sAPP modulates iron efflux from brain microvascular endothelial cells by stabilizing the ferrous iron exporter ferroportin

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Abstract

A sequence within the E2 domain of soluble amyloid precursor protein (sAPP) stimulates iron efflux. This activity has been attributed to a ferroxidase activity suggested for this motif. We demonstrate that the stimulation of efflux supported by this peptide and by sAPP α is due to their stabilization of the ferrous iron exporter, ferroportin (Fpn), in the plasma membrane of human brain microvascular endothelial cells (hBMVEC). The peptide does not bind ferric iron explaining why it does not and thermodynamically cannot promote ferrous iron autoxidation. This peptide specifically pulls Fpn down from the plasma membrane of hBMVEC; based on these results, FTP, for ferroportin-targeting peptide, correctly identifies the function of this peptide. The data suggest that in stabilizing Fpn via the targeting due to the FTP sequence, sAPP will increase the flux of iron into the cerebral interstitium. This inference correlates with the observation of significant iron deposition in the amyloid plaques characteristic of Alzheimer's disease.

Keywords blood-brain barrier; brain iron metabolism; ferroportin; iron transport; sAPP

Subject Categories Membrane & Intracellular Transport; Molecular Biology of Disease; Neuroscience

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Introduction

Deposition of iron (and copper) within the amyloid plaques associated with Alzheimer's disease suggests that misregulation of such redox-active, first-row transition metals is a significant if not causative factor in the progression of this neurodegenerative disease [1–7]. A mechanistic connection between iron and plaque formation in Alzheimer's was recently attributed to a ferrous iron oxidation activity within a relatively short sequence of the sAPP protein; based on this putative activity, this 22-amino acid peptide was designated FD1 for ferroxidase domain 1 [8]. This

motif is illustrated in Fig 1 based on the sequence of APP695, the most dominant sAPP form expressed in the brain [9]. The conclusion was argued that this peptide, specifically the REWEE motif within it (Fig 1A), and its suggested 'ferroxidase' activity could explain the relationship between iron and β -amyloid aggregation [8].

Ferroxidase activity is taken as the ability of a species to accelerate the electron transfer from Fe^{II} to O_2 ; this reaction is commonly referred to as autoxidation [10]. Physiologically, in mammals, the oxidation of ferrous iron by dioxygen is due to the multicopper ferroxidases, ceruloplasmin (Cp) and hephaestin (Hp) [11–15]. Cp is expressed in both a soluble, secreted form (sCp) and a GPI-linked and therefore membrane-bound form [16–18]; the orientation of GPI-Cp places the ferroxidase domain in the exo-cytoplasmic space. Hp is expressed exclusively as a type I membrane protein, tethered to the plasma membrane with an exo-cytoplasmic orientation of the ferroxidase domain [13–15]. Both proteins are known to support iron efflux from cells via the ferrous iron exporter, ferroportin (Fpn) [19–23]. In regards to ferroxidase activity provided by FD1, this conclusion has been challenged [24,25]. The putative ferroxidase activity of this APP-derived peptide was postulated to mimic the ferroxidase activity of Cp and Hp in support of iron efflux through Fpn [8]. However, a more direct analysis of the capacity of 'FD1' [25] or of the APP E2 domain [24] to support ferrous iron autoxidation failed to detect any such activity associated with this region of the sAPP protein.

This negative result, however, did not address the compelling cell biologic observation that sAPP did, in fact, stimulate ^{59}Fe -efflux through the Fpn expressed in either HEK293T cells or primary mouse neurons. The authors presenting these results correlated this stimulation with the ferroxidase activity they had claimed for their sAPP species [8]. Thus, one is left with the significant result that sAPP does modulate cell iron efflux but without any insight into the mechanism of this important physiologic regulatory effect. Here, we demonstrate that this small APP domain binds to Fpn and that it and sAPP α stabilize Fpn in the plasma membrane of hBMVEC. This stabilization accounts for sAPP-dependent stimulation of Fe-efflux. Based on these findings, this APP sequence is designated FTP, ferroportin-targeting peptide.

Figure 1. FTP sequence within the E2 domain of amyloid precursor protein does not bind or oxidize iron.

- A Amino acid numbering is based on the sequence of APP695, the most prevalent splice variant expressed in the brain [9].
- B The autoxidation of 200 μ M ferrous ammonium sulfate in 100 mM MOPS, pH 7.2 was determined by polarographic quantification of O_2 consumption. Additions to the buffer were citrate, 250 μ M; glutamate, 50 μ M; HEDTA, 250 μ M; FTP, 10 μ M. All traces were corrected for the addition to the 1 ml air-equilibrated reaction buffer of 20 μ l of the N_2 -equilibrated Fe^{II} -substrate stock solution. Initial velocity values for ferrous iron autoxidation (dioxxygen consumption) are illustrated. The sole statistically significant difference ($P < 0.01$) was the initial rate supported by HEDTA. All other values were not different from buffer alone.
- C Isothermal titration calorimetry quantifies the lack of detectable formation of an FTP, Fe^{III} complex. A MicroCal VP-ITC unit was used to examine the heat content changes associated with the addition of 10 μ l aliquots of a 1:3 mixture of ferric ammonium sulfate (7 mM) and disodium citrate (21 mM) to FTP (70 μ M) in 100 mM MES buffer, pH 6.0. Heat content changes were converted to binding enthalpies and equilibrium binding constants using the Origin software operating system.
- D As a positive control for transition metal ion binding, the association constant and enthalpy of association of Cu^{II} to the substrate binding site in the yeast metallo-oxidase, Fet3, was quantified by ITC. The conditions for this titration were as above with 10 μ l injections of 2 mM cupric chloride and 20 μ M Fet3. In both experiments, values are corrected for control additions of metal ion to buffer alone.

Results

The APP peptide FTP is not a 'ferroxidase' catalyst as it has no measurable affinity for Fe^{III}

'Catalysis' of ferrous iron autoxidation (electron transfer directly to O_2) correlates directly to $\log K_{spII}/K_{spIII}$ or $\log \beta_{III}/\beta_{II}$ (where β is the overall stability constant of a ferrous or ferric iron complex); this relationship holds since the relative stability of a ferric iron complex modulates the standard reduction potential of the Fe^{III}/Fe^{II} couple and thus the driving force for electron transfer to O_2 [10]. The classic example of this relationship is the pH dependence of aqueous iron's reduction potential, +771 mV at pH = 0, and -540 mV at pH = 14 due solely to the 10^{22} -fold greater insolubility of $Fe(OH)_3$ in comparison to $Fe(OH)_2$ under

standard basic conditions. Kinetically, Fe^{II} is a better reductant at neutral or alkaline pH values because ferric iron is relatively so insoluble.

Thus, to catalyze ferrous iron oxidation by O_2 (ferroxidation), a species has to bind at least ferric iron; if it does not, then any O_2 -consumption linked to Fe^{II} oxidation is due to other factors that contribute to the thermodynamic distribution of iron forms in solution, for example, the $[H^+]/[OH^-]$ ratio. At pH = 7.2, the rate of O_2 -consumption in the absence of factors that potentiate electron transfer from Fe^{II} is low since at that pH, Fe^{II} is a weak reductant ($E^0 = +400$ mV). Addition of HEDTA, a common Fe^{III} chelating agent ($\log \beta_{III} = 19.7$), strongly 'catalyzes' Fe^{II} oxidation as quantified by O_2 consumption (Fig 1B). Citrate, a weaker Fe^{III} ligand ($\log \beta_{III} = 11.8$), exhibits little activity in stimulating O_2 -consumption when added at 250 μ M, its concentration in the

cerebrospinal fluid, CSF [26]. This relationship between ligand strength and rate of ferrous iron autoxidation has been illustrated previously [27]. The FTP, as reported [25], exhibits a comparable lack of activity in this direct assay of ferrous iron autoxidation; its activity is not different than that exhibited by 50 μ M glutamic acid, the concentration of glutamate found in the CSF [26]. In summary, even at the likely non-physiologic concentration of 10 μ M (value for sAPP [28,29]), FTP exhibits no more ferroxidase activity in this assay than that due to common carboxyl group containing metabolites found in the CSF; the composition of this compartment is directly dependent on the metabolite production rate in the brain itself [30]. Using ferrozine as an indicator of ferrous iron in solution, we confirmed that FTP was not a ferroxidase (Supplementary Fig S1).

FTP does not potentiate ferrous iron oxidation because it does not bind Fe^{III} as determined by ITC analysis of the interaction between FTP and Fe^{III} (Fig 1C). Based on the concentrations of Fe^{III} and FTP used in this experiment, an upper limit on the association constant of the Fe^{III} and FTP was estimated to be 1 mM. As a positive example of transition metal ion binding data, data for Cu^{II} binding to the metallo-oxidase substrate site in the yeast ferroxidase, Fet3 [31], are shown as well (Fig 1D). The Cu^{II} -Fet3 association constant derived from these data was 1.3×10^5 M.

FTP binds to Fpn in hBMVEC

Fpn pull-down assays using human or mouse brain homogenates indicated that full-length sAPP interacted with endogenous Fpn [8]. To test the hypothesis that FTP was a motif in sAPP associated with this apparent binding, we used FTP-FLAG in a co-immunoprecipitation assay probing for an interaction between FTP and endogenous human brain microvascular endothelial cell (hBMVEC) Fpn. Together with underlying glial cells, hBMVEC make up the blood-brain barrier that regulates the transport of circulating iron into the brain interstitium [32–35]; regulation of this transcellular iron trafficking occurs at the basolateral (brain) side of hBMVEC primarily as a result of the presence and activity of the ferrous iron exporter, ferroportin (Fpn) [36,37]. In these cells, Fpn (arrow) and Fpn:Fpn dimer (open arrowhead) were co-immunoprecipitated with FTP-FLAG as demonstrated by the retention of Fpn on a Protein A/G affinity matrix programmed with anti-FLAG IgG (Fig 2A). Fpn was not retained on the matrix lacking FLAG-specific IgG (-Flag antibody). Fpn species of higher molecular mass (Fig 2A, filled arrowheads) were also observed in the unbound fractions. Internalized Fpn that has undergone post-translational modification and migrates with an apparent molecular mass ≥ 100 kDa has been detected previously [38,39]. In comparison to these intracellular Fpn species, unmodified, monomeric Fpn is thought to dominate in the plasma membrane; significantly, in this pull-down experiment, this latter Fpn form was effectively captured by Fpn-FLAG. The Western blot of a 10-fold concentrated input sample shown in Fig 2B demonstrates that this form of Fpn represented a small fraction of the cell total of this protein; this finding was consistent with the model that surface expression of Fpn is controlled at the level of cycling in and out of the plasma membrane [40]. Specificity of our antibody was confirmed via immunoblots of lysates from HEK293T cells transfected with pFpn-GFP (Supplementary Fig S2).

FTP stimulation of ^{59}Fe -efflux from hBMVEC requires a multicopper ferroxidase working in *trans*

Previous studies on the putative role of sAPP as a ferroxidase in cell iron efflux were compromised in that endogenous ferroxidase activity likely supported the reported efflux behavior [8]. For our experiments, copper depletion (via bathocuproine disulfonate (BCS) treatment) was used to down-regulate the expression of hephaestin (Hp), or GPI-Cp, in the hBMVEC plasma membrane. Loss of Fpn from the plasma membrane accompanies this loss of endogenous multicopper ferroxidase(s), leading to a knockdown of Fe-efflux activity [19].

The knockdown of plasma membrane Fpn and Fe-efflux due to BCS treatment of hBMVEC is documented in Fig 3A and B, respectively. Addition of sCp to the efflux medium (thus supplementing for the absence of endogenous Hp or Cp) stabilizes Fpn in the membrane and supports Fe-efflux. In contrast, while 10 nM FTP or sAPP α stabilizes membrane Fpn, neither supports Fe-efflux (Fig 3A and B). In the context of the demonstrated lack of FTP ferroxidase activity, the simplest interpretation of this result is that cell Fe-efflux depends on the presence of Fpn in the plasma membrane and on a ferroxidase activity, an inference fully consistent with the current model for ferroportin-mediated iron trafficking [19,20,41]. Note that the immunofluorescence images in Fig 3A were obtained on unpermeabilized hBMVEC using an Fpn-specific antibody that recognized an extracellular loop [42,43]; thus, the images specifically demonstrate the loss of surface Fpn; the species that the pull-down assay suggests is uniquely recognized by FTP-FLAG (Fig 2).

The interpretation that a ferroxidase activity was required to support Fpn-mediated Fe-efflux was further supported by the results of the experiment in which FTP was complemented with the addition of a fungal ferroxidase, that is, the yeast Fet3. Fet3 alone neither stabilized Fpn in the membrane (Fig 3A) nor rescued iron efflux in BCS-treated hBMVEC (Fig 3B). In contrast, addition of Fet3 to FTP-treated cells resulted in the iron efflux that FTP-treated cells alone did not exhibit (Fig 3B). This level of iron efflux was equivalent to that supported by sCp. This FTP-Fet3 combination allowed us to demonstrate directly the requirement for ferroxidase activity in Fpn-supported iron efflux. That is, addition of the ferroxidase-inactive Fet3 C484S mutant, Fet3-T1D [44], to the BCS- and FTP-treated cells failed to elicit the iron efflux exhibited by those cells treated with wild-type Fet3. We propose that FTP, by interacting with Fpn, acts as a co-factor to achieve the first of these requirements but, lacking ferroxidase activity of its own, fails to fulfill the second. These data indicate that the activities documented for sAPP in regard to cell iron efflux are due at least in part to the FTP motif [8].

Discussion

This brief report makes three significant contributions to our understanding of the mechanism of Fpn-supported iron efflux from brain microvascular endothelial cells and the regulation of this iron trafficking process by sAPP. First, our data, together with previous reports [24,25], demonstrate directly that FTP is not a ferroxidase; we specifically demonstrate the lack of Fe^{III} -FTP interaction that thermodynamically predicts this outcome. Second, we demonstrate that FTP and sAPP α stabilize the iron exporter, Fpn, in the plasma

Figure 2. FTP interacts with Fpn in hBMVEC.

- A hBMVEC were loaded for 24 h with 10 μM FeCl₃ plus 50 μM citrate to promote endogenous Fpn expression. FTP-FLAG (2 μM) was then added to the iron-loaded hBMVEC for 1 h after which the cells were lysed and processed for Protein A/G affinity analysis. Bound and unbound fractions from the experimental Protein A/G matrix (+Flag antibody) and control matrix (–Flag antibody) are displayed in a Western blot probing for Fpn. Control is Protein A/G columns devoid of FLAG-specific IgG (–). Both monomeric (arrow) and multimeric/modified (arrowheads) forms of Fpn were detected; however, no monomeric Fpn was detected in input and unbound samples.
- B Note that the binding and elution protocols used in (A) resulted in an eight-fold concentration of Fpn in the bound fraction. The Western blot shown in (B) was consistent with this prediction; that is, concentration (10×) of the input demonstrated the presence of monomeric Fpn (arrow).

membrane; this is a novel finding. Thus, in the presence of the ferroxidase activity provided by either or both Cp (either sCp or GPI-CP) and Hp, FTP (or sAPP) increases Fe-efflux by increasing the number of Fe-exporters in the membrane, not by increasing the molecular activity of the exporters that are present in the membrane as has been suggested [8].

Last, using the combination of FTP and yeast Fet3 in ferroxidase-negative hBMVEC, we provide evidence that Fpn-supported efflux of ferrous iron requires coupling to a ferroxidase reaction as has been proposed [19]. In effect, we have separated the two functions Hp and Cp contribute in *cis* in support of Fpn-mediated trafficking of ferrous iron out of a cell: stabilization of Fpn in the membrane and ferroxidase activity. By adding either active or inactive Fet3 in *trans* to BCS- and FTP- or sAPPα-treated cells, we have demonstrated directly that the ferroxidase activity provided by wild-type but not mutant Fet3-T1D is required for this iron efflux process.

In conclusion, sAPP forms that contain the E2 domain and its FTP sequence will modulate (increase) Fe-efflux from any cell type in the brain that expresses Fpn and, also, either expresses a ferroxidase, or is exposed to sCp secreted by another cell type. To the extent that sAPP increases Fpn at the hBMVEC basolateral (brain) membrane, iron uptake into the brain interstitium will be enhanced. Both hBMVEC and astrocytes express APP [45–47]. Indeed, Aβ accumulation is first seen in the vicinity of BMVEC (cerebral amyloid angiopathy) where it appears to induce toxic effects such as oxidative stress, ion channel dysfunction, inflammation, and apoptosis [48,49]. The abundance of data that indicates an association between amyloid plaque and iron deposition [50–52], together with the results presented here, suggests that dys-regulation of brain iron due to sAPP potentiates the aggressiveness of the pathophysiology underlying Alzheimer's disease. A fundamental question in iron biology that remains to be answered is: in regards to normal brain iron homeostasis, what role does this sAPP Fe-efflux modulation serve? Certainly, the fact that APP expression appears to be translationally regulated by iron status via the canonical iron regulatory

protein 1 pathway [53,54] indicates the likelihood that it does play such a role. The work presented here suggests an entirely new direction in the investigation of the factors that regulate iron trafficking in mammals.

Materials and Methods

Detailed methods can be found in Supplementary Methods.

Polarographic analysis of ferrous iron autoxidation

Oxygen consumption was quantified using an Oxygraph (Hansatech, Norfolk, UK) and the OXYG32 software provided by Hansatech.

Isothermal titration calorimetry

A MicroCal VP-ITC (GE Healthcare Life Sciences, Piscataway, NJ) unit was used for these analyses.

hBMVEC ⁵⁹Fe-efflux

All ⁵⁹Fe-efflux assays were performed using confluent monolayers of hBMVEC grown in 24-well tissue culture dishes as previously described [36]. Additions to the efflux media included either BCS (500 μM), FTP (10 nM), sCp (6.6 nM), wild-type Fet3 (6.6 nM), Fet3-T1D (6.6 nM), sAPPα (10 nM), or a combination of components.

Indirect immunofluorescence

hBMVEC were processed for Fpn indirect immunofluorescence as previously described with modifications [37]. Briefly, cells were fixed, blocked, and exposed to primary Fpn antibody (ab85370)

Figure 3. FTP and the fungal ferroxidase Fet3 synergistically support ⁵⁹Fe-efflux from ferroxidase-deficient hBMVEC.

A hBMVEC monolayers were probed for Fpn by indirect immunofluorescence. Prior to fixing, the cells had been Cu-depleted by treatment with BCS (500 μ M) for 24 h, followed by an additional 24 h with BCS plus either FTP (10 nM), WT-Fet3 (6.6 nM), sCp (6.6 nM), or sAPP α (10 nM). Images are 63 $\times$ (scale bar, 20 μ m). See Supplementary Fig S3 for DAPI counterstain.

B hBMVEC treated as in (A) were loaded with ⁵⁹Fe-citrate for 24 h after which point ⁵⁹Fe-efflux assays were performed; the concentrations of the additions are as noted above (Fet3-T1D; 6.6 nM). The percent loss of hBMVEC-associated ⁵⁹Fe at 24 h relative to $t = 0$ h was quantified. One-way ANOVA statistical analyses were used to determine significance of the differences relative to control BCS-treated cells at *** $P < 0.001$. Data are represented as means $\pm$ SD ($n = 3$ –6, experimental replicates).

(Abcam, Cambridge, MA) (1:100) followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:1000, Invitrogen Life Technologies, Carlsbad, CA). Images were obtained at 63 $\times$.

Co-immunoprecipitation assay

hBMVEC were incubated with 10 μ M FeCl₃ plus 50 μ M citrate for 24 h. After the 24 h incubation, 2 μ M FTP-Flag was added to each dish, and the hBMVEC were incubated for 1 h. hBMVEC were lysed, and the lysates were pre-cleared and added to protein A/G

agarose containing anti-Flag antibody (+) or not (–). Flow-through and eluate were resolved on a 10% SDS-PAGE gel and probed for Fpn.

Statistical analyses

All statistical analyses were performed using Prism 4.0 or 5.0 software (GraphPad, La Jolla, CA).

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

DJK conceived the project and wrote the manuscript; RCM reviewed and made suggested changes to this draft. RCM and Y-HP planned and carried out experiments and analyzed the resulting data.

Conflict of interest

The authors declare that they have no conflict of interest.

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