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IER family proteins are regulators of protein phosphatase PP2A and modulate the phosphorylation status of CDC25A

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ABSTRACT

Proteins encoded by immediate-early response (IER) family genes, *IER2*, *IER5*, and *IER5L*, share homology at their N-terminal regions. IER5 binds to protein phosphatase 2A (PP2A) and enhances dephosphorylation of PP2A target proteins such as heat shock factor HSF1. Here, we show the expression of IER family genes and the target protein-specific function of IER proteins. The IER homology regions of IER2 and IER5L are required for the interaction with PP2A. Expression of IER2 and IER5L in cells leads to reduced phosphorylation of HSF1 and derepression of its transcriptional activity. Although IER5 and IER5L enhance dephosphorylation of ribosomal protein S6 kinase, IER2 fails to do so. IER2, IER5, and IER5L all bind to the cell cycle regulator CDC25A and convert it to the hypophosphorylated form, which causes dissociation from 14-3-3 regulatory protein. IER5 differentially regulates CDC25A levels in cells under normal and thermal stress conditions. These results suggest that IER proteins are target protein-specific regulators of PP2A activity and modulate cell proliferation through CDC25A activity.

1. Introduction

Protein phosphorylation, which is reversibly controlled by protein kinases and phosphatases, plays a key role in regulating various cellular processes. Protein phosphatase 2A (PP2A) is the most abundant serine/threonine phosphatase and is responsible for the majority of serine/threonine phosphatase activity in a cell. The core dimer consists of a catalytic C subunit and a scaffold A subunit and forms a trimeric holoenzyme with a regulatory B subunit. The B subunits belong to four distinct gene families, B (B55/PR55), B′ (B56/PR61), B″ (B72/PR72), and B‴ (PR93/PR110) and are responsible for widespread substrate specificity and for regulation of activity [1–3]. PP2A plays an important role as a regulator of cell proliferation, differentiation, and death [4–6]. PP2A has been suggested to dephosphorylate over 300 substrates, and most of those are involved in cell cycle regulation, in which PP2A has anti-mitotic activity: PP2A activates WEE1 kinase, a negative regulator of cyclin-dependent kinase (CDK), and inhibits CDC25 phosphatase, a positive regulator of CDK [4,7,8].

Immediate early response 5 (*IER5*) is one of the growth factor-inducible genes [9]. *IER5* is also known as a heat shock factor (HSF1) target gene upon heat shock and as a p53 target gene upon γ-ray irradiation [10–12]. Upregulation of IER5 expression leads to downregulation of CDC25B expression [13,14]. The IER5 protein enhances the survival and/or recovery of heat-shocked cells and supports cell proliferation under anchorage-independent cell growth [10,12]. We have previously demonstrated that IER5 interacts with the B55 family regulatory subunits (B55α, B55β, B55γ, and B55δ) of PP2A through its N-terminal region [15,16]. IER5 also binds to HSF1, a key transcriptional regulator of heat shock protein (HSP) and chaperone genes. Thus, IER5 enhances PP2A/B55 phosphatase activity toward HSF1 at the inhibitory phosphorylation sites and derepresses HSF1 transcription activity [15,16]. High IER5 expression is associated with various cancers and causes HSF1 activation, which contributes to the proliferation of cancer cells under stressed conditions [12]. IER5 also binds to the PP2A/B55 target protein ribosomal protein S6 kinase (S6K), which controls several factors involved in the processing and translation of mRNAs, and converts S6K to hypophosphorylated forms [16]. These findings suggest that IER5 regulates cell proliferation through modulation of PP2A activity.

The N-terminal region of IER5 shares homology to the other IER proteins IER2 and IER5L (Fig. 1A) [9,17]. IER2 participates in the regulation of cell motility and adhesion in normal embryonic development, angiogenesis, and tumor cell invasion and metastasis [18–22]. Upon overexpression, IER2 elicits cell death [23]. IER2 is a potential

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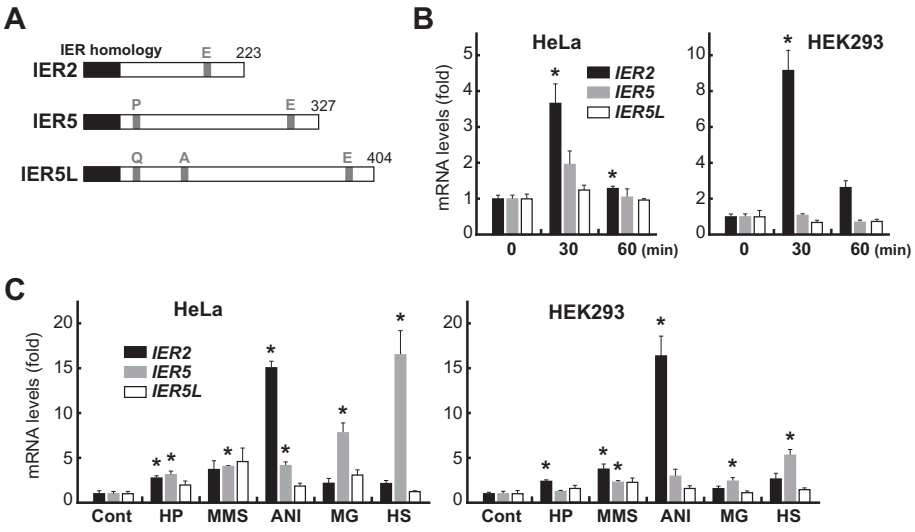

Fig. 1. Regulated expression of IER genes.
(A) Schematic representation of IER proteins. IER2, IER5, and IER5L are 223-, 327-, and 404-amino acids proteins. IER proteins share homology in their N-terminal region, and each protein contains alanine (A), glutamic acid (E), proline (P), and glutamine (Q) stretches.
(B) Serum-induced expression of IER genes. HeLa (left panel) and HEK293 (right panel) cells grown in serum-free medium for 1 day were stimulated by 10% serum for 0, 30, and 60 min. Total RNA was prepared from the cells and analyzed by qPCR. Fold activation relative to time-0 is expressed as the mean ± SE of three independent experiments (* $p < .05$).
(C) Stress-induced expression of IER genes. HeLa cells (left panel) were treated with 0.2 mM hydrogen peroxide (HP) for 4 h, 100 μg/ml MMS for 4 h, 50 ng/ml anisomycin (ANI) for 1 h, and 10 μM MG132 (MG) for 4 h, or were subjected to heat shock (HS) at 42.5 °C for 1 h. HEK293 cells (right panel) were treated as above, except that MG132 was 20 μM. Total RNA was prepared from the cells and analyzed by qPCR. Fold activation relative to the control (Cont) is expressed as the mean ± SE of three independent experiments (* $p < .05$).

transcription factor; however, how this protein regulates transcription initiation of genes is not well-understood. *IER5L* is named as an IER5-like gene, and its expression and cellular roles have not been elucidated.

In this report, we show that IER2 and IER5L interact with PP2A/B55 through their N-terminal IER homology regions. IER proteins differentially regulate the phosphorylation status of various PP2A target proteins, including S6K, HSF1, and CDC25A. These observations demonstrate that the substrate specificity of PP2A is regulated by IER proteins, which bind both PP2A and its target proteins.

2. Materials and methods

2.1. DNA constructs

The coding regions of IER genes were amplified by PCR from the genomic DNA of HeLa cells and cloned into the gene expression vector pcDNA3.1(+) (Invitrogen). The coding regions of B56γ1, PR72, CDC25A, CDC25C, and 14-3-3ε were amplified by RT-PCR from HeLa total RNA and cloned into pcDNA3.1(+). The HA-tag, Myc-tag, and FLAG-tag sequences were created at the N- and C-terminal regions [15,16]. CDC25A mutant (CDC25A*) exhibiting reduced binding ability to CDC2, CDK2, Cyclin B1, and Cyclin A contained R446L, R450L, and Y455A substitutions [24]. In Fig. 5C, pEBMulti-Hyg (Wako Pure Chemical) containing HA-CDC25A* was used for the analysis. The expression constructs of B55-HA, HA-S6K, HSF1-HA, and HSF1 shRNA and the firefly reporter construct *HSP70-LUC* were described previously [15,16].

2.2. Cell culture and transfection

HeLa and HEK293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B suspension (Wako Pure Chemical). Transfection was conducted using HilyMax (Dojindo Laboratories), Lipofectamine 3000 (Invitrogen), and RNAiMAX (Invitrogen) reagents. For silencing HSF1, HeLa cells carrying the HSF1 shRNA expression construct were cultured in medium containing 1 μg/ml puromycin [10]. The siRNA sequences are as follows: siIER5, 5′-CCGGGAACGUGGCUAACCUTT-3′ and 5′-AGGUUAGCCACGUUCCCGGTT-3′; siScramble, 5′-CCUACGCCA CCAAUUUCGUTT-3′ and 5′-ACGAAAUUGGUGGCGUAGGTT-3′.

2.3. Quantitative PCR analysis

Total RNA was prepared from HeLa and HEK293 cells using ISOGEN II reagent (Nippon Gene). The cDNA was synthesized by PrimeScript reverse transcriptase (Takara Bio) using oligo(dT) and random primers [10]. The real-time quantitative PCR (qPCR) was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems) and a LightCycler 96 system (Roche Applied Science). *β-actin* was used as an endogenous normalization control. Using ΔΔCt analysis protocol, relative fold induction was determined. All data represent three biological replicates with two technical replicates. The primer sequences are as follows: *IER2*, 5′-CGCCTTTCCCAACCTGG-3′ and 5′-GTTGAGCATGCT GTCCGC-3′; *IER5* [10]; *IER5L*, 5′-GGACTTCTGCCCGGACT-3′ and 5′-CACAGCTGCCCGTTGAG-3′; *CDC25A*, 5′-ATGCCAGTCTTACTGTG AGC-3′ and 5′-GCCCTCAGAGCTTCTTCAG-3′; *β-actin*, 5′-ACTGGGACG ACATGGAGAAA-3′ and 5′-GTCTCAAACATGATCTGGGT-3′.

2.4. Western blot analysis

Cell extracts were subjected to SDS-PAGE with/without Phos-tag (Wako Pure Chemical) and analyzed by western blotting as described previously [15,16]. The antibodies used were anti-IER2 (#23849–1-AP, Proteintech Group), anti-IER5 (AP17351c, Abgent), anti-pan-B55 (sc-365,282, Santa Cruz Biotechnology), anti-PP2A-Cα (GTX113523, GeneTex), anti-PP1-Cγ (GTX105618, GeneTex), anti-phospho p70 S6 kinase (T421/S424) (AF8965, R&D Systems), anti-HSP70 (SMC-100, StressMarq Biosciences), anti-HSF1 (ADI-SPA-901, Enzo Life Sciences), anti-phospho HSF1 (Ser307) (#11195, Signalway Antibody), anti-CDC25A (sc-7389, Santa Cruz Biotechnology), anti-phospho CDC25A (Thr507) (AP3051a, Abgent), anti-HA (H9658, Sigma-Aldrich), anti-Myc (M192, MBL), anti-FLAG (M185, MBL), and anti-GAPDH (G9545, Sigma-Aldrich). All data are representative of at least three biological replicates.

2.5. Immunoprecipitation analysis

Cells were lysed in IP buffer (20 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Nacalai Tesque), and phosphatase inhibitor cocktail (Nacalai Tesque)) [16]. Cleared cell extracts were incubated with an antibody at 4 °C for > 2.5 h and mixed with protein A Sepharose CL-4B (GE Healthcare), protein G magnetic beads (New England Biolabs), or anti-

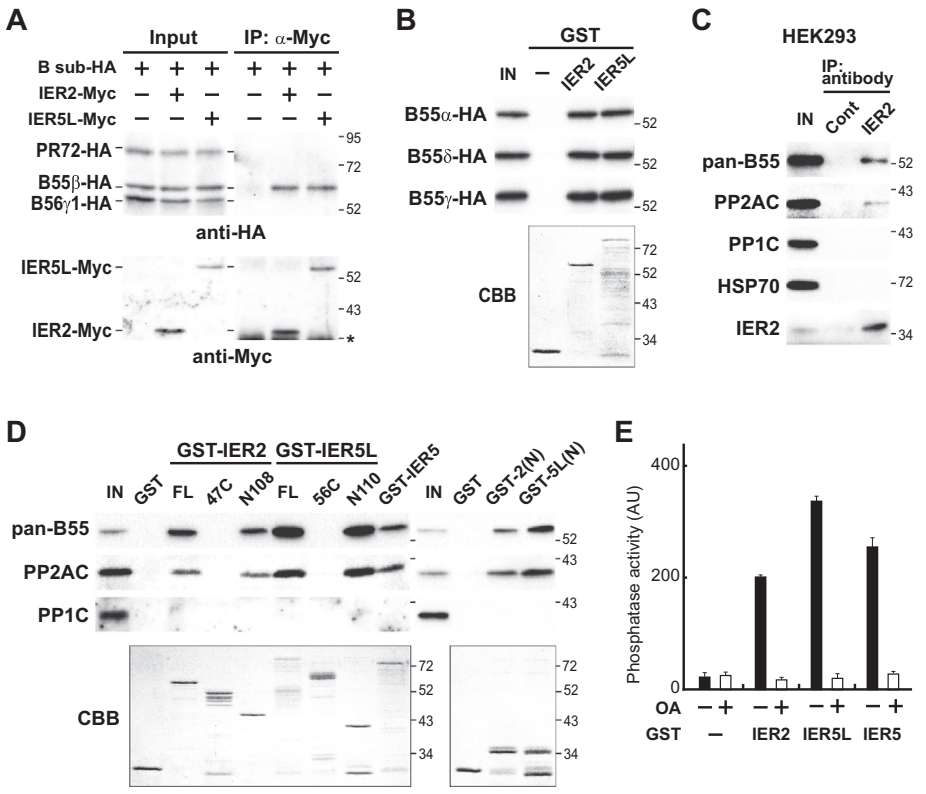

Fig. 2. Interactions of IER2 and IER5L with PP2A.
(A) Co-immunoprecipitation analysis of IER proteins and B subunits. B55β-HA, B56γ1-HA, and PR72-HA expression constructs were transfected along with IER2-Myc or IER5L-Myc expression constructs. HeLa cell extracts were subjected to immunoprecipitation analysis using an anti-Myc antibody, and input and immunoprecipitated (IP) proteins were analyzed by western blotting. Positions of proteins and molecular mass markers are shown on the left and right, respectively. An asterisk shows the protein G bands.
(B) GST pull-down analysis of IER proteins and B55 isoforms. In vitro-synthesized B55α-HA, B55δ-HA, and B55γ-HA polypeptides were incubated with GST (−) or GST-IER2 and GST-IER5L fusion proteins immobilized on glutathione Sepharose. Input (IN) and bound proteins were subjected to western blot analysis using an anti-HA antibody. Lower panel shows GST and GST-IER fusion proteins analyzed by SDS-PAGE and Coomassie brilliant blue (CBB) staining.
(C) Co-immunoprecipitation analysis of IER2 and PP2A. HEK293 cell extracts were subjected to immunoprecipitation analysis using anti-IER2 and control (Cont) antibodies. Extracts (IN: input) and immunoprecipitated (IP) proteins were analyzed by western blotting using anti-pan-B55, anti-PP2A-Cα, anti-PP1-Cγ, anti-HSP70, and anti-IER2 antibodies.
(D) GST pull-down analysis of IER proteins and PP2A. HeLa cell extracts were incubated with GST or GST-IER2 (FL), GST-IER2-47C, GST-IER2-N108, GST-IER5L (FL), GST-IER5L-56C, GST-IER5L-N110, GST-IER5, GST-2(N) (N-terminal 59 amino acids of IER2), and GST-5 L(N) (N-terminal 59 amino acids of IER5L) fusion proteins immobilized on glutathione Sepharose. Cell extracts (IN) and bound proteins were subjected to western blot analysis using anti-pan-B55, anti-PP2A-Cα, and anti-PP1-Cγ antibodies. Lower panels show GST and GST-IER fusion proteins analyzed by SDS-PAGE and Coomassie brilliant blue staining.
(E) Protein phosphatase activity of proteins bound to GST-IER fusions. GST pull-down analysis was done as in (D). Bound proteins were subjected to a phosphatase assay in the absence or presence of 15 nM okadaic acid (OA). The activities are given as arbitrary units (AU) and expressed as the mean ± SE of three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rabbit IgG Sepharose (Cell Signaling Technology) in a rotating wheel for > 1.5 h. Beads were washed twice with IP buffer, and bound proteins were eluted and analyzed by western blotting. All data are representative of at least three biological replicates.

2.6. Glutathione S-transferase (GST) pull-down and phosphatase assay

The coding regions of IER were cloned into the GST gene fusion vector pGEX-6P-1 (GE Healthcare). GST fusion proteins were prepared and approximately 200 ng of protein was immobilized on glutathione-Sepharose 4B (GE Healthcare). HA-tagged polypeptides synthesized in vitro (Promega) were incubated with the beads in binding buffer (10 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 2 mM EDTA, 0.2% NP-40, and 6% glycerol) for 20 min. After two washes using binding buffer, bound proteins were analyzed by western blotting using an anti-HA antibody [15]. All data are representative of at least three independent experiments. For analysis of cellular proteins that bind to the GST fusions, HeLa cell extracts were incubated with the beads in binding buffer for 1 h, and the bound proteins were analyzed by western blotting or were subjected to the phosphatase assay using 6,8-difluoro-4-methylumbelliferyl phosphate (Molecular Probes) as a substrate [15]. Phosphatase activity was calculated from three independent experiments.

2.7. Luciferase assay

HeLa and HSF1-silenced HeLa cells [10] were used for the analysis. The gene expression plasmids were each co-transfected with firefly luciferase reporter plasmid and *Renilla* luciferase control plasmid (pRL-TK, Promega). Firefly luciferase activity (arbitrary units) was calculated from at least three independent experiments after the normalization to *Renilla* luciferase values, as described previously [10,15].

2.8. Statistical analysis

The data are representative of at least three independent experiments. Significant differences were determined by Student's *t*-test.

3. Results

3.1. Regulated expression of IER family genes

Changes in IER expression upon serum stimulation of HeLa and HEK293 cells were analyzed by quantitative RT-PCR (Fig. 1B). When serum-starved cells were activated by 10% serum, the *IER2* mRNA levels strongly increased after 30 min and returned to nearly initial levels after 60 min. *IER5* expression was also transiently activated in HeLa cells, but the levels were lower than that of *IER2*. Therefore, *IER2* and *IER5* are serum-inducible genes [9,17]. Of note, the serum response of mouse *IER2* is mediated by serum response factor and Elk1 [25]. However, the serum addition had a negligible effect on the expression of *IER5L* (*IER5L* is named as an IER5-like gene, and its serum response had not been tested).

Treatment of HeLa and HEK293 cells with hydrogen peroxide (an oxidative stress inducer) and methyl methanesulfonate (MMS, a DNA damage inducer) caused an increase in the *IER2* mRNA levels (Fig. 1C). These stresses induce p38 and JNK mitogen-activated protein kinases and lead to activation of Elk1 [26,27]. Consistently, *IER2* expression was robustly activated by exposure of cells to anisomycin, a potent activator of p38 and JNK [28]. HSF1 is a transcriptional regulator of *IER5* [10], and *IER5* expression was induced under proteotoxic

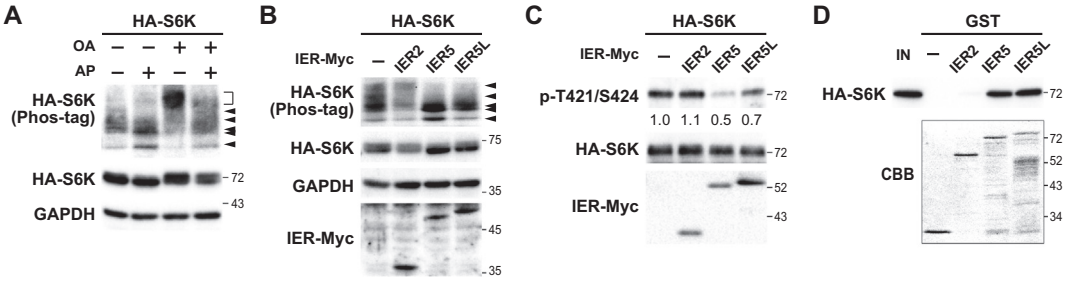

Fig. 3. Effects of IER proteins on the phosphorylation status of S6K.
(A) Phos-tag western blot analysis of S6K. HeLa cells transfected with HA-S6K expression construct were treated with 150 nM okadaic acid (OA) for 1 h. Cells were lysed in IP buffer, and cleared extracts were treated with alkaline phosphatase (AP). Extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting. Closed arrowheads indicate hyper- and hypo-phosphorylated proteins, and bracket shows hyperphosphorylated proteins in okadaic acid-treated cells. Positions of molecular mass markers are shown on the right.
(B) Phosphorylation of S6K in cells expressing IER proteins. HA-S6K expression construct was transfected along with IER-Myc expression constructs. Cell extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting.
(C) Phosphorylation of S6K at T421/S424 in cells expressing IER proteins. HA-S6K expression construct was transfected along with IER-Myc expression constructs. Expression of IER-Myc proteins was analyzed by western blotting using an anti-Myc antibody. HA-S6K was immunoprecipitated by an anti-HA antibody, and total and T421/S424-phosphorylated HA-S6K were analyzed by western blotting using anti-HA and anti-p-T421/S424 antibodies. Numbers show the phosphorylated T421/S424 levels after normalization to the total HA-S6K levels with comparison to the levels of control cells.
(D) GST pull-down analysis of IER proteins and S6K. In vitro-synthesized HA-S6K polypeptides were incubated with GST (−) or GST-IER fusion proteins, and input (IN) and bound proteins were subjected to western blot analysis using an anti-HA antibody. Lower panel shows GST and GST-IER fusion proteins analyzed by SDS-PAGE and Coomassie brilliant blue (CBB) staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions, where cells were exposed to the proteasome inhibitor MG132 or heat shock. *IER5L* expression was moderately elevated by MMS treatment in both cells. These results suggest that IER gene expression is differentially regulated: although MMS induces the expression of all IER family genes, *IER2* expression is induced under p38 and JNK activating conditions, and *IER5* expression is up-regulated by proteotoxic stress such as heat shock.

3.2. Interactions of IER2 and IER5L with PP2A

We conducted co-immunoprecipitation analysis to examine the interactions of IER2 and IER5L with PP2A regulatory subunits. Three classes of B subunits, B55β, B56γ1, and PR72, were tagged with HA, whereas IER2 and IER5L were tagged with Myc. These proteins were expressed in HeLa cells, and immunoprecipitation was performed using an anti-Myc antibody. As shown in Fig. 2A, immunoprecipitation of IER2-Myc caused co-precipitation of B55β-HA, but not B56γ1-HA or PR72-HA. Similarly, IER5L-Myc co-precipitated B55β-HA. The B55 family consists of four members, α, β, γ, and δ [1]. The glutathione *S*-transferase (GST) pull-down assay was conducted by using in vitro-synthesized B55-HA polypeptides and GST-IER fusion proteins (Fig. 2B). The B55α-HA, B55δ-HA, and B55γ-HA subunits were pulled-down with GST-IER2 and GST-IER5L, but not GST alone. Therefore, IER2 and IER5L bind to all four B55 subunits.

Immunoprecipitation of endogenous IER2 from HEK293 cell extracts resulted in co-precipitation of the B55 subunits (pan-B55) and PP2A catalytic subunit (PP2AC), but not the protein phosphatase PP1 catalytic subunit (PP1C) or control heat shock protein 70 (HSP70), showing the specific interaction of IER2 and PP2A in cells (Fig. 2C). When HeLa cell extracts were incubated with immobilized GST fusion proteins in vitro, the B55 and catalytic subunits of PP2A, but not the PP1 catalytic subunit, were pulled-down with GST-IER2, GST-IER5L, and GST-IER5 (Fig. 2D). Both PP2A subunits failed to bind a deletion derivative lacking the N-terminal 46 amino acids of IER2 (IER2-47C) but did bind the N-terminal 108 amino acids of IER2 (IER2-N108). In case of IER5L, deletion of the N-terminal 55 amino acids (IER5L-56C) resulted in a loss of binding to PP2A and the N-terminal 110 amino acids (IER5L-N110) were sufficient for the binding. The binding region was located at the N-terminal IER homology regions of IER2 and IER5L (GST-2(N) and GST-5L(N)). The pulled-down proteins of GST-IER2, GST-IER5L, and GST-IER5 had phosphatase activity; however, the activity was almost completely inhibited by the addition of the PP2A inhibitor okadaic acid (Fig. 2E). The slight differences in the activity were due to the differences in the amounts of pulled-down PP2A, and the higher levels of PP2A in GST-IER5L compared with GST-IER2 and GST-IER5 would be due to the presence of degraded GST-IER5L proteins (see Fig. 2D). These results show that IER2 and IER5L interact with catalytically active PP2A through their N-terminal IER homology regions.

3.3. IER family proteins differentially affect phosphorylation status of S6K

We examined the effects of IER proteins on the phosphorylation status of PP2A target proteins. Cells were transfected with HA-tagged S6K, and extracts were separated on SDS-polyacrylamide gel in the presence or absence of a phosphate-binding molecule, Phos-tag (Fig. 3A). On Phos-tag SDS-PAGE, HA-S6K was separated into five bands. The different mobility was due to the different phosphorylation states of HA-S6K, and hyperphosphorylated forms migrated slower than hypophosphorylated forms, as judged by the alkaline phosphatase treatment of cell extracts. Pre-treatment of cells with okadaic acid caused further hyperphosphorylation of HA-S6K, suggesting the involvement of PP2A in dephosphorylation of cellular S6K. Co-expression of IER5-Myc or IER5L-Myc led to the disappearance of two slowly migrating hyperphosphorylated bands and enhancement of three faster-migrating hypophosphorylated bands (Fig. 3B). In contrast, IER2-Myc failed to induce hypophosphorylation of HA-S6K but rather enhanced hyperphosphorylation for unknown reasons (see Discussion). It has been reported that PP2A is involved in dephosphorylation at threonine 421 (T421) and S424 [29]. The phosphorylation levels at these residues of HA-S6K were reduced by the expression of IER5-Myc and IER5L-Myc, but not IER2-Myc, as judged by the western blot analysis using a phospho-T421/S424 antibody (Fig. 3C). GST pull-down analysis using the in vitro-synthesized HA-S6K polypeptides and GST-IER fusions showed binding of HA-S6K to GST-IER5 and GST-IER5L, but not GST-IER2 or GST alone (Fig. 3D). Therefore, IER proteins exhibit different effects on the phosphorylation status of S6K: interactions of IER5 and IER5L with S6K induce hypophosphorylation of S6K, but IER2 fails to do so.

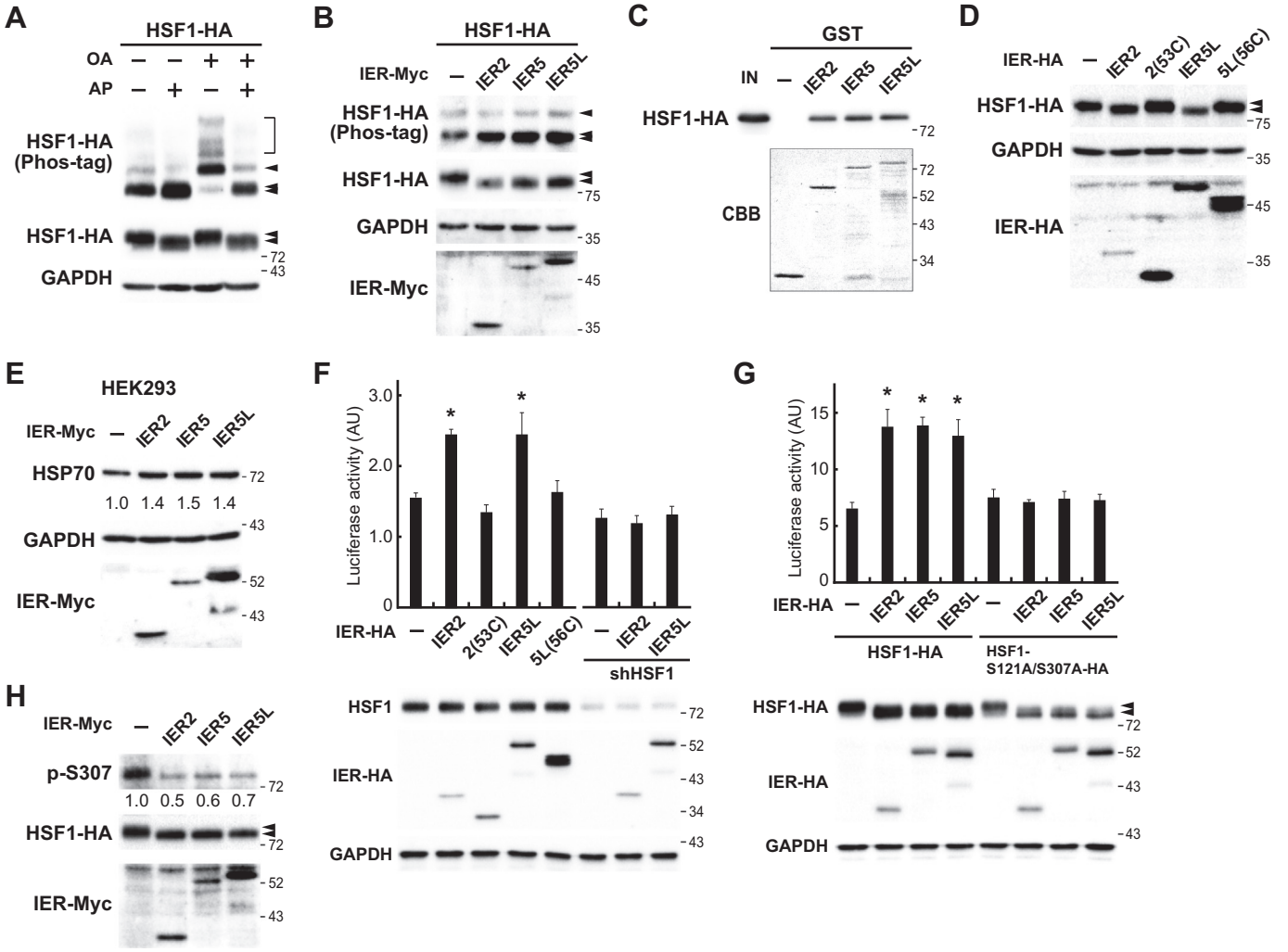

Fig. 4. Effects of IER proteins on the transcriptional activity of HSF1.

(A) Phos-tag western blot analysis of HSF1. HeLa cells transfected with HSF1-HA expression construct were treated with 150 nM okadaic acid (OA) for 1 h. Cells were lysed in IP buffer, and cleared extracts were treated with alkaline phosphatase (AP). Extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting. Closed arrowheads indicate hyper- and hypo-phosphorylated proteins, and bracket shows hyperphosphorylated proteins in okadaic acid-treated cells. Positions of molecular mass markers are shown on the right.

(B) Phosphorylation of HSF1 in cells expressing IER proteins. HSF1-HA expression construct was transfected along with IER-Myc expression constructs. Cell extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting.

(C) GST pull-down analysis of IER proteins and HSF1. In vitro-synthesized HSF1-HA polypeptides were incubated with GST (−) or GST-IER fusion proteins, and input (IN) and bound proteins were subjected to western blot analysis using an anti-HA antibody. Lower panel shows GST and GST-IER fusion proteins analyzed by SDS-PAGE and Coomassie brilliant blue (CBB) staining.

(D) Western blot analysis of HSF1 in cells expressing IER derivatives. HSF1-HA expression construct was transfected along with IER2-HA, IER2-53C-HA, IER5L-HA, or IER5L-56C-HA expression constructs. Cell extracts were subjected to western blot analysis.

(E) Levels of HSP70 in cells expressing IER proteins. Extracts of HEK293 cells expressing IER2-Myc, IER5-Myc, or IER5L-Myc were subjected to western blot analysis. Numbers show the HSP70 levels determined after normalization to the GAPDH levels with comparison to the levels of control cells.

(F) Luciferase assay of *HSP70-LUC* in cells expressing IER derivatives. HeLa and HSF1-silenced HeLa (shHSF1) cells were transfected with *HSP70-LUC* firefly luciferase reporter construct and various IER-HA expression constructs. Firefly luciferase activities (arbitrary units) are expressed as the mean ± SE of five experiments (* $p < .05$). Lower panels show the levels of HSF1 and IER-HA proteins analyzed by western blotting. Note that the mobility shifts of endogenous HSF1 were not evident on SDS-PAGE without Phos-tag.

(G) Luciferase assay of *HSP70-LUC* in cells expressing HSF1 and IER proteins. *HSP70-LUC* firefly luciferase reporter and HSF1-HA or HSF1-S121A/S307A-HA expression constructs were transfected along with IER-HA expression constructs. Firefly luciferase activities (arbitrary units) are expressed as the mean ± SE of five experiments (* $p < .05$). Lower panels show the levels of HSF1-HA and IER-HA proteins analyzed by western blotting. Note that IER5-HA migrated as a 55-kDa band, a lower electrophoretic mobility as compared with the predicted molecular mass of 38 kDa.

(H) Phosphorylation of HSF1 at S307 in cells expressing IER proteins. HSF1-HA expression construct was transfected along with IER-Myc expression constructs. Expression of IER-Myc proteins was analyzed by western blotting using an anti-Myc antibody. HSF1-HA was immunoprecipitated by an anti-HA antibody, and total and S307-phosphorylated HSF1-HA were analyzed by western blotting using anti-HA and anti-p-S307 antibodies. Numbers show the phosphorylated S307 levels determined after normalization to the total HSF1-HA levels with comparison to the levels of control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

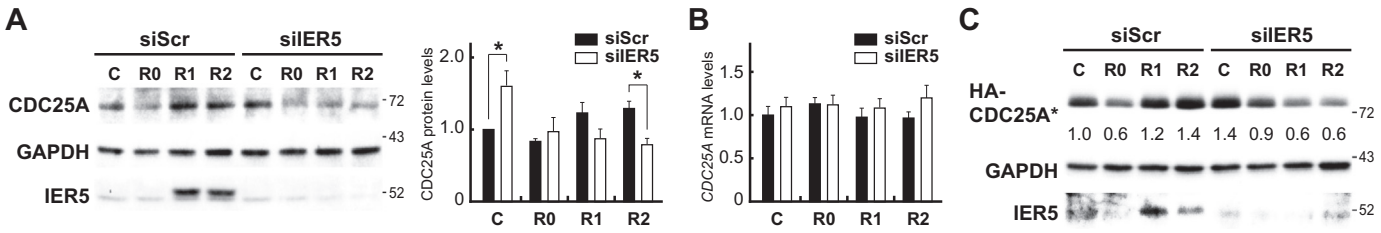

Fig. 5. Effects of IER5 on the expression of CDC25A.

(A) CDC25A protein levels in IER5-silenced cells. HeLa cells transfected with IER5 siRNA (siIER5) or scrambled siRNA (siScr) were cultured at 37 °C (C), heat-shocked at 42.5 °C for 40 min, and allowed to recover (R) at 37 °C for 0, 1, or 2 h. Cell extracts were subjected to western blotting. Positions of molecular mass markers are shown on the right. The relative CDC25A levels were determined after normalization to the GAPDH levels, compared to the levels of unheated siScr cells, and expressed as the mean ± SE of three independent experiments (* $p < .05$).

(B) *CDC25A* mRNA levels in IER5-silenced cells. Cells were cultured as in (A), and total RNA prepared was analyzed by qPCR. The relative *CDC25A* mRNA levels were determined after normalization to the *β-actin* mRNA levels, compared to the levels of unheated siScr cells, and expressed as the mean ± SE of three independent experiments.

(C) HA-CDC25A protein levels in IER5-silenced cells. siIER5- and siScr-treated cells were transfected with HA-CDC25A* expression construct. Cells were cultured as in (A), and extracts were subjected to western blotting. Numbers show the relative HA-CDC25A* levels determined after normalization to the GAPDH levels with comparison to the levels of unheated siScr cells.

3.4. IER2 and IER5L activate transcriptional activity of HSF1

HSF1 is a target protein of PP2A [12,15]. When extracts of cells expressing HA-tagged HSF1 were subjected to Phos-tag western blotting, HSF1-HA was separated into two bands (Fig. 4A). Although dephosphorylation of HSF1-HA by alkaline phosphatase resulted in the formation of much faster migrating bands on Phos-tag gels, the mobility shifts were more evident on gels without Phos-tag. In cells expressing IER2-Myc, IER5-Myc, or IER5L-Myc, HSF1-HA migrated slightly faster than that in control cells (Fig. 4B). As shown in Fig. 4C, HSF1-HA polypeptides were pulled-down with GST-IER2, GST-IER5, and GST-IER5L. Therefore, three IER members bind to HSF1 and convert HSF1 into hypophosphorylated forms.

To gain insight into the roles of IER2 and IER5L in the regulation of HSF1 function, we used their N-terminally truncated derivatives, IER2-53C-HA and IER5L-56C-HA. These derivatives lacking the PP2A-binding regions failed to generate hypophosphorylated HSF1-HA, as judged by the reduced migration on SDS-PAGE (without Phos-tag) (Fig. 4D). *HSP70* is an HSF1-controlled gene, so we analyzed the effects of IER on the expression of HSP70. As shown in Fig. 4E, expression of IER2-Myc, IER5-Myc, or IER5L-Myc caused an increase in the HSP70 protein levels in HEK293 cells. A *HSP70* promoter-driven luciferase reporter construct (*HSP70-LUC*) was used to estimate the transcriptional activity of HSF1. The luciferase activity was increased 1.6-fold by the expression of IER2-HA; however, the activity was not significantly affected by IER2-53C-HA (Fig. 4F). Similarly, IER5L-HA, but not its N-terminally truncated derivative (IER5L-56C-HA), induced the expression of the reporter construct. The effects of IER2-HA and IER5L-HA were not observed when HSF1 was knocked-down by shRNA. Therefore, IER2- and IER5L-mediated hypophosphorylation of HSF1 is related to the derepression of HSF1.

Constitutive phosphorylation of HSF1 on several serine and threonine residues is involved in repression of its transcriptional activity [30]. We and others have previously found that IER5 is involved in the hypophosphorylation of inhibitory phosphorylation sites, including S121, S307, S314, T323, S363, and T367, and enhances HSF1 activity [12,15]. HSF1-HA induced the expression of *HSP70-LUC* in cells (~4.2-fold), and introduction of IER2-HA, IER5-HA, or IER5L-HA caused further 2.0–2.1-fold increases in luciferase activity (Fig. 4G). HSF1-HA containing serine to alanine substitutions at S121 and S307 (S121A/S307A) would be more active than wild type HSF1-HA. HSF1-S121A/S307A-HA also induced the reporter expression, although the mutant HSF1-HA was expressed at lower levels than the wild type for unknown reasons. Notably, IER-HA failed to increase luciferase activity in the presence of the mutant. These results could be explained by a loss of inhibitory phosphorylation sites that would be hypophosphorylated by the expression of IER proteins. The western blot analysis using a phopsho-S307 antibody showed reduced phosphorylation levels at S307 in cells expressing IER2-Myc, IER5-Myc, and IER5L-Myc (Fig. 4H). We suggest that similar to IER5, IER2 and IER5L induce HSF1 transcriptional activity through enhancing hypophosphorylation at S307.

3.5. IER5 regulates CDC25A expression

Three CDC25 family members, CDC25A, CDC25B, and CDC25C, regulate progression of cell division cycle; however, genotoxic and non-genotoxic stresses induce destabilization of CDC25 [31,32]. It has been shown that IER5 negatively regulates the expression of CDC25B [13,14]. We examined whether IER5 affects CDC25A expression under normal and heat shock conditions. In control cells (siScr), the levels of CDC25A were slightly decreased by exposure of cells to 42.5 °C for 40 min (R0) then increased 1.3-fold after 2 h of recovery at 37 °C (R2) (Fig. 5A). Transfection of IER5 siRNA resulted in a 1.6-fold increase in the CDC25A levels in unheated cells. In contrast, the CDC25A levels were remained low during heat recovery periods where heat-induced expression of IER5 was inhibited. The mRNA levels of *CDC25A* were not significantly affected by silencing IER5 or by heat shock (Fig. 5B). To examine whether this is due to changes in the stability of CDC25A, the levels of exogenously expressed HA-CDC25A were analyzed. To avoid the effects on cell cycle progression, a CDC25A mutant (CDC25A*) exhibiting reduced binding ability to CDC2, CDK2, Cyclin B1, and Cyclin A was used for the analysis [24]. In siIER5 cells, the steady-state levels of HA-CDC25A* were slightly higher than unheated siScr cells, whereas under heat-shocked conditions the levels were lower than siScr cells (Fig. 5C). Therefore, IER5 differentially regulates the CDC25A levels under normal and thermal stress conditions.

3.6. IER family proteins inhibit interactions of CDC25A with 14-3-3

On Phos-tag SDS-PAGE, phosphorylated HA-CDC25A was separated into four bands, and further hyperphosphorylated forms were observed in okadaic acid-treated cells (Fig. 6A). Although the most slowly migrating HA-CDC25A band was prominent in control cells, expression of IER2-Myc, IER5-Myc, or IER5L-Myc led to shift of HA-CDC25A to the hypophosphorylated forms (Fig. 6B). We also analyzed CDC25C and found that HA-tagged CDC25C was phosphorylated in cells and that PP2A was involved in dephosphorylation of HA-CDC25C (Fig. 6C). In contrast to HA-CDC25A, the phosphorylation status of HA-CDC25C was not notably affected by the expression of IER family proteins (Fig. 6D). Therefore, IER proteins exhibit differential effects on proteins belonging

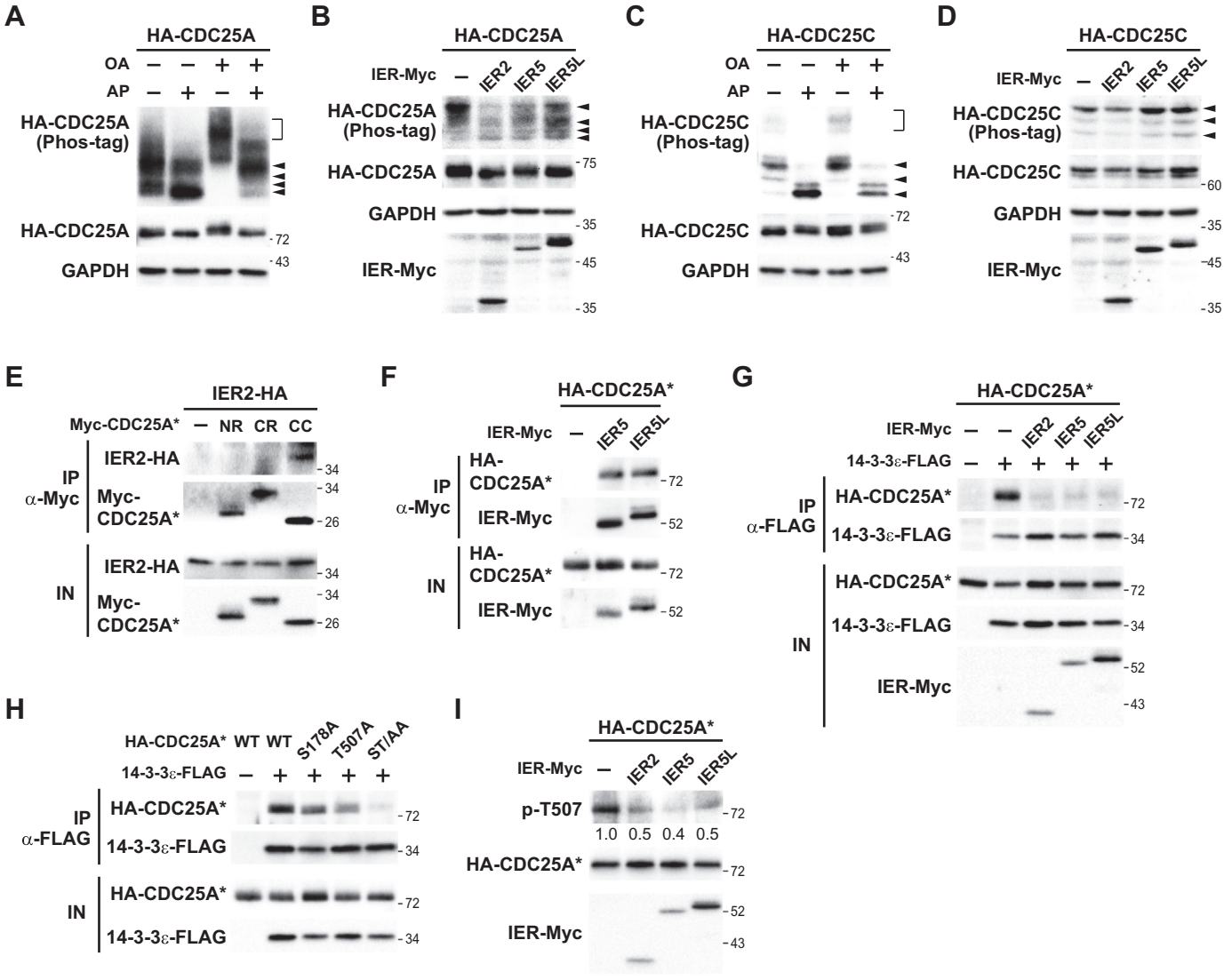

Fig. 6. Effects of IER proteins on interactions of CDC25A and 14-3-3.

(A and C) Phos-tag western blot analysis of CDC25. HeLa cells transfected with HA-CDC25A (A) and HA-CDC25C (C) expression constructs were treated with 150 nM okadaic acid (OA) for 1 h. Cells were lysed in IP buffer, and cleared extracts were treated with alkaline phosphatase (AP). Extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting. Closed arrowheads indicate hyper- and hypo-phosphorylated proteins, and bracket shows hyperphosphorylated proteins in okadaic acid-treated cells. Positions of molecular mass markers are shown on the right.

(B and D) Phosphorylation of CDC25 in cells expressing IER proteins. HA-CDC25A (B) and HA-CDC25C (D) expression constructs were transfected along with IER-Myc expression constructs. Cell extracts were subjected to SDS-PAGE with/without Phos-tag and analyzed by western blotting.

(E) Co-immunoprecipitation analysis of IER2 with CDC25A. IER2-HA expression construct was transfected along with Myc-CDC25A* expression constructs containing the N-terminal regulatory region (NR, residues 1 to 185), central regulatory region (CR, residues 151 to 336) or C-terminal catalytic domain (CC, residues 336 to 524). Cell extracts were subjected to immunoprecipitation analysis using an anti-Myc antibody, and input (IN) and immunoprecipitated (IP) proteins were analyzed by western blotting.

(F) Co-immunoprecipitation analysis of IER5 and IER5L with CDC25A. HA-CDC25A* expression construct was transfected along with IER5-Myc or IER5L-Myc expression constructs. Cell extracts were subjected to immunoprecipitation analysis using an anti-Myc antibody, and input (IN) and immunoprecipitated (IP) proteins were analyzed by western blotting.

(G) Co-immunoprecipitation analysis of CDC25A with 14-3-3ε in cells expressing IER proteins. HA-CDC25A* and 14-3-3ε-FLAG expression constructs were transfected along with IER-Myc expression constructs. Cell extracts were subjected to immunoprecipitation analysis using an anti-FLAG antibody, and input (IN) and immunoprecipitated (IP) proteins were analyzed by western blotting.

(H) Co-immunoprecipitation analysis of CDC25A mutants with 14-3-3ε. HA-CDC25A* expression constructs containing S178A, T507A, and both substitutions (ST/AA) were transfected along with 14-3-3ε-FLAG expression construct. Cell extracts were subjected to immunoprecipitation analysis using an anti-FLAG antibody, and input (IN) and immunoprecipitated (IP) proteins were analyzed by western blotting.

(I) Phosphorylation of CDC25A at T507 in cells expressing IER proteins. HA-CDC25A* expression construct was transfected along with IER-Myc expression constructs. Expression of IER-Myc proteins was analyzed by western blotting using an anti-Myc antibody. HA-CDC25A* was immunoprecipitated by an anti-HA antibody, and total and T507-phosphorylated HA-CDC25A* were analyzed by western blotting using anti-HA and anti-p-T507 antibodies. Numbers show the phosphorylated T507 levels determined after normalization to the total HA-CDC25A* levels with comparison to the levels of control cells.

to the CDC25 family.

We examined the interaction of IER2 with CDC25A. CDC25A constructs consisting of the Myc-tagged N-terminal regulatory region, central regulatory region, and C-terminal catalytic domain were expressed in cells along with IER2-HA. As shown in Fig. 6E, co-immunoprecipitation analysis demonstrated the binding of IER2 to the C-terminal catalytic domain of CDC25A. The interactions of IER5 and IER5L with CDC25A were also analyzed, and HA-CDC25A* was co-precipitated with IER5-Myc or IER5L-Myc by an anti-Myc antibody (Fig. 6F). Therefore, all three IER members bind to CDC25A.

14-3-3 proteins, which interact with proteins through recognition of a phosphoserine or phosphothreonine motif, are known to bind CDC25A [33]. We examined the effects of IER proteins on the interaction of CDC25A and 14-3-3. As shown in Fig. 6G, HA-CDC25A* was co-precipitated with FLAG-tagged 14-3-3ε by an anti-FLAG antibody. The levels of co-precipitated HA-CDC25A* were decreased when IER2-Myc, IER5-Myc, and IER5L-Myc were expressed in cells. The interaction of CDC25A and 14-3-3 is mediated by phosphorylated residues S178 and T507 [33]. Consistently, HA-CDC25A* containing either a S178A or T507A substitution exhibited reduced binding to 14-3-3ε-FLAG, and the combination of both substitutions resulted in a loss of binding (Fig. 6H). The western blot analysis using a phopsho-T507 antibody showed reduced phosphorylation levels at T507 of HA-CDC25A* in cells expressing IER2-Myc, IER5-Myc, and IER5L (Fig. 6I). Therefore, all IER proteins enhance dephosphorylation of T507 and inhibit the interactions of CDC25A with 14-3-3.

4. Discussion

Gene expression of IER family proteins, IER2, IER5, and IER5L, is differentially regulated under various growth conditions. Particularly, IER2 expression is upregulated under p38 and JNK activating conditions, whereas IER5 expression is upregulated by proteotoxic stress. Similarly to IER5, IER2 and IER5L interact with PP2A/B55 through their N-terminal homology regions. IER5 and IER5L, but not IER2, also bind to PP2A/B55 target S6K and enhance hypophosphorylation of S6K. All three IER members bind to HSF1 and CDC25A, and thus enhance dephosphorylation of the proteins. Dephosphorylation of HSF1 leads to induction of HSF1 transcriptional activity and that of CDC25A causes dissociation from 14-3-3 regulatory protein. We suggest that differentially expressed IER proteins regulate the specificity of PP2A toward different target proteins.

Both IER2 and IER5L bind to the α, β, δ, and γ isoforms of B55, but not B56γ1 or PR72. The IER family members share homology at their N-terminal 50 amino acids and this region is required for the interactions with PP2A; four different isoforms of B55 share high levels of sequence similarity. The B subunits determine the substrate specificity, subcellular localization, and enzymatic activity of the PP2A holoenzyme. IER proteins regulate the phosphorylation status of PP2A/B55 targets HSF1 and CDC25A, but not CDC25C. It has been shown that PP2A targets CDC25C via the B56δ regulatory subunit [34]. Although IER5 and IER5L also enhance hypophosphorylation of S6K, IER2 rather contributes to hyperphosphorylation of S6K. This effect of IER2 would be indirect, because it fails to bind S6K. It is possible that IER2 enhances the activity of a protein kinase phosphorylating S6K. There are various inhibitor proteins of PP2A: the inhibitors interact with the catalytic C subunit, scaffold A subunit, and regulatory B subunit [35]. CIP2A interacts with both the B56 subunits and PP2A target c-Myc and inhibits PP2A activity at phosphorylated S62, which causes stabilization of c-Myc [36]. There are only a few proteins that enhance PP2A phosphatase activity toward the specific substrate [37,38]. Eya3 interacts with both the B55α subunit and c-Myc and enables PP2A to dephosphorylate T58, which also causes stabilization of c-Myc [38]. The IER proteins are new members of PP2A activators, which interact with both PP2A and its targets and enhance phosphatase activity.

CDC25A phosphatase is an activator of the CDK-cyclin complex for cell cycle progression. CDC25A is a potential oncogene and is overexpressed in various human cancers [31]. 14-3-3 binding impairs the ability of CDC25A to interact with CDK1/cyclin B1 [33]. Expression of IER proteins causes dephosphorylation at T507 and dissociation of CDC25A from 14-3-3, suggesting that IER enhances the CDC25A activity on CDK1/cyclin B1. IER2 is known to be a regulator of the G_1-S transition, and siRNA-mediated knockdown of IER2, as CDC25A, leads to G_1 arrest of breast cancer cells [39]. However, IER5 downregulates the CDC25A levels under normal culture conditions. CDK-cyclin complexes are known to induce CDC25A degradation in a negative feedback loop [40,41]. It is possible that active CDC25A is more labile than 14-3-3-bound inactive form; therefore, the CDC25A levels decrease in IER5-expressing cells. In heat-shocked cells, however, IER5 is involved in a rapid increase in the CDC25A levels. Consistent with this, IER5 is required for the efficient recovery of cells from heat stress [10]. IER proteins also enhance the transcriptional activity of HSF1, a master regulator of the heat shock response, through dephosphorylation of the inhibitory phosphorylated residues. HSF1 has a pro-oncogenic role and supports cancer cell growth, survival, and metastasis [30,42]. Therefore, IER proteins could promote cell growth via activating CDC25A and HSF1.

Although PP2A functions as an antagonist of many signaling pathways associated with cell growth and proliferation, it also functions as a promoter, depending on the context, by dephosphorylating a residue that negatively regulates the function of proteins in oncogenic pathways [37,38,43]. It is noteworthy that high expression of IER5 is associated with poor prognosis of cancer patients [12]. IER2 expression in tumor cells correlates with metastatic potential [19]. We speculate that IER proteins may play an important role in various signaling pathways through regulating the substrate specificity of PP2A.

5. Conclusions

IER proteins differentially regulate the phosphorylation status of various PP2A target proteins, including S6K, HSF1, and CDC25A. The substrate specificity of PP2A is regulated by IER proteins, which bind both PP2A and its target proteins.

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Author contributions

T.U. and Y.K. contributed equally to this work; T.U. and Y.K. performed experiments and analyzed the data; H.S. planned the experiments and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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