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Generation of intracellular domain of insulin receptor tyrosine kinase by γ-secretase

K. Kasuga [a,b], H. Kaneko [a,b], M. Nishizawa [b], O. Onodera [a], T. Ikeuchi [a,*]

[a] *Department of Molecular Neuroscience, Brain Research Institute, Niigata University, 1 Asahimachi, Niigata 951-8585, Japan*
[b] *Department of Neurology, Brain Research Institute, Niigata University, 1 Asahimachi, Niigata 951-8585, Japan*

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Abstract

The proteolytic cleavage of a precursor protein into α- and β-subunits by furin is required to form functional insulin receptor (IR). In this study, we examined if IR undergoes the additional presenilin (PS)/γ-secretase-dependent processing. In cells treated with γ-secretase inhibitors or expressing the dominant-negative PS1 variant led to the accumulation of an endogenous IR C-terminal fragment. In the presence of proteasome inhibitors, we detected a PS/γ-secretase cleavage product of the IR, termed the IR intracellular domain (ICD). Cellular fractionation and confocal microscopy analyses showed that the IR-ICD is predominantly detected in the nucleus. These data indicate that IR is a tyrosine kinase receptor, which undergoes PS/γ-secretase-dependent processing. We also show that the autophosphorylation levels of the IR β-subunit upon insulin stimulation were decreased by the inactivation of PS/γ-secretase, raising the possibility that the PS/γ-secretase proteolysis of IR may play a modulatory role in insulin signaling.

Keywords: Regulated intramembranous proteolysis; Presenilin-dependent γ-secretase; Metalloprotease; Insulin signalling; Tyrosine phosphorylation; Tyrosine kinase

The insulin receptor (IR) that belongs to a large family of growth factor receptors with intrinsic tyrosine kinase activity, is a tetrameric transmembrane protein composed of two α- (135 kDa) and β- (95 kDa) subunits [1]. Both subunits are derived from a single-chain precursor protein, which undergoes glycosylation, disulphide isomerization, and dimerization in the endoplasmic reticulum (ER). The proteolytic cleavage of the precursor protein into the α- and β-subunits by furin occurs in the Golgi apparatus [2,3]. The IR β-subunit is composed of an extracellular part (194 amino acids), a transmembrane domain (23 amino acids), and a cytoplasmic domain (403 amino acids) [4]. When insulin binds to IR at the plasma membrane, this activates the tyrosine-specific kinase of the intracellular domain of the IR β-subunit, which subsequently phosphorylates intracellular substrates. These events are necessary for the signal transduction to elicit various insulin actions [5].

Several lines of evidence suggest that a process known as regulated intramembrane proteolysis (RIP) acts on a number of type I membrane proteins and links intracellular signalling events [6,7]. Presenilin (PS)-dependent RIP, termed γ-secretase, is a unique aspartyl protease that cleaves a growing number of substrates, including the amyloid precursor protein (APP) [8], Notch [9] and the Notch ligands Delta-1 and Jagged2 [10] within a predicted transmembrane domain. PS/γ-secretase generates the intracellular domain (ICD) of the protein, resulting in the release of ICD from the membrane. The released intracellular domain in turn is translocated to the nucleus where it acts as a transcription regulator or performs non-nuclear signaling functions [7].

Recently, several tyrosine kinase receptors of the type I membrane protein have been shown to undergo sequential proteolytic cleavages including PS/γ-secretase cleavage. On

* Corresponding author. Fax: +81 25 227 0682.
E-mail address: ikeuchi@bri.niigata-u.ac.jp (T. Ikeuchi).

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the basis of the similarity that IR is the tyrosine kinase receptor of type I membrane proteins, which is cleaved by furin at ectodomain, generating a membrane-tethered stub, we examined whether the resulting stub is further cleaved by PS/γ-secretase to generate ICD. Here, we report that IR is a novel substrate that undergoes PS/γ-secretase-dependent proteolysis and the resulting soluble intracellular domain of IR localizes to the nucleus.

Material and methods

Construct. Human IR cDNA [11] was inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate pcDNA3.1-hIR. The cDNA encoding the β-subunit of IR was generated by polymerase chain reaction (PCR) using the following primers: IRβ forward, ggggtacctcccttggc gatgttgggaat, and IRβ-ICD reverse, cgggatccggaaggattggaccgaggcaa. The PCR product was subcloned into the reading frame of the KpnI/BamHI sites of the pEGFP vector, in which the signal peptide sequence composed of N-terminal 27 amino acid residues of IR was inserted. The cDNA encoding the intracellular domain of IR was generated using the following primers: IR-ICD forward, cggaattccgccatgagaaaagaggcagccagac, and the IRβ-ICD reverse. The PCR product was subcloned into the EcoRI/BamHI sites of the pEGFP vector. The resulting cDNA constructs were verified by sequence analysis.

Cell culture and transfection. Human embryonic kidney (HEK) 293 or mouse embryonic fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fatal bovine serum (FBS). The transient transfection of cDNA into cells was performed using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's recommendations. Cells were treated with the γ-secretase inhibitor L-685,458 (Calbiochem, San Diego, CA) or compound E (Calbiochem) dissolved in Me_2SO, or an equivalent concentration of Me_2SO as a vehicle control. Proteasomes in cells were inhibited by treating cells with lactacystin (10 μM), MG132 (5 μM), or epoxomycin (0.05 μM) (Sigma–Aldrich, St. Louis, MO) for 16 h. For primary neuronal culture, cortical neuronal cultures were prepared from mouse brains on embryonic day 18 according to the manufacturer's instruction (Sumitomo Bakelite, Japan), and maintained for 7 days before they were used for experiments.

Western blot. Cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Sigma–Aldrich). Solubilized proteins were fractionated by electrophoresis on Tris–glycine SDS–polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The IR derivatives were detected using CT-3 (NeoMarkers, Fremont, CA), a monoclonal antibody that recognizes the C-terminal amino acid residues of human IR. The polyclonal antibody $PS1_{NT}$ [12] was used to detect the full-length PS1 and the PS1 N-terminal fragment. The full-length APP and C-terminal fragment of APP were visualized by staining with the antiserum CT15 [13]. Bound antibodies were visualized using an enhanced chemiluminescence (ECL) detection system (Millipore).

Insulin stimulation and immonuprecipitation. Cells were treated with 10 nM human insulin (Sigma–Aldrich) for 10 min at 37 °C. Cells were immediately washed and solubilized with IP buffer containing 0.5 mM sodium orthovanidate and 1 mM sodium fluoride. Phosphorylated proteins were immunoprecipitated with PY20 (Zymed Laboratories, South San Francisco, CA), a monoclonal anti-phosphotyrosine antibody, at 4 °C overnight. The immune complexes collected on Protein-G-conjugated agarose beads (Pierce, Rockford, IL) were eluted by boiling for 5 min in Laemmli SDS sample buffer.

Subcellular fractionation. The membrane fractions were purified as described previously [14]. Briefly, membrane fractions were prepared from 10-cm dishes. Each dish was washed twice with 5 mL of Buffer A (250 mM sucrose and 20 mM Hepes, pH 7.4) and cells were collected by centrifugation at 1400*g* at 4 °C for 5 min. The resulting cell pellets were homogenized in Buffer A containing a protease inhibitor cocktail (Sigma–Aldrich) using a teflon pestle with 20 strokes. The postnuclear supernatant (PNS) was collected by centrifugation at 1000*g* at 4 °C for 10 min. Membrane and soluble fractions were collected by further ultracentrifugation. Nuclear fraction was purified using the NE-PER Nuclear Extraction kit (Pierce) according to the manufacturer's instruction.

Immunostaining of cells. Cos-7 cells were transfected with the IRβ-GFP or IR-ICD-GFP construct. After 48 h, GFP fluorescence in living cells grown on glass bottom dishes was analyzed by confocal laser scanning using a Nikon TE300NT inverted microscope.

Results

PS/γ-secretase mediated proteolytic processing of IR

We first determined whether PS/γ-secretase plays any role in IR processing. HEK293 cells were treated with a highly potent γ-secretase inhibitor, L-685,458 or compound E. We probed for endogenous IR derivatives using an IR C-terminus-specific monoclonal antibody, CT-3. The levels of full-length IR migrating at ~190 kDa and the IR β-subunit migrating at ~95 kDa were unchanged irrespective of the γ-secretase inhibitor used (Fig. 1A). However, a novel IR derivative migrating at ~51 kDa, termed IR-C-terminal fragment (CTF), is accumulated in the presence of L-685,458 or compound E in a dose-dependent manner (Fig. 1A). We next compared cells stably expressing either human wild-type PS1 or a dominant-negative human PS1 variant that harbors the D385A mutation [15,16]. Similarly to the cells treated with the γ-secretase inhibitors, IR-CTF accumulation was observed in cells expressing PS1 D385A (Fig. 1B, left panel). To examine whether IR-CTF production occurs in neurons, we cultured primary cortical neurons prepared from mouse embryos, and then treated them with the γ-secretase inhibitor, L-685,458. The addition of L-685,458 resulted in the accumulation of IR-CTF (Fig. 1C, left panel), indicating that IR-CTF was processed in a similar manner in neurons. Since it has been reported that ectodomain shedding induced by metalloprotease precedes PS/γ-secretase cleavage [7], we examined the effects of metalloprotease on the production of IR-CTF. Treating cells with 100 ng/ml phorbol 12-myristate 13-acetase (PMA), a potent protein kinase C activator that rapidly activate metalloproteases such as ADAM17, resulted in an enhanced production of IR-CTF (Fig. 1D). Collectively, these results suggest that IR undergoes a series of proteolysis including PS-dependent γ-secretase cleavage.

Detection of IR-ICD in soluble fraction

Although γ-secretase inactivation led to the accumulation of IR-CTF, a putative substrate derivative of IR, the predicted intracellular domain of IR, termed IR-ICD, was not readily detected by Western blot analysis, suggesting that IR-ICD is unstable in intact cells. We, therefore, attempted to detect IR-ICD by inhibiting a proteasomal pathway that has been shown to mediate the degradation of various γ-secretase-dependent ICDs [7]. HEK293 cells were treated with various proteosomal inhibitors including

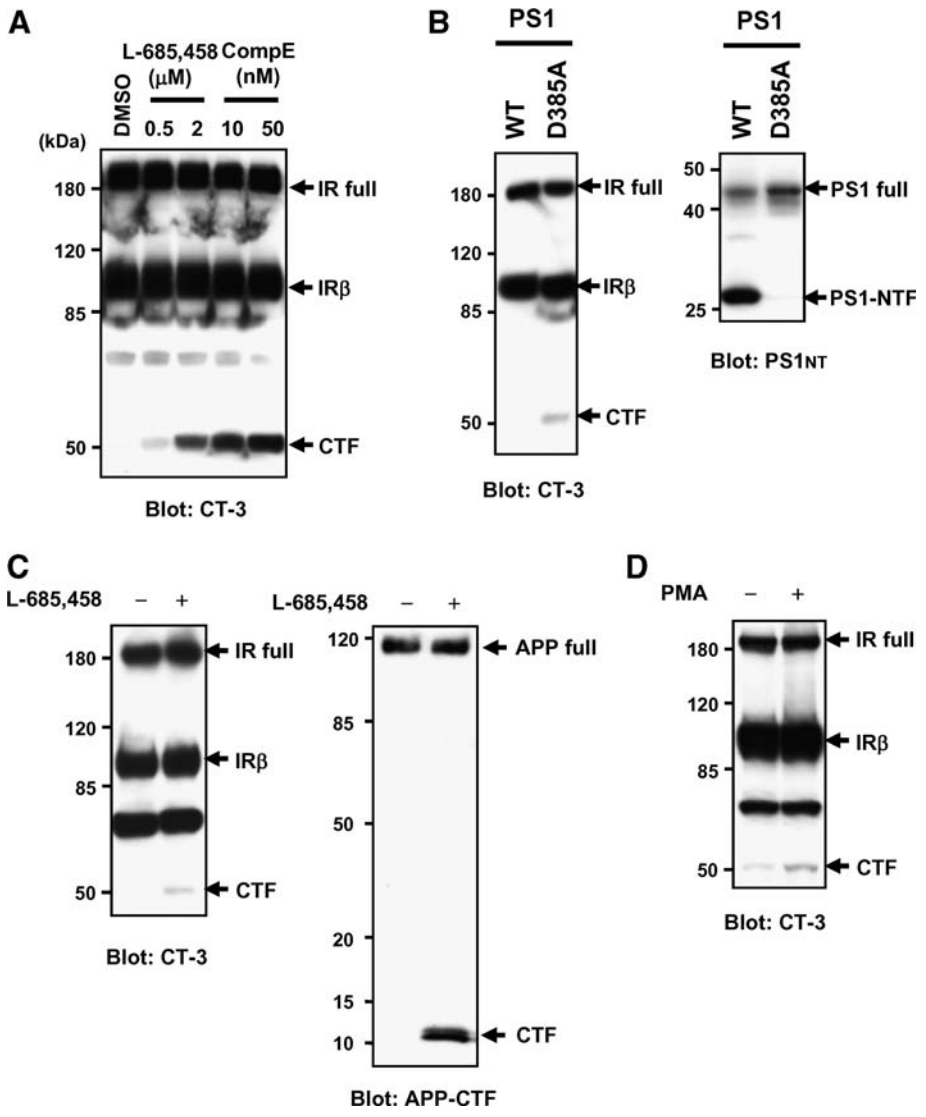

Fig. 1. Detection of accumulated endogenous IR-CTF by PS/γ-secretase inactivation. (A) HEK293 cells were incubated in 0.5% Me_2SO (vehicle), or different concentrations of the γ-secretase inhibitor L-685,458 or compound E. Detergent lysates were analyzed by immunoblotting with the anti-IR C-terminal antibody, CT-3. The bands corresponding to ~190 kDa full-length IR, ~95 kDa IRβ, and ~51 kDa IR-CTF are indicated on the right side. Molecular weight markers (in kilodalton) are shown on the left side. (B) Detergent lysates extracted from HEK293 cells stably expressing human wild-type PS1 or PS1 D385A variant were analyzed by immunoblotting to detect endogenous IR derivatives using the CT3 antibody (left panel). The same lysates were analyzed by immunoblotting using the anti-$PS1_{NT}$ antibody (right panel). The fragments corresponding to ~45 kDa full-length PS1 and ~29 kDa NTF are indicated on the right side. (C) Primary neurons prepared from mouse embryos were cultured for 7 days and treated with L-685,458 for 18 h. IR-CTF is detected in neurons treated with L-685,458 (left panel). Same lysates were analyzed with antiserum CT15, which revealed that APP-CTF accumulation was similarly observed in cells treated with L-685,458 (right panel). (D) Activation of metelloprotease by PMA treatment of 293 cells resulted in an enhanced production of endogenous IR-CTF.

lactacystin, expoxomycin, or MG132 for 16 h. These experiments revealed that the inhibition of proteasomes led to the accumulation of an additional fragment with an apparent molecular weight of ~48 kDa (Fig. 2A). On the basis of the findings that the fragment accumulated at high levels with proteasomal inhibitors treatment and not present in cells treated with a γ-secretase inhibitor (Fig. 2A), we considered this fragment as a γ-secretase-generated IR derivative, IR-ICD. These results indicate that endogenous IR-ICD levels in intact cells are low, but become stabilized when the proteasomes are inhibited.

To determine the localization of IR derivatives, we fractioned the cell extracts into the membrane and soluble fractions. The endogenous IRβ fragment and IR-CTF were predominantly detected in the membrane fraction as expected (Fig. 2B). In contrast, the putative IR-ICD fragment that lacks the transmembrane domain was largely present in the soluble fraction. Importantly, the generation of IR-ICD in the soluble fraction was barely detected in cells expressing PS1 D385A (Fig. 2B), confirming that IR-ICD generation is PS/γ-secretase dependent.

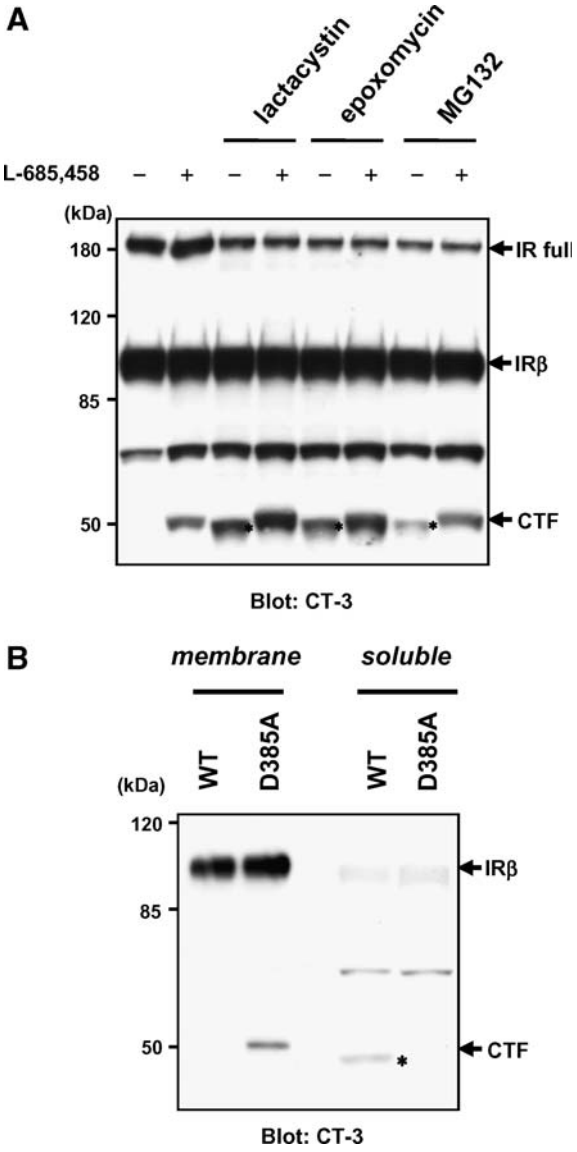

Fig. 2. Soluble IR-ICD is rapidly degradated by proteosomes. (A) HEK293 cells were treated with or without proteasome inhibitors for 16 h, and endogenous IR derivatives were analyzed by immunoblotting with the CT3 antibody. Treatment with proteasome inhibitors resulted in the accumulation of IR-ICD migrating at ~48 kDa (asterisk). In the presence of L-685,458, the accumulation of IR-CTF (arrowhead), showing a slightly slower migration than the putative IR-ICD, was detected. (B) HEK293 cells were treated with epoxomycin for 16 h, and cellular extracts were separated into the membrane and soluble fractions. Note that the IR-ICD fragments were predominantly detected in the soluble fraction, and the production of IR-ICD was nearly diminished in cells expressing PS1 D385A.

Preferential nuclear localization of intracellular domain of IR

γ-Secretase dependent ICDs have been previously reported to translocate into the nucleus where they act as a transcriptional regulator or perform non-nuclear signaling functions [7]. To examine the ability of IR-ICD to translocate to the nucleus, we analyzed Cos-7 cells transfected with a cDNA encoding IRβ or IR-ICD with a GFP tag at the N-terminus (Fig. 3A). We examined the expression patterns of GFP fusion proteins by Western blot analysis after subcellular fractionation. The IRβ-GFP fusion pro-

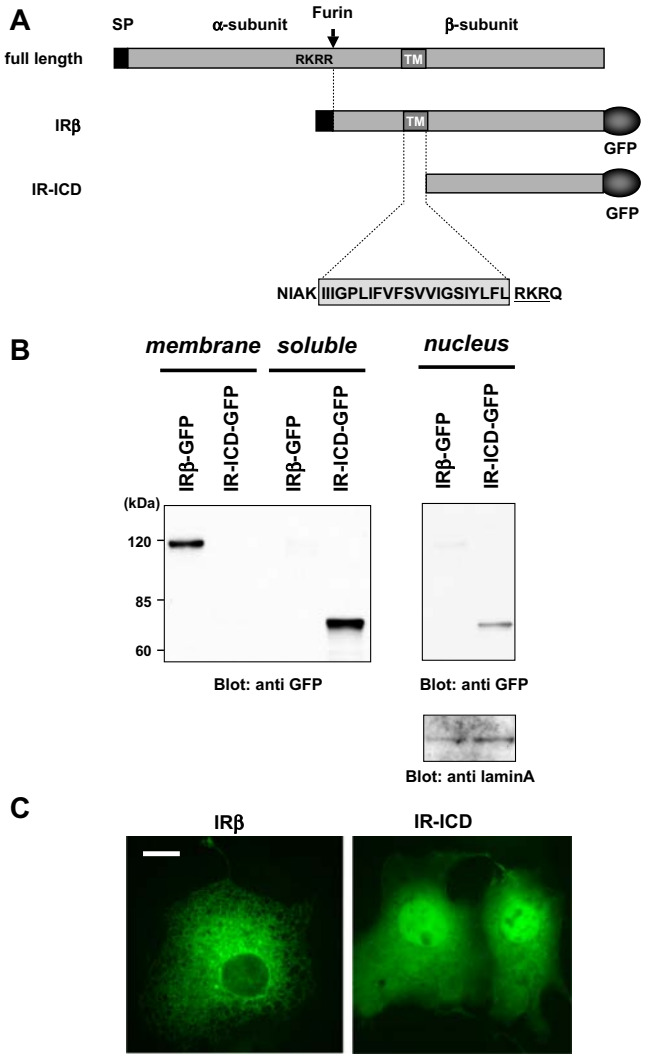

Fig. 3. Subcellular localization of the IRβ and IR-ICD. (A) Schematic structure of GPF tagged IR derivatives, namely, IRβ-GFP and IR-ICD-GFP, used in this study. Signal peptides (SP) containing the first 27 amino acid residues are removed by a peptidase. IR is further cleaved at the RKRR motif by furin to generate the IR α- and β-subunits. The amino acid sequences of transmembrane (TM) domain and juxtamembrane region are indicated. The *box* represents the predicted TM domain. Similarly to other γ-secretase substrates, the stop-transfer amino acid sequence (underlined) is observed in the IR sequences. (B) Cos-7 cells were transiently transfected with cDNA encoding the IRβ - or ICD-GFP fusion protein. Cells were fractionated into membrane, soluble, and nuclear fractions to examine the distribution of the fusion proteins. Western blots probed with the anti-GFP antibody revealed that the IRβ-GFP fusion protein migrating at ~120 kDa is predominantly detected in the membrane fraction, whereas the ICD-GFP fusion protein migrating at ~74 kDa was observed in the soluble and nuclear fractions. The fractionated nuclear proteins were further blotted with the polyclonal anti-lamin A antibody. (C) Cos-7 cells transiently transfected with IRβ-GFP (left panel) and IR-CTF (right panel) were analyzed by confocal microscopy. Scale bar, 10 μm.

tein migrating at the predicted size of ~120 kDa was predominantly detected in the membrane fraction, whereas the ICD-GFP fusion protein was largely present in the soluble fraction (Fig. 3B). Notably, the ICD-GFP fragment was also detected in the nuclear fraction where a nuclear envelop protein, lamin A, is also distributed (Fig. 3B). Confocal imaging performed on live cells revealed that IRβ-GFP accumulated in the cytoplasmic region with a reticular formation (Fig. 3C). In contrast, IR-ICD predominantly localized within the nucleus as well as diffusely in the cytoplasmic region.

Phosphorylation of intracellular domain of IR is affected by PS/γ-secretase activity

To explore the possible physiological roles of PS/γ-secretase activity in insulin signaling, we examined the levels of tyrosine autophosphorylation of IR. Cells were treated with human insulin for 10 min at 37 °C, and the immunoprecipitates obtained using the anti-phosphorylated tyrosine antibody PY20 were blotted with CT3. In cells expressing PS1 WT, insulin treatment induced a rapid autophosphorylation of the IR β-subunit (Fig. 4A, upper panel). Notably, the levels of the phosphorylated IR β-subunit were significantly lower in cells expressing the dominant-negative PS1 D385A variant than in PS1 WT cells (Fig. 4B), although the levels of the IR β-subunit are comparable between the two cell lines (Fig. 4A, lower panel).

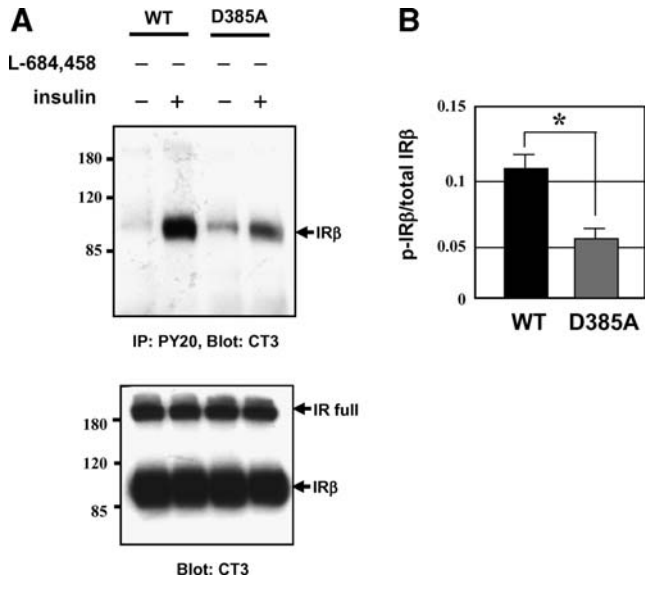

Fig. 4. Decreased autophosphorylation of IR by inactivation of PS/γ-secretase activity. (A) HEK293 cells were stimulated with insulin for 10 min. Detergent lysates were immunoprecipitated with monoclonal anti-phosphotyrosine antibody PY20, followed by immunoblotting with the CT-3 antibody (upper panel). Note that the levels of the phosphorylated IR β-subunit decreased in cells expressing PS1 D385A. The same lysates were immunoblotted with the CT-3 antibody, which revealed that comparable levels of IR derivatives were observed in cells expressing PS1 WT and D385A (lower panel). (B) The semiquantitative analysis of the expression level of IR β-subunit was performed by densitometry, and the ratio of phosphorylated/total IR β-subunit levels was determined. Results from three independent experiments are shown as mean ± standard error ($^*p < 0.05$ by Student's t test).

Discussion

PS/γ-secretase belongs to the unique aspartic protease family and is able to cleave membrane proteins within the transmembrane domains [17]. A growing number of type I membrane proteins as the substrates of PS/γ-secretase have been identified [7]. In this report, we provide several lines of evidence that IR represents a novel substrate for PS/γ-secretase. We identified a novel endogenous IR C-terminal fragment migrating at ~51 kDa, which is accumulated in cells treated with γ-secretase inhibitors or expressing the dominant-negative PS1 variant D385A. IR-CTF production was enhanced upon the activation of metalloprotease by PMA treatment. IR-CTF is further cleaved by γ-secretase-like activity, generating soluble IR-ICD, which is highly labile and localizes to the nucleus. Collectively, these data strongly suggest that γ-secretase has the capacity to cleave endogenous IR.

Tyrosine kinase receptors are type I membrane proteins expressed at the cell surface to detect the presence of cognate growth factors. IR belongs to tyrosine kinase receptor family whose activation is essential for insulin signaling in target tissues. Insulin binding to IR leads to a rapid autophosphorylation of the receptor, followed by the tyrosine phosphorylation of the insulin receptor substrate (IRS) family, inducing the activation of downstream pathways such as the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades [5]. Previous reports showed that several tyrosine kinase receptors including ErbB-4 [18], p75 [19,20], and ephrinB [21,22] undergo proteolytic cleavage to generate ICDs, which potentially modulate the signaling events [23]. In ErbB4, neuregulin (NRG) binding results in a series of proteolysis including PS/γ-secretase cleavage to generate ErbB4-ICD [18]. The maturation of oligodendrocytes mediated by NRG-ErbB4 signaling is impaired when γ-secretase activity is inhibited [24]. Recently, it has been reported that ErbB4-ICD released from an activated receptor by ligand binding forms a complex with the adaptor TAB2 and the corepressor N-CoR that translocate into the nucleus and bind to promoters of astrocytic genes in a PS/γ-secretase-dependent manner [25]. Our observation that PS/γ-secretase inactivation resulted in reduced levels of tyrosine autophosphorylation of the IR β-subunit upon insulin stimulation, raises the possibility that PS/γ-secretase may act as a modulator of insulin signaling. Very recently, insulin-like growth factor I receptor (IGF-IR) that shares a highly structural homology with IR, was reported to be a substrate for PS/γ-secretase activity [26]. Further studies of the effects of PS/γ-secretase activity on downstream signaling events of IR phosphorylation that include the phosphorylation of IRS, Akt and GSK3β will be required to clarify this possibility.

Although the central nervous system has been classically considered to be an insulin-insensitive tissue, recent emerging evidence has suggested that insulin signaling plays an important role in neuronal survival and synaptic plasticity. Insulin has been shown to induce changes in synaptic strength, which are assumed to form the basis of learning and memory [27]. Moreover, an insulin-induced increase in tyrosine phosphorylated IR levels has been observed in rat hippocampal neurons following a water maze training, suggesting that learning may improve receptor sensitivity [28]. In primary neuronal cultures, we detected the accumulation of a metalloprotease-cleaved endogenous IR fragment, indicating that the PS/γ-secretase cleavage of IR may play a physiological role in the central nervous system. More recently, disturbances of insulin signaling in the brain have been implicated in the pathophysiology of neurodegenerative diseases. In the brain of neuron-specific insulin receptor knockout mice, the loss of insulin-mediated activation of PI3K, leading to reduced levels of phosphorylation of Akt and glycogen synthase kinase (GSK) 3β, results in a substantial increase in levels of the phosphorylated microtubule-associated protein Tau, a pathological hallmark of Alzheimer disease [29]. Further studies will be required to clarify the association of insulin signaling with the molecular pathology in Alzheimer's disease with reference to altered PS/γ-secretase activity.

In conclusion, our findings demonstrated that IR undergoes sequential proteolysis by furin, metalloprotease and PS/γ-secretase to generate IR-ICD, which potentially translocates into the nucleus. Further characterization of the physiological consequences of the nuclear translocation of IR-ICD and the identification of downstream target genes, if present, may provide new insights into the insulin signaling pathways in the central nervous system.

Acknowledgments

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