Homer2 interacts with Plasma Membrane Ca^{2+} ATPase and regulates Its Activity in Mouse Parotid Gland Acinar Cells

Eun Ho Yang

Department of Dental Science

The Graduate School, Yonsei University

Homer2 interacts with Plasma Membrane Ca^{2+} ATPase and regulates Its Activity in Mouse Parotid Gland Acinar Cells

Directed by Professor Dong Min Shin

The Master's Thesis

Submitted to the Department of Dental Science,

The graduate School of Yonsei University

in partial fulfillment of

the requirements for the degree of

Master of Medical Science

Eun Ho Yang

November, 2007

This certifies that the Master's Thesis

of Eun Ho Yang is approved.

_____

Thesis Supervisor: Prof. Dong Min Shin

_____

Thesis Committee: Prof. Syng-Ill Lee

_____

Thesis Committee: Prof. Jeong Taeg Seo

The Graduate School

Yonsei University

November, 2007

Acknowledgements

I would like to appreciate Professor Dong Min Shin, Vice Director of Medical Research affairs, Yonsei Health System. He always supported through out my training and graduate school course with warm concerns and academic knowledge. I would like to express my deepest appreciations to Professor Syng-Ill Lee, Director of Brain Korea 21 Project, College of Dentistry, for thoughtful advice and guide from start to the end of my doctoral degree. I would like to have a deepest appreciation to Professor Jeong Taeg Seo, a chairman of Oral Biology. Also, I would like to thank so much to research assistant Hae Jo. Without his help, I could not accomplish my experiments effectively. Finally I would like to thank my family, parents, father-in-law and mother-in-law, my loving wife, Jung Min Park, daughter, Jung Eun, and son, In Sung.. Whenever I was in difficulty, they always gave me energy to overcome.

Contents

List of Figures

ABSTRACT

Homer2 interacts with Plasma Membrane Ca^{2+} ATPase and regulates Its Activity in Mouse Parotid Gland Acinar Cells

Yang, Eun Ho

Department of Dental Science

The Graduate School, Yonsei University

(Directed by Professor Dong Min Shin)

The Homers are scaffold proteins and consist of an N-terminal Ena/VASP homology 1 (EVH) protein-binding domain and C-terminal leucin zipper/coiled-coil domain. The EVH domain recognizes the proline-rich motifs (PPXXF, PPXF, and LPSSP) and binds many Ca^{2+} signaling proteins including G protein-coupled receptors (GPCRs), inositol 1,4,5-triphosphate (IP_3) receptors (IP_3Rs), ryanodine receptors (RyRs), and TRP channels. Therefore, Homers critically control Ca^{2+} signaling and thereby regulate

dendritic spine morphogenesis, remodeling of synapses, and synaptic clustering of CNS neurons, and lead to changes in neuronal transcriptional activity. However, their role in Ca^{2+} signaling of non-neuronal cells is not well known, except that Homer2 tunes GPCRs stimulus intensity by regulating RGS proteins and PLCβ GAP activities in pancreatic acinar cells. In the present work, the role of Homer2 in Ca^{2+} signaling in parotid gland acinar cells using Homer2$^{-/-}$ mice was investigated with microfluororimeter, immunofluoroscence, and co-immunoprecipitation. Homer2 showed polarized luminal localization in these cells, but the deletion of Homer2 did not affect a localization of IP_3Rs or IP_3R channel activity. The protein expression level of plasma membrane Ca^{2+} ATPase (PMCA) was increased in Homer2$^{-/-}$, whereas sarco/endo plasmic reticulum Ca^{2+} ATPase was not. Moreover, deletion of Homer2 increased PMCA activity and co-immunoprecipitation showed that Homer2 interacted with PMCAs. These results suggest that Homer2 may play an important role in regulation of PMCA expression and PMCA-mediated Ca^{2+} signaling in mouse parotid gland acinar cells.

Keywords: Homer2; Plasma membrane Ca^{2+} ATPase; Proline-rich motif; Parotid Gland Acinar Cells

Homer2 interacts with Plasma Membrane Ca^{2+} ATPase and regulates Its Activity in Mouse Parotid Gland Acinar Cells

Eun Ho Yang

Department of Dental Science

The Graduate School, Yonsei University

(Directed by Professor Dong Min Shin)

I. INTRODUCTION

Homers are scaffolding proteins that consists of three members, Homer 1, Homer 2, and Homer 3, and several splice variants (Fagni *et al*., 2000; Szumlinski *et al*., 2006). Homers are composed of an EVH protein-binding domain, a coiled-coil multimerization domain, and leucine zipper (Fagni *et al*., 2002). The EVH domain is a protein-protein binding module that recognizes the proline-rich motifs PPXXF, PPXF, and LPSSP (Brakeman *et al*., 1997;

Kato *et al*., 1998; Tu *et al*., 1998; Yuan *et al*., 2003), and binds the GPCR mGluR1/5, canonical transient receptor potential (TRPC) channels, inositol 1,4,5-triphosphate (IP_3) receptors (IP_3Rs), ryanodine receptors, and the Shank family of scaffolding proteins (Tu *et al*., 1998; Xiao *et al*., 1998; Tu *et al*., 1999; Feng *et al*., 2002; Shin *et al*., 2003; Yuan *et al*., 2003). These binding receptors are known to consist of a biochemical component in receptor-mediated Ca^{2+} signaling.

Intracellular Ca^{2+} is a common second messenger which has close relationships to fertilization, muscle contraction, neurotransmitter release, exocytosis, learning and memory (Berridge *et al*., 2003). Especially, there is a close connection between GPCRs and Ca^{2+} signaling in parotid acinar cell and these cells also represent an excellent model system not only for the study of Ca^{2+} signaling in general but also to investigate cross-talk between cAMP and Ca^{2+} signaling (Brini & Carafoli, 2000). Ca^{2+} release from the endoplasmic reticulum (ER) leads to activation of store-operated Ca^{2+} channels in the plasma membrane, and Ca^{2+} release and influx increase the intracellular Ca^{2+}

concentration ($[Ca^{2+}]_i$). Subsequently, the plasma membrane Ca^{2+} ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) removes Ca^{2+} from the cytosol to reduce Ca^{2+} toward resting levels until $[Ca^{2+}]_i$ stabilizes at a plateau (Kiselyov *et al*., 2003). Several previous reports demonstrated that raising cAMP potentiates Ca^{2+} release from intracellular Ca^{2+} through PKA-mediated phosphorylation of IP_3Rs and enhances the rate of Ca^{2+} clearance by complex modulation of PMCA activity in parotid acinar cells (Bruce *et al.*, 2002a; Bruce *et al.*, 2002b). Specially, IP_3Rs, which include proline-rich sequence, can be bound to Homer proteins. Accordingly, it is possible that Homer proteins has regulated effects for Ca^{2+} signaling in cells. However, their role in Ca^{2+} signaling of non-neuronal cells is not well known. Therefore, in the present work, the role and functions of Homer 2 in Ca^{2+} signaling using parotid acinar cells from wild-type (WT) and Homer 2 mutant (Homer $2^{-/-}$) mice was investigated.

II. MATERIALS AND METHODS

1. Animals

Wild-type (WT) and Homer 2 mutant (Homer $2^{-/-}$) mice have been previously described (Shin *et al*., 2003). Homer $2^{-/-}$ mice have a normal life span similar to WT littermates. All animal protocols were performed according to institutional guidelines.

2. Preparation of parotid acinar cells from WT and Homer $2^{-/-}$

WT (25-28g) and Homer $2^{-/-}$ (25-28g) mice were sacrificed by cervical dislocation. The cells were prepared from the parotids of WT and Homer $2^{-/-}$ mice by limited collagenase digestion as previously described (Zeng *et al*., 1997). After isolation, the acinar cells were resuspended in an extracellular physiologic salt solution (PSS), the composition of which was as follows (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. The osmolality of the extracellular solution

(measured with a FISKE 110 osmometer), was 310 mOsm.

3. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Cells, which were from both types, were incubated for 40 min in PSS containing 5 μM Fura-2/AM (Teflabs Inc., Austin, TX) with pluronic F-127 to enhance dye loading. Changes in $[Ca^{2+}]_i$ were measured by means of Fura-2 fluorescence, with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm at room temperature. Background fluorescence was subtracted from the raw signals at each excitation wavelength before calculating the fluorescence ratio as follows: Ratio = F_{340}/F_{380}. The emitted fluorescence was monitored with a CCD camera (Photon Technology International Inc., Lawrenceville, NJ) attached to an inverted microscope. Fluorescence images were obtained at 2 s intervals. All data are presented as means ± SEM.

4. Immunocytochemistry

The immunostaining procedure was described previously (Shin *et al*., 2003). In brief, cells from WT and Homer $2^{-/-}$ mice attached to glass coverslips were fixed and permeabilized with 0.5 ml of cold methanol for 10 min at –20℃. After removal of methanol, the cells were fixed with 4% formaldehyde for 20 min at room temperature, followed by permeabilization with 0.05 % Triton X-100. After removal of methanol or Triton X-100, the cells were washed with PBS and incubated in 0.5 ml of PBS containing 50 mM glycine for 10 min at room temperature. This buffer was aspirated and the nonspecific sites were blocked by 1-h incubation at room temperature with 0.25 ml of PBS containing 5% goat serum, 1% BSA, and 0.1% gelatin (blocking medium). The medium was aspirated and replaced with 50 μl of blocking medium containing control serum or a 1:50 dilution of Ab against Homer 2, or a 1:100 dilution of Abs against IP_3R1, 2, or 3. After incubation with the primary Ab overnight at 4℃ and three washes with the incubation buffer (same as blocking buffer, but with serum), the Abs were detected with goat anti-rabbit

or anti-mouse IgG tagged with fluorescein or rhodamine. Images were collected with a confocal LSM 510 laser scanning microscope (Zeiss, Göttingen, Germany).

5. Western blot

Protein extracts were prepared by parotid acini from WT and Homer $2^{-/-}$ mice as follows. Pure acinar cells were washed with ice-cold PBS and then resuspended in lysis buffer containing (in mM): 150 NaCl, 10 Tris (pH 7.8 with HCl), 1 EDTA, 1% NP-40, and 0.1% SDS. Protease and phosphatase inhibitors were added to the lysis buffer containing (in mM): 2 Na_3VO_4, 10 NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptine, and 10 μg/ml PMSF. The samples were then centrifuged at 12,000 rpm for 20 min at 4℃, and separated by SDS–PAGE. Proteins were probed with a 1:500 dilution of Ab against SERCA2b; 1:2000 dilution of Abs against IP_3R1, 2, and 3; and 1:200 dilution of Ab against PMCA.

6. Co-immunoprecipitation

The co-immunoprecipitation procedure was modified from Shin *et al.* (2000). Parotid microsomes of WT mice were prepared by homogenizing a minced parotid in a buffer containing (in mM): 20 Mops, 250 sucrose, 1 EDTA, 1 $MgCl_2$, 10 benzamidine, and 0.2 PMSF, pH-adjusted to 6.7 with KOH. The homogenate was centrifuged at 1,200 rpm for 10 min. The supernatant was collected and centrifuged at 1,800 rpm for 10 min at 4℃. The pellet was resuspended in the same buffer while avoiding suspension of the hard, white-colored granular fraction in the bottom of the tube. When needed, the fraction enriched in secretory granules was collected in homogenization buffer into a separate tube. To avoid protein degradation by digestive enzymes, IP was initiated immediately after completion of microsomal preparation. Parotid microsomes were extracted by a 1-h incubation on ice with a buffer containing (in mM): 50 Tris (pH 6.8 with HCl), 150 NaCl, 3 EDTA, 2 EGTA, and 0.5 % Triton X-100 supplemented with protease inhibitors (0.2 mM PMSF, 10 μg/ml leupeptin, 15 μg/ml aprotinin, 1 mM benzamidine). The

lysate was cleared by centrifugation at 7,000 rpm for 10 min. About 300 μl of the extract was further incubated with 15 μl of Sepharose A beads for 1 h at 4℃ and centrifuged for 2 min at 7,000 rpm to remove the beads. The cleared supernatant was incubated with 5 μl anti-PMCA, or 5 μl anti-Homer 2 Abs for 30 min before addition of 30 μl Sepharose A beads and an overnight incubation at 4℃ under gentle agitation. The beads were washed five times with 0.8 ml lysis buffer and stripped of proteins by boiling in a 50 μl of SDS sample buffer. To test the effect of the actin cytoskeleton on the binding of the Homer 2 and PMCA, buffer or 20 μg/ml of the NH2-terminal fragment of gelsolin was added to equal portions of beads after the second wash. After 20-min incubation at 4℃, the beads were washed three times with lysis buffer and the proteins remaining attached to the beads were released by boiling in a sample buffer. Released proteins were separated by an SDS-PAGE using 7.5% polyacrylamide gels. The separated proteins were transferred to 0.2 μm polyvinylidene difluoride membranes, and the membranes were blocked by a 1-h incubation at room temperature in 5% nonfat dry milk in a solution

containing 150 mM NaCl, 20 mM Tris (pH 7.5 with HCl), and 0.05% Tween 20 (TTBS). The Homer 2 and PMCA were detected by a 1~2-h incubation of individual membranes with the respective Abs diluted in TTBS.

7. Measurement of extracellualr calcium concentration ($[Ca^{2+}]_o$)

To measure directly the rate of Ca^{2+} efflux by PMCA, I measured the appearance of Ca^{2+} in the external medium using the procedure modified from Zhang *et al*. (1992). Intact parotid acini were washed once and then suspended in medium containing (in mM): 120 KCl, 20 NaCl, 10 glucose, 0.002 free acid fura-2/AM, and 10 HEPES, pH-adjusted to 7.4 with KOH. After initiation of fluorescence recording, 7.5 μM EGTA was added to reduce the extracellular Ca^{2+} concentration to ~100 nM. After establishing a baseline leak for ~1 min, the cells were stimulated with 1 mM carbachol. At the end of experiment, the signals were calibrated simply by adding 1 mM $CaCl_2$ and then 1 mM $MnCl_2$ to the medium as previously described (Zhang & Muallem, 1992).

8. Measurement of Ca^{2+} uptake and release from internal stores

IP_3-mediated Ca^{2+} release from internal stores was measured in SLO-permeabilized cells as described before (Xu *et al*., 1996). Cells washed with a high K^+, Chelax-treated medium were added to the same medium containing an ATP regeneration system (comprised of 3 mM ATP, 5 mM $MgCl_2$, 10 mM creatine phosphate, and 5 U/ml creatine kinase), a cocktail of mitochondrial inhibitors, 2 μM Fluo3 and 3 mg/ml SLO (Difco). In this medium, the cells were almost instantaneously permeabilized so that Ca^{2+} uptake into the ER could be measured immediately. Uptake of Ca^{2+} into the ER was allowed to continue until medium [Ca^{2+}] was stabilized. Then IP_3 was added in increasing concentrations to measure the extent of Ca^{2+} release and the potency of IP_3 in mobilizing Ca^{2+} from the ER.

9. Data analysis and statistics

All numeric values are represented as the mean ± S.E. The statistical significance of the data was determined using Student's unpaired *t*-test.

Statistical significance was set at $p < 0.05$ level.

III. RESULTS

1. Deletion of Homer 2 does not affect polarized expression of IP_3Rs and IP_3-mediated Ca^{2+} release.

The mGluRs and its related receptors are well known the targeting and binding receptors with Homer proteins (Tu *et al*., 1998; Yuan *et al*., 2003). Therefore, I first examined the localization and expression of Homer 2 and IP_3Rs in parotid acinar cells of wild-type (WT) and Homer $2^{-/-}$ mice. In WT mice, the immunoreactivity of Homer 2 and IP_3Rs was primary observed in the apical pole of parotid acinar cells, whereas this immunoreactivity did not affect expression and localization of any IP_3R isoform except for Homer 2 in Homer $2^{-/-}$ mice (Fig. 1). I next examined whether a lack of Homer 2 affects activity of IP_3Rs that regulate the Ca^{2+} content in the stores by the concentration of IP_3 and $[Ca^{2+}]_i$ (Xu *et al*., 1996; Thrower *et al*., 2001). Parotid acinar cells of WT and Homer $2^{-/-}$ mice are fully permeabilized to SLO within 10-15 sec and reduced $[Ca^{2+}]$ of the incubation medium to the 50-

80 nM range within 2 min of incubation at 37 ℃. Addition of increasing concentrations of IP_3 and the muscarinic agonist carbachol resulted in the same potency of [Ca^{2+}] increase from stores of SLO-permeabilized WT and Homer $2^{-/-}$ cells (Fig. 2A-B). Thus, no compensatory effect in localization and activity of IP_3Rs were observed in Homer $2^{-/-}$ mice.

2. Level of PMCA is selectively increased in the parotid acini of Homer $2^{-/-}$ mice

To establish whether expression of Ca^{2+} transporters is normal in Homer $2^{-/-}$ mice, I examined protein expression by Western blotting using parotid acinar cells from WT and Homer $2^{-/-}$ mice. The expression level of PMCA was significantly increased to 2.5 ± 0.1-fold of WT in Homer $2^{-/-}$ mice (n = 4, p < 0.001, Fig. 3). However, there was no significant change to SERCA2b between WT and Homer $2^{-/-}$ mice (1.2 ± 0.2 fold of WT, n = 4, p = 0.4). The results in Fig. 1 and Fig. 2 suggest that lack of Homer 2 has no effect on expression and activity of IP_3Rs. Similarly, the levels of IP_3Rs were

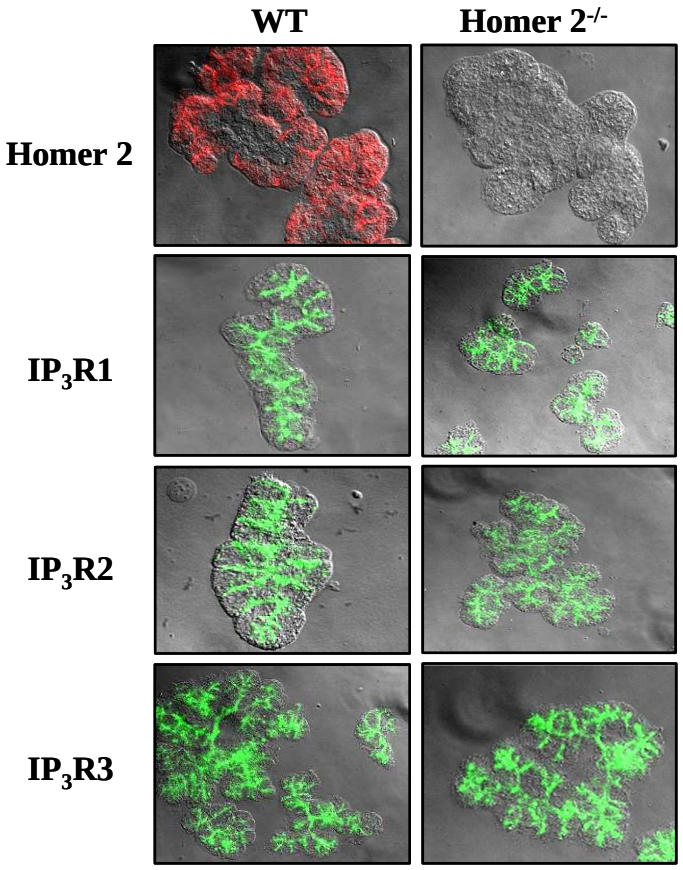

Fig. 1. Localization of Homer2 and IP_3 receptors in parotid gland acinar cells from WT and Homer $2^{-/-}$ cells.

Parotid acini from WT and Homer $2^{-/-}$ mice were stained for Homer 2, IP_3R1, IP_3R2, and IP_3R3. Note lack of effect of Homer 2 deletion on expression and localization of the IP_3Rs.

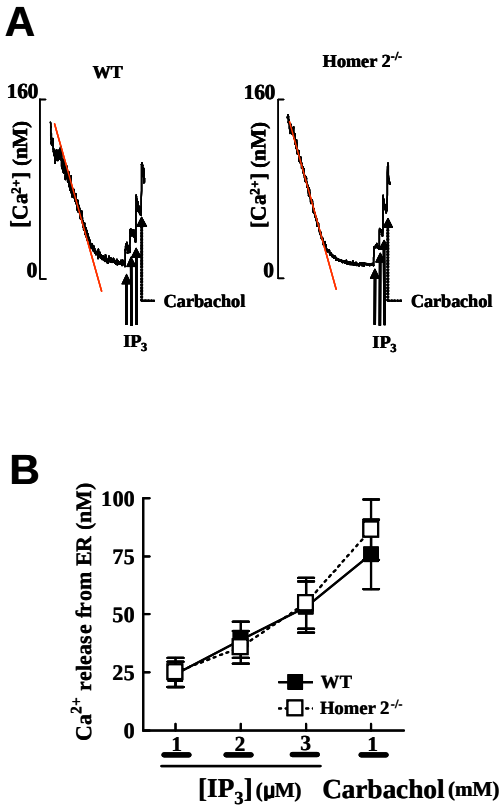

Fig. 2. Ca^{2+} uptake and IP_3-mediated Ca^{2+} release from WT and Homer $2^{-/-}$ cells.

A, Cells from WT and Homer $2^{-/-}$ mice were permeabilized with SLO and allowed to reduce $[Ca^{2+}]_i$ of the incubation medium to ~75 nM. Next, Ca^{2+} release was measured by adding increasing concentrations of IP_3 (arrows of solid lines) and carbachol (arrows of dashed lines). B, Summarizes the results obtained in the experiment with cells prepared from each of WT (solid lines) and Homer $2^{-/-}$ (dashed lines) mouse. The results were expressed as the mean ± S.E.

unaffected in Homer $2^{-/-}$ cells (n = 4, data not shown). These finding suggest that Homer 2 has effect on the Ca^{2+} signaling by changing of component that transports Ca^{2+} into and out of the cytosol.

3. The rates of [Ca^{2+}] efflux are increased in Homer $2^{-/-}$ cells.

The major routes for Ca^{2+} clearance in non-excitable cells, such as parotid acinar cells, are believed to be Ca^{2+} uptake into the ER by the SERCA, Ca^{2+} efflux across the plasma membrane by the PMCA, and Ca^{2+} uptake into mitochondria (Bruce *et al.*, 2002b; Gorr *et al.*, 2005; Homann *et al.*, 2006). The results in Fig. 2B and Fig. 3 suggest that deletion of Homer 2 has unaffected on expression and activity of SERCA. Therefore, I measured the change of $[Ca^{2+}]_o$ at low external Ca^{2+} concentration during stimulation with 1 mM carbachol. As shown in Fig. 4A, $[Ca^{2+}]_o$ was significantly increased by carbachol in both cell types and the change of $[Ca^{2+}]_o$ in Homer $2^{-/-}$ cells was approximately 1.5-fold more abundant than in WT cells (n = 4, $p < 0.01$, Fig. 4B). To examine the effect on the Ca^{2+} clearance by PMCA between WT and

Homer $2^{-/-}$ mice, I performed the experiment following inhibition of SERCA activity by 100 μM CPA. The increase in $[Ca^{2+}]_i$ reached a maximum and then declined slowly to new steady state, representing a balance of Ca^{2+} efflux and Ca^{2+} influx. Exchange the external solution, addition of 1 mM carbachol and 10 μM atropine in Ca^{2+} free solution, evoked an immediate clearance of Ca^{2+} that was primarily because of the PMCA (Fig. 5A). Then, I compared with slope of Ca^{2+} clearance during the exchanged the external solution in parotid acinar cells of WT and Homer $2^{-/-}$ mice. Under these conditions, Homer $2^{-/-}$ cells induced 1.5-fold increase in the slope of Ca^{2+} clearance (1.5 ± 0.3-fold of slope, $n = 4$, $p < 0.05$, Fig. 5B), indicating that the level of Ca^{2+} clearance due to the increase can be responsible for the potentiation of PMCA activity in the Homer $2^{-/-}$ mice.

4. Homer 2 is interacted with** PMCA **in parotid acinar cells.

To identify the specificity of interaction between the PMCA subtypes and PPXXF motif which was interacted EVH domain of Homers, I investigated

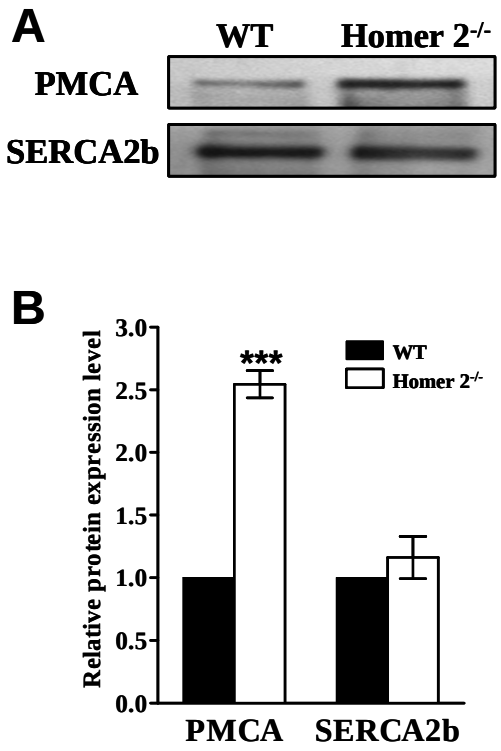

Fig. 3. Expression of Ca^{2+} transporters in WT and Homer $2^{-/-}$ cells.

A, The level of PMCA and SERCA2b were analyzed in extracts prepared from parotid acini of four WT and four Homer $2^{-/-}$ mice. Expression was analyzed by densitometry. B, Means of protein expression levels between WT and Homer $2^{-/-}$ cells. Expression level was significantly increased for PMCA, except SERCA2b in Homer $2^{-/-}$ cells. Data were normalized to the expression level in cells of WT mice and expressed as the mean ± S.E. *** $p < 0.001$ (compared with WT).

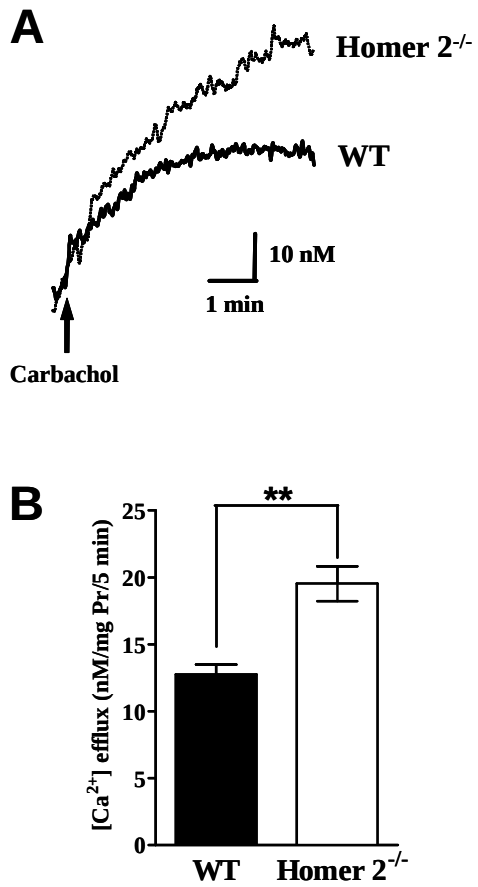

Fig. 4. Plasma membrane Ca^{2+} ATPase activity in WT and Homer $2^{-/-}$ cells.

A, The parotid acinar cells of WT and Homer $2^{-/-}$ mice were exposed to 1 mM carbachol while measuring $[Ca^{2+}]_o$. Homer $2^{-/-}$ cells increased the rate of $[Ca^{2+}]_o$ to a maximal state more activated by PMCA than cells from WT mice. Similar results were obtained using cells prepared from four WT and four Homer $2^{-/-}$ mice. B, An average rate of $[Ca^{2+}]_o$ was significantly increased in Homer $2^{-/-}$ cells and data were expressed as the mean ± S.E.

amino-acid sequences (Fig. 6). The amino-terminal 91~98 residues of PMCA subtypes are identical to the PPXXF motif. This finding suggests that Homer 2 has an interaction with PMCA subtypes. I then examined whether Homer 2 selectively binds to PMCA in parotid acinar cells of WT mice using an *in vivo* co-immunoprecipitation assay. After cell lysates were prepared, immunoprecipitated with anti-PMCA antibodies, and subsequently immunoblotted with anti-Homer 2 antibodies. As shown in left of Fig. 7, the PMCA was able to bind to endogenous Homer 2 (left of Fig. 7, *fourth lane*), whereas no signal was detected from antibodies non- treated cells (*third lanes*). When co-immunoprecipitation assay was performed in reverse with anti-Homer 2 antibodies followed by immunoblotting with anti-PMCA antibodies, the result was the same (right of Fig. 7, *fourth lane*). These results strongly suggest that the Homer 2 is associated (directly or indirectly) with PMCA subtypes.

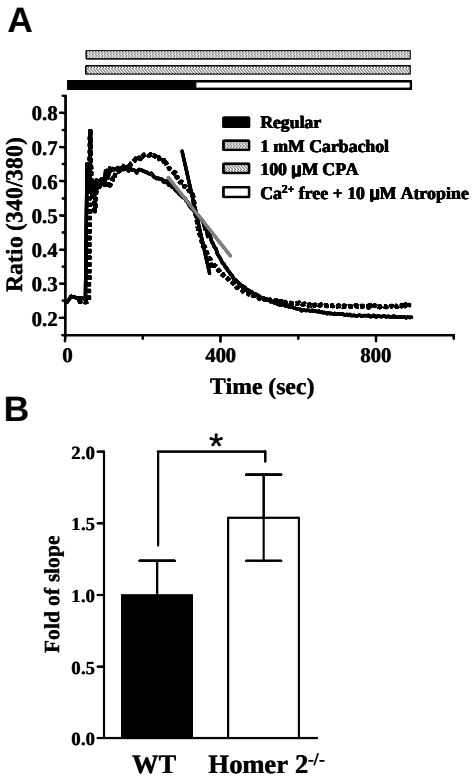

Fig. 5. Characterization of Ca^{2+} signaling in WT and Homer $2^{-/-}$ cells.

A, $[Ca^{2+}]_i$ of parotid acini of four WT and four Homer $2^{-/-}$ mice were elevated by 1 mM carbachol and inhibition of SERCA with 100 μM CPA. The increase in $[Ca^{2+}]_i$ maximum and then declined to a new steady state, representing a balance of Ca^{2+} efflux and Ca^{2+} influx. Ca^{2+} clearance was initiated by removal of external Ca^{2+} and addition of 10 μM atropine. B, An average of Ca^{2+} clearance rate was significantly increased in Homer $2^{-/-}$ cells. Data were normalized to the fold of slope in cells of WT mice and expressed as the mean ± S.E. * $p < 0.05$, ** $p < 0.01$ (compared with WT).

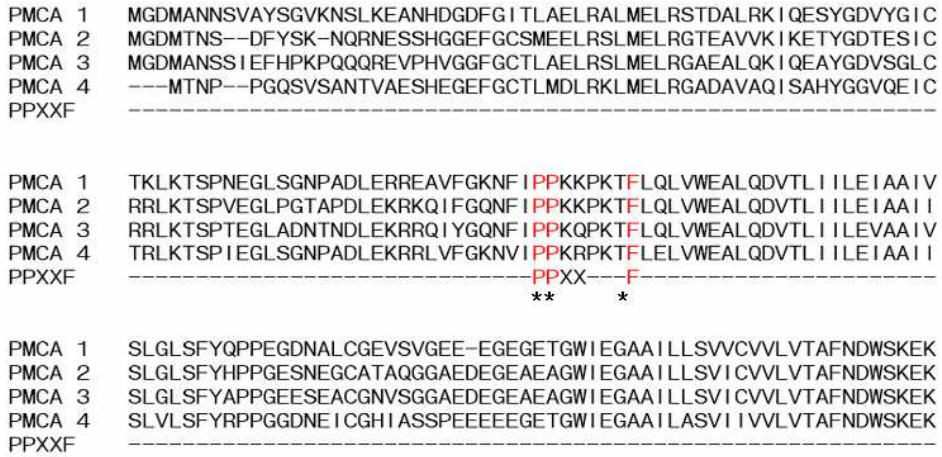

Fig. 6. Alignment of PMCAs with proline-rich motif, PPXXF, interacting with EVH domain of Homers.

Amino-acid sequence of mouse PMCA subtypes were aligned and compared with proline-rich motif, PPXXF, which was interacted with EVH domain of Homers. Colored sequences (red) indicate areas of amino-acid similarity between PMCA subtypes and PPXXF motif.

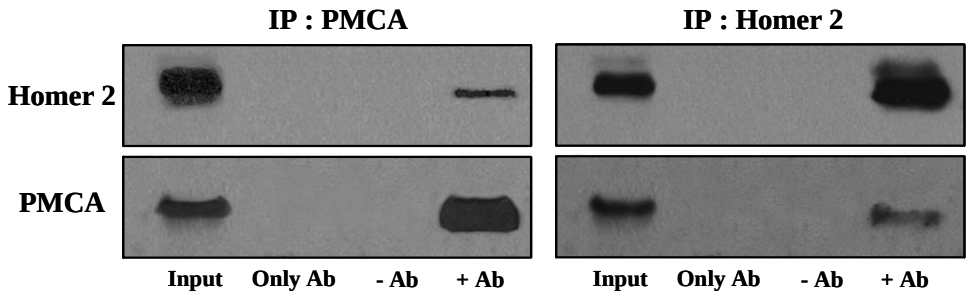

Fig. 7. Co-immunoprecipitation between Homer2 and PMCA.

The interaction between PMCA and Homer 2 were co-immunoprecipitated (IP) using cell lysates, which were prepared from parotid acini of four WT mice. *Left panel* showed IP of PMCA and probing for Homer 2, and *right panel* showed IP of Homer 2 and probing for PMCA. In IP experiments, Input donated extract samples used for Western blot, Only Ab donated control IP using Ab without extract in the IP reaction, –Ab donated control IP using extract without Ab in the IP reaction, and +Ab donated IP using extract and Ab in the IP reaction.

IV. DISCUSSION

In the present study I demonstrated a novel interaction between the EVH domain of Homer 2 and proline-rich motif of PMCA using parotid acinar cells of Homer 2-deficient mice and provided firm evidence in support of a critical role for Homer 2 in PMCA-mediated Ca^{2+} signaling using several assays.

Based on the previous reports concerning the modular structure of Homer proteins in CNS, Homer proteins have ability to bind the GPCR receptors such as mGluR1/5 and IP_3Rs, and act as scaffolding proteins to assemble Ca^{2+} signaling complexes in cellular microdomains (Kato *et al*., 1998; Tu et al., 1998; Xiao *et al*., 1998; Kammermeier *et al*., 2000; Fagni *et al*., 2002). I, therefore, hypothesized that there is also a functional relationship through the interaction between Homer 2 and IP_3Rs in exocrine cells. First, I found that Homer 2 and IP_3Rs were expressed in the apical pole of parotid acinar cells using immunocytochemistry. In a variety of exocrine cells, the apical region is regarded as the “trigger zone” from which Ca^{2+} waves are initiated (Lee *et al.*,

1997b; Tojyo *et al.*, 1997). Therefore, the apical region seems relative abundance of Ca^{2+} release channels and/or expression of the most sensitive channels. Immunocytochemical studies have reported that the extrame apical region is highly enriched in all IP_3R types (Takemura *et al.*, 1999; Zhang *et al.*, 1999; Shin *et al.*, 2003). However, I also found that deletion of Homer 2 has no effect on expression of any IP_3R isoform by measurement of Ca^{2+} uptake and release from internal stores. The only effect observed is increased PMCA protein expression in parotid acinar cells of Homer 2-deficient mice. These Homer 2 effects were originally reported in pancreatic acinar cells by Shin and his collaborators, but they observed increase of SERCA2b protein expression and activity in pancreatic acini and brain of Homer 2-deficient mice (Shin *et al.*, 2003). It might be possible for Homer 2 to play roles via interactions with several proteins such as PMCA or SERCA2b that are modulated by the activation of Ca^{2+} signaling system.

Second, measurements of PMCA activity and co-immunoprecipitation binding assays confirmed that Homer 2 is interacted with PMCA and

increased rate of $[Ca^{2+}]_i$ clearance by PMCA in parotid acinar cells of Homer 2-deficient mice. The PMCA is a ubiquitously expressed P-type ATPase with a high affinity for Ca^{2+} and has been thought to be the major mechanism for the maintenance of resting $[Ca^{2+}]_i$ (Monteith *et al.*, 1998; Kiselyov *et al.*, 2003). The PMCA consists of four members, PMCA 1, PMCA 2, PMCA 3, and PMCA 4, and PMCA isoforms (PMCA 1, 2, and 4, especially PMCA 2) are expressed in apical region of parotid and submandibular gland cells (Homann *et al.*, 2006). Furthermore, PMCA and SERCA2a are highly expressed in the apical region, while SERCA2b is mostly expressed in the basal pole and basolateral region in salivary gland and pancreatic acinar cells (Lee *et al.*, 1997a). Therefore, it is possible that the Homer 2 in the apical region of parotid acinar cells is physically associated with PMCA and potentially allowing for functional modulation between the proteins in Ca^{2+} signaling by GPCRs. Sgambato-Faure *et al.* (2006), recently, reported that endogenous Ania-3, a member of the Homer 1 family, and Homer 1 are co-expressed with PMCAs, especially PMCA 2 in the soma and dendrites of rat

hippocampal neurons. Similarly, PMCA 2 significantly decreases the expression levels of mGluR1, IP_3R1, and Homer proteins (Homer 1b/c and Homer 3) in cerebellar Purkinje neurons of PMCA-deficient mice (Kurnellas *et al.*, 2007). It might be possible that Homer 2 cross-talk with IP_3Rs-mediated Ca^{2+} signaling via regulation of PMCA, serving as the basis for its negative regulator effect. This study provides a novel modular model in Ca^{2+} signaling for examining the role and functions of Homer 2 in the non-neuronal exocrine cells.

V. REFERENCES

Berridge MJ, Bootman MD & Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 2003;4:517-529.

Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL & Worley PF. Homer: a protein that selectively binds metabotropic glutamate receptors. Nature 1997;386:284-288.

Brini M & Carafoli E. Calcium signalling: a historical account, recent developments and future perspectives. Cell Mol Life Sci 2000;57:354-370.

Bruce JI, Shuttleworth TJ, Giovannucci DR & Yule DI. Phosphorylation of inositol 1,4,5-trisphosphate receptors in parotid acinar cells. A mechanism for the synergistic effects of cAMP on Ca^{2+} signaling. J Biol Chem 2002a;277:1340-1348.

Bruce JI, Yule DI & Shuttleworth TJ. Ca^{2+}-dependent protein kinase--a modulation of the plasma membrane Ca^{2+}-ATPase in parotid acinar cells. J Biol Chem 2002b;277:48172-48181.

Fagni L, Chavis P, Ango F & Bockaert J. Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. Trends Neurosci 2000;23:80-88.

Fagni L, Worley PF & Ango F. (2002). Homer as both a scaffold and transduction molecule. Sci STKE 2002; RE8.

Feng W, Tu J, Yang T, Vernon PS, Allen PD, Worley PF & Pessah IN. Homer regulates gain of ryanodine receptor type 1 channel complex. J Biol Chem

2002;277:44722-44730.

Gorr SU, Venkatesh SG & Darling DS. Parotid secretory granules: crossroads of secretory pathways and protein storage. J Dent Res 2005;84:500-509.

Homann V, Kinne-Saffran E, Arnold WH, Gaengler P & Kinne RK. Calcium transport in human salivary glands: a proposed model of calcium secretion into saliva. Histochem Cell Biol 2006;125:583-591.

Kammermeier PJ, Xiao B, Tu JC, Worley PF & Ikeda SR. Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels. J Neurosci 2000;20:7238-7245.

Kato A, Ozawa F, Saitoh Y, Fukazawa Y, Sugiyama H & Inokuchi K. Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. J Biol Chem 1998;273:23969-23975.

Kiselyov K, Shin DM & Muallem S. Signalling specificity in GPCR-dependent Ca^{2+} signalling. Cell Signal 2003;15:243-253.

Kurnellas MP, Lee AK, Li H, Deng L, Ehrlich DJ & Elkabes S. Molecular alterations in the cerebellum of the plasma membrane calcium ATPase 2 (PMCA2)-null mouse indicate abnormalities in Purkinje neurons. Mol Cell Neurosci 2007;34:178-188.

Lee MG, Xu X, Zeng W, Diaz J, Kuo TH, Wuytack F, Racymaekers L & Muallem S. Polarized expression of Ca^{2+} pumps in pancreatic and salivary gland cells. Role in initiation and propagation of $[Ca^{2+}]_i$ waves. J Biol Chem 1997a;272:15771-15776.

Lee MG, Xu X, Zeng W, Diaz J, Wojcikiewicz RJ, Kuo TH, Wuytack F, Racymaekers L & Muallem S. Polarized expression of Ca^{2+} channels in

pancreatic and salivary gland cells. Correlation with initiation and propagation of $[Ca^{2+}]_i$ waves. J Biol Chem 1997b;272:15765-15770.

Monteith GR, Wanigasekara Y & Roufogalis BD. The plasma membrane calcium pump, its role and regulation: new complexities and possibilities. J Pharmacol Toxicol Methods 1998;40:183-190.

Sgambato-Faure V, Xiong Y, Berke JD, Hyman SE & Strehler EE. The Homer-1 protein Ania-3 interacts with the plasma membrane calcium pump. Biochem Biophys Res Commun 2006;343:630-637.

Shin DM, Dehoff M, Luo X, Kang SH, Tu J, Nayak SK, Ross EM, Worley PF & Muallem S. Homer 2 tunes G protein-coupled receptors stimulus intensity by regulating RGS proteins and PLCbeta GAP activities. J Cell Biol 2003;162:293-303.

Shin DM, Zhao XS, Zeng W, Mozhayeva M & Muallem S. The mammalian Sec6/8 complex interacts with Ca^{2+} signaling complexes and regulates their activity. J Cell Biol 2000;150:1101-1112.

Szumlinski KK, Kalivas PW & Worley PF. (2006). Homer proteins: implications for neuropsychiatric disorders. Curr Opin Neurobiol 2006;16:251-257.

Takemura H, Yamashina S & Segawa A. Millisecond analyses of Ca^{2+} initiation sites evoked by muscarinic receptor stimulation in exocrine acinar cells. Biochem Biophys Res Commun 1999;259:656-660.

Thrower EC, Hagar RE & Ehrlich BE. Regulation of $Ins(1,4,5)P_3$ receptor isoforms by endogenous modulators. Trends Pharmacol Sci 2001;22:580-586.

Tojyo Y, Tanimura A & Matsumoto Y. Imaging of intracellular Ca^{2+} waves

induced by muscarinic receptor stimulation in rat parotid acinar cells. Cell Calcium 1997;22:455-462.

Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M & Worley PF. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron 1999;23:583-592.

Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ & Worley PF. Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP_3 receptors. Neuron 1998;21:717-726.

Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ & Worley PF. Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. Neuron 1998;21:707-716.

Xu X, Zeng W & Muallem S. Regulation of the inositol 1,4,5-trisphosphate-activated Ca^{2+} channel by activation of G proteins. J Biol Chem 1996;271:11737-11744.

Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S & Worley PF. Homer binds TRPC family channels and is required for gating of TRPC1 by IP_3 receptors. Cell 2003;114:777-789.

Zeng W, Lee MG, Yan M, Diaz J, Benjamin I, Marino CR, Kopito R, Freedman S, Cotton C, Muallem S & Thomas P. Immuno and functional characterization of CFTR in submandibular and pancreatic acinar and duct cells. Am J Physiol 1997;273:C442-455.

Zhang BX & Muallem S. Feedback inhibition of Ca^{2+} release by Ca^{2+} is the

underlying mechanism of agonist-evoked intracellular Ca^{2+} oscillations in pancreatic acinar cells. J Biol Chem 1992;267:24387-24393.

Zhang BX, Zhao H, Loessberg P & Muallem S. Activation of the plasma membrane Ca^{2+} pump during agonist stimulation of pancreatic acini. J Biol Chem 1992;267:15419-15425.

Zhang X, Wen J, Bidasee KR, Besch HR, Jr., Wojcikiewicz RJ, Lee B & Rubin RP. (1999). Ryanodine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland. Biochem J 1999;340 (Pt 2):519-527.

국문요약

생쥐 이하선 세포에서 세포막 칼슘펌프와 결합하여 칼슘펌프 활성도를 조절하는 Homer2.

연세대학교 대학원 치의학과

양 은 호

(지도교수: 신 동 민)

호머들은 지주단백이며 이들은 N-터미널에 Ena/VASP homology 1 (EVH) 단백-결합 도메인과 C-터미널에 leucin zipper/coiled-coiled 도메인들로 구성되어 있다. EVH 도메인은 proline-rich motif (PPXXF, PPXF, 그리고 LPSSP)를 인식하는데 이를 통해 G-단백 연계 수용체, inositol 1,4,5-triphosphate (IP_3) 수용체, ryanodine 수용체, TRP 통로 등과 결합한다. 따라서, 호머들은 세포내 칼슘을 조절하는데, 이와 같은 기전을 이용하여 dendritic spine의 형태형성, 신경에서 시냅스의 개조, 연합신경 중추에서 시냅스의 집중, 신경세포의 유전자 전사활성 등을 조절한다. 그러나 호머들의 비신경 세포에서의 역할은 잘 알려져 있지 않다. 다만 췌장 선세포에서 RGS 단백과 PLCβ의 GAP 활성도를 조절하여 G-단백연계 신호를 조절한다는 보고가 있을 뿐이다. 이에 본 연구에서는 호머 제

이형 유전자 결여 생쥐를 이용하여 이하선세포에서 호머 제 이형의 역할을 미세형광분석법, 면역형광염색법, 면역 침강법 등을 통해 알아보고자 하였다. 이하선 세포에서 호머 제 이형은 내강 측으로 발현하고 있었다. 그런데 IP_3 수용체의 발현위치와 수용체의 활성도에는 영향을 주지 못 하였다. 호머 제 이형 결여 생쥐에서 소포체 칼슘펌프의 발현에는 변화가 없었으나 세포막 칼슘펌프 (PMCA)의 발현을 증가시켰다. 더불어 호머 제 이형 결여 생쥐에서 세포막 칼슘펌프의 활성도가 증가하였으며, 면역침강 결과는 호머 제 이형과 PMCA가 서로 단백결합을 함을 보여 주었다. 이상의 결과는 생쥐 이하선 세포에서 호머 제 이형은 PMCA의 발현과 PMCA- 연계 칼슘신호 전달에서 중요한 역할을 수행하고 있음을 의미한다.

핵심되는 말: 호머 제 이형, 세포막 칼슘펌프, 이하선 선세포