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The prolyl isomerase Pin1 interacts with a ribosomal protein S6 kinase to enhance insulin–induced AP–1 activity and cellular transformation

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朝鮮大學校 大學院

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(국문 초록)

Pin1 과 p70S6K 의 상호작용에 의한 insulin 유도성 AP-1 활성 및 세포 형질 변환의 조절 기전 연구

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조선대학교 대학원 약학과

펩티딜-프롤린 이성화효소인 Pin1 은 serine 이나 threonine 뒤에 proline 이 따르는 잔기가 인산화(pSer/Thr-Pro)된 단백질에 결합해서 cis/trans 이성화효소로 작용하여, 세포 증식과 세포형질전환에 중추적인 역할을 하는 것으로 알려져 있다. 비록 Pin1 이 간암 세포주에서 과발현 되었다고 보고되었지만, 그 분자적 기전은 밝혀지지 않았다. 이 논문에서 본인은 in vitro, ex vivo 에서 Pin1 이 p70S6K 와 결합한다는 것을 밝혔다.

Pin1 을 과발현시켰을 때, 간암세포(SK-HEP-1)에서 인슐린 자극에 의한 p70S6K 의 인산화가 더욱 증가되었고, 반대로 Pin1 knockout mouse embryonic fibroblasts (MEFs)에서는 정상세포와 비교시 인슐린에 의한 p70S6K 의 인산화가 현저히 감소되었다. 그 뿐만 아니라, 인슐린 자극시 Pin1 은 p70S6K 와의 상호작용을 통해 ERK1/2 의 인산화를 증가시켰다. 이러한 현상은 p70S6K 활성을 억제하는 rapamycin 처리를 통해 억제되었다. 따라서, Pin1 은 간암세포에서 p70S6K-ERK1/2 신호를 통해서 AP-1 의 활성에 영향을 준다는 것을 규명하였다. 이러한 기전을 통해서, Pin1 이 infection 된 JB6 Cl41 세포는 GFP 만 infection 된 JB6 Cl41 세포에 비해서 인슐린 자극에 의한 세포 형질전환이 훨씬 유도된다는 것을 확인하였다. 이러한 결과들은 간암세포에서 Pin1 이 p70S6K 와의 상호작용을 통해 인슐린 신호를 증폭시켜 발암 과정에 중요한 역할을 하는 단백질이라는 것을 시사하며, 이를 항암 치료의 타겟으로 설정할 수 있다는 가능성을 보여준다.

ABSTRACT

The prolyl isomerase Pin1 interacts with a ribosomal protein S6 kinase to enhance insulin-induced AP-1 activity and cellular transformation

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Phosphorylation of proteins on serine or threonine residues that immediately precede proline (pSer/Thr-Pro) is specifically catalyzed by the peptidyl-prolyl cis/trans isomerase Pin1 and Pin1 is a central signaling mechanism in cell proliferation and transformation. Although Pin1 is frequently overexpressed in hepatocellular carcinoma (HCC), the molecular mechanism of Pin1 in HCC has not been completely elucidated. Here, we show that Pin1 interacts with p70S6K *in vitro* and *ex vivo*. Overexpression of Pin1 resulted in enhanced p70S6K phosphorylation induced by insulin in SK-HEP-1 cells. In contrast, $Pin1^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited significantly

decreased insulin-induced p70S6K phosphorylation compared to $Pin1^{+/+}$ MEFs. Furthermore, Pin1 enhanced the insulin-induced ERK1/2 phosphorylation through its interaction with p70S6K, whereas the inhibition of p70S6K activity by rapamycin suppressed insulin-induced ERK1/2 phosphorylation in SK-HEP-1 cells. Hence, Pin1 affected AP-1 activity through p70S6K-ERK1/2 signaling in SK-HEP-1 cells. Most importantly, Pin1-overexpressing JB6 Cl41 cells enhanced neoplastic cell transformation promoted by insulin much more than GFP-overexpressing JB6 Cl41 control cells. These results imply that Pin1 amplifies insulin signaling in hepatocarcinoma cells through its interaction with p70S6K, suggesting that Pin1 plays an important role in insulin-induced tumorigenesis and is a potential therapeutic target in hepatocarcinoma.

Keywords: Pin1, p70S6K, ERK1/2, insulin, AP-1, hepatocarcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1,2). HCC is an aggressive tumor with a generally poor prognosis (3). Oncogenesis is a multistep process that ultimately results in uncontrolled cell proliferation and neoplastic cell transformation. A pivotal signaling mechanism regulating cell proliferation is the phosphorylation of proteins on serine or threonine residues preceding proline (pSer/Thr-Pro) (4). The recent identification and characterization of a peptidyl-prolyl cis/trans isomerase, Pin1, has led to the discovery of a new phosphorylation regulatory mechanism in cell signaling (5-8). Pin1 is an enzyme that specifically binds phosphorylated serine or threonine immediately preceding proline (7) and then regulates protein function, including regulation of catalytic activity levels, phosphorylation status, protein interaction, subcellular location, and/or protein stability by promoting cis/trans isomerization of the peptide bond (9,10). Pin1 contains both an NH_2-terminal WW domain and a COOH-terminal isomerase domain (11). The WW domain binds specific pSer/Thr-Pro motifs to enable the isomerase domain to function by inducing conformational changes in the

pSer/Thr-Pro bond (11). Recently, it was reported that Pin1 is overexpressed in HCCs (11-13). A better understanding of the role of Pin1 in hepatocarcinogenesis may lead to the identification of molecular targets for prevention and therapeutic intervention.

One potential target that has emerged as a new therapeutic target in the management of hepatocellular carcinoma is the signal transduction pathway centered on the mammalian target of rapamycin (mTOR) protein kinase (14). The mTOR signaling pathway is integrated with the insulin-regulated phosphatidyl-inositide-3-OH-kinase (PI3K) pathway to control nutrient and energy homeostasis (15,16). Rapamycin and its derivatives, RAD001 (Novatis) and CCI-779 (Wyeth Ayerst), which are presently in clinical trials for the treatment of solid tumors, block mTOR signaling by forming an inhibitory complex with the immunophilin FKBP12; FKBP12 binds to and inhibits the ability of mTOR to phosphorylate downstream substrates, including the ribosomal S6 kinases (p70S6K), S6K1 and S6K2, and translation initiation factor 4E binding proteins (17-19). The importance of mTOR as a therapeutic target in the treatment of HCC is underscored by reports that this pathway is activated in a large number of these tumors and that mTOR and p70S6K are overexpressed in a

significant proportion of hepatocellular carcinomas (20,21). However, the molecular mechanism of mTOR action in hepatocellular carcinoma is not fully understood.

Our data provide the first evidence that Pin1 regulates the insulin-signaling pathway in such a way as to increase the phosphorylation of ribosomal protein S6 kinase (p70S6K). In this signaling pathway, Pin1 binds phosphorylated p70S6K, which is downstream of mTOR signaling in hepatocarcinoma cells. Furthermore, Pin1 affects the interaction of p70S6K with ERK1/2, resulting in increased AP-1 transactivation activity and neoplastic cellular transformation. These results suggest that Pin1 plays an important role in promoting insulin-induced hepatocarcinogenesis.

2. Materials and Methods

2-1. Reagent and Antibodies

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Insulin (NovoLet® R) and rapamycin was purchased from Novo Nordisk (Bagsvaerd, Demark) and Calbiochem (EMD Chemicals, Inc. Gibbstown, NJ, USA), respectively. Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA, USA). jetPEI cationic polymer transfection reagent was obtained from Polyplus-transfection Inc. (New York, NY, USA). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI, USA). Antibodies against phospho-p70S6K, -Akt, -ERK1/2, -Thr-Pro and total antibodies for each respective protein were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA); phospho-GSK3-β and total GSK3 were from Upstate (Temecula, CA, USA); anti-Pin1, goat anti-mouse IgG HRP and goat anti-rabbit IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Xpress was from Invitrogen (Carlsbad, CA, USA).

2-2. Cell culture and transfection

SK-HEP-1 human hepatocellular carcinoma cells, HEK 293 human embryonic kidney cells, and *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mouse embryonic fibroblast (MEF) cells were grown in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics (100U/mL penicillin, 100μg/mL streptomycin). The *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells were kindly provided by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School, MA). Cells were incubated at 37°C in humidified air containing 5% CO_2. DNA transfection of cells was performed using jetPEI cationic polymer transfection reagent (Polyplus-transfection Inc., New York, NY, USA).

2-3. Construction of mammalian expression and small interfering RNA vectors

For the mammalian two-hybrid (M2-H) system, the cDNAs of 50 human kinases were amplified by polymerase chain reaction (PCR) and each introduced into the pACT or pBIND two-hybrid system vector. In this system, pBIND and pACT (Promega) are fusion vectors used to link proteins to the GAL4 DNA binding domain and to the VP16

transactivation domain. pBIND-Pin1 was generated by PCR and subcloned into the pcDNA4/*Xpress* vector (Invitrogen, Carlsbad, CA). A segment encoding the full coding sequence of the selected human kinase candidate, in this case p70S6K, was amplified by PCR and cloned in-frame into the *BamHI/XbaI* sites of the pACT fusion vector or the *BamHI/XhoI* sites of the pcDNA3.1/*Myc* fusion vector to produce plasmid pACT-p70S6K or pcDNA3.1/*Myc*-p70S6K, respectively. pBIND-Pin1-wild type (WT), -WW domain (WW), and -PPIase domain (PPIase) were a gift from by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School, MA). For the knockdown of human Pin1, a validated Stealth^{TM} RNAi DuoPak of human Pin1 was purchased from Invitrogen (Carlsbad, CA) and was introduced using jetSI^{TM}-ENDO according to the recommended protocols (Polyplus-transfection Inc., New York, NY).

2-4. Infection of GFP and Pin1 into JB6 Cl41 cells

Pin1 was stably expressed in JB6 Cl41 cells using the MSCV-GFP retrovirus system. Human Pin1 was subcloned into the MSCV-GFP retroviral vector (Clontech Inc., Mountain View, CA) and phoenix cells (a packaging cell line) were transfected with MSCV-GFP (control)

or MSCV-Pin1-GFP (Pin1-overexpressing) plasmid. Supernatants containing amphotropic replication-incompetent retroviruses were collected and then stored at -80°C until required. JB6 Cl41 cells (20% confluent) were multiply infected (8 times) with retrovirus particles. Intensities of infection were monitored by GFP-fluorescence and western blotting analysis using the Pin1-specific antibody.

2-5. Mammalian two-hybrid assay

pACT-p70S6K, pBind-Pin1-WT, pBind-Pin1-WW or pBind-Pin1-PPIase was combined with pG5-luciferase in equal molar ratio and the total amount of DNA was at most 100 ng/well. Transfections were performed using the jetPEI reagent as described by the manufacturer. The cells were disrupted by direct addition of 100 μL of passive lysis buffer (Promega) into each well of the 48-well plates and then incubated at 4°C for 1 hour. The cells were completely lysed by freezing and thawing 2 times. After centrifugation, 50 μl of cell lysates were added to individual wells of a 96-well luminescence plate. The luminescence activity was measured using a luminometer (Berthold Technologies LB941, Bad Wildbad, Germany). The relative luciferase activity was calculated and normalized to the pG5-luciferase basal

control. To assess transfection efficiency and protein concentration, the *Renilla* luciferase activity assay and Lowry protein assay were used, respectively.

2-6. Immunoblotting

Cells were disrupted in lysis buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride and 1x protease inhibitors cocktail]. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. Protein bands were visualized by the chemiluminescence detection kit (Amercham HRP Chemiluminescent Substrates, Amersham) after hybridization with horseradish peroxidase (HRP)–conjugated secondary antibody from rabbit or mouse. For chemiluminescence detection, the LAS3000-mini (Fujifilm, Japan) was used.

2-7. In vitro binding assay

To evaluate the biochemical interaction between Pin1 and

p70S6K *in vitro*, DNA transfections and immunoprecipitations were performed. The Xpress-epitope tagged Pin1-WT (pcDNA4/Xpress-Pin1-WT) was co-transfected with myc-epitope tagged p70S6K (pcDNA3/myc-p70S6K) into human embryonic kidney cells (HEK293) using the jetPEI reagent as described by the manufacturer. pcDNA4/Xpress-Pin1-WT or pcDNA3/myc-p70S6K was immunoprecipitated with antibody against anti-Xpress or anti-myc antibody, respectively, and then immunoblotting was performed. The bound proteins were denatured in sample buffer and separated by 10-20% SDS-PAGE; expression was detected by the LAS3000-mini (Fujifilm, Japan).

2-8. Anchorage-independent cell transformation assay (soft agar assay)

Insulin-induced cell transformation was investigated in GFP-, Pin1-infected JB6 Cl41 or JB6 Cl41 cells. In brief, cells (8×10^3/ml) were exposed to insulin (0, 25, 50, 100 nM) in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO2 atmosphere for 3 weeks, and cell colonies larger than 80 μm in diameter were scored using an Axiovert

200M fluorescence microscope and AxioVision software (Carl Zeiss Inc., Thornwood, NY).

2-9. Reporter gene assays

The reporter gene assay for firefly luciferase activity was performed using lysates from transfected cells. The reporter gene vector *phRL-SV40* was also co-transfected into each cell line and the *Renilla* luciferase activity generated by this vector was used to normalize for transfection efficiency. Cell lysates were prepared by first washing the transfected SK-HEP-1 cells (grown in 12-well plates) once in PBS. After complete removal of the PBS, passive lysis buffer (200 μL; Promega Dual Luciferase Reporter Assay System) was added; the cells were then incubated for 1 hour with gentle shaking. Cell lysates (80 μL each) were mixed with 50 μL Luciferase Assay II reagent and firefly luciferase light emission was measured by TriStar (Berthold Technologies LB941, Bad Wildbad, Germany). Subsequently, 50 μL of *Renilla* luciferase substrate was added in order to normalize the firefly luciferase data. The AP-1 luciferase reporter plasmid was kindly provided by Dr. Jeong (College of Pharmacy, Chosun University, Gwangju, Korea).

3. Results

3-1. Pin1 interacts with p70S6K and enhances the activity of p70S6K.

To confirm the interaction between Pin1 and p70S6K, we co-transfected HEK 293 cells with Myc-tagged p70S6K and Xpress-tagged Pin1. Then, we immunoprecipitated cell lysates using normal mouse IgG or anti-Myc antibody and blotted with anti-Xpress antibody. Reciprocal immunoprecipitation/immunoblotting using anti-Xpress and anti-Myc antibodies showed that the exogenously expressed p70S6K interacted with Xpress-tagged full-length Pin1 (Fig. 1*A* and *B*). To determine the region of Pin1 that was responsible for its interaction with p70S6K, full-length Pin1 (Pin1-WT), the WW domain of Pin1 (Pin1-WW), or the PPIase domain of Pin1 (Pin1-PPIase) (Fig. 1*C*) was co-transfected with p70S6K, and the interaction was examined by a mammalian two-hybrid assay (Promega). The Pin1-binding site on p70S6K encompasses amino acids 1-44, located in the WW domain (Fig. 1*C*). To examine whether the expression of Pin1 affected the activity of p70S6K, various doses of pcDNA4/Xpress-Pin1 were transfected into HEK 293 cells and the cells were incubated for 48 h under normal culture condition. Pin1 enhanced the phosphorylation of

p70S6K, as well as that of a downstream molecule, GSK3-β (Fig. 1*D*). These results suggest that the WW domain of Pin1 may recognize specific pSer/Thr-Pro motifs in p70S6K while the PPIase domain catalyzed the *cis/trans* isomerization of pSer/Thr-Pro bonds.

3-2. Pin1 enhance insulin-induced phosphorylation of p70S6K.

Given that all known Pin1 substrates are phosphoproteins (6,7) and the phosphorylation of the potential Pin1 binding site occurs on serine or threonine residues preceding proline (pSer/Thr-Pro) (4), we examined whether the interaction between Pin1 and p70S6K was phosphorylation-dependent. The result showed that insulin induced the phosphorylation of p70S6K at a threonine residue preceding proline, suggesting that pThr/Pro in p70S6K was necessary for Pin1 binding (Fig. 2*A*). Next, we examined the time course for the insulin-induced interaction of Pin1 and p70S6K in human hepatocarcinoma SK-HEP-1 cells to determine whether endogenous Pin1 and p70S6K also interact *ex vivo*. An antibody against p70S6K was used to immunoprecipitate p70S6K, and then Pin1 was detected by immunoblot. The results indicated that Pin1 was detectable in p70S6K immunoprecipitates from 10 min to 30 min after insulin treatment (Fig. 2*B*). To assess whether

insulin signaling was regulated by Pin1, we exposed *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells to insulin (50 nM) and immunoblotted with the respective antibodies. We found significantly decreased phosphorylation of p70S6K and GSK3-β in *Pin1*$^{-/-}$ MEF cells. However, phosphorylation of Akt in *Pin1*$^{-/-}$ cells was not significantly changed compared to that in *Pin1*$^{+/+}$ cells (Fig. 2*C*). To further determine the effect of Pin1, we exposed mock- or pcDNA4-Pin1-transfected SK-HEP-1 cells to insulin (50 nM) and performed immunoblotting with the respective antibodies. We found significantly increased phosphorylation of p70S6K and GSK3-β, but not of Akt, in pcDNA4-Pin1-transfected cells (Fig. 2*D*). Taken together, these data indicate that, upon insulin stimulation, Pin1 interacts with phosphorylated p70S6K at a threonine preceding proline and enhances the activity of p70S6K.

3-3. Pin1 induces ERK1/2 phosphorylation through activation of p70S6K.

Insulin induces the mTOR-dependent phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 (22), suggesting molecular crosstalk between p70S6K and MAPK cell signaling pathways (23). To determine whether Pin1 regulates insulin-induced

ERK1/2 phosphorylation, we exposed *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ MEF cells to insulin (50 nM) and performed immunoblotting with antibody against phospho-ERK1/2. We found significantly decreased phosphorylation of ERK1/2 in *Pin1*$^{-/-}$ MEF cells compared to *Pin1*$^{+/+}$ cells (Fig. 3*A*). Next, we examined ERK1/2 phosphorylation in Pin1-overexpressing SK-HEP-1 cells to further confirm the role of Pin1 in insulin signaling; we exposed mock- or pcDNA4-Pin1-transfected SK-HEP-1 cells to insulin (50 nM) and performed immunoblotting with anti-phospho-ERK1/2. We found increased phosphorylation of ERK1/2 in pcDNA4-Pin1-transfected cells compared to mock-transfected cells (Fig. 3*B*). These data indicate that Pin1 enhances insulin-induced ERK1/2 phosphorylation.

To determine whether the phosphorylation of ERK1/2 is mediated by direct interaction between p70S6K and ERK1/2, we examined the time course for the insulin-induced interaction of p70S6K and ERK1/2 in SK-HEP-1 cells. An antibody against ERK1/2 was used to immunoprecipitate ERK1/2, and then p70S6K was detected by immunoblotting. p70S6K was detectable in ERK1/2 immunoprecipitates at 10 min and 30 min, but not at 60 min after insulin treatment (Fig. 3*C*). Under the same conditions,

phosphorylation of p70S6K and ERK1/2 also increased in whole cell lysates at 10 – 30 min after insulin treatment (Fig. 3*C*, *cell lysate panals*).

3-4. Rapamycin suppresses insulin-induced ERK1/2 phosphorylation.

To determine whether Pin1 induces ERK1/2 phosphorylation through activation of p70S6K, insulin-induced phosphorylation of p70S6K was completely blocked by the mTOR inhibitor rapamycin (10 nM), confirming that regulation of p70S6K requires mTOR. We found significantly decreased phosphorylation of ERK1/2 in rapamycin-treated, Pin1-overexpressing cells. These data indicate that Pin1 enhances insulin-induced ERK1/2 phosphorylation through indirect activation of mTOR/p70S6K (Fig. 4*A*). To assess the effect of rapamycin on the insulin-induced interaction of p70S6K and ERK1/2, we treated SK-HEP-1 cells to insulin with rapamycin. An antibody against ERK1/2 was used to immunoprecipitate ERK1/2, and then p70S6K was detected by immunoblotting. As in Fig. 3*C*, p70S6K was detectable in ERK1/2 immunoprecipitates 30 min after insulin treatment. However, the interaction between p70S6K and ERK1/2 was almost completely suppressed due to the inhibition of p70S6K activity

by rapamycin (Fig. 4*B*). Moreover, phosphorylation of p70S6K and ERK1/2 also decreased in whole cell lysates after rapamycin treatment (Fig. 4*B*, *cell lysate panals*). Taken together, these results indicate that Pin1 augmented the crosstalk between p70S6K and ERK1/2 through its binding and isomerizaton activities toward p70S6K.

3-5. Pin1 and p70S6K have a synergistic effect on insulin-induced AP-1 activity.

The Activator protein-1 (AP-1) transcription factor is a complex of homo- or heterodimers of the Jun, Fos, activating transcription factor, and musculoaponeurotic fibrosarcoma protein families (24). Regulation of cell proliferation by AP-1 might be crucial in the multistage development of tumors (25). When Pin1 was transiently overexpressed in SK-HEP-1 cells, AP-1 luciferase activity was dose-dependently increased by Pin1 overexpression (Fig. 5*A*, *left graph*). Also, when p70S6K was transiently overexpressed in SK-HEP-1 cells, AP-1 luciferase activity was dose-dependently increased by overexpression of p70S6K (Fig. 5*A*, *right graph*). We next assessed insulin-induced AP-1 activation in control siRNA or Pin1 siRNA-treated SK-HEP-1 cells. AP-1 activity in SK-HEP-1 cells was increased

at 24 h after treatment with insulin (0, 25, 50, and 100 nM), but knockdown of Pin1 expression significantly suppressed insulin-induced AP-1 activation compared with control siRNA (Fig. 5*B*). To determine whether the overexpression of p70S6K in SK-HEP-1 was responsible for AP-1 activation in response to insulin, we co-transfected p70S6K-overexpressing or control SK-HEP-1 cells with the AP-1 luciferase reporter plasmid and the *phRL-SV40* gene. At 24 hours after transfection, cells were starved for an additional 24 hours in serum-free DMEM at 37°C in a 5% CO_2 incubator. They were then treated with or without insulin (0, 25, 50, and 100 nM). Insulin-induced AP-1 activation in SK-HEP-1 cells was much more pronounced in p70S6K-overexpressing cells than in mock vector control cells (Fig. 5*C*). Next, to determine whether Pin1 and p70S6K had a synergistic effect on the insulin-induced AP-1 activity, we co-transfected Pin1-, p70S6K-, or Pin1/p70S6K-overexpressing SK-HEP-1 cells with the AP-1 luciferase reporter plasmid and the *phRL-SV40* gene. At 24 hours after transfection, cells were starved for an additional 24 hours in serum-free DMEM at 37°C in a 5% CO_2 incubator. They were then treated with or without insulin (50 nM). The insulin-induced AP-1 activation in cells overexpressing p70S6K and Pin1 was dramatically increased compared

with that in cells overexpressing only p70S6K or Pin1, suggesting that Pin1 and p70S6K have a synergistic effect on insulin-induced AP-1 activation (Fig. 5*D*).

3-6. Pin1 enhances insulin-induced neoplastic cell transformation through mTOR/p70S6K signaling.

The mouse skin epidermal JB6 Cl41 cell system is a well-developed model for studying tumor promotion under anchorage-independent growth conditions (26). To determine whether JB6 Cl41 cells were susceptible to insulin-induced cell transformation, the cells were treated with insulin (50 nM) in soft agar and incubated at 37°C in a 5% CO_2 incubator for 3 weeks; colony numbers were then determined as described. The colony numbers were increased in cells treated insulin compared with those in untreated cells (Fig 6*A*). To assess whether Pin1 enhanced insulin-induced cell transformation, GFP-overexpressing JB6 Cl41 control cells (MSCV-GFP) and Pin1-overexpressing JB6 Cl41 cells (MSCV-Pin1) were treated with insulin (25, 50, 100 nM) in soft agar and incubated under the same conditions. Colony numbers and colony sizes were significantly increased in insulin-treated MSCV-Pin1 cells compared with those in MSCV-GFP

cells (Fig 6*B*). These results indicated that Pin1 plays an important role in enhancing the insulin-induced neoplastic cell transformation in JB6 Cl41 cells. Next, to establish whether the mTOR-specific inhibitor, rapamycin, inhibited Pin1-enhanced cell transformation induced by insulin, MSCV-Pin1 cells were treated with insulin (50 nM) in the presence or absence of rapamycin (5 and 10 nM) in soft agar and incubated under the same conditions. Rapamycin decreased colony numbers as well as colony sizes in MSCV-Pin1 cells compared with insulin-treated control cells (Fig 6*C*), suggesting that the Pin1-p70S6K signaling pathway mediates the insulin-induced neoplastic cell transformation.

4. Discussion

Although the phospho-Ser/Thr-Pro-specific prolyl-isomerase Pin1 is overexpressed in more than 50% of hepatocellular carcinomas (HCCs) (27), a molecular mechanism of tumorigenic effect for Pin1 in hepatocarcinoma cells has not been established. Pin1 promotes the *cis-trans* isomerization of specific proteins that are phosphorylated at a Ser/Thr-Pro motif (8). Such conformational changes can profoundly affect the function of many Pin1 substrates (5,28,29). In fact, two important oncogenic proteins, β-catenin and cyclin D1, are regulated by Pin1 in this way. However, specific Pin1 substrates have yet to be identified in hepatocarcinogenesis, although such substrates very likely exist. To identify new specific substrates that might be associated the distinct role of Pin1 in hepatocarcinogenesis, we screened for interaction between Pin1 and 50 kinases using the *in vitro* mammalian two-hybrid assay (*data not shown*). Our results show that p70S6K strongly interacted with Pin1 *in vitro* (Fig. 1*A* and *B*). We also show that the WW domain of Pin1 is required for its interaction with p70S6K *in vitro* (Fig. 1*C*). Indeed, our results confirm that overexpression of Pin1 also enhances the phosphorylation of p70S6K and GSK3-β in

unstimulated HEK 293 cells (Fig. 1*D*). These findings suggest that Pin1 may regulate the insulin signaling pathway through interaction with phosphorylated p70S6K.

Multiple serine and threonine phosphorylation sites in p70S6K are believed to be essential for its activation (30,31). The first step of p70S6K activation is mediated by phosphorylation of the four Ser/Thr-Pro sites in the carboxy-terminal tail and thus facilitates the next step, phosphorylation of Thr^{389} in a PI3K-dependent manner (32). Phosphorylation of Thr^{389} in p70S6K, a site specifically phosphorylated by mTOR, is crucial for its activation (30). p70S6K interferes with insulin receptor signaling through a negative feedback mechanism by direct phosphorylation of IRS-1 (33). The mTOR-p70S6K pathway is essential to the emergence of insulin resistance, which is preceded by IRS-1 serine phosphorylation (34). Constitutive activation of p70S6K by unrestrained mTOR signaling was sufficient to cause mouse embryo fibroblast resistance to insulin and IGF-1 signaling, although this resistance can be rescued by rapamycin treatment (35). On the other hand, mice lacking p70S6K are protected from high-fat diet-induced insulin resistance (34). Insulin resistance, which is the cause of type 2 diabetes and other metabolic disorders, is associated with an increased

risk of tumor development in several tissues, including hepatocellular carcinoma (36,37). In a case-control study, El-Serag et al. found that diabetes increased the risk of HCC in the presence of hepatitis B, hepatitis C, or alcoholic cirrhosis (38). The temporal relationships between diabetes and HCC are important, because a significant proportion of patients with cirrhosis have overt diabetes or impaired glucose tolerance. In general, most cases of HCC are diagnosed in patients affected by long-standing cirrhosis. Interestingly, as hepatic mitochondrial production of reactive oxygen species is increased significantly in ob/ob mice, oxidant stress can be involved in the generation of hepatic hyperplasia (39). However, the molecular mechanism of the association between hepatic-insulin resistance and hepatocarcinogenesis is unclear. Our analysis demonstrates that *Pin1*$^{-/-}$ MEFs exhibit dramatically suppressed phosphorylation of p70S6K after treatment with insulin, concomitant with decreased phosphorylation of GSK3-β (Fig. 2*C*). In contrast, overexpression of Pin1 in SK-HEP-1 hepatocarcinoma cells leads to enhanced phosphorylation of p70S6K in response to insulin stimulation compared to that in control cells (Fig. 2*D*). Also, Pin1 prolonged the insulin-induced phosphorylation of GSK3-β in Pin1-overexpressing SK-HEP-1 cells. In mammals, insulin-

stimulated inhibition of glycogen synthase kinase 3 (GSK3) by Akt plays a role in maintaining blood glucose levels by increasing glycogen synthesis in insulin-responsive tissues such as liver and skeletal muscle (40,41). However, under the conditions of cellular insulin resistance linked to activation of mTOR-p70S6K signaling, GSK3 can be regulated by mTOR-p70S6K, rather than by Akt, in HepG2 cells (42).

The ERK signaling pathway has been studied extensively and is a central MAPK pathway that plays a role in cell proliferation, differentiation and transformation (43). The ERK cascade is activated by a large number of extracellular stimuli including EGF. The first model that was proposed to explain signaling specificity by the ERK cascade involved changes in the duration and strength of the signals transmitted by the cascade (44). The PI3K/Akt and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated protein kinase (ERK) 1/2 pathways are two intracellular pathways involved in insulin signal transduction (45,46). Insulin stimulates ras/raf/MEK/ERK1/2 in an insulin-receptor-dependent and a PI3K-independent pathway. This effect has been demonstrated in several cell types. This pathway can activate mTOR, which in turn activates p70S6K. These pathways are important in the modulation of protein

synthesis and of other cellular processes such as apoptosis, cell cycle progression, vascular tone, and angiogenesis (47). However, the direct crosstalk between p70S6K and ERK1/2 induced by insulin is not fully understood. Our analysis demonstrated that insulin dramatically induced phosphorylation of ERK1/2 in *Pin1*$^{+/+}$ MEF or Pin1-overexpressing SK-HEP-1 cells, whereas insulin-induced phosphorylation of ERK1/2 was suppressed in *Pin1*$^{-/-}$ MEF cells (Fig. 3*A* and *B*), suggesting that Pin1 may mediate the phosphorylation of ERK1/2 by insulin. Furthermore, the insulin-induced phosphorylation of p70S6K and of ERK1/2 is totally inhibited by rapamycin, an mTOR inhibitor (Fig. 4*A*). Interestingly, the insulin-induced interaction of p70S6K with ERK1/2 (Fig. 3*C*) was also blocked by rapamycin (Fig. 4*B*), suggesting that insulin-mediated crosstalk exists between p70S6K and ERK1/2, and Pin1 thereby regulates ERK1/2 signaling by controlling p70S6K.

Activator protein-1 (AP-1) is a well-characterized transcription factor composed of homodimers and/or heterodimers of the Fos and Jun protein families and plays a key role in “preneoplastic-to-neoplastic transforma-tion” and proliferation (24). A diverse variety of stimuli induce AP-1 binding to various genes that govern cellular processes

such as transformation and proliferation (48). AP-1 activity is important and required for neoplastic cell transformation in JB6 Cl41 cells (49). As a sequence-specific transcriptional activator, AP-1 mediates a broad range of external stimuli that lead to gene transcription. Many stimuli, including TPA, EGF, and UV radiation that induce AP-1, are associated with tumorigenesis (48). Furthermore, insulin has been shown to enhance TPA-induced AP-1 activation and cell transformation in JB6 Cl41 cells (50). Our results demonstrated that overexpression of p70S6K induced AP-1 activity (Fig. 5*A*, *right graph*) and enhanced insulin-induced AP-1 activity in SK-HEP-1 cells (Fig. 5*C*). Also, overexpression of Pin1 induced AP-1 activity (Fig. 5*A*, *left graph*), whereas knockdown of Pin1 suppressed insulin-induced AP-1 activity (Fig. 5*B*). Interestingly, Pin1 and p70S6K had a synergistic effect on AP-1 activity in SK-HEP-1 cells (Fig. 5*D*). Moreover, the synergistic effect on AP-1 activity is highly increased in insulin-treated SK-HEP-1 cells compared to untreated control cells. In addition, we found that insulin alone induced neoplastic cell transformation in JB6 Cl41 cells (Fig. 6*A*) and overexpression of Pin1 in JB6Cl41 cells enhanced insulin-promoted neoplastic cell transformation much more than that in control cells (Fig. 6*B*). Interestingly, there was a wide difference

between GFP-overexpressing cells (control) and Pin1-overexpressing cells, particularly in colony size. The data support our hypothesis that Pin1 is required for insulin-induced cell transformation of JB6 Cl41 cells. We also confirmed that the mTOR inhibitor, rapamycin, dramatically suppressed insulin-induced cell transformation in Pin1-overexpressing cells, suggesting that Pin1 enhanced insulin-induced cell transformation through its interaction and prolonged activity toward p70S6K (Fig. 6*C*).

In this study, we found that Pin1 plays a pivotal role in insulin-induced AP-1 activation through its interaction with p70S6K and prolongs activity of p70S6K in hepatocarcinoma cells. In addition, the interaction of the two proteins results in activation of ERK1/2 signaling pathway by insulin, leading to synergistic augmentation of its downstream target genes such as AP-1, which in turn promotes cell proliferation and cellular transformation. However, the molecular mechanism leading to a significant association between diabetes and HCC clinically observed (38) remain to be elucidated. One possibility is that aberrantly high p70S6K activity by Pin1 leads to IRS-1 serine/threonine phosphorylation and its degradation and thereby desensitize hepatic insulin response, suggesting that detailed

understanding of the molecular mechanisms of Pin1 in the insulin-signaling cascade may improve current treatments for HCC associated with diabetes.

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6. Figure and Legends

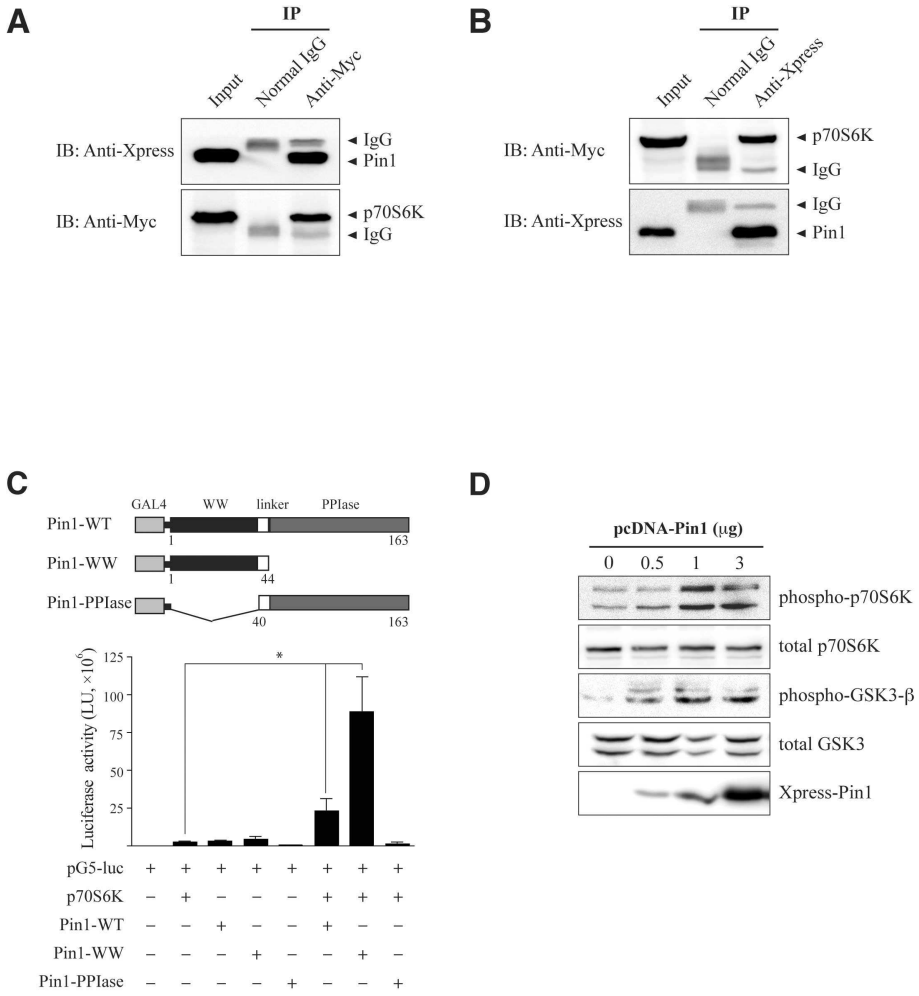


Figure 1. The interaction of Pin1 with p70S6K. (A and B) Pin1 co-immunoprecipitated with p70S6K. The pcDNA4-Xpress-Pin1 plasmid was co-transfected with pcDNA3-Myc-p70S6K into HEK293 cells and then cultured for 48 h at 37℃ in a 5% CO_2 incubator. The proteins were extracted as described in Materials and Methods and were used for immunoprecipitation (IP) with anti-Myc against p70S6K *(A)* or anti-Xpress against Pin1 *(B)*. Normal IgG antibody from mouse was used as a negative control for the IP. Pin1 or p70S6K was visualized by immunoblotting with anti-Xpress or anti-Myc antibody, respectively, using the ECL detection kit (Amersham Biosciences). (C) *Ex vivo* interaction of pACT-p70S6K with pBIND-Pin1-WT, -WW, or -PPIase. Schematic diagram of full-length (1-163) pBIND-pin1 WT (Pin1-WT), the WW domain (1-44) of pBIND-pin1 (Pin1-WW), or the PPIase domain (40-163) of pBIND-pin1 (Pin1-PPIase) was shown in *upper panel***.** As a negative control, HEK293 cells were transfected with pACT-p70S6K plasmid along with the pG5-*luc* reporter plasmid (10,000 cells/well). The pACT-p70S6K and pBIND-Pin1 plasmids (WT, WW, or PPIase) were co-transfected with the pG5-*luc* plasmid to confirm the binding site for p70S6K in Pin1. After 48 h incubation, the *firefly* luciferase activity was measured in cell lysates and normalized

against *Renilla* luciferase activity. All experiments were performed at least twice with triplicate samples and are depicted as means ± S.E. The asterisk (*) indicates a significant increase in activity compared to the negative control, pACT-p70S6K only ($p < 0.05$). The data are shown as relative luciferase activity units as measured by the TriStar LB 941. (D) HEK293 cells were transfected with 0.5 - 3 μg of pcDNA4-Xpress-Pin1 or pcDNA4 plasmid. Cells were harvested at 48 h after transfection. Proteins in whole cell lysates were separated by SDS-PAGE, and western blot analyses were performed using antibodies against the respective proteins.

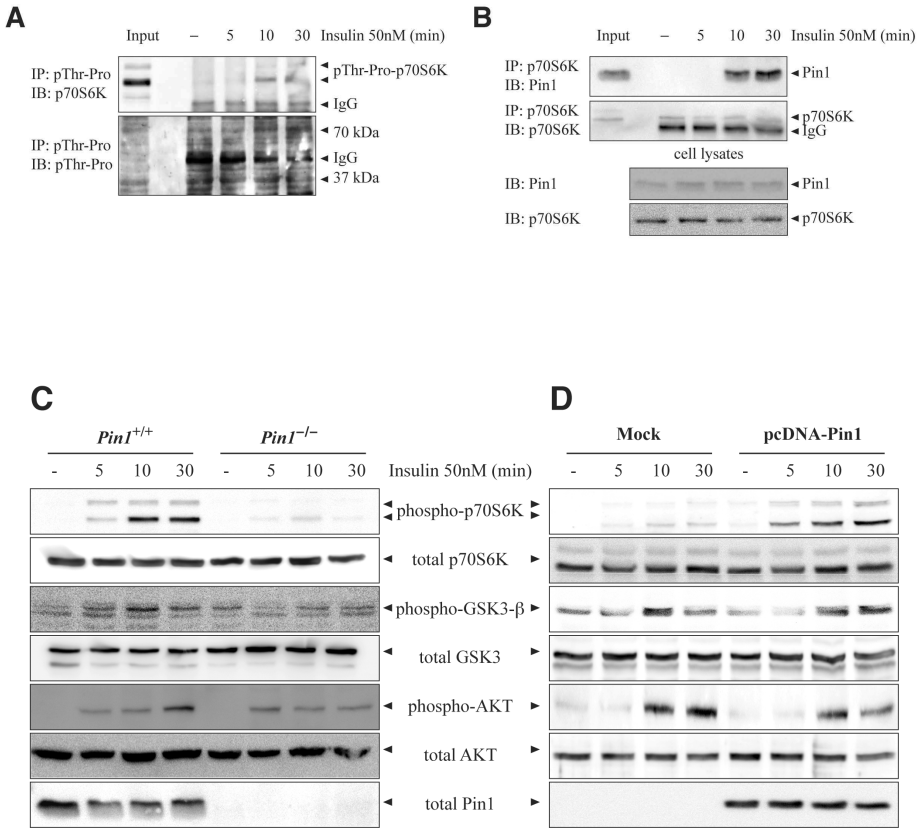
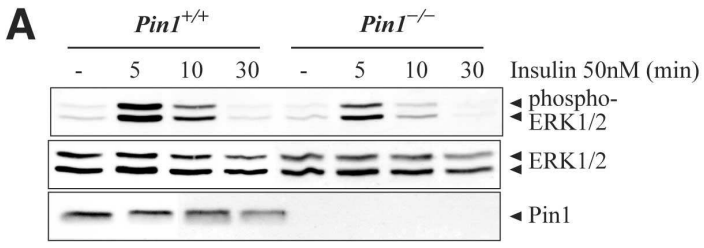

A
Input
–
5
10
30
Insulin 50nM (min)
IP: pThr-Pro
IB: p70S6K
pThr-Pro-p70S6K
IgG
IP: pThr-Pro
IB: pThr-Pro
70 kDa
IgG
37 kDa
B
Input
–
5
10
30
Insulin 50nM (min)
IP: p70S6K
IB: Pin1
Pin1
IP: p70S6K
IB: p70S6K
p70S6K
IgG
cell lysates
IB: Pin1
Pin1
IB: p70S6K
p70S6K
C
Pin1+/+
Pin1–/–
-
5
10
30
-
5
10
30
Insulin 50nM (min)
phospho-p70S6K
total p70S6K
phospho-GSK3-β
total GSK3
phospho-AKT
total AKT
total Pin1
D
Mock
pcDNA-Pin1
-
5
10
30
-
5
10
30

Figure 2. Pin1 regulates the insulin-induced phosphorylation of p70S6K. (A) p70S6K is phosphorylated at the phospho-Thr-Pro site in response to insulin. SK-HEP-1 cells were cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator. The cells were then starved in serum-free DMEM for 24 h, then exposed or not exposed to insulin (50 nM), and harvested after incubation for the time indicated. Immunoprecipitation (IP) was performed to precipitate phosphorylated proteins at threonine residue immediately preceding proline and p70S6K protein was detected by immunoblotting (IB) with anti-p70S6K antibody. (B) The interaction of endogenous p70S6K with Pin1 is stimulated by insulin. SK-HEP-1 cells were cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator. The cells were then starved in serum-free DMEM for 24 h, then exposed or not exposed to insulin (50 nM), and harvested after incubation for the time indicated. Immunoprecipitation (IP) was performed to precipitate endogenous p70S6K. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotting (IB) analyses were performed using antibodies against Pin1 and p70S6K. (C) Time-course analysis of insulin-induced phosphorylation of p70S6K after gene ablation of Pin1. *Pin1*$^{+/+}$ and

Pin1$^{-/-}$ MEFs were seeded and cultured 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator, and then starved in serum-free DMEM for 24 h. Cells treated with or without insulin (50 nM) for the indicated times were harvested and lysed, and lysates were resolved by SDS-PAGE. Immunoblotting analyses were performed using antibodies against the respective proteins. (D) Time-course analysis of insulin-induced phosphorylation of p70S6K in Pin1-overexpressing SK-HEP-1 cells. SK-HEP-1 cells were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator, and then transfected with pcDNA4-Xpress-Pin1 (5 μg) or pcDNA4 plasmid (5 μg). After 24 h, transfected cells were starved in serum-free DMEM for 24 h. Cells treated or not treated with insulin (50 nM) for the indicated times were harvested and lysed, and lysates were resolved by SDS-PAGE. Western blot analyses were performed using antibodies against the respective proteins.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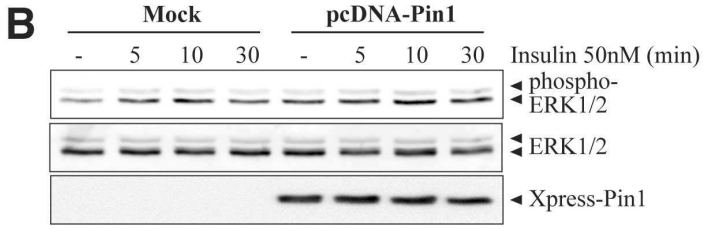
Pin1+/+
Pin1−/−
- 5 10 30 - 5 10 30
Insulin 50nM (min)
phospho-ERK1/2
ERK1/2
Pin1


B
Mock
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- 5 10 30 - 5 10 30
Insulin 50nM (min)
phospho-ERK1/2
ERK1/2
Xpress-Pin1

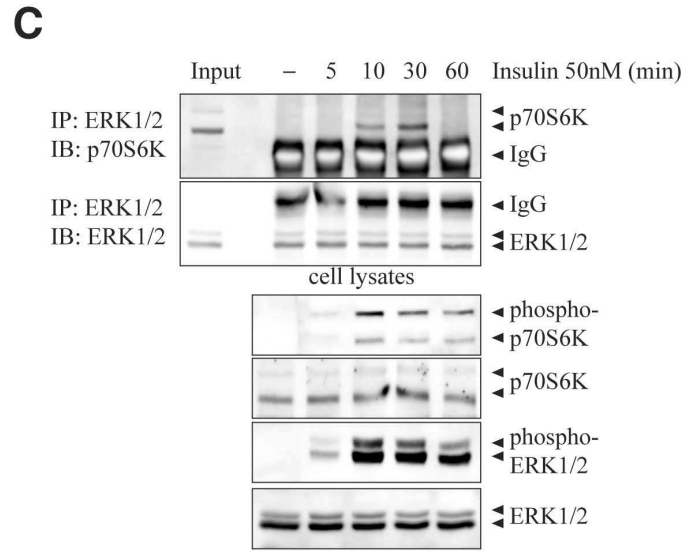

C
Input – 5 10 30 60
Insulin 50nM (min)
IP: ERK1/2
IB: p70S6K
p70S6K
IgG
IP: ERK1/2
IB: ERK1/2
IgG
ERK1/2
cell lysates
phospho-p70S6K
p70S6K
phospho-ERK1/2
ERK1/2

Figure 3. Pin1 mediates the crosstalk of ERK1/2 and p70S6K by insulin. (A) Time-course analysis of insulin-induced phosphorylation of ERK1/2 after gene ablation of Pin1. *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEFs were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator, and then starved in serum-free DMEM for 24 h. Cells treated with or without insulin (50 nM) for the indicated times were harvested, lysed, and the lysates were resolved by SDS-PAGE. Immunoblotting analyses were carried out using antibodies against the respective proteins. (B) Time-course analysis of insulin-induced phosphorylation of ERK1/2 in Pin1-overexpressing SK-HEP-1 cells. Cells were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator, and then transfected with pcDNA4-Xpress-Pin1 (5 μg) or pcDNA4 plasmid (5 μg). At 24 h after transfection, cells were starved in serum-free DMEM for 24 h. Cells treated with or without insulin (50 nM) for the indicated times were harvested, lysed, and lysates were resolved by SDS-PAGE. Immunoblotting analyses were carried out using antibodies against the respective proteins. (C) Insulin-stimulated interaction of endogenous p70S6K and ERK1/2. SK-HEP-1 cells were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2

incubator. The cells were then starved in serum-free DMEM for 24 h, then exposed or not exposed to insulin (50 nM), and harvested after incubation for the time indicated. Immunoprecipitation (IP) was performed to precipitate endogenous ERK1/2, and p70S6K protein was detected by immunoblotting (IB) with anti-p70S6K antibody. ERK1/2 protein was also detected by immunoblotting (IB) with anti-ERK1/2 antibody. Proteins in whole cell lysates were separated by SDS-PAGE and western blot analyses were performed using antibodies against the respective proteins.

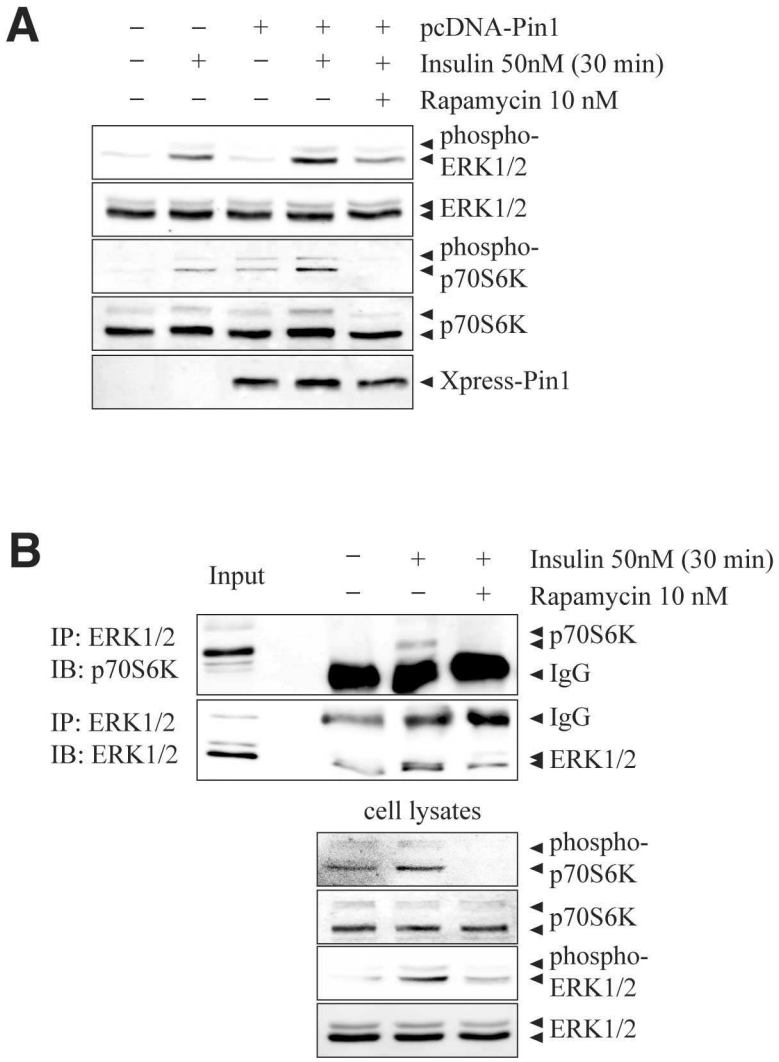
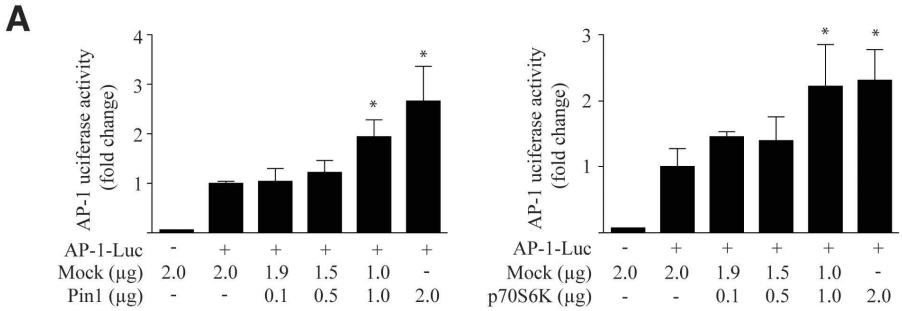
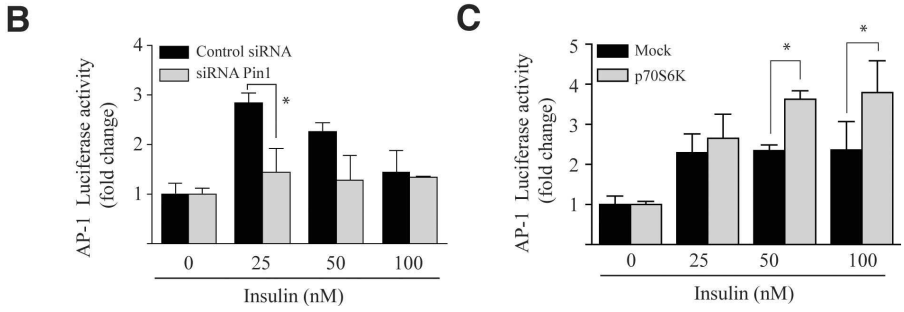

A
− − + + + pcDNA-Pin1
− + − + + Insulin 50nM (30 min)
− − − − + Rapamycin 10 nM
phospho-ERK1/2
ERK1/2
phospho-p70S6K
p70S6K
Xpress-Pin1
B
Input
− + + Insulin 50nM (30 min)
− − + Rapamycin 10 nM
IP: ERK1/2
IB: p70S6K
p70S6K
IgG
IP: ERK1/2
IB: ERK1/2
IgG
ERK1/2
cell lysates
phospho-p70S6K
p70S6K
phospho-ERK1/2
ERK1/2

Figure 4. ERK1/2 phosphorylation is suppressed by rapamycin. (A) Effect of rapamycin on insulin-induced ERK1/2 phosphorylation in Pin1-overexpressing SK-HEP-1 cells. Cells were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator, and then transfected with pcDNA4-Xpress-Pin1 (5 μg) or pcDNA4 plasmid (5 μg). At 24 h after transfection, cells were starved in serum-free DMEM for 24 h and then pretreated with or without rapamycin (10 nM). After 2 h, cells were stimulated with or without insulin (50 nM) for 30 minutes, harvested, and lysed, and cell lysates were resolved by SDS-PAGE. Immunoblotting analyses were performed using antibodies against the respective proteins. (B) Effect of rapamycin on insulin-stimulated interaction of endogenous p70S6K with ERK1/2. SK-HEP-1 cells were seeded and cultured for 24 h in 10% FBS/DMEM in a 37℃, 5% CO_2 incubator. The cells were then starved in serum-free DMEM for 24 h, and then pretreated with or without rapamycin (10 nM). After 2 h, cells were stimulated with or without insulin (50 nM) and harvested after 30 minutes. Immunoprecipitation (IP) was performed to precipitate endogenous ERK1/2, and p70S6K protein was detected by immunoblotting (IB) with anti-p70S6K antibody. ERK1/2 protein was

also detected by immunoblotting (IB) with anti-ERK1/2 antibody. Proteins in whole cell lysates were separated by SDS-PAGE and western blot analyses were carried out using antibodies against the respective proteins.


A
AP-1 uciferase activity (fold change)
AP-1-Luc - + + + + +
Mock (μg) 2.0 2.0 1.9 1.5 1.0 -
Pin1 (μg) - - 0.1 0.5 1.0 2.0
AP-1 uciferase activity (fold change)
AP-1-Luc - + + + + +
Mock (μg) 2.0 2.0 1.9 1.5 1.0 -
p70S6K (μg) - - 0.1 0.5 1.0 2.0


B
Control siRNA
siRNA Pin1
AP-1 Luciferase activity (fold change)
0 25 50 100
Insulin (nM)
C
Mock
p70S6K
AP-1 Luciferase activity (fold change)
0 25 50 100
Insulin (nM)

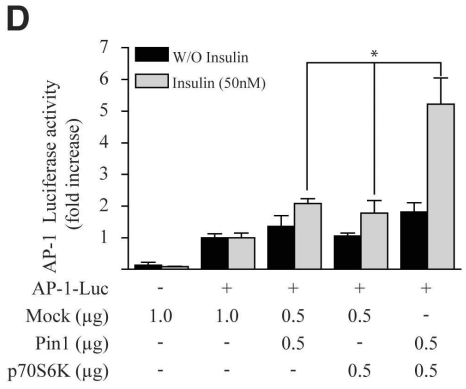

D
W/O Insulin
Insulin (50nM)
AP-1 Luciferase activity (fold increase)
AP-1-Luc - + + + +
Mock (μg) 1.0 1.0 0.5 0.5 -
Pin1 (μg) - - 0.5 - 0.5
p70S6K (μg) - - - 0.5 0.5

Figure 5. The synergistic effect of Pin1 and p70S6K on insulin-induced AP-1 activity. (A) SK-HEP-1 cells were transfected with a mixture containing the *AP-1* luciferase reporter gene (0.5 μg), pcDNA4-Xpress-Pin1 *(left)* or pcDNA3-Myc-p70S6K *(right)* with *phRL-SV40* gene (0.5 ng). At 48 h after transfection, the *firefly* luciferase activity was measured in cell lysates and normalized against *Renilla* luciferase activity. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and significant increases in AP-1 activity were observed in cells transiently transfected with Pin1 *(left)* or p70S6K *(right)* compared with mock cells. (B) Control siRNA- or Pin1 siRNA-treated SK-HEP-1 cells were co-transfected with the *AP-1* luciferase reporter gene (0.5 μg) and the phRL-SV40 gene (0.5 ng). At 24 h after transfection, cells were starved for 24 h in serum-free DMEM at 37℃ in a 5% CO_2 atmosphere and then incubated in the presence or absence of insulin (25, 50, 100 nM) for 24 h. The *firefly* luciferase activity was measured in cell lysates and normalized against *Renilla* luciferase activity. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and a significant decrease of insulin-induced AP-1 activity was observed in Pin1 siRNA- treated cells

compared with control siRNA-treated cells. (C) Mock (1 μg) and pcDNA3-Myc-p70S6K (1 μg) transiently transfected SK-HEP-1 cells were co-transfected with the *AP-1* luciferase reporter gene (0.5 μg) and the *phRL-SV40* gene (0.5 ng). At 24 h after transfection, cells were starved for 24 h in serum-free DMEM at 37℃ in a 5% CO_2 atmosphere and then incubated in the presence or absence of insulin (25, 50, 100 nM) for 24 h. The *firefly* luciferase activity was measured in cell lysates and normalized against *Renilla* luciferase activity. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and significant increases in insulin-induced AP-1 activity observed in cells transiently transfected with p70S6K compared with mock cells. (D) SK-HEP-1 cells were transfected with the *AP-1* luciferase reporter gene (0.5 μg) and pcDNA4-Xpress-Pin1 and/or pcDNA3-Myc-p70S6K with *phRL-SV40* gene (0.5 ng). At 24 h after transfection, cells were starved for 24 h in serum-free DMEM at 37℃ in a 5% CO_2 atmosphere and then incubated in the presence or absence of insulin (50 nM) for 24 h. The *firefly* luciferase activity was measured in cell lysates and normalized against *Renilla* luciferase activity. Data were recorded as relative luciferase activity (fold) using the TriStar LB 941. All experiments were performed at least twice. *Columns*, mean of

triplicate samples; *bars*, SE. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and a significant synergistic increase of insulin-induced AP-1 activity was observed in cells transiently transfected with Pin1 and p70S6K compared with cells transfected with only Pin1 or p70S6K.

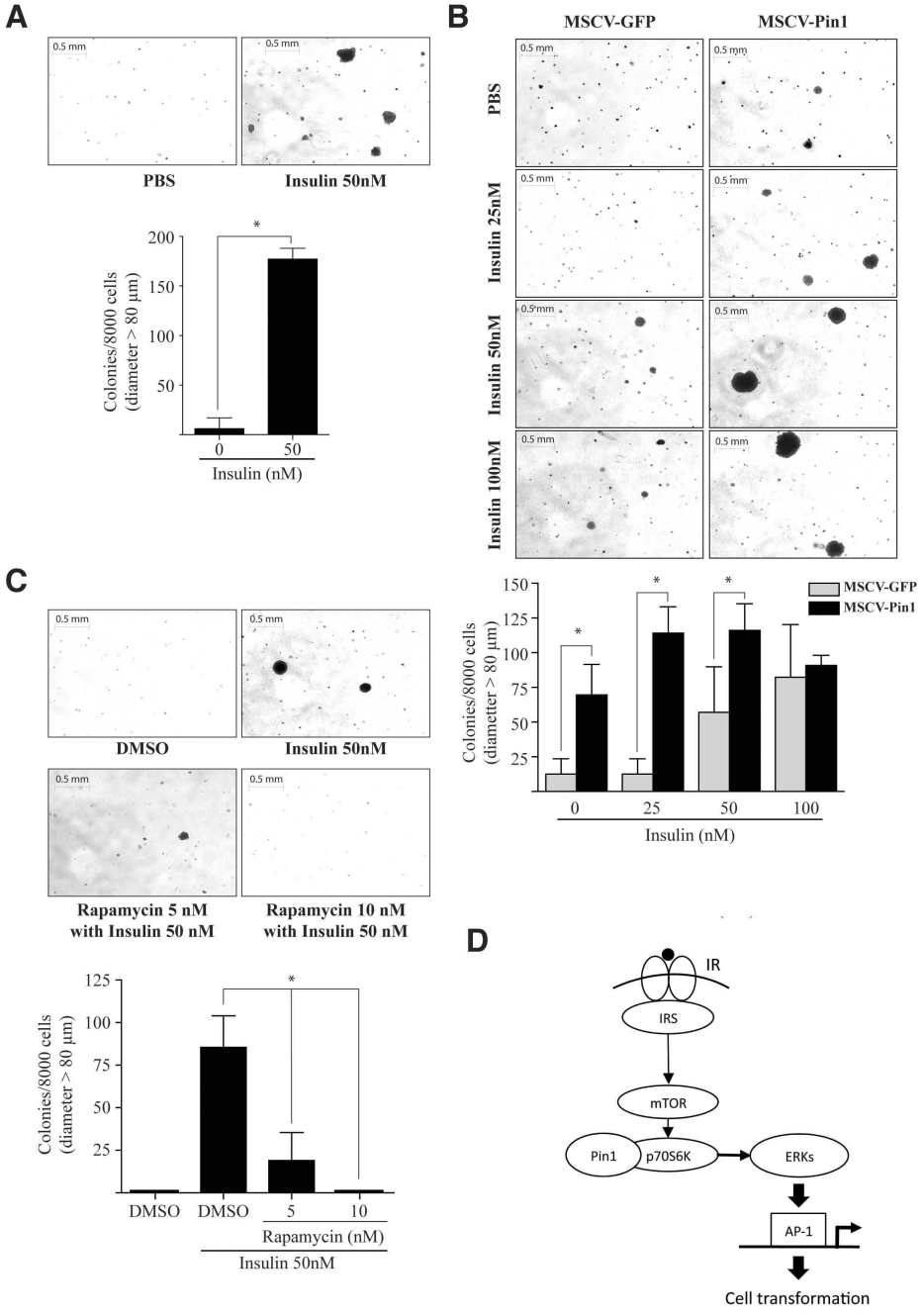

A
0.5 mm
PBS
Insulin 50nM
Colonies/8000 cells (diameter > 80 µm)
200
150
100
50
0
50
Insulin (nM)
*
B
MSCV-GFP
MSCV-Pin1
PBS
Insulin 25nM
Insulin 50nM
Insulin 100nM
MSCV-GFP
MSCV-Pin1
150
125
100
75
50
25
0
25
50
100
Insulin (nM)
C
DMSO
Insulin 50nM
Rapamycin 5 nM with Insulin 50 nM
Rapamycin 10 nM with Insulin 50 nM
125
100
75
50
25
DMSO
DMSO
5
10
Rapamycin (nM)
Insulin 50nM
D
IR
IRS
mTOR
Pin1
p70S6K
ERKs
AP-1
Cell transformation

Figure 6. Pin1 enhances insulin-induced neoplastic cell transformation. (A) JB6 Cl41 cells were used to assess cell transformation in a soft agar assay. Cells (8×10^3/ml) were exposed to insulin (50 nM) or PBS in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. The cultures were maintained at 37℃ in a 5% CO_2 atmosphere for 3 weeks. The average colony number (diameter > 80μM) was calculated and colonies were photographed in three separate experiments. *Columns*, mean of triplicate samples; *bars*, SE. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and a significant increase in insulin-induced cell transformation was observed in JB6 Cl41 cells. (B) GFP- (MSCV-GFP) or Pin1-infected (MSCV-Pin1) JB6 Cl41 cells were subjected to soft agar assays in the presence or absence of insulin. Cells (8×10^3/ml) were exposed to insulin (0, 25, 50, 100 nM) in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. The culture was maintained at 37°C in a 5% CO_2 atmosphere for 3 weeks. The average colony number (diameter > 80μM) was calculated and colonies were photographed from three separate experiments. *Columns*, mean of triplicate samples; *bars*, SE. Significant differences were evaluated using the Student's *t* test (*, $p <$

0.05), and a significant increase in the insulin-induced cell transformation was observed in MSCV-Pin1-JB6 cells compared to MSCV-GFP-JB6 cells. (C) Pin1-infected (MSCV-Pin1) JB6 Cl41 cells were subjected to soft agar assays in the presence or absence of insulin with/without rapamycin. Cells (8×10^3/ml) treated with/without rapamycin (5 and 10 nM), were exposed to insulin (0 or 50 nM) in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. The culture was maintained at 37℃ in a 5% CO_2 atmosphere for 3 weeks. The average colony number (diameter > 80μM) was calculated and colonies were photographed from three separate experiments. *Columns*, mean of triplicate samples; *bars*, SE. Significant differences were evaluated using the Student's *t* test (*, $p < 0.05$), and significant inhibition of the insulin-induced cell transformation was observed in MSCV-Pin1-JB6 cells treated with rapamycin compared to control cells. (D) The model of insulin signaling regulated by Pin1. The binding of insulin to the insulin receptor (IR) causes phosphorylation of insulin receptor substrate (IRS). When Pin1 is overexpressed, aberrantly high p70S6K activity leads to enhance insulin-induced AP-1 activity and neoplastic cell transformation through the interaction of p70S6K with ERK1/2.

저작물 이용 허락서

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논문제목	한글 : Pin1 과 p70S6K 의 상호작용에 의한 insulin 유도성 AP-1 활성 및 세포 형질 변환의 조절 기전 연구 영어 : The prolyl isomerase Pin1 interacts with a ribosomal protein S6 kinase to enhance insulin-induced AP-1 activity and cellular transformation				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2009 년 2 월 25 일

저작자: 이 나 연 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

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you, Prem. 맞은편 물리약학 실험실에 선자 언니, 선님이 언니, 로버쓰, 푸스파, 상완이, 그리고 졸업한 지영이 언니, 항상 따뜻하게 대해주셔서 고맙습니다. 이런 저런 시약을 빌리거나 기계를 쓰려고 수시로 드나들었던 독성학실에 사람들에게도 고맙고, 약물학실에 갈 때마다 마음 편하게 대화할 수 있었던 옥이 언니, 정우 오빠, 지금은 졸업한 창엽이 오빠한테도 고맙다는 인사를 하고 싶습니다. 대학원 총무라는 일까지 맡아서 항상 바빠 보이는 운정이, 대학원에서는 자주 볼 수 없는 형기, 모두 고맙습니다. 2년간 학부실험을 하면서 만났던 05, 06학번 학생들 덕에 매주 심심하진 않았습니다. 내년에 실험실 후배로 들어오는 광모와 해국이도 잘 해 나가길 바랍니다.

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모두들 진심으로 감사합니다. 행복하세요.

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