [^{125}I]sAPP-binding (Fig. 3E) whereas, surprisingly, the E2-domain with its RERMS sequence did not compete for receptor binding at all.

To confirm that the high-affinity receptor-binding site is located in the GFLD, radiolabeled [^{125}I]GFLD (specific activity ~20 cpm/fmol) was added to cells. Again, saturated cell-surface-binding similar to that of sAPP (Fig. 3F) was obtained, with a curve fitting that is best represented by a single-binding-site model. In this case, the K_D (130 nM±60 nM, six independent experiments) is slightly higher than the K_D observed for sAPP (81 nM) but is clearly in the same range, which is consistent with our conclusion that the main receptor-binding site of sAPP is located in the GFLD.

sAPP behaves like a classic heparin-binding growth factor

We tested to what extent the GFLD is involved in sAPP-HSPG-binding by comparing GFLD-specific binding to CHO-WT cells and to GAG-deficient CHO-745 cells. Interestingly, binding was found to be similar in both cases (Fig. 4A), demonstrating that the GFLD alone is not sufficient to bind to membrane anchored HSPG. We confirmed that the GFLD binds to heparin (not shown); however, heparin is structurally quite different from HS-chains in HSPGs. Whereas heparin is a homogenous molecule with fully sulfated disaccharide units, HSPGs cluster their modifications along the HS chain, resulting in highly *N*-sulfated domains that alternate with less-modified *N*-acetyl-rich domains. Heparin-binding properties should, therefore, not necessarily be considered as a proof for binding to HSPGs. Most probably, the positive-charged surface patch of the GFLD, which is dominated by the β-hairpin loop and part of the most-mobile region in the GFLD-structure, enables the GFLD to bind to heparin but does not allow tight binding to HSPGs.

A second putative HSPG-binding site in sAPP was identified in the E2-domain. The conserved, basic amino acid residues involved in heparin-binding seem to form a groove on the protein surface (Wang and Ha, 2004), and its elongated shape makes it more suitable for tight HSPG-binding compared to the positive surface patch identified in the GFLD. We, therefore, tested the possibility whether the E2-domain binds to membrane-anchored HSPGs on CHO-WT cells. CHO-WT and CHO-745 cells were incubated with 100 nM [^{125}I]E2-domain (specific activity of ~30 cpm/fmol) in the presence or absence of 20 μM non-labeled E2-domain. CHO-WT cells displayed considerable binding of the

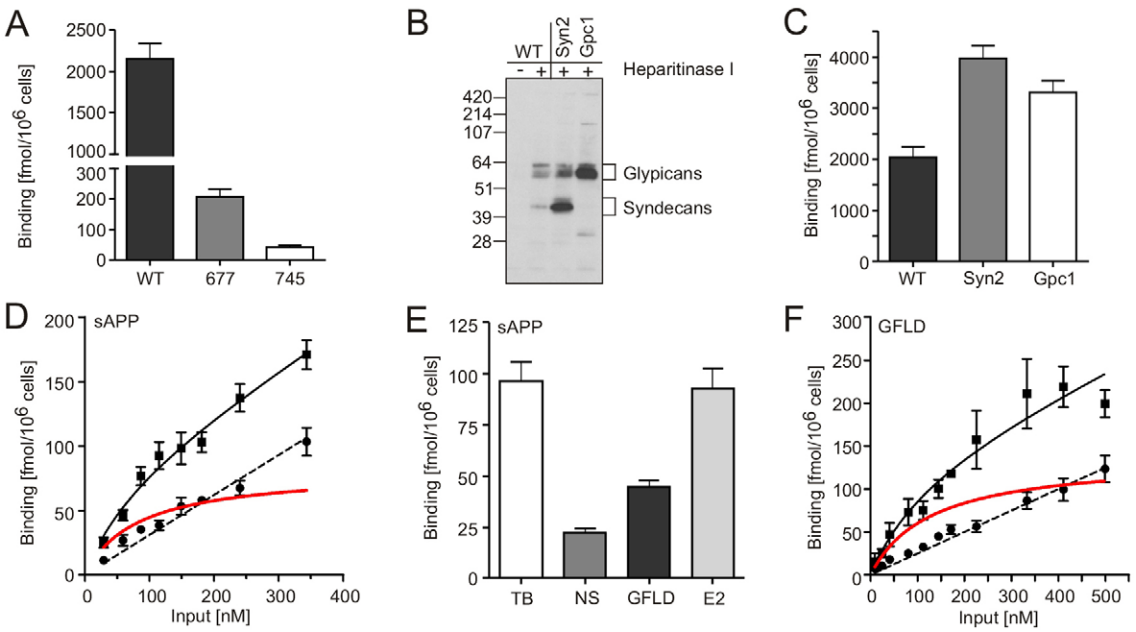

Fig. 3. sAPP-binding occurs via membrane-anchored heparan-sulfate proteoglycans and a protein-protein-mediated interaction located in the GFLD. (A) CHO-WT cells, CHO-677 cells defective in heparan-sulfate proteoglycans (HSPGs) and CHO-745 cells that fail to produce any GAGs were incubated with [^{125}I]sAPP at 4℃ for 1.5 hours. After washing the cells two times with PBS containing 1% bovine serum albumine (BSA), bound material was analyzed using a γ-counter. Specific binding to each cell line from six independent experiments (n=12) is shown. (B) After heparitinase I digestion, HSPGs present on CHO-WT cells were probed with antibody 3G10. The main bands (second lane from left) correspond to glypicans and syndecans. Overexpression of syndecan 2 (Syn2; lane 3) or glypican 1 (Gpc1; lane 4). (C) Overexpressing Syn2 or Gpc1 in CHO-WT cells results in increased sAPP-binding (n=8 in four independent experiments). (D) Graph, showing a sAPP-binding curve. CHO-745 cells were incubated with increasing amounts of [^{125}I]sAPP in the absence or presence of a 200-fold excess of unlabeled protein. Specific binding (red curve) was determined by subtracting non-specific from total binding (n=4). (E) Competition studies with individual sAPP-domains. CHO-745 cells were incubated with a constant amount of [^{125}I]sAPP in the absence (total binding, TB) or presence of a 200-fold excess of unlabeled sAPP (non-specific binding, NS). In addition, binding of [^{125}I]sAPP was measured in the presence of a 200-fold excess of unlabeled GFLD or E2-domain (n≥30 in twelve independent experiments). (F) Graph, showing a GFLD-binding curve, using [^{125}I]GFLD (n=6). Total binding, solid line; non-specific binding, dashed line; specific binding, red line.

E2-domain, whereas only residual binding to cells that were lacking GAGs (CHO-745) was observed (Fig. 4B). Thus, we concluded that the E2-domain is, indeed, mainly involved in the interaction of sAPP with HSPGs. Since the E2-domain does not contribute to protein-receptor binding (Fig. 3E), residual binding in CHO-745 cells most probably indicates the second low-affinity binding site that was observed with sAPP, possibly provided through glycolipids. To further confirm that the E2-domain provides the main HSPG-binding site, we performed competition studies with 100 nM [^{125}I]sAPP in the absence (total binding) or presence of 20 μM sAPP, GFLD or E2-domain in CHO-WT cells. Under these conditions the sAPP-protein receptor is saturated and the majority of the observed binding derived from interaction with HSPG. As expected, high total sAPP-binding could be competed with using non-labeled sAPP but no significant reduction was observed with the GFLD – which only interacts with the protein receptor (Fig. 4C). The E2-domain, however, strongly competes for binding to CHO-WT cells confirming that the E2-domain is responsible for the interaction with HSPGs. It should be noted that E2-binding to CHO-WT cells is much lower than binding of sAPP to CHO-WT cells (compare Fig. 4B with Fig. 3A, respectively), reflecting a lower affinity of the isolated E2-domain to HSPGs.

To test whether sAPP obeys a model of co-receptor-induced cooperative binding, we tested whether sAPP-binding affinity is affected by the presence of HSPGs. To this end, we measured binding of [^{125}I]sAPP to CHO-WT (Fig. 4D). In contrast to the results obtained with CHO-745 cells, the Scatchard plot of the specific binding data (Fig. 4E) revealed at least two binding sites with quite different K_D and B_{Max} values. We therefore believe that, on the basis of our previous experiments, the high-affinity binding site represents the protein-receptor interaction and the low-affinity site the binding to HSPGs. Using non-linear, global curve fitting, we obtained a K_D for the sAPP-receptor in CHO-WT cells that is lower (~10 nM) than that in CHO-745 cells (81 nM), which is in agreement with a cooperative effect between sAPP, its protein-receptor and HSPGs. Moreover, B_{Max} of sAPP is considerably increased.

Somewhat unexpectedly, our data did not confirm results of earlier *in vitro* studies that had described the peptide RERMS (a.a. 328–332) as an essential part of the receptor-binding site in APP (Ninomiya et al., 1993; Roch et al., 1993; Ninomiya et al., 1994). Short linear peptides like the RERMS sequence but also the more-potent 17-mer peptide used in these studies rarely adopt a stable structure in solution but have the propensity to adopt stable conformation upon ligand- and/or receptor-binding (induced fit, see Uversky et al., 2005 for more details). X-Ray analysis of the E2-domain revealed that the RERMS sequence is part of an α-helix that contributes to the two-stranded coiled-coil motive in the E2-domain. Moreover, two amino acid residues of the RERMS sequence directly participate in the dimerization of the E2-domain, which then conceals the RERMS motif from interactions with other proteins (Wang and Ha, 2004). Thus, the RERMS sequence is likely to represent an important APP-dimerization motif rather

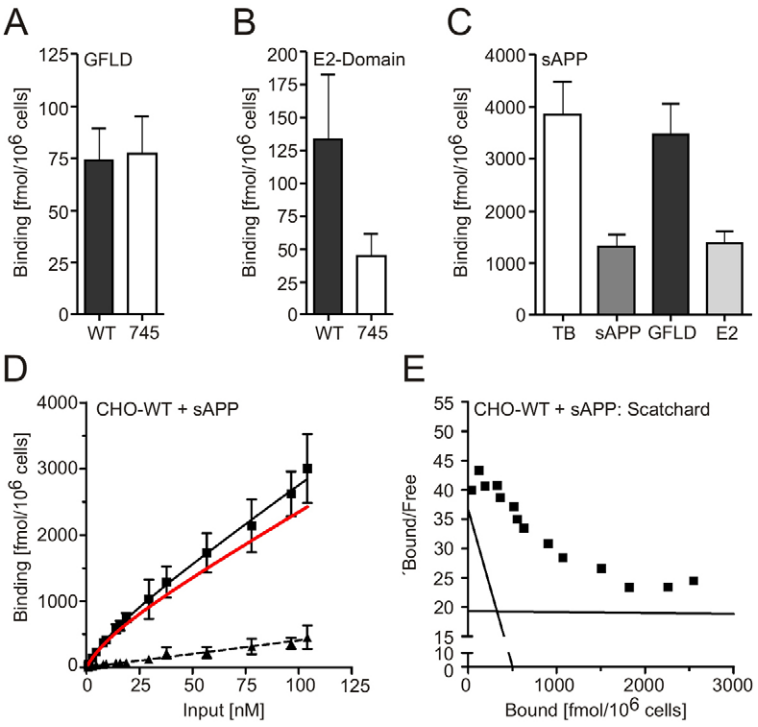

Fig. 4. Binding of sAPP to HSPGs increases the affinity of sAPP to its protein receptor. (A) Comparison of [^{125}I]GFLD binding to CHO wild type (WT) and CHO-745 cells. The GFLD lost the ability to interact with HSPGs (n=12 in six independent experiments). (B) Similar comparison performed with [^{125}I]E2-domain (n=10 in five independent experiments). (C) Competition studies with individual sAPP-domains: CHO-WT cells were incubated with 100 nM [^{125}I]sAPP in the absence (total binding, TB) or presence of a 200-fold excess of unlabeled sAPP, GFLD or E2-domain (n=6 in three independent experiments). (D) Graph showing binding of APP to CHO-WT cells. Cells were incubated with increasing amounts of [^{125}I]sAPP in the absence (total binding=solid line) or presence of a 200-fold excess of unlabeled protein (non-specific binding=dashed line). Specific binding (red curve) was determined by subtracting non-specific from total binding (n=6). (E) Specific binding of sAPP to CHO-WT cells displayed in a Scatchard plot including K_D and B_{Max} for both binding sites.

than a binding-site for a protein receptor – which, however, not excludes an indirect influence on APP-activity.

In conclusion, we provide evidence that binding of sAPP to the plasma membrane strictly follows the binding characteristics of classic heparin-binding growth factors, such as FGF. Like all heparin-binding growth factors, sAPP contains two binding sites. The GFLD is necessary and sufficient for protein-receptor binding, and the E2-domain mediates interaction with HSPGs. Many growth factors are synthesized as type I membrane precursor proteins, and shedding of the ectodomain is the crucial event to release the active growth factor. APP shows a strikingly similar behavior and, in order to understand the biological function of APP, it is important to distinguish between membrane-bound and soluble APP. Indeed, membrane-bound growth factor precursor proteins can exert biological functions that are distinct from their soluble counter parts – as demonstrated, for example, for proHB-EGF and HB-EGF (Iwamoto and Mekada, 2000). Whereas the biological role of membrane-anchored APP is dedicated to cell-adhesion and cell-migration, sAPP acts as a classic heparin-binding growth factor that regulates cell proliferation and differentiation. Our work, thus, clarifies the different roles of the subdomains in sAPP in HSPG binding, and provides evidence for an sAPP protein receptor, whose identification is anticipated to shed more light on the function of APP.

Materials and Methods

Antibodies

Monoclonal antibodies anti-Xpress™ (Invitrogen) and 3G10 (David et al., 1992), and polyclonal anti-Map2 (Santa Cruz Biotechnology), peroxidase-labeled anti-mouse (Bio-Rad Laboratories) and Alexa-488- and Alexa-568-labeled antibodies (Invitrogen) were used in this study.

Purification of His-tagged proteins

sAPP (amino acids 19–612), GFLD (amino acids 19–128) or E2-domain (amino acids 299–494) were cloned in the pET30b(+) (Novagen) and pTrcHisB vectors (Invitrogen), and expressed in the *E.coli* Origami2™(DE3) host strain (Novagen). Expression of recombinant proteins was induced by 0.5 mM IPTG. Cells were collected by centrifugation, lysed in BugBuster® Master Mix (Novagen) and recombinant proteins purified using the ÄKTAprime™ system (GE Healthcare Life science). Clarified protein extracts were loaded on a HisTrap™ HP column (GE Healthcare Life Science), equilibrated with PBS (20 mM NaH_2PO_4 pH 7.4) containing 300 mM NaCl and 40 mM imidazole. His-tagged proteins were eluted by a linear gradient from 40–500 mM imidazole. Fractions containing the purified protein were identified using SDS-PAGE analysis, combined and dialysed against PBS containing 150 mM NaCl. Protein concentration was measured using the Bio-Rad protein assay and aliquots were stored at −80°C.

Analysis of sAPP binding using indirect immunofluorescence

Primary mouse hippocampal neurons and CHO-WT cells, plated on coverslips 2 days before the experiment, were washed with 1×DPBS without Ca^{2+}, Mg^{2+} and Phenol Red (DPBS$^{-/-}$; Invitrogen) and incubated on ice with 1 µM Xpressed-tagged sAPP in the presence or absence of 10 µM S-tagged sAPP in DPBS$^{-/-}$ containing 1% BSA (PBS-BSA) for 1.5 hours. Cells were washed with DPBS$^{-/-}$ and processed for indirect immunofluorescence. Images were captured on a confocal microscope (Radiance 2100; Carl Zeiss Microimaging, Inc.) connected to an upright microscope (Eclipse E800; Nikon) and using an oil-immersion plan Apo 60×A/1.40 NA objective lens. Final processing was done using Lasersharp 2000 (Carl Zeiss Microimaging, Inc.) and Photoshop (Adobe) restricted to limited linear color balance adjustments.

^{125}I-binding assays

sAPP, GFLD and the E2-domain were labeled with [^{125}I]iodine using Iodo-Gen® precoated tubes (Pierce) according to manufacturer's instructions and purified with PD-10 desalting columns. For binding studies, cells were detached with 0.5 mM EDTA in DPBS$^{-/-}$, collected by centrifugation and adjusted to a final concentration of 1×10^6 cells per 30 µl PBS-BSA. The reaction was carried out for 1 hour on a rotation wheel in 300 µl PBS-BSA containing 1×10^6 cells, 0.5 µM EDTA, the iodinated protein with or without proteins for competition as indicated. Cells were washed and bound material was analyzed using a γ-counter (PerkinElmer). All steps were carried out at 4°C and with pre-chilled buffers. Data were analyzed using GraphPad 4.0 and a global non-linear fitting approach that allowed fit of total and non-specific binding at one time to the raw, experimentally determined binding data. Bars represent standard errors obtained from at least three independent experiments.

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Author contributions

C.R. and B.D.S. conceived the study. C.R. and M.B. performed all experiments, G.D. assisted with the proteoglycan experiments, C.R. wrote the first version of the MS. All authors interpreted and discussed data, and wrote and read the manuscript.

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