A Single Tyrosine Residue in the Amyloid Precursor Protein Intracellular Domain Is Essential for Developmental Function*

Received for publication, January 10, 2011, and in revised form, January 22, 2011
Published, JBC Papers in Press, January 25, 2011, DOI 10.1074/jbc.C111.219873

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The Aβ-precuror protein (APP) intracellular domain is highly conserved and contains many potentially important residues, in particular the 683YENPTY687 motif. To dissect the functions of this sequence in vivo, we created an APP knock-in allele mutating Tyr682 to Gly (Y682G). Crossing this allele to APP-like protein 2 (APLP2) knock-out background showed that mutation of Tyr682 results in postnatal lethality and neuromuscular synapse defects similar to doubly deficient APP/APLP2 mice. Our results demonstrate that a single residue in the APP intracellular region, Tyr682, is indispensable for the essential function of APP in developmental regulation.

The pathogenic model of Alzheimer disease (AD) posits that aggregates of Aβ, one of the many products of Aβ-precursor protein (APP) processing, cause dementia (1). However, it cannot be excluded that alterations of normal APP functions contribute to AD pathogenesis, and it is therefore important to understand the role of APP in vivo. Mice deficient in APP exhibit subtle phenotypes (2–5) and have given limited information about the functions of APP. APP is a member of a gene family that includes APP-like protein 1 and 2 (APLP1 and APLP2); functional redundancy compensates for the loss of essential gene functions in APP knock-out mice as documented by the evidence that APLP1−/−, APLP2−/−, and APP−/− mice are viable, whereas combined APP−/−APLP2−/− double KO (dKO) mice (6, 7) die shortly after birth. Analysis of APP/APLP2 dKO mice identified an essential role for the APP family of proteins and for the highly conserved APP intracellular domain in the patterning of neuromuscular junction (NMJ) (8–11), suggesting that the intracellular region of APP is functionally important in vivo.

Numerous cytosolic proteins bind this region of APP, and these protein-protein interactions have been reported to regulate both processing and functions of APP polypeptides (12). Most interactions of APP with cytosolic partners involve the YENPTY sequence of APP, which is conserved from Caenorhabditis elegans to mammalian APP/APLPs. Phosphorylation of Tyr682 is consequential. Some proteins interact with APP only when Tyr682 is phosphorylated (13–16), others only when this tyrosine is not phosphorylated (17). These data suggest that phosphorylation/dephosphorylation on Tyr682 modulates APP interactions and function.

The evidence points to an important role for Tyr682 and its potential functional regulation by phosphorylation. The early postnatal lethality and the diffused synaptic distribution of the NMJ present in the APP/APLP2 double knock-out animals provide sensitive and specific readouts for us to definitely determine the role of this amino acid in vivo. We have created APP knock-in (ki) mice in which Tyr682 is replaced by a glycine (we will refer to these mice as APPYG). The Y682G mutation was chosen to destroy interactions mediated by those involving both phospho-Tyr682 and non-phospho-Tyr682. The evidence that the APPYG mutant protein is still phosphorylated in vivo on Thr668 (18) indicates that the structure of the AID is not grossly altered by the mutation. We report that APPYG/YG/APLP2−/− mice present NMJ deficits and early lethality similar to APP/APLP2 double KO mice, demonstrating that Tyr682 is indispensable for the essential function of APP in developmental regulation.

EXPERIMENTAL PROCEDURES

Mice and Ethics Statement—Mice were maintained on a C57BL/6 background for several generations (at least 15). Mice were handled according to the Ethical Guidelines for Treatment of Laboratory Animals of Albert Einstein College of Medicine. APP-ki generation and genotyping have been described (18). Genotyping for the APP and APLP2 KO alleles were performed as described on the Jackson Laboratory web site.

Mouse Brain Preparations and GST Pulldown Experiments—Brains were homogenized (w/v = 10 mg of tissue/100 ml of buffer) in tissue homogenization buffer (20 mM Tris base, pH 7.4, 1 mM EDTA, 1 mM EGTA) supplemented with protease and phosphatase inhibitors. The postnuclear supernatant was prepared by precipitating the nuclei and debris by centrifuging the homogenates at 1000 × g for 10 min. GST fusion proteins were produced and purified as described (19). The binding experiments were performed using ~6 μg (200 pmol) of GST or GST-Mint1 phosphotyrosine binding (PTB) domain (16) following the methods described previously (19). To detect the bound APP, we used the 22C11 (Chemicon) antibody in Western blot analysis.

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**Immunofluorescence Staining—** The muscle dissection, preparation, staining, and quantification of the neuromuscular synapses have been previously described by Wang et al. (8, 10). Confocal images were obtained with a Zeiss 510 laser-scanning microscope, and quantification was done using the ImageJ program from the National Institutes of Health. Antibodies were: anti-synaptoophysin (Dako, 1:250); anti-neurofilament (Developmental Studies Hybridoma Bank (DSHB) 1:500); anti-APP (Epitomics Inc., Y188, 1:250); and α-Alexa Fluor 488/555/647-conjugated secondary antibodies and α-bungarotoxin (Molecular Probes).

**Statistical Analysis—** Genotyping analysis of the offspring from APP$^{+/-}$ APLP2$^{+/-}$ male and female intercrosses was performed using $\chi^2$ analysis. The Student’s $t$ test was used for all other analyses ($*, p < 0.05; **, p < 0.01; ***, p < 0.001$). Data were presented as the average ± S.E.

**RESULTS**

**Expression of APP$^{YG}$ on APLP2-null Background Leads to Early Postnatal Lethality—** To assess whether Tyr682 mediates the essential functions of APP, we tested whether APLP2 KO mice carrying the APP$^{YG}$ mutation have a lethal phenotype, similar to the APP/APLP2 2KO mice. We intercrossed double heterozygous mice harboring one allele each of the APP and APLP2-null mutations (APP$^{YG/-}$ APLP2$^{+/-}$). We then determined the genotypes of the offspring at postnatal day 1 (P1) and day 28 (P28) and compared the number observed against the number expected (Fig. 1, A and B). Genotyping of P1 pups revealed a close to Mendelian distribution of all genotypes, indicating no embryonic lethality as expected (χ-square analysis at P1: $\sum d^2/e = 3.531, df = 8, p > 0.95$). However, genotyping of offspring from the same cross at P28 identified very few surviving APP$^{YG/-}$ APLP2$^{+/-}$, APP$^{YG/-}$ APLP2$^{+/-}$, or APP$^{+/-}$ APLP2$^{+/-}$ mice, and the number of homozygous mutant mice significantly deviated from the predicted Mendelian ratio (χ-square analysis at P28: $\sum d^2/e = 31.406, df = 8, p < 0.001$). To confirm these data, we intercrossed APP$^{YG/+}$ APLP2$^{+/-}$ mice. Again, all nine possible genotypes were represented according to the predicted Mendelian distribution at P1. However, at P28, we only identified one surviving APP$^{YG/+-}$ APLP2$^{+/-}$ mouse. These results demonstrate that expression of APP$^{YG}$ cannot rescue the postnatal lethality of the APP/APLP2 double deficient mice.

**Defective Neuromuscular Synapse Development in APP$^{YG}$ Knock-in Animals—** Using a monoclonal antibody that recognizes the C-terminal sequences of APP (Y188), we previously reported that APP is localized to the synaptic sites of NMJ (10). We performed immunostaining of the homozygous APP$^{YG}$ knock-in mice and found that the knock-in product can be recognized by the Y188 antibody and that the staining pattern was indistinguishable from that of wild-type APP (Fig. 2A), suggesting that the APP$^{YG}$ mutant protein can be transported and targeted to the synaptic terminals similar to the wild-type APP. Despite the apparent synaptic expression of APP$^{YG}$, analysis of the neuromuscular synapse at P0 stage showed that as compared with APP$^{+/-}$ APLP2$^{+/-}$ littermate controls, the APP$^{YG/+-}$ APLP2$^{+/-}$ mutants exhibited widened neuromuscular synapses (Fig. 2B and quantified in Fig. 2D), with significant localization of synaptic vesicle proteins in extrasynaptic compartments and a correspondingly reduced apposition of presynaptic markers with postsynaptic receptors (Fig. 2C and quantified in Fig. 2E). The degree of the defects was comparable with the APP$^{+/-}$ APLP2$^{+/-}$ animals reported previously (8, 10). These results demonstrate that the Tyr682 residue is indispensable for APP-mediated survival and neuromuscular synapse function.
Presynaptic differentiation induced by APP may involve intracellular association with Cask and Mint1 (10) (also termed X11/H9251, herein referred as Mint1), similarly to neurexin/neuroligin (NX/NL) and the SynCAM class of synaptic adhesion proteins (20–22). Because the PTB motif of Mint1 binds the YENPTY sequence of the APP intracellular domain (23), we asked whether mutation of Tyr682 interfered with the formation of a Mint1-APP complex. To test for this, we produced a recombinant protein in vitro in which the PTB domain of Mint1 was fused to GST for production and purification from bacterial cultures. As a control, we produced GST on its own (16). These recombinant proteins were used for pulldown experiments from mouse brain lysates. GST-Mint1 interacts with APP in samples isolated from WT mice. The interaction is specific because GST does not bring down APP (data not shown), and a molecule reacting with the Y9251-APP antibody is not isolated by GST-Mint1 when brain lysates from APP KO mice are used (Fig. 2F).

Some residual binding between GST-Mint1 and APP is only visible after long exposure. Mint1 belongs to a gene family that also comprises Mint3. Both Mint1 and Mint3 bind APP and have opposite effects on the localization of AID (24). Here we focused on Mint1 because only Mint1 interacts with Cask. In the trans-synaptic interaction.
tion model, we have previously proposed that for APP function in synaptogenesis, APP-Mint1-CASK is likely the central complex mediating APP effect (10). As discussed, Mint1-CASK complex has also been implicated in neurexin-neuroligin-mediated signaling in presynaptic organization (20–22). Altogether, these results and considerations support the view that the Tyr682 mutation abolishes presynaptic functions of APP by impairing the recruitment of Mint1.

**DISCUSSION**

We report here that expression of an APP moiety bearing a mutation on Tyr682 fails to rescue the early postnatal lethality and neuromuscular synapse defects of the APP/APLP2-null mice. These data indicate that Tyr682 of the highly conserved APP intracellular region is required for APP-mediated survival and neuromuscular synapse assembly in vivo. Genetic studies in *C. elegans* and mammals have suggested that the ectodomain of APP is necessary and sufficient to recapitulate essential functions of APP in development and have argued for a dispensable role of the APP intracellular domain. The APL-1 extracellular domain rescues the apl-1-null lethality (25), and the α-secretase-cleaved APP ectodomain complements the anatomical and behavioral abnormalities of the APP-deficient mice (26). However, more recent studies present contrasting results. Analysis of mutant mice expressing either the sAPPβ (27) or an APP mutant lacking the intracellular domain (11) suggests that APP intracellular sequences are required to rescue the early postnatal lethality and neuromuscular synapse defects of the APP/APLP2-null mice. It needs to be pointed out that in the latter study, the human Aβ sequence with familial AD mutations and the C-terminal mutation were simultaneously introduced in mice. Therefore, it is conceivable that changes in the Aβ region contribute to the lethality and NMJ defects.

Given these opposing results, we have made use of an APP knock-in mutant that bears a single amino acid mutation in Tyr682 of the intracellular domain (18). The specific nature of this mutation allows for a direct and precise analysis of the functional requirements of APP. Our data not only clearly show this mutation allows for a direct and precise analysis of the functional requirements of APP. The APL-1 extracellular domain and the functional motif including this tyrosine may contribute to AD pathogenesis. In this perspective, it will be important to unveil the biological mechanisms that regulate phosphorylation of APP on Tyr682 and the signaling pathways that are controlled by this functional domain of APP.

Mounting evidence supports a role for synaptic dysfunction in AD. Our finding that Tyr682 of APP plays a role in synaptogenesis makes it a legitimate possibility that the APP intracellular domain and the functional motif including this tyrosine may contribute to AD pathogenesis. In this perspective, it will be important to unveil the biological mechanisms that regulate phosphorylation of APP on Tyr682 and the signaling pathways that are controlled by this functional domain of APP.

ACKNOWLEDGMENT—We thank Erhan Ma for help in the genotyping and PCR procedures on the APP knock-in mice.

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