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Background: Oligomeric complexes of APP, APLP1, and APLP2 contribute to synapse formation and structure.
Results: Zinc binding to the E2 domain of APP and APLPs promotes their oligomerization in the cell, most notably with APLP1.
Conclusion: Extracellular zinc is a regulator for structure and function of APP and APLPs.
Significance: Novel insight into how APP and APLP function is regulated at the molecular level.

The amyloid precursor protein (APP) and the APP-like proteins 1 and 2 (APLP1 and APLP2) are a family of multidomain transmembrane proteins possessing homo- and heterotypic

surface, whereas APP and APLP2 are primarily found in intracellular compartments. APLP1 is exclusively found in the nervous system (18, 20), and APLP1-heparin complexes are only formed by the E2 domain (21, 22). In contrast, APP and APLP2 are ubiquitously expressed and can bind heparin through their E1 and E2 domains (23–25). The ectodomains of APP and APLPs are not only mediators of cell-extracellular matrix interactions (26). All APP family members have been shown to bind metal ions (copper, zinc) through their extracellular E1 domain (27–29). Moreover, we have previously shown that metal ions can also bind to the extracellular E2 domain of APP, thereby affecting the conformation and flexibility of the domain (30).

Because little is known about how extracellular ligand binding to APP, APLP1, and APLP2 affects these proteins in the cell, we have now examined zinc and copper binding to isolated domains of APP and APLPs, as well as to the full-length proteins at the cellular level. Our detailed biophysical findings have determined that zinc and copper can bind to the E2 domain of all APP family members, but zinc binding is sterically restricted in the case of APLP2. Live cell FRET analyses and confocal microscopy revealed that zinc ions mediate the oligomerization of APP and APLPs, but APLP1 is predominantly regulated by zinc among the three family members. Notably, we have determined that APLP1 oligomerization is mediated by a novel zinc-binding site in its E2 domain. We also present detailed biochemical evidence that zinc binding and oligomerization are intimately linked, impacting the structural and functional properties of APLP1 as well as of APP and APLP2.

EXPERIMENTAL PROCEDURES

—APP, APLP1, APLP1-ΔE1-AcD, and APLP2 were cloned in pcDNA3-CFP, pcDNA3-YFP, and pcDNA3-FLAG custom-made vectors as described previously (7). Point mutations in the APLP1 sequence were introduced by PCR-based site-directed mutagenesis using partially overlapping primers (31). APLP1 deletion constructs were generated from the full-length sequence by PCR-mediated deletion (32). Amino acids 290–494, 290–580, or 614–650 were deleted to generate APLP1 ΔE2, APLP1 ΔE2-JMR, or APLP1 ΔCT, respectively. Recombinant E2 and E2-JMR proteins of APP, APLP1, and APLP2 were expressed in with the pPICZα vector system (Invitrogen). Sequences encoding amino acids 365–566 of APP, 290–495 or 290–566 of APLP1, and 370–565 of APLP2 were introduced into the pPICZα vector via the EcoRI restriction site at the 5′ end and with an additional stop codon at the 3′ end. For APP E2, an additional ATT codon was introduced between the EcoRI restriction site and the APP sequence. Sequences of all vectors were verified by DNA sequencing (GATC).

cultures were precultured in BMGY, pelleted, and resuspended in expression medium BMMY (pH 6 or 7) with 0.5% methanol at $_{600}$ of 1.0. Supernatants containing APP E2, APLP1 E2, APLP1 E2-JMR or APLP2 E2 were collected 24 h after methanol induction and purified in a two-step chromatography procedure using HiTrap Blue HP (GE Healthcare) columns and a HiLoad 16/60 Superdex 200 column (GE Healthcare).

In the presence of buffer (“native” protein scan), $ZnCl_2$, and/or EDTA (inhibit metal-binding), fluorescence spectra were recorded using an excitation wavelength of 295 nm (slit width □ 5 nm), and emission was recorded from 305 to 400 nm (slit width □ 5 nm; scanning speed □ 50 nm/min). The ActA peptide (Ac-SFEWPPPPT-NH_2) was kindly provided by Dr. Robert Opitz (FMP Berlin). For each set of experimental conditions, the acquired spectra were corrected for dilution effects and for background emission of the buffer.

—For gel filtration-coupled SEC-SLS analyses, 100 μ

reversed by addition of a 10-fold excess of EDTA. To exclude a generic effect of zinc ions on the Trp residue in the E2 domains in APP, APLP1, and APLP2, we repeated our fluorescence assay using a derivative of the ActA class of EVH1 ligands (38). As a negative control, this peptide contained a single Trp residue but no histidines or cysteines, which are the major zinc-binding residues of proteins (39). Titrating zinc to even higher concentrations (100 μM) did not significantly alter the emission spec-

predictions for monomeric (theoretical mass of 24.4 kDa) or dimeric APLP1 E2. Subsequent shape- and conformation-independent SLS analyses confirmed an absolute $mass_{abs}$ of 26.6 ± 0.1 kDa (Fig. 2*A*), which is in good agreement with the theoretical mass of monomeric APLP1 E2. In the absence of zinc, we conclude that recombinantly purified APLP1 E2 is monomeric at the employed experimental conditions. Similarly to APLP1 E2, the E2 domain of APP was also shown to exist in a monomeric form in solution (40). In the presence of 10 μM $ZnCl_2$, a significant peak broadening was observed with APP E2 and

cases) was increased in the presence of zinc (Fig. 4). In contrast, APP/APLP2 hetero-oligomerization was affected at significantly higher zinc concentrations (EC_{50} □ 231 □ 76 μM). This finding suggests that APLP2 preferentially forms hetero-oligomers with APLP1 (rather than homo-oligomers) at low zinc concentrations.

Overall, we have demonstrated that zinc increases the oligomerization of APP family members in the plasma membrane of living cells. Moreover, we have shown that the zinc-induced oligomerization of these full-length proteins resembles oligomerization of the isolated E2 domains of APP, APLP1, and APLP2 at low micromolar zinc concentrations. Based upon its subcellular localization and maximal sensitivity, oligomerization of APLP1 appears to be predominantly affected by extracellular zinc ions among the APP family members.

—To analyze whether zinc binding and oligomerization of APP family proteins in the plasma membrane affect their localization, we examined YFP-tagged APP, APLP1, and APLP2 in living HEK293 cells with confocal laser scanning microscopy (cLSM). At basal conditions, APLP1 was primarily localized to the cell surface, whereas APLP2 was strongly retained in intracellular compartments (Fig. 5). APP levels at the plasma membrane were below the limit of detection, preventing an analysis of membrane-localized APP by cLSM. The addition of zinc ions to the incubation buffer resulted in the formation of large, distinguishable clusters of APLP1 or APLP2 at the plasma membrane (Fig. 5). The effect occurred within 30 s after the addition of zinc and was stronger for APLP1 than for APLP2, consistent with a larger impact of zinc on APLP1 oligomerization compared with APLP2. Chelation of the metal ions by EDTA reversed the Zn^{2+} effect, and the cluster-like structures disappeared (Fig. 5). As a negative control, GypA was used. GypA distribution at the plasma membrane was not altered by zinc addition (Fig. 5). Thus, zinc ions specifically triggered the redistribution of APLP1 at the plasma membrane, with an attenuated effect on APLP2.

To further assess the metal ion specificity of APLP1 or APLP2 clustering, we tested other divalent metal ions. Neither 50 μM Co^{2+} nor concentrations as high as 1 mM Ca^{2+} or 1 mM Mg^{2+} ions altered the cellular localization of APLP1 or APLP2 (Fig. 5). The only other metal ion tested that induced APLP1 and APLP2 clustering was copper (Fig. 5), which was consistent with copper effecting APLP1 oligomerization as recorded by SE-FRET measurements.

Because APLP1 expression in contrast to APP and APLP2 is restricted to the nervous system (18), we tested whether zinc could induce clustering of APLP1 in the plasma membrane of rat primary hippocampal neurons. Within seconds, zinc ions

the candidate histidines to alanines and then re-evaluated zinc-

APP and its paralogs APLP1 and APLP2. Through a series of complementary biochemical and cellular studies, we have now shown the following: (i) the E2 domains of APP, APLP1, and APLP2 can all bind zinc and copper, but the zinc ion-binding site of APLP2 E2 is less surface-exposed; (ii) zinc binding can mediate oligomerization of the E2 domains in APP and APLP1 but not APLP2; (iii) zinc ions can rapidly induce homo- and hetero-oligomerization of APP, APLP1, and APLP2 on the cell surface with a concomitant redistribution of APLPs into protein clusters observed in two different cell types, HEK293 cells and primary hippocampal neurons; and (iv) a novel zinc-binding site in the E2 domain of APLP1 promotes its oligomerization.

Based upon APP homology (21), it was proposed that APLP1

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g . g s g .*C e . Re .* **108,** 1 1 1
3. s , ., , ., , ., , ., , ., , ., s -
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