Supplemental Data

Reciprocal negative regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and apoptosis signal-regulating kinase 1 (ASK1) signaling by direct interaction and phosphorylation

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Generation of Stable Cell Lines for Overexpression and Knockdown of Endogenous PDK1

To generate HEK293 cells [PDK1 (OE)] stably expressing PDK1, we performed a PCR reaction using the full-length PDK1 cDNA as the template. The primers used for PCR were as follows: sense (5'-GCGAATTCATGGCCAGGACCACCAGCCAG-3', *Eco*RI site underlined) and antisense (5'-GCCAGCTGTCACTGCACAGCGGCGTCCGG-3', *Sal*I site underlined). The amplified PCR products were cut with *Eco*RI plus *Sal*I and cloned into pcDNA3.1-HisC vector, cut with *Eco*RI and *Xho*I, to generate pcDNA-His-PDK1. HEK293 cells were then transfected with pcDNA-His-PDK1. To prepare HEK293 cells (PDK1 shRNA) stably expressing PDK1-specific shRNA, a double-stranded oligonucleotide (forward, 5'-GATCCCCGAGACCTCGTGGAGAAACTTTCAAGAGAAGTTTCTCCACGAGGTCTCTTTTTGGAAA-3'; reverse, 5'-AGCTTTTCCAAAAAGAGACCTCGTGGAGAAACTTCTCTTGAAAGTTTCTCCACGAGGTCTCGGG-3'; PDK1 sequence underlined) was cloned into the pSuper vector. For inducible knockdown of endogenous PDK1 expression, a double-stranded oligonucleotide (forward, 5'-TCGAGGGAGACCTCGTGGAGAAACTTTCAAGAGAAGTTTCTCCACGAGGTCTCCTTTTTTA-3'; reverse, 5'-AGCTTAAAAAAGGAGACCTCGTGGAGAAACTTCTCTTGAAAGTTTCTCCACGAGGTCTCCC-3'; PDK1 sequence underlined) was cloned into the pSingle-tTS-shRNA vector as described previously (38).

Construction of PDK1 Mutants

PDK1 mutants were generated by PCR mutagenesis, as described previously (17), on PDK1-C (amino acids 249 to 556) or wild-type PDK1 using the following primers: PDK1-C forward primer 5'-GCGAATTCGTTTCTCCAGAGCTGCTCACG-3' (*Eco*RI site underlined), reverse primer 5'-GCCAGCTGTCACTGCACAGCGGCGTCCGG-3' (*Sal*I site underlined); wild-type

PDK1 forward primer 5'-GCGAATTCATGGCCAGGACCACCAGCCAG-3' (*EcoR*I site underlined), reverse primer 5'-GCCAGCTGTCACTGCACAGCGGCGTCCGG-3' (*Sal*I site underlined); for S258A/S262A, sense 5'-ACGGAGAAGGCCGCCTGTAAGGCTTCAG ACCTT-3', antisense 5'-AAGGTCTGAAGCCTTACAGGCGGCCTTCTCCGT-3'; for S394A /S398A, sense 5'-TCCTCCTCCGCACACTCCCTGGCAGCCTCCGAC-3', antisense 5'-GTCGGAGGCTGCCAGGGAGTGTGCGGAGGAGGA-3'; for T518A/T522A, sense 5'-TTT GTCCACGCGCCTAACAGGGCGTATTATCTG-3', antisense 5'-GTCGGAGGCTGCCAGG GAGTGTGCGGAGGAGGA-3'.

Small Interfering RNA (siRNA)

The PDK1 siRNA (#1) oligonucleotide (5'-GGACUCGAACUCCUUUGAATT-3') targeting the coding region (amino acids 420 to 425) on human PDK1 (GenBank^{TM} accession number Y15056) and the PDK1 siRNA (#2) oligonucleotide (5'-GAGACCUCGUGGAGAAACU-3') corresponding to the coding region (amino acids 310 to 316) were used for the siRNA experiments.

Immuno-depletion of Akt

HEK293 cell extracts expressing Myc-PDK1 were incubated with approximately 4 μg of anti-Akt antibody for 1 h at 4 ^{0}C, and protein A-Sepharoses were subsequently added and incubated for an additional 3 h at 4 ^{0}C, as described previously (38). After immuno-depletion twice with an anti-Akt antibody, the immuno-depletion of Akt was confirmed by immunoblot analysis using an anti-Akt antibody.

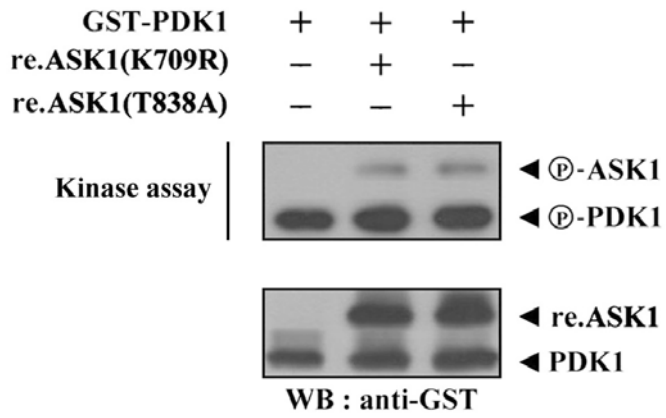
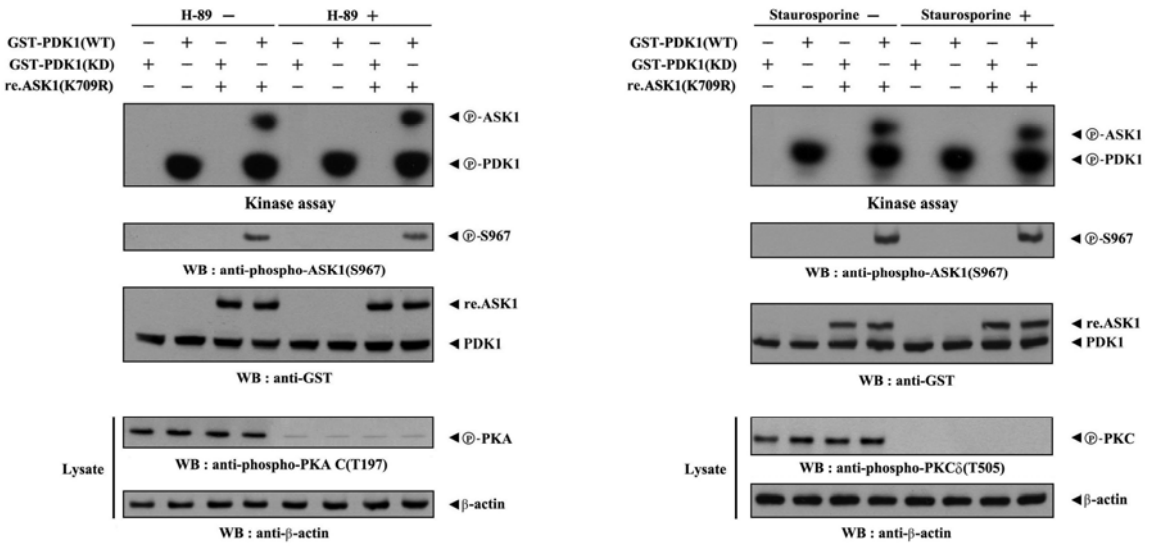


FIGURE 1. **PDK1 phosphorylates ASK1 *in vitro*.** GST-tagged wild-type PDK1, precipitated using glutathione-Sepharose beads from HEK293 cells transiently expressing GST-PDK1, was assayed for its kinase activity in the presence of kinase buffer containing approximately 3 to 4 μg of recombinant GST-tagged ASK1(K709R) or ASK1(T838A) substrates.

A **Seong et al.**



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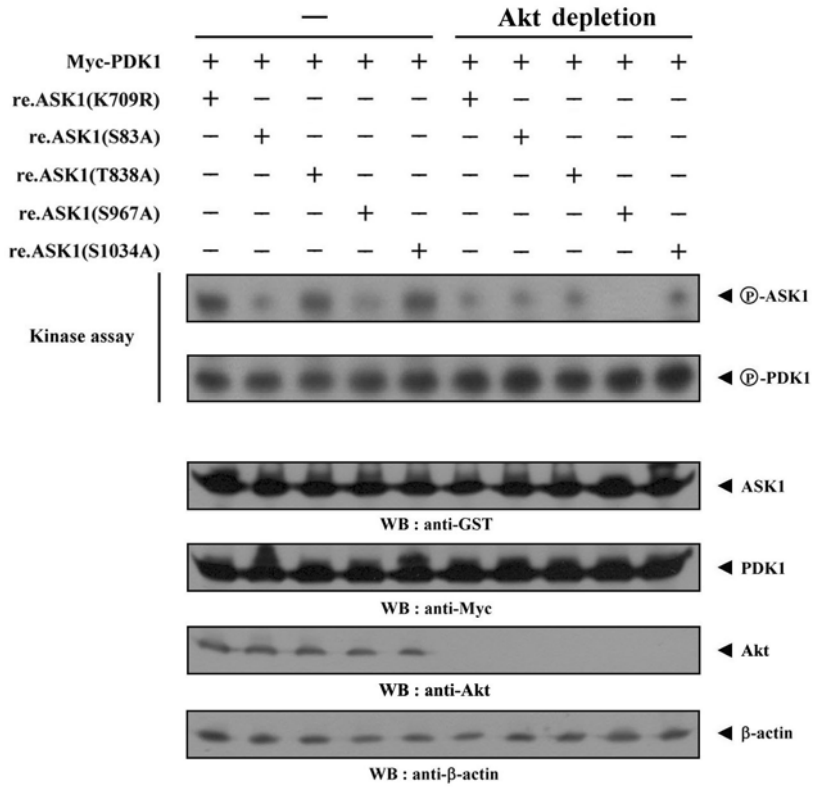


FIGURE 2. **PDK1 directly phosphorylates ASK1 at Ser^{967}.** *A*, GST-tagged PDK1(WT and KD), precipitated using glutathione-Sepharose beads from HEK293 cells expressing GST-PDK1(WT and KD) treated or untreated with specific inhibitors for PKA (H-89; 10 μM, 30 min) and PKC (staurosporine; 100 nM, 30 min), was analyzed for its kinase activity by an *in vitro* kinase assay using approximately 3 to 4 μg of recombinant GST-tagged ASK1(K709R) as a substrate. *B*, Myc-PDK1, immunoprecipitated using an anti-Myc antibody from control cell extracts (-) or Akt-depleted extracts (*Akt depletion*) of HEK293 cells expressing Myc-PDK1, was assayed for its kinase activity in the presence of kinase buffer containing recombinant GST-tagged ASK1(K709R) and one of its substitution mutants, ASK1(S83A), ASK1(T838A), ASK1(S967A), or ASK1(S1034A), as substrates (each 4 μg). The level of immuno-depleted Akt in total cell lysates was determined by immunoblot analysis using an anti-Akt antibody (*5th panel*).

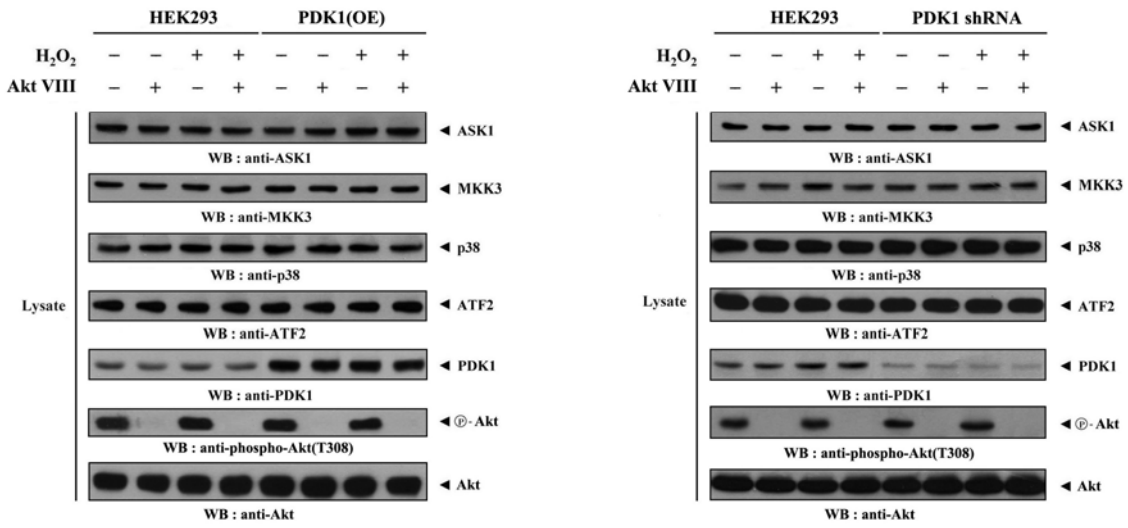
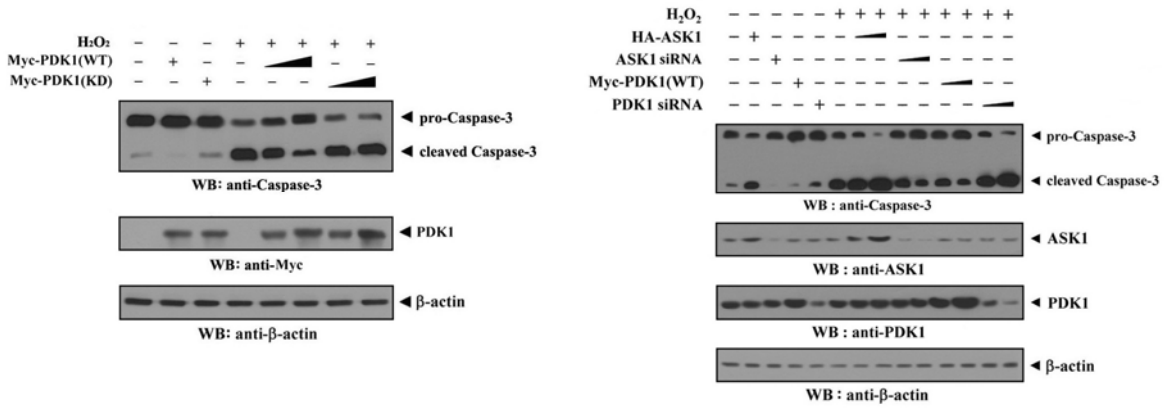


FIGURE 3. **Determination of the expression levels of ASK1 and its downstream targets used in immunoblot analyses.** The expression levels of ASK1, MKK3, p38, and ATF2 in total cell lysates were evaluated by immunoblot analyses using the indicated antibodies (see Fig. 4*B* and *C*, *lower right*). The overexpression or knockdown level of endogenous PDK1 was determined by anti-PDK1 immunoblotting.

A



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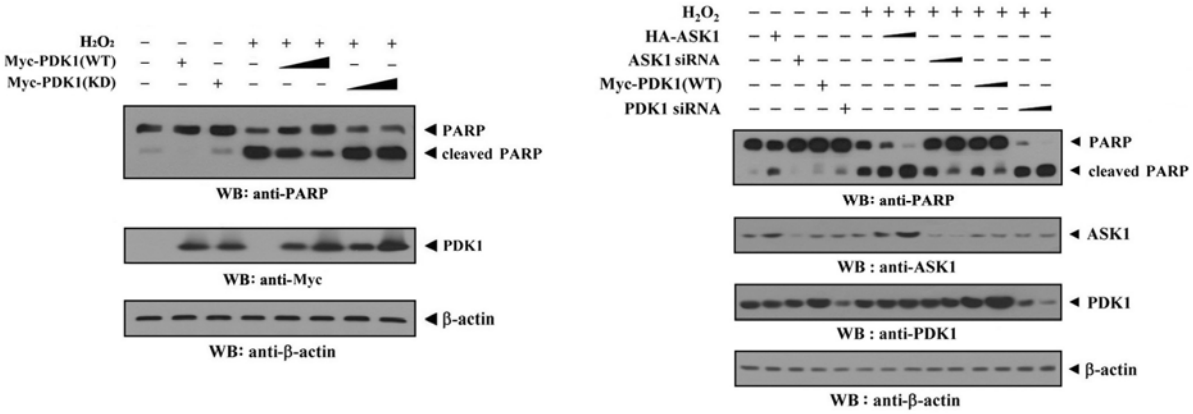
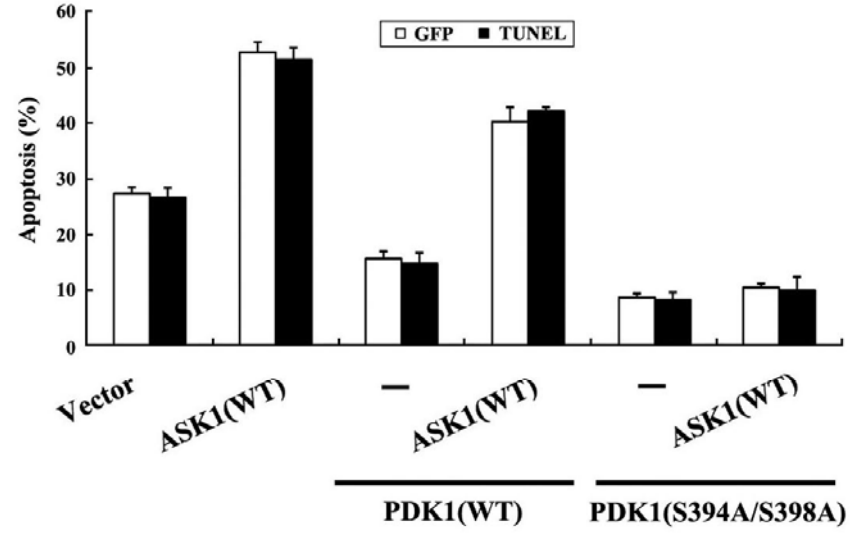


FIGURE 4. **Effect of PDK1 on caspase-3 activity and PARP cleavage.** HEK293 cells were transiently transfected with increasing amounts of PDK1(WT and KD) (2 and 4 μg), wild-type ASK1 (2 and 4 μg), or PDK1 and ASK1 siRNAs (100 and 200 nM) in the presence or absence of H_2O_2. Cell lysates were then analyzed by immunoblot analysis using an anti-caspase-3 antibody (Calbiochem) to determine the degradation of caspase-3 (*A*). PARP degradation was also measured by immunoblotting with an anti-PARP antibody (Santa Cruz Biotechnology) (*B*).



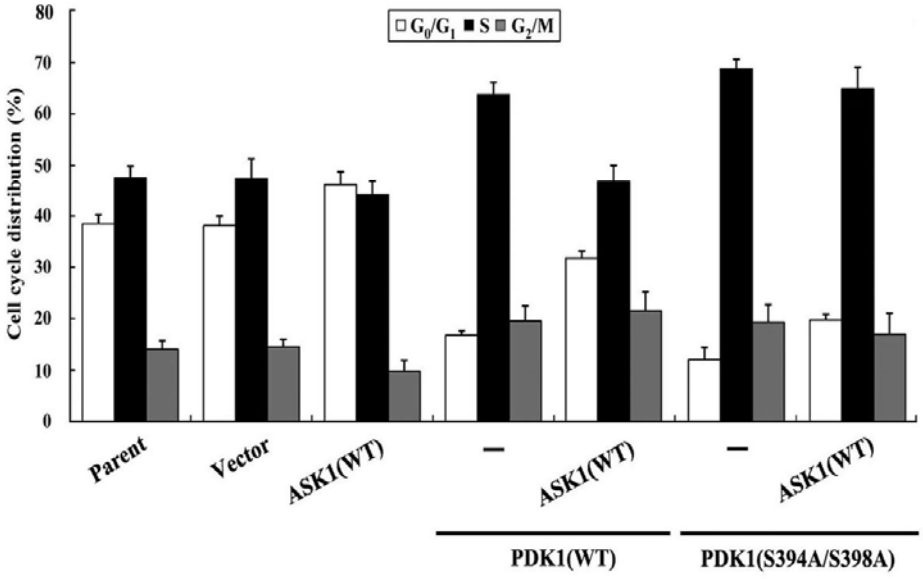
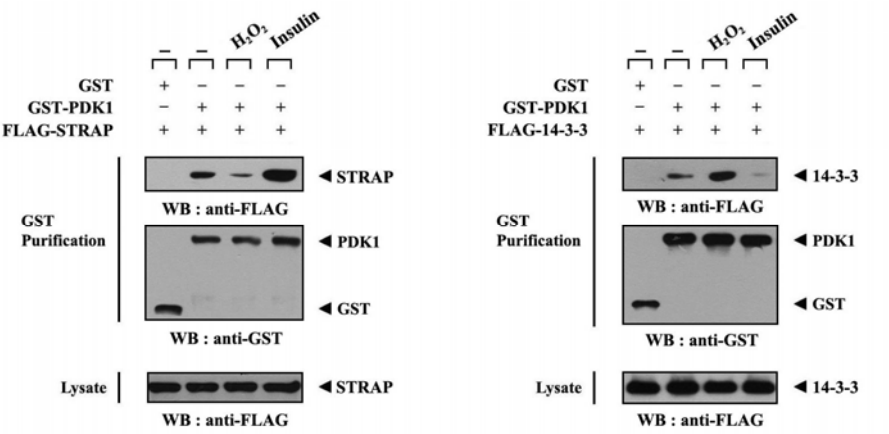


FIGURE 5. **Effect of ASK1-mediated phosphorylation of PDK1 at Ser^{394} and Ser^{398} on PDK1-mediated apoptosis and cell cycle progression.** Assays were done using 293T (*upper*) or HaCaT (*lower*) cells transfected with the indicated expression vectors, as described in Fig. 8*A* and *B*.

A


– – H_2O_2 Insulin
GST + – – –
GST-PDK1 – + + +
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STRAP
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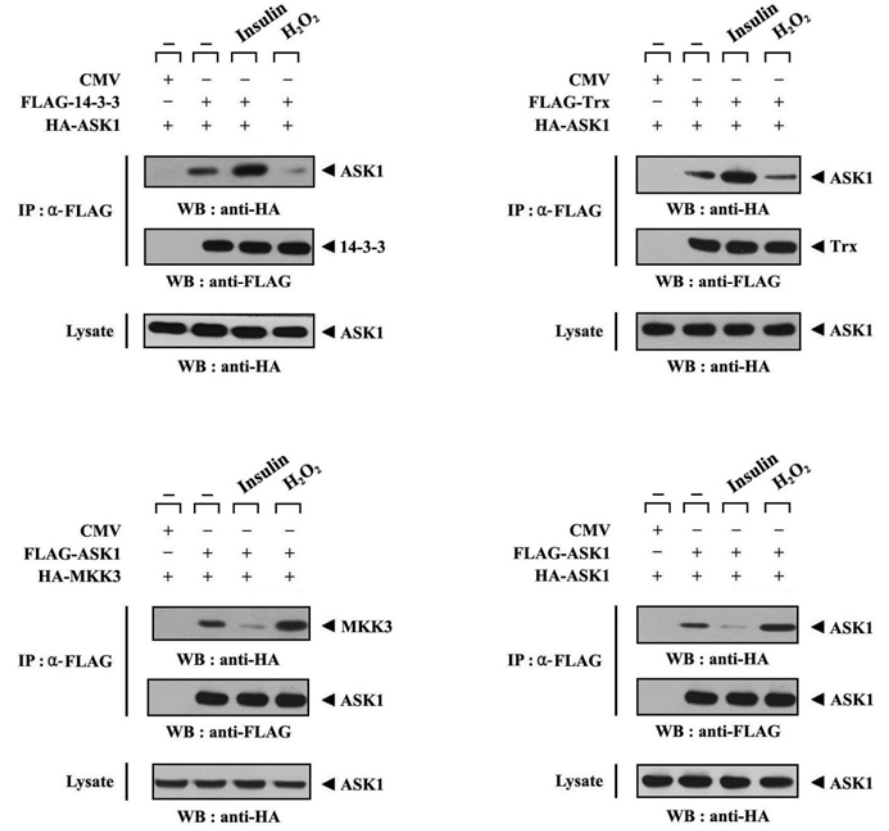
GST Purification
PDK1
GST
WB : anti-GST
Lysate
STRAP
WB : anti-FLAG
– – H_2O_2 Insulin
GST + – – –
GST-PDK1 – + + +
FLAG-14-3-3 + + + +
14-3-3
WB : anti-FLAG
GST Purification
PDK1
GST
WB : anti-GST
Lysate
14-3-3
WB : anti-FLAG

B


– – Insulin H_2O_2
CMV + – – –
FLAG-14-3-3 – + + +
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WB : anti-FLAG
Lysate
ASK1
WB : anti-HA
– – Insulin H_2O_2
CMV + – – –
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HA-ASK1 + + + +
ASK1
IP : α-FLAG
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FIGURE 6. **Regulation of the association between PDK1 or ASK1 and its regulators by H_2O_2 and insulin.** *A*, Modulation of the association between PDK1 and its regulators, STRAP and 14-3-3 protein, by H_2O_2 and insulin. HEK293 cells were transfected with expression vectors encoding FLAG-STRAP, FLAG-14-3-3 protein and GST-PDK1, as indicated. After 48 h of transfection, the cells were incubated with 100 nM insulin for 20 min. For H_2O_2 treatment, the cells were incubated with 2 mM H_2O_2 for 30 min. The cell lysates were subjected to precipitation with glutathione-Sepharose beads (*GST purification*), followed by immunoblotting with an anti-FLAG antibody to determine the association between PDK1 and STRAP or 14-3-3 protein. *B*, Modulation of the association between ASK1 and its regulators, including 14-3-3 protein, Trx, MKK3, and ASK1, by H_2O_2 and insulin. HEK293 cells, transfected with the plasmids indicated, were treated with or without H_2O_2 or insulin as described in *A*, and the cell lysates were immunoprecipitated with an anti-FLAG antibody (*α-FLAG*), followed by immunoblotting with an anti-HA antibody to determine the association between ASK1 and its regulators.