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Regulation of Type 1 Protein Phosphatase/Inhibitor-2 Complex by Glycogen Synthase Kinase-3β in Intact Cells

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Inhibitor 2 (I-2) is a ubiquitous regulator of type 1 protein phosphatase (PP1). Previous *in vitro* studies suggested that its inhibitory activity towards PP1 is regulated by phosphorylation at Thr72 by glycogen synthase kinase-3β (GSK-3β), and at Ser86, Ser120, and Ser121 by casein kinase 2 (CK2). Here we report that GSK-3β expressed in COS-7 cells phosphorylates wild-type I-2 but not an I-2 mutant carrying a T to A substitution at residue 72, showing that GSK-3β phosphorylates I-2 at T72 *in vivo* as well. Co-immunoprecipitation study demonstrated that HA-GSK-3β and I-2-FLAG co-exist in a same complex in the intact cells, but they do not bind directly. It is noteworthy that co-expression of Myc-PP1C significantly increased co-precipitation of HA-GSK-3β with I-2-FLAG, showing a complex formation of HA-GSK-3β/Myc-PP1C / I-2-FLAG *in vivo*. Further studies using a GSK-3β kinase–dead mutant and LiCl, an inhibitor of GSK-3β, showed that the enzyme activity of GSK-3β is required for co-precipitation. IP-Western study using several I-2 mutants substituted at phosphorylation sites (T72, S86, S120, and S121) suggested that phosphorylation of I-2 by CK2 is also involved in enhancement of association between GSK-3β and I-2 *in vivo*. This study is the first demonstration that GSK-3β associates with PP1C/I-2 complex and phosphorylates I-2 at T72 in the intact cells.

Key words: glycogen synthase kinase-3β, inhibitor-2, type 1 protein phosphatase.

Abbreviations: PP1, type-1 Ser/Thr protein phosphatase; PP1C, catalytic subunit of PP1; I-2, inhibitor 2; GSK-3β, glycogen synthase kinase-3β; CK2, casein kinase 2; I-1, inhibitor-1; DARPP-32, dopamine and cAMP regulated phosphoprotein-32; NIPP-1, nuclear inhibitor of protein phosphatase-1; PP1M, myosin-associated phosphatase; I-4, inhibitor-4; GFP, green fluorescent protein; HA, hemagglutinin; GST; glutathione S-transferase; MBP, maltose-binding protein; APC, adenomateous polyposis coli gene product; PP2A, type-2A Ser/Thr protein phosphatase; PAK, p21-activated kinase; AKAP, A-kinase–anchoring protein.

The reversible serine/threonine phosphorylation of proteins is a crucial component of the intracellular signaling machinery. Type-1 protein phosphatase (PP1), one of the most abundant Ser/Thr protein phosphatases, regulates cell cycle progression, protein synthesis, muscle contraction, glycogen synthesis, transcription, translation and neuronal signaling by dephosphorylating key phosphoproteins in eukaryotic cells (*1–2*). The functional diversity of PP1 is thought to be achieved by formation of various heterooligomers (mostly dimeric) composed of PP1 catalytic subunit, PP1C, and numerous regulatory subunits which target PP1C to subcellular compartments, define substrate specificity, and regulate enzyme activity (*3*). There are four mammalian PP1C isoforms, PP1Cα, PP1Cγ1, PP1Cγ2, and PP1Cδ, which exhibit similar biochemical properties with approximately 90% identity with a strong sequence diversity in their COOH-terminal 30 amino acid residues (*4–6*).

PP1C is regulated by several endogenous protein inhibitors including inhibitor-1 (I-1), dopamine and cAMP regulated phosphoprotein-32 (DARPP-32), nuclear inhibitor of protein phosphatase-1 (NIPP-1) and inhibitor-2 (I-2). For the past two decades, we have extensively studied on neoplastic alteration and the significance of the four PP1 catalytic subunits and their regulatory subunits including NIPP-1, M110, I-1, and I-2 in various hepatomas and leukemias (*7–9*). We found that PP1α and NIPP-1 levels were increased in hepatomas in a manner closely correlated with malignant phenotype (*10–13*). We also suggested that PP1M, a holoenzyme of PP1 located myofibrils and composed of PP1Cδ and M110, is involved in maintenance and regulation of cytoskeletal structure in B cell leukemias (*9*). Then we investigated an interaction of PP1 with I-2. Recently, we reported a novel PP1 inhibitor, I-4, which is 44% identical to I-2 (*14*). I-4 inhibits PP1C through a multiple point interaction as previously reported in I-2 (*15–17*).

As a mechanism by which PP1C recognizes its regulatory subunits, it was reported that several PP1 regulatory subunits bind PP1C through the (R/K) (V/I) XF motif

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(*18*, *19*). Although there is no sequence analogous to the consensus (R/K) (V/I) XF motif in I-2 and I-4, they can bind PP1C through the IKGI motif (*14*, *16*, *19*) and the KLHY motif (*17*). The β12-β13 loop within PP1 was identified as an important region for binding inhibitor proteins (*20*).

I-2 was initially isolated as a heat-stable protein from skeletal muscle extracts (*21*), which specifically binds PP1C to form an inactive PP1 heterodimer, PP1I. In vitro studies showed that PP1I can be activated by phosphorylation of I-2 at T72 residue by glycogen synthase kinase-3β (GSK-3β) (*22–24*). I-2 is also phosphorylated at S86, S120 and S121 residues by casein kinase 2 (CK2) (*25*). This phosphorylation by CK2 does not alter the inhibitory activity of I-2, but the phosphorylation at Ser86 greatly accelerates the subsequent phosphorylation at Thr72 by GSK-3β (*15*, *26*). So far, two genes for I-2 have been identified, as I-2α and I-2β, and these phosphorylation sites were conserved (*27*). Although extensive in vitro studies were carried out, the biological function of I-2 still remains to be fully elucidated. However, it is of interest that the protein level of I-2 fluctuates during the cell cycle, peaking at S phase and mitosis (*28*). Experiments using I-2 fused to the green fluorescent protein (GFP) showed that I-2 is cytosolic during G1 phase but translocates into the nucleus in S phase (*29*). Moreover, phosphorylation at the four sites was supposed to be a determinant in its subcellular localization (*29*). Changes in the phosphorylation of I-2 during G1 and S phases also argued for a role of I-2 phosphorylation in its subcellular translocation (*29*).

To further our understanding of the regulation and function of I-2 *in vivo*, we have transfected I-2-FLAG, HA-GSK-3β and myc-PP1C into COS-7 cells and examined the association among them. This is the first demonstration of an association between GSK-3β and PP1C/I-2 complex *in vivo*.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—COS-7 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum. Cells were maintained in a 5% CO_2 incubator at 37°C. For transient transfection, cells were transfected using FuGene-6 (Roche Molecular Biochemicals) according to the manufacturer's recommendation. For LiCl treatment, cells were treated with medium containing 40 mM LiCl just before transfection.

Plasmid—Human cDNA for I-2 was isolated from Daudi cells as described (*30*), and the sequence for FLAG epitope was added at its C-terminus by polymerase chain reaction with two primers, 5′-GCCATGGCGGCCTCGACGGCCTCGCA-3′ and 5′-GATGAACAGCAGTAGCAGAAACATCAGACTTGAAGCATTAAACA-3′. The I-2-FLAG cDNA was subcloned into pGEM T-Easy vector (CLONTECH) and its sequence was verified. To construct pI-2-FLAG, an EcoRI fragment encoding the I-2 FLAG was then ligated to *Eco*RI-digested pCXN2 vector. Mutations were introduced by site-directed mutagenesis by polymerase chain reaction, as described (*31*). Constructs encoding I-2 mutant proteins T72A, T72D, S86A, S120/121A, and S86/120/121A, were constructed by PCR and subcloned into pCXN2 vector. Plasmids of pCGN / GSK-3β (*32*), pCGN/GSK-3β K85M (*33*), pCDNA3/Myc-PP1Cα (*34*), pCDNA3/Myc-PP1Cγ1 (*34*), and pCDNA3/Myc-PP1Cδ (*34*) are for expression vectors for HA-GSK-3β, HA-GSK-3β K85M, Myc-PP1α, Myc-PP1Cγ1, and Myc-PP1Cδ, respectively, as described previously. The pGEX-2T/GSK-3β (*32*) is an expression vector for GST-GSK-3β as described previously.

Preparation of Recombinant Protein—GST-GSK-3β was expressed in *E. coli* BL21 strain (Stratagene) and purified with Glutathione Sepharose 4B (Amersham Biosciences) for GST-fusion proteins according to the manufacturer's instruction.

Immunoprecipitation and Western Blot Analysis—Twenty-four hours after transfection, the transfected COS-7 cells in a 60-mm dish were washed twice with phosphate-buffer saline and lysed with 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 40 mM NaF, and 10 μg/ml leupeptin). Extracts were prepared by centrifugation at 20,000 ×*g* for 10 min. Each sample (500 μg of protein) was incubated with 8 μg of anti-FLAG M2 antibody (Sigma), 5 μg of anti-HA antibody (Roche Molecular Biochemicals), or 5 μg of anti-Myc antibody (Santa Cruz) at 4°C for 1 h, then with 30 μl of Protein G-Sepharose (Amersham Pharmacia Biotech) at 4°C for 3 h. The immune complexes were washed three times with lysis buffer and subjected to Western blot analysis. I-2-FLAG, HA-GSK-3β, and Myc-PP1 were detected with anti-FLAG M2 antibody, anti–GSK-3β antibody (Santa Cruz), anti-Myc antibody, respectively using ECL reagents (Amersham Pharmacia Biotech).

Preparation of I-2—COS-7 cells were transiently transfected with WT I-2-FLAG or T72A I-2-FLAG. Twenty-four h after transfection, the cells were harvested and suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 10 μg/ml leupeptin). After centrifugation at 20,000 ×*g* for 10 min, the supernatant was heated at 100°C for 10 min, then kept on ice for 10 min. The samples were then centrifuged at 20,000 ×*g* for 10 min, and the supernatant containing I-2-FLAG was used.

In Vitro Phosphorylation and Binding Assay—For in vitro phosphorylation of I-2 and binding between I-2 and GSK-3β, I-2 was incubated with 6 μg of GST or GST-GSK-3β in 50 μl of kinase reaction mixture (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, and 0.1 mM ATP) for 60 min at 30°C. To stop the reaction, 450 μl of lysis buffer and 2 μl of 500 mM EDTA were added to the reaction mixtures on ice. To detect phosphorylation of I-2, the samples were immunoprecipitated by anti–FLAG M2 antibody and subjected to western blot analysis using anti-phospho threonine antibody. To detect binding between I-2 and GSK-3β, the same samples were incubated with 30 μl of glutathione-Sepharose 4B for 2 h at 4°C. Precipitates were washed three times with the lysis buffer and subjected to Western blot analysis using anti–FLAG M2 antibody.

RESULTS

Phosphorylation of FLAG-Tagged I-2 by HA-GSK-3β in Intact Cells—To examine whether I-2 is phosphor-

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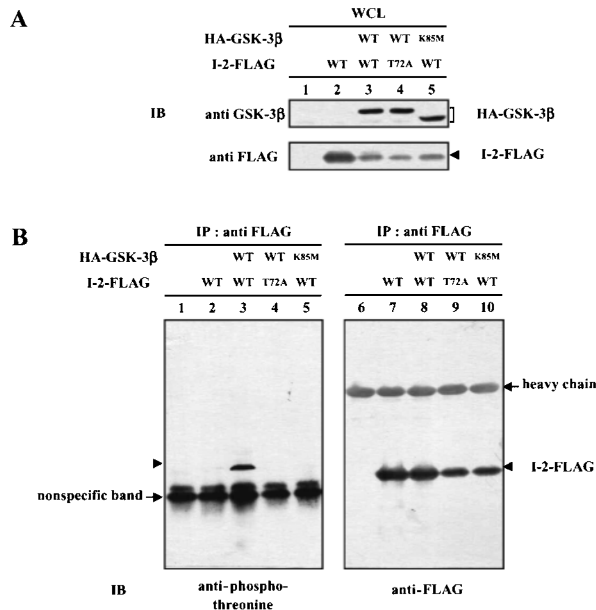

Fig. 1. **Phosphorylation of I-2-FLAG by HA-GSK-3β in intact cells.** COS-7 cells were transfected with 2.6 μg of pCGN (lane 1), 1.3 μg of pCGN (lane 2), 2.6 μg of pCGN/GSK-3β (lanes 3 and 5) or pCGN/GSK-3β K85M (lane 4), together with 1.3 μg of pCXN2 (lane 1), 2.6 μg of pI-2-FLAG (lane 2), 1.3 μg of pI-2-FLAG (lanes 3 and 5), or 1.3 μg of pI-2-FLAG T72A (A and B). Lysates (40 μg of protein) were analyzed by immunoblot analysis with anti–GSK-3β antibody or anti-FLAG M2 antibody (A). Immunoprecipitation (IP)-Western bloting (WB) was done using anti-FLAG antibody for immunoprecipitation and anti–phospho-threonine antibody for blotting (B lanes 1–5), and then reprobed with anti-Flag antibody M2 (B lane 6–10). Similar results were obtained in three separate experiments.

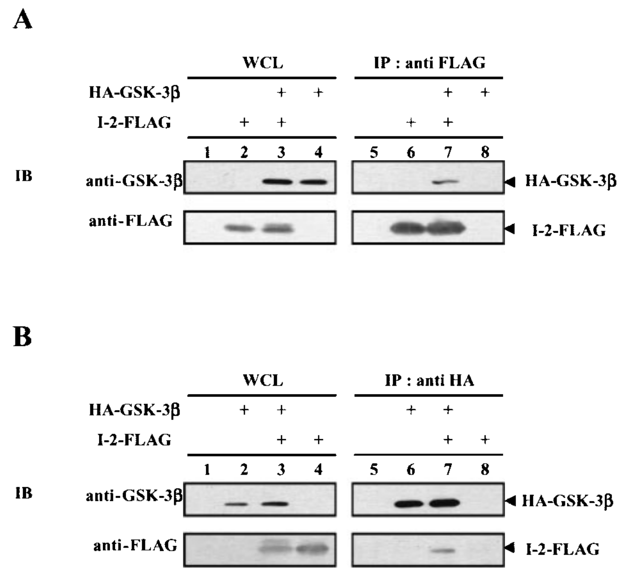

Fig. 2. **Co-precipitation of FLAG-tagged I-2 with HA-GSK-3β in intact cells.** COS-7 cells were transfected with 2.6 μg of pCGN (lanes 1, 2, 5, and 6) or 2.6 μg of pCGN/GSK-3β (lanes 3, 4, 7, and 8), together with 1.3 μg of pCXN2 (lanes 1, 4, 6, and 8) or 1.3 μg of pI-2-FLAG (lanes 2, 3, 6, and 7) (A). COS-7 cells were transfected with 2.6 μg of pCGN (lanes 1, 4, 5, and 8) or 2.6 μg of pCGN/GSK-3β (lanes 2, 3, 6, and 7), together with 1.3 μg of pCXN2 (lanes 1, 2, 5, and 6) or 1.3 μg of pI-2-FLAG (lanes 3, 4, 7, and 8) (B). Lysates (40 μg of protein) were analyzed by immunoblot analysis with anti-GSK-3β antibody or anti-FLAG M2 antibody (A and B). The lysates (500 μg of protein)s were immunoprecipitated with anti-FLAG M2 antibody (A, lanes 5–8) or with anti-HA antibody (B, lanes 5–8). The immunoprecipitates were subjected to immunoblot analysis with anti–GSK-3β antibody or with anti-FLAG M2 antibody. Similar results were obtained in two separate experiments.

ylated by GSK-3β in intact cells, I-2-FLAG constructs with or without HA-GSK-3β constructs were transiently transfected in COS-7 cells. Expression levels of HA-GSK-3β protein and I-2-FLAG protein were confirmed in the whole cell lysate (Fig. 1A). When lysates from the COS-7 cells expressing both wild-type I-2-FLAG and wild-type HA-GSK-3β were immunoprecipitated with anti–FLAG M2 antibody and blotted with anti–phospho-threonine antibody, a single band was detected at the position of I-2-FLAG (Fig. 1B, lanes 3 and 8), suggesting that one or more Thr residues of I-2 are phosphorylated *in vivo*. Since I-2 is known to be phosphorylated by GSK-3β at T72 *in vitro*, we examined whether T72 is also phosphorylated in intact cells. The I-2 mutant with Ala substituted for Thr at was expressed with HA-GSK-3β (Fig. 1A, lane 4). Wild-type HA-GSK-3β at a concentration sufficient to induce phosphorylation of wild-type I-2-FLAG (Fig. 1A, lanes 3 and 4) did not induce phosphorylation of the mutant I-2-FLAG (Fig. 1B, lane 4). Furthermore, HA-GSK-3β K85M, a kinase-dead mutant, did not induce phosphorylation of wild type I-2-FLAG (Fig. 1B, lane 5). These data demonstrate that GSK-3β induces phosphorylation of I-2 at T72 in intact cells.

Co-Precipitation of HA-GSK-3β with FLAG-Tagged I-2 in Intact Cells—GSK-3β was found to induce phosphorylation of I-2 at T72 in the intact cells, but it was still uncertain whether GSK-3β phosphorylates the T72 directly. We therefore examined whether GSK-3β is physically associated with I-2. As shown in Fig. 2A, HA-GSK-3β was detected in I-2-FLAG immune complex from COS-7 cells (lane 7). A similar result was observed in Hela cells (data not shown). Similarly, I-2- FLAG was detected in HA-GSK-3β immunocomplex from COS-7 cells (Fig. 2B lane 7). These data demonstrate that HA-GSK-3β and I-2-FLAG are present in the same complex in the intact cells, suggesting that GSK-3β can phosphorylate I-2 *in vivo*.

Co-Precipitation of GSK-3β with I-2 Requires Its Kinase Activity—Phosphorylation of Axin by GSK-3β is known to affect the stability of the signaling complex composed of Axin, APC, GSK-3β, and β-catenin (*35*, *36*). Thus, we examined whether the co-precipitation of GSK-3β with I-2 is affected by the activity of GSK-3β (Fig. 3). As shown in Fig. 3 (lanes 6 and 8), LiCl, an inhibitor of GSK-3β, abrogated the formation of an immunocomplex between HA-GSK-3β and I-2-FLAG (*37*, *38*). HA-GSK-3β K85M was not co-precipitated with I-2-FLAG (Fig. 3, lane 7). These results suggest that co-precipitation of GSK-3β with I-2 requires intact enzyme activity of GSK-3β.

Mutational Analysis of Phosphorylation Sites on I-2—We next examined how the kinase activity of GSK-3β affects its association with I-2. Since T72 of I-2 is a target of GSK-3β, one possibility was that T72-phosphorylated I-2 has higher affinity to GSK-3β than dephosphorylated

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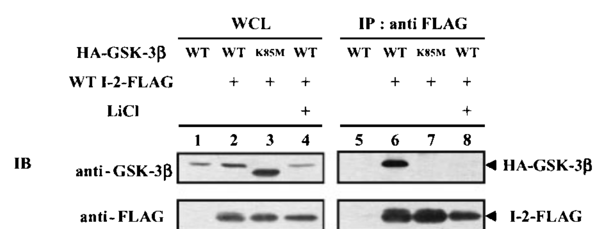

Fig. 3. **Co-precipitation of HA-GSK-3β with I-2-FLAG requires its kinase activity.** COS-7 cells were transfected with 2.6 μg of pCGN/GSK-3β (lanes 1, 2, 4, 5, 6, and 8) or pCGN / GSK-3β K85M (lanes 3 and 7), together with 1.3 μg of pCXN2 (lanes 1 and 5) or 2.6 μg of pI-2-FLAG (lanes 2, 3, 4, 6, 7, and 8). Cells were treated with 40 mM LiCl for 24 h (lanes 4 and 8). Lysates (40 μg of protein) were analyzed by immunoblot analysis with anti-GSK-3β antibody or anti-FLAG M2 antibody (lanes 1–4). The lysates (500 μg of protein) were immunoprecipitated with anti-FLAG M2 antibody, then the immunoprecipitates were subjected to immunoblot analysis with anti-GSK-3β antibody or with anti-FLAG M2 antibody (lanes 5–8). Similar results were obtained in two separate experiments.

I-2. But this was not the case. Compared with wild-type I-2-FLAG, the T72A mutant of I-2-FLAG (mimicking a dephosphorylated type) was slightly more co-precipitated with HA-GSK-3β, whereas the T72D mutant (mimicking a phosphorylated type) was not (Fig. 4, lanes 10 and 11).

It has been reported that I-2 is phosphorylated at S86, S120, and S121 by CK2, and that phosphorylation at S86 is required for efficient phosphorylation of T72 by GSK-3β *in vitro* (*25*, *26*). However, the physiological significance in the intact cells of the phosphorylation at S86, as well as at S120 and S121, remained uncertain. We therefore examined whether phosphorylation of S86, S120, and S121 affects the phosphorylation level of Thr in I-2 (Fig. 4). The level of the phospho-threonine I-2 in all of the three I-2 mutants S86A, S120/121A, and S86/120/121A was significantly inhibited (Fig. 4, lanes 12–14). These data suggest that phosphorylation of S86, S120, and S121 is prerequisite for phosphorylation of I-2 at T72 by GSK-3β *in vivo*. It should be noted that destruction of the CK2 phosphorylation sites abrogated the co-precipitation of GSK-3β with I-2.

GSK-3β Does Not Interact Directly with I-2 In Vitro but Was Co-Precipitated with PP1C/I-2 Complex in Intact Cells—We next examined whether GSK-3β interacts directly with I-2. GST- GSK-3β could phosphorylate the I-2-FLAG at T72 (Fig. 5A). Under the same conditions as used in Fig. 5A, GST-GSK-3β did not pull down the I-2-FLAG (Fig. 5B), suggesting that GSK-3β does not interact directly with I-2. Since PP1C is known to interact directly with I-2, we then examined an effect of PP1C on the co-precipitation of GSK-3β with I-2 in COS-7 cells. As shown in Fig. 6A, Myc-PP1C was co-immunoprecipitated with I-2-FLAG. It should be noted that Myc-PP1C significantly increased the immunoprecipitation of HA-GSK-3β with I-2-FLAG (Fig. 6A, lanes 5 and 6). We next examined whether HA-GSK-3β is co-precipitated with Myc-PP1C *in vivo* (Fig. 6B). As shown in Fig. 6B, HA-GSK-3β was not co-precipitated with Myc-PP1C, whereas I-2-FLAG was co-precipitated with Myc-PP1C (lanes 8 and 9). However, HA-GSK-3β was immunoprecipitated with Myc-PP1C when I-2- FLAG was co-expressed (lane 10). These data suggested that GSK-3β associates with PP1C/I-2 complex in intact cells. In these experiments, there was no difference in immunoprecipitation among the isotypes of PP1C (not shown).

DISCUSSION

In this study, we investigated the association of phosphatase inhibitor-2 and glycogen synthase kinase-3β the effects of PP1C on the association. The results are as follows: (i) HA-GSK-3β phosphorylated I-2-FLAG at T72 in intact cells. (ii) HA-GSK-3β was co-precipitated with wild-type and T72A mutant of I-2-FLAG, but not with T72D I-2-FLAG. (iii) Co-precipitation of GSK-3β with I-2 requires its kinase activity. (iv) S86A I-2-FLAG and S120/121A I-2-FLAG were neither phosphorylated at T72 nor co-precipitated with GSK-3β. (v) GSK-3β is co-precipitated with PP1C/I-2 in intact cells.

We demonstrated that GSK-3β is co-precipitated with I-2 in intact cells. Furthermore, T72A I-2 was co-precipitated with GSK-3β slightly more than WT I-2, whereas T72D I-2 was not co-precipitated, suggesting that phosphorylation of I-2 at T72 induces dissociation of I-2 from GSK-3β. Phospho-threonine I-2 seems to be present as a

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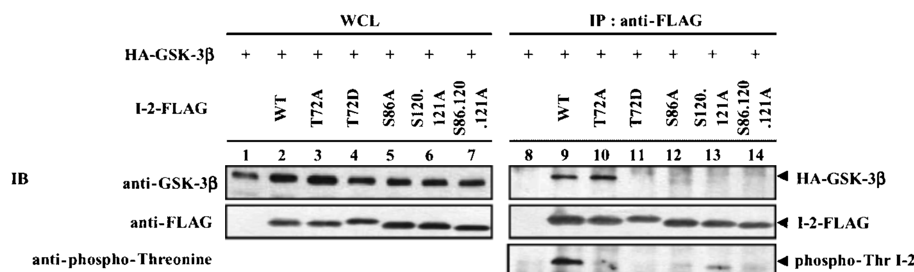

Fig. 4. **Co-precipitation of I-2-FLAG with HA-GSK-3β does not require phosphorylation at T72 but S86, S120, and S121.** COS-7 cells were transfected with 2.6 μg of pCGN/GSK-3β together with 1.3 μg of pCXN2 (lanes 1 and 8) or 1.3 μg of the indicated plasmids (lanes 1–7 and 8–14). The expression of HA-GSK-3β and I-2-FLAG wild type or mutants was verified by use of anti-GSK-3β and anti-FLAG M2 antibody, respectively (lane 1–7). The lysates (500 μg of protein) were immunoprecipitated with anti-FLAG M2 antibody, then the immunoprecipitates were subjected to immunoblot analysis with anti-GSK-3β antibody, anti–phospho-threonine antibody or with anti-FLAG M2 antibody (lane 8–14). Similar results were obtained in three separate experiments.

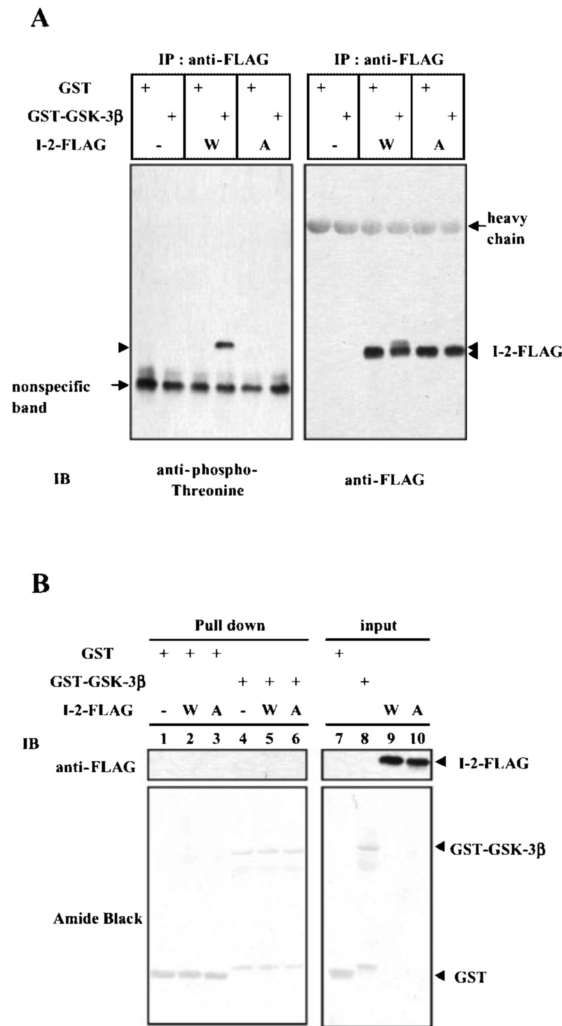

Fig. 5. **GST-GSK-3β does not associate with I-2-FLAG directly *in vitro*.** GST and GST-GSK-3β were prepared as described in "MATERIALS AND METHODS". Wild type (W) and T72A (A) of I-2-FLAG were prepared by transient transfection of 2.6 μg of pI-2-FLAG or pI-2-FLAG T72A into COS7 cells followed by heat treatment as described in "MATERIALS AND METHODS". (A) I-2-FLAG was incubated with 6 μg of GST or GST-GSK-3β in the presence of 15 mM $MgCl_2$ at 30°C for 60 min. Then the samples were precipitated with anti-FLAG M2 antibody and subjected to immunoblot analysis with anti–phospho-threonine antibody or anti-FLAG M2 antibody. (B) I-2-FLAG was incubated with 6 μg of GST or GST-GSK-3β under the same conditions as in A. The samples were precipitated with Glutathione Sepharose and subjected to immunoblot analysis with anti–FLAG M2 antibody (lanes 1–6). The input lanes represent 100% (lanes 7 and 8) and 10% (lanes 9 and 10) of the materials used for pull-down assay. The presence of precipitated GST or GST-GSK-3β was verified by staining the membrane with Amide Black (lanes 1–10). Similar results were obtained in seven separate experiments.

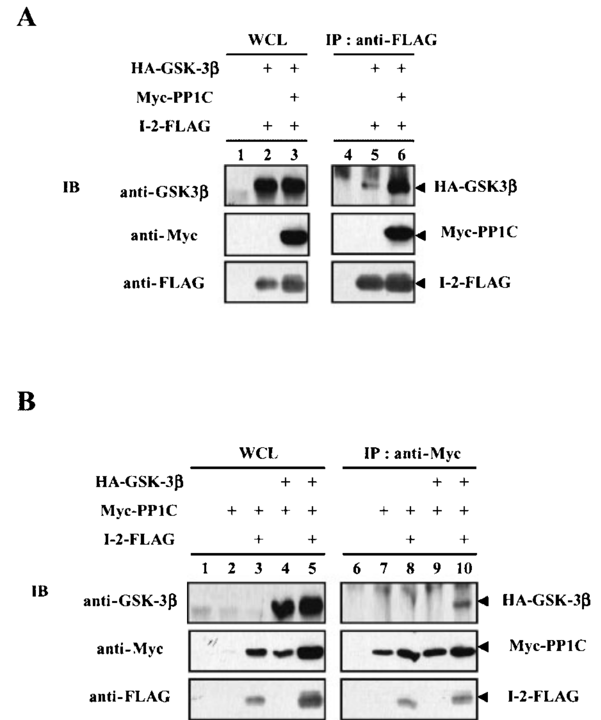

Fig. 6. **HA-GSK-3β is co-precipitated with PP1C/I-2 complex in intact cells.** COS-7 cells were transiently transfected with 1.3 μg of the plasmids as indicated. The expression of HA-GSK-3β, Myc-PP1Cα, and I-2-FLAG in the extracts was verified by use of anti-GSK-3β, anti-Myc, and anti-FLAG M2 antibody, respectively (A, lanes 1–3; and B, lanes 1–5). The extracts were immunoprecipitated with anti-FLAG M2 antibody (A, lanes 4–6) or with anti-Myc antibody (B, lanes 6–10), then subjected to immunoblot analysis with anti-GSK-3β, anti-Myc, or anti-FLAG M2 antibody. Similar results were also observed when PP1Cγ1 and PP1Cδ were used (not shown). Similar results were obtained in two separate experiments.

PP1C/I-2 complex after dissociation from GSK-3β (Fig. 4, lane9).

Previous biochemical studies have demonstrated that I-2 is phosphorylated by CK2 at S86, S120, and S121 (*25*, *26*). The phosphorylation of S86 was shown to promote phosphorylation of T72 by GSK-3β *in vitro*, but, the roles of the phosphorylation at S120 and 121 were not clarified (*25*, *26*). We showed that phosphorylation at S86 in I-2 is important for the phosphorylation of T72 by GSK-3β in intact cells consistent with the *in vitro* observation. In addition, we demonstrated that the phosphorylation at S120 and 121 is also important for the phosphorylation of T72 by GSK-3β in intact cells. Moreover, the phosphorylation at S86, 120, and 121 in I-2 also affected the co-precipitation of GSK-3β with I-2 in intact cells. Taken together, these results suggest that (i) GSK-3β easily accesses to PP1C / I-2 complex when I-2 is phosphorylated by CK2 at S86, S120, and S121, and then phosphorylates I-2 at T72. (ii) After phosphorylation at T72, GSK-3β loses its affinity to PP1C / I-2 complex, resulting in its dissociation from this complex *in vivo*.

As shown in Fig. 5, I-2-FLAG did not bind HA-GSK-3β directly *in vitro*, whereas Myc-PP1C increased co-precipitation of HA-GSK-3β with I-2-FLAG in intact cells. These data suggested that GSK-3β associates not with I-2 alone but with PP1C / I-2 complex. However, we could not reconstitute this heterotrimer complex among bacterially expressed PP1C, I-2-FLAG, and GST-GSK-3β *in vitro* (not shown). Additional intercalated protein(s) may be required for the complex formation. If this is the case, given that the co-precipitation of GSK-3β with I-2

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requires its kinase activity, this protein may be phosphorylated by GSK-3β.

Recent evidence has demonstrated the existence of molecular devices for the feedback regulation of signaling pathways, the so-called signaling modules. In these structures, a kinase, which is regulated by autophosphorylation, interacts with a phosphatase, for which it can become a substrate (or *vice versa*). As such, kinases and phosphatases can regulate their own activities within a signaling complex. For example, CK2 interacts with the C subunit of PP2A in resting cells and stimulates PP2A activity towards Raf-phosphorylated MEK1 (*39*). Furthermore, PP2A was found in complexes with p70 S6 kinase (*40*, *41*) and p21-activated kinases PAK1 and PAK3 (*40*). A complex presented here composed of GSK-3β and PP1 may be one such signaling complex.

GSK-3β is known to be involved in many cellular events including glycogen metabolism, embryonic development, protein synthesis, and cell differentiation (*42*). GSK-3β is one of a few protein kinases in two points. First, GSK-3β is inactivated by phosphorylation. Second, GSK-3β has a unique specificity in requiring a C-terminal priming phosphate for efficient phosphorylation of several of its substrates. It is suggested that the phosphorylated N-terminus acts as a pseudo-primed substrate that competes for the binding of primed substrates to the phosphate-binding site (*43*, *44*). It is presently unknown how the phosphorylated N-terminus of GSK-3β is dephosphorylated for its activation. Tanji *et al.* recently reported that PP1 co-exists in GSK-3β/AKAP220 complex in PC12 cells (*45*). In this study we demonstrated that GSK-3β is co-localized with PP1/I-2 complex. These results also suggest a novel regulatory mechanism of GSK-3β signaling by PP1. Further experiments are necessary to address how the GSK-3β/PP1/I-2 complex is assembled and to elucidate potential roles of the complex in the regulation of GSK-3β and PP1.

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