Sak Serine-Threonine Kinase Acts as an Effector of Tec Tyrosine Kinase*

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The murine sak gene encodes a putative serine-threonine kinase which is homologous to the members of the Plk/Polo family. Although Sak protein is presumed to be involved in cell growth mechanism, efforts have failed to demonstrate its kinase activity. Little has been, therefore, elucidated how Sak is regulated and how Sak contributes to cell proliferation. Tec is a cytoplasmic protein-tyrosine kinase (PTK) which becomes activated by the stimulation of cytokine receptors, lymphocyte surface antigens, heterotrimeric G protein-linked receptors, and integrins. To clarify the in vivo function of Tec, we have tried to isolate the second messengers of Tec by using the yeast two-hybrid screening. One of such Tec-binding proteins turned out to be Sak. In human kidney 293 cells, Sak became tyrosine-phosphorylated by Tec, and the serine-threonine kinase activity of Sak was detected only under the presence of Tec, suggesting Sak to be an effector molecule of Tec. In addition, Tec activity efficiently protects Sak from the “PEST” sequence-dependent proteolysis. Internal deletion of the PEST sequences led to the stabilization of Sak proteins, and expression of these mutants acted suppressive to cell growth. Our data collectively supports a novel role of Sak acting in the PTK-mediated signaling pathway.

The murine sak gene was shown to encode two isoforms of a putative serine-threonine kinase, namely, Sak-a and Sak-b (1). The predicted Sak-a protein has a kinase domain at the N terminus followed by a long unique region of ~680 amino acid residues. The amino acid sequence of Sak kinase domain has a moderate sequence similarity (~50%) to those of the Plk/Polo family proteins. Furthermore, the amino acid sequence at the ATP-binding site (Gly-Xaa-Gly-Xaa-Phe-Ala) is conserved throughout Sak and Plk/Polo family members, which is a hallmark of this family kinases (2, 3). These results support the idea that, among a variety of serine-threonine kinases, Sak may belong to the Plk/Polo family composed of Plk (4), Fnk (2), Prk (5), Snk (3), Drosophila Polo (6), Xenopus laevis Plx1 (7), Saccharomyces cerevisiae Cdc5 (8), and Schizosaccharomyces pombe Plo1 (9).

Although neither the regulation mechanisms nor the in vivo substrates of murine Sak kinases have been elucidated yet, accumulating evidence suggests that Sak is involved in cell growth or cell division process. First, transcriptional regulation of the sak gene seems to be linked to the cell growth status. Northern analysis revealed that the sak-a expression is abundant in organs containing dividing cells, such as testis, thymus, and spleen, but is not found in liver, heart, or brain (1). It has been also demonstrated that, in 3T3 cells, expression of sak-a transcripts is high through S-G2-M-phases of cell cycle but declines in G1 (10). Second, forced expression of sense or antisense RNA of sak-a gene both acts suppressive to the growth of CHO cells (1, 10). Thus, a tightly regulated Sak activity may be required for normal cell growth. However, efforts have failed to demonstrate the kinase activity of mouse Sak either by autophosphorylation or by using exogenous substrates, which has seriously hampered the characterization of this protein. Hence, nothing is yet known for the molecular mechanism by which Sak contributes to cell growth or cell division.

Plk, a well studied representative of this subfamily, is an M phase-specific serine-threonine kinase whose activity peaks at the onset of mitosis. Plk is co-localized with CHO1/MKLP-1, a kinesin-like protein, at the midbody in late M-phase, and was shown capable of phosphorylating CHO1/MKLP-1 in vitro (11). Without the activity of Cdc5 (another member of the Plk/Polo family), S. cerevisiae cells no longer complete the M-phase and result in a dumbbell-shaped terminal morphology with the nuclei almost divided but still connected by a thin bridge of chromatin (8). Interestingly, ectopic expression of mouse Plk in these cells rescued the inappropriate cell division cycle, indicating that Plk is a functional homologue of Cdc5 in higher eukaryotes (8). In contrast, introduction of a kinase-inactive Plk could not compensate for the defect of Cdc5, and the S. cerevisiae cells accumulated at G2/M in cell cycle. Therefore, Plk and Cdc5 may be required for cytokinesis in late M-phase, especially for the coordinated movement of microtubules. Such physiological role would be also applicable to Drosophila Polo. The polo gene product shows a cyclin-like kinase activity which peaks at late anaphase/ telophase during the rapid cycles of...

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mitosis in syncytiotrophoblasts of Drosophila embryos. Mutations of the polo gene cause abnormal mitotic and meiotic divisions (12). Taken together, these results suggest an in vivo function common to the Plk/Polo family kinases as a controller of mitotic spindles in late M-phase.

It is yet obscure whether Sak also regulates microtubules in M-phase of cell cycle. Fode et al. (10) reported that overexpression of Sak-a leads to the suppression of CHO cell growth without the accumulation of cells in any phase of cell cycle. This is in contrast to the cell cycle arrest caused by the loss of Cdc5/Plk activities. Therefore, the intracellular targets of Sak and Plk kinases are likely to be different. In addition, a low-grade sequence homology (20–30%) can be found among the C-terminal non-catalytic regions of the Plk/Polo family kinases, whereas the non-catalytic region of Sak has no sequence similarity to them. Importantly, Sak lacks the polo homology domain, an amino acid stretch of ~20 residues highly conserved within the non-catalytic regions of the Plk/Polo family kinases. The murine Sak kinases instead have “PEST” regions in its C terminus (13), which is a unique character to Sak. Therefore, the non-catalytic domain of Sak is structurally distinct from those of the Plk/Polo family members. This non-catalytic C-terminal region should be important for the appropriate function of Sak, since the expression of Sak-b, lacking most of the C-terminal part of Sak-a, could not suppress the growth of CHO cells. Thus, it would not be surprising if Sak is assigned a totally distinct role in vivo from those of the Plk/Polo family kinases, and we believe it is still an open question whether Sak should be included in the Plk/Polo family kinases.

Tec is a non-receptor type protein-tyrosine kinase (PTK) that belongs to a recently identified subfamily of PTKs, including Tec, Btk, Itk/Tsk/Emt, Txk, and Bmx (14, 15). Tec has been shown to become activated and tyrosine-phosphorylated by the stimulation of cell surface receptors on blood cells, and that Tec, Btk, Itk/Tsk/Emt, Txk, and Bmx belong to a recently identified subfamily of PTKs, including Tec, Btk, Itk/Tsk/Emt, Txk, and Bmx (14, 15). Tec has been shown to be involved in the cytokine-driven activation mechanism of c-fos transcription (16). To identify downstream effectors of Tec, we searched for Tec-interacting proteins (TIPs) by using the yeast two-hybrid system. Interestingly, one of such proteins turned out to be human Sak. The analysis of the Tec-Sak interaction in 293 cells has revealed that Sak is tyrosine phosphorylated by Tec. Furthermore, we also demonstrate that co-expression of Tec strongly increases the protein amount of Sak in cells. Our data indicate a novel role of Sak as an effector molecule of PTKs.

EXPERIMENTAL PROCEDURES

Cell Lines—The mouse hematopoietic cell lines, BA/F3 cells (17) and 32D cells (18), were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum and 25 units/ml of mouse IL-3. Human kidney 293 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium/EMR (Life Technologies) containing 10% fetal calf serum and 2 mM L-glutamine. SF21 cells (Invitrogen, San Diego, CA) were grown in suspension at 28 °C in the SF-900 II serum-free medium (Life Technologies) without CO2 supply.

Isolation of Human Sak cDNAs and Construction of Expression Plasmids—The cDNA corresponding to the kinase domain of human Tec (amino acids 357–630) was inserted into the yeast GALA DNA-binding domain vector, pGBT9 (CLONTECH, Palo Alto, CA), giving rise to pGAL4bd-TecKD. Since this construct covers the ATG-binding site of Tec, the resultant fusion protein should have a PTK activity. Indeed, one of the isolated TIP clones, TIP4/BRDG1, turned out to be a phosphorylation-dependent binding protein to Tec (19). The pGAL4bd-TecKD plasmid was then used in the screening as described previously (19, 20). The TIP6 cDNA obtained by the two-hybrid screening was labeled with [α-32P]dCTP, and used as a probe to screen the K562 cDNA library which was constructed in the λZAP II phage vector (Stratagene, La Jolla, CA). After the second round of screening, positive phage clones were converted to pBlueScript II plasmids (Stratagene) by the in vitro excision protocol according to the manufacturers instructions. Insert cDNAs of the plasmids were compared by restriction map analysis, and the longest one was subjected to nucleotide sequencing.

A cDNA fragment containing the full coding region of human Sak was amplified from the TIP1 cDNA by polymerase chain reaction, and inserted into the BamHI-XbaI sites of the pcdNA3-FLAG vector to encode the Sak protein with a C-terminal FLAG epitope tag. With the pcDNA/Sak-F plasmid as a template, pcDNA/Sak-HA-F was generated by using the QuickChange mutagenesis kit (Stratagene) to express Sak-HA-F in which Lys176 at the ATP-binding site is replaced with Met. The expression plasmids for ΔPEST1 (lacking aa 272–311 of Sak-F) and ΔPEST2 (lacking aa 286–325 of Sak-F) were constructed with the ExSite mutagenesis kit (Stratagene) based on the pcDNA/Sak-F and pcDNA/Sak-HA-F plasmids, respectively. The expression plasmid for ΔPEST2 (lacking aa 805–883) was also constructed by the same kit with pcDNA/Sak-F as a template. Desired mutations in these constructs were confirmed by nucleotide sequencing.

Transfection and Protein Analysis—The 293 cells (2 × 10⁴) were transfected with 10 μg each of expression plasmids by the calcium phosphate method. After 2 days of incubation, cells were rinsed once with ice-cold phosphate-buffered saline (PBS) supplemented with 0.1 mM Na2VO4, and resuspended into the lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na3P04, 1 mM Na2VO4, 200 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After incubation for 30 min on ice, cell extracts were centrifuged to remove insoluble materials. Sak and its mutants were immunoprecipitated from 1.5 to 2 mg of the lysates by anti-FLAG antibody (αFLAG Ab) (M2; Eastman Kodak, New Haven, CT) and protein G-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden).

For immunoblotting, samples were separated through SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were incubated for 1 h at room temperature in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) with 4% bovine serum albumin (Fraction V; Sigma). The membranes were then incubated with α-FLAG Ab or anti-phosphotyrosine antibody (αTyr(P) Ab) (4G10; Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature in TBST. Specific bindings of the antibodies were visualized by the ECL detection system (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer’s instruction.

For the in vitro assay, the immune complexes were washed twice with the lysis buffer, three times with the kinase buffer (20 mM Tris-HCl, 7.4, 50 mM NaCl, 10 mM MgCl2, 2 mM MnCl2), and finally incubated with 0.37 MBq of [γ-32P]ATP (Amersham Pharmacia Biotech) for 1 h at 30 °C. For the phosphoamino acid analysis, the samples were electrophoresed by SDS-PAGE, and blotted onto the polyvinylidene difluoride membrane as described above. A piece of the membrane containing the Sak protein was cut out, and subjected to acid hydrolysis and the electrophoresis through a thin layer chromatography (TLC) as described previously (21).

The cDNAs of Sak-F was inserted into the pFastBac1 plasmid (Life Technologies). Recombinant baculovirus based on this plasmid was generated by the Bac-to-Bac baculovirus expression systems (Life Technologies), and was used to infect SF21 cells with the baculovirus expressing Tec or TecΔ106 (22). After 48–72 h of culture, cells were harvested and lysed as described above.

GST Fusion Proteins—The Sak cDNA fragment encoding the domain 1 (aa 1–297), domain 2 (aa 326–569), domain 3 (aa 889–970) was polymerase chain reaction-amplified and inserted into pGEX2T vector (Amersham Pharmacia Biotech) to produce the GST fusion protein in BL21 cells (Stratagene). Anti-Tec immune complexes, prepared from BA/F3 cells, were washed extensively by the kinase buffer, mixed with 3 μg each of GST or the GST fusion proteins, and finally incubated with 100 μM cold ATP for 30 min at 37 °C. The samples were eluted into the SDS-PAGE sample buffer and immunoblotted with α-Tyr(P) Ab or anti-GST antibody (AMRAD, Kew Victoria, Australia).

BA/F3 cells Expressing Sak Variants—With the BOSC23 packaging cell line, ecotropic retrovirus was generated from pMX-iresCD8 (see “Results”), pMX-ΔPEST1-iresCD8, or pMX-ΔPEST1-iresCD8, and was used to infect BA/F3 cells. After the infection, the trypsinized, 10⁶ cells of each population were resuspended in 90 μl of the MACS buffer (PBS containing 2 mM EDTA, 0.5% bovine serum albumin, and 0.01% sodium azide) and incubated with anti-CD8 antibody conjugated with magnetic microbeads (Miltenyi Biotec, Germany). After washing with the MACS buffer, cells were loaded onto the mini-MACS separation columns (Miltenyi Biotec). The CD8⁺-positive population of each set was then eluted into the MACS buffer according to the manufacturers protocol.
For the analysis of DNA contents, BA/F3 cells expressing CD8 (BA/F3-CD8), CD8 + ΔPEST1 (BA/F3-ΔPEST1), or CD8 + ΔPEST1KM (BA/F3-ΔPEST1KM) cells were resuspended into the propidium iodide (PI) suspension buffer (0.05 mg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100, and 80 units/ml RNase A). DNA profiles of each cell fraction was determined by the ModFIT program (Becton Dickinson, San Jose, CA) for 1 h. Then, the tissues were postfixed in a 1% osmium tetroxide solution for 90 min at 4 °C, dehydrated through graded ethanol and propylene oxide, and embedded in epoxy resin. The ultra thin sections were made with an LKB 8800 Ultrotome III (Amersham Pharmacia Biotech) and were observed under Hitachi H-7000 electron microscope (Hitachi, Tokyo, Japan) after staining with uranyl acetate and lead citrate.

RESULTS

Identification of TIP 6 as Human Sak—To investigate the downstream signaling system of Tec, we looked for its substrates by using the yeast two-hybrid system. With the Tec kinase domain containing its ATP-binding site as a "bait," a total of six TIPs were identified from various cDNA expression libraries of human origin. 19 Among them, TIP6 was isolated from a human cDNA library from bone marrow mononuclear cells, and its predicted amino acid sequence was highly homologous to the C-terminal portions of mouse Sak-a, raising the possibility that TIP6 is the human homologue of Sak-a.

We then examined whether TIP6 is expressed in hematopoietic cell lines by Northern blot analysis. A major transcript of 4.2 kilobases long was identified in 32D and BA/F3 cells (both of mouse origin), and two major transcripts of 5.5 and 4.0 kilobases long were in K562 cells (human origin) (data not shown). A cDNA library of K562 cells was then constructed in λZAPII phage vector (Stratagene), and was used to isolate the full-length human TIP6 cDNA. The longest TIP6 cDNA contains an open reading frame encoding a protein of 970 amino acid residues with a calculated molecular mass of 109 kDa (Fig. 1A). The homology of the amino acid sequences between TIP6 and mouse Sak-a is 94.4% in the kinase domain, 75.9% in the C-terminal region, and 78.3% in overall. The putative PEST sequences identified in mouse Sak-a are also found in TIP6 at the corresponding positions (Fig. 1, A and B). Therefore, TIP6 is likely to be the human homologue of Sak.

It should be noted that, compared with mouse Sak-a, human Sak has a 35-amino acid insertion (shown boxed in Fig. 1A) at the position where alternative exons were reported in mouse (1). Thus, Sak variants generated by alternative splicing of the mRNAs may be present also in the human system. Although the kinase domain of human Sak has moderate sequence similarity to that of human Plk/Polo, 38.1% with that of human Drosophila Plk, we could not detect significant sequence similarity between the C-terminal non-catalytic region of human Sak and those of the Plk/Polo family members.

Sak Is Tyrosine Phosphorylated by Tec—It is not known whether Sak receives inputs from PTKs. To investigate the functional interaction between Tec and human Sak, we tested if Sak becomes tyrosine phosphorylated by Tec in cells. The
Binding of the second antibody to the immunoglobulin heavy chain is shown as an asterisk.

proteins were immunoprecipitated by Ssion vector (V) or in combination with the expression plasmid for Tec (T) or TecKM (Tδ4). Sak proteins were immunoprecipitated by αFLAG Ab, separated through 7.5% SDS-PAGE and probed with α-Tyr(P) Ab (α-Tyr) or αFLAG Ab (αFLAG). The positions of Sak-F and SakKM-F are indicated by arrows. Binding of the second antibody to the immunoglobulin heavy chain is shown as an asterisk. The positions of molecular weight standards (×10^-5) are indicated at the left. B, RNA was extracted from 293 cells (2 × 10^6 each) transfected with pcDNA-FLAG-pSRα (vector), pcDNA/Sak-F+pSRα (sak), or pcDNA/Sak-F+pSRα/Tec (sak+tectec). pcDNA/Sak-F plasmid, which produces the human Sak protein with a C-terminal FLAG tag (Sak-F), was introduced into human kidney 293 cells together with an expression plasmid for Tec. Sak-F was immunoprecipitated by αFLAG Ab and probed with α-Tyr(P) Ab. Interestingly, as shown in Fig. 2A, Sak-F became profoundly tyrosine phosphorylated when co-expressed with Tec (lane 3 in the upper panel). Phosphorylation of tyrosine residues was also evident when a kinase-inactive SakKM-F was used instead of Sak-F (lane 6). In both cases, loss of Tec activity led to the disappearance of Sak phosphorylation (lanes 4 and 7).

Furthermore, from Fig. 2A we could deduce the following information for Sak protein. First, as evident from the lower panel, the amount of Sak-F protein was far more abundant when co-expressed with Tec than expressed alone (compare lanes 2 and 3). This effect was almost canceled when a kinase-inactive Tec (TecKM) in which Lys^397 at the ATP-binding site is replaced with Met (23) was used (lane 4). Thus, Tec activity should protect Sak protein from degradation in cells or should induce the Sak mRNA. It is not yet clear why the protein amount of kinase-inactive Sak (SakKM) was more abundant than that of wild Sak (compare lanes 2 and 5). We believe the Sak protein is prone to degradation in a dependent manner to its PEST sequences (see the following paragraphs), and it could be possible that the kinase activity of Sak affects its structure, thereby changing the accessibility of the ubiquitination machinery.

The second interesting finding was that, under the presence of Tec, electrophoretic mobility of Sak-F became significantly slower than that of SakKM-F (compare lanes 3 and 6). This mobility shift was not owing to the phosphorylation on tyrosine residues, since tyrosine-phosphorylated SakKM-F in lane 6 does not have the slow mobility. Rather, the shift is likely to arise from the activity of Sak-F itself; in other words, phosphorylation on serine/threonine residues.

Sak Kinase as an Effector of Tec

To decipher the mechanism underlying the accumulation of Sak proteins, we examined whether co-expression of Tec induces the transcription of Sak. RNA was extracted from 293 cells transfected with the mock vector, the Sak-F expression plasmid, or the Sak-F expression plasmid plus Tec expression plasmid. Northern blot analysis with the Sak cDNA probe revealed that co-expression of Tec slightly affected the expression level of the Sak message (upper panel, Fig. 2B). We confirmed that more than 70% of 293 cells incorporated the DNAs in our transient expression system (data not shown), and, thus, the difference of the message level observed in Fig. 2B should be too little to account for the vast change in the protein amounts of Sak. Hybridization of the same RNA set with the β-actin probe demonstrated that an equivalent amount of RNA was loaded in each lane (lower panel). These results imply that Tec may increase the amount of Sak proteins by a translational or post-translational mechanism.

We next investigated whether tyrosine phosphorylation of Sak is mediated specifically by Tec. Sak-F was transiently expressed in 293 cells either alone or in combination with Tec, Jak2, or Lyn, and analyzed for its tyrosine phosphorylation. As shown in Fig. 2C, Sak became phosphorylated only when co-expressed with Tec, but not with the other kinases examined. Therefore, Sak is not a common substrate of various PTKs, but may be an effector molecule only to a subset of PTKs.

We then examined the Tec-Sak interaction in the insect cell system. SF21 cells derived from Spodoptera frugiperda were infected with the recombinant baculovirus expressing human Sak-F alone or in combination with the Tec-expressing or Tec^KM-expressing virus. After 2 days of incubation, Sak was immunoprecipitated from the cells and probed with αFLAG Ab (upper panel of Fig. 3A). Interestingly, Sak protein was abundantly expressed in SF21 cells even without the introduction of Tec. In parallel with the ample expression, we could detect the tyrosine phosphorylation of Sak, which is presumably carried out by endogenous PTKs in SF21 cells (lower panel). As expected, in response to the introduction of Tec proteins, Sak phosphorylation significantly increased. This result favors the idea that Sak is a direct substrate of Tec in vivo.

To study which part of the Sak protein contains the phosphorylation targets of Tec, immunoprecipitated Sak protein was mixed with glutathione S-transferase (GST) or GST fusion proteins of domain 1 (aa 1–297), domain 2 (aa 326–569), domain 3 (aa 586–830), or domain 4 (aa 889–970) of Sak (see Fig. 3).
membrane was re-probed with anti-GST antibody to estimate the amounts of GST fusion proteins used in the assay (right panel). We could not prevent the GST-domain 2 and GST-domain 3 proteins from proteolysis during the incubation period with MgCl₂ and ATP.

Previous efforts have been unsuccessful to detect the kinase activity of Sak proteins. Here we examined whether human Sak has a demonstrable activity in an in vitro kinase assay and whether its activity is controlled by Tec. Sak-F was transiently expressed in 293 cells either alone or in combination with Tec, immunoprecipitated and incubated with [γ³²P]ATP. As shown in Fig. 3C, autophosphorylation of human Sak was not detectable when it was precipitated from 293 cells expressing Sak alone. However, in parallel with the induction of tyrosine phosphorylation, co-expression of Tec strongly increased the intensity of phosphorylated Sak band. In contrast, a kinase-negative Tec could not achieve this enhancement (lane “S+T³⁴⁷”). Similar results were obtained when casein was used as an exogenous substrate (data not shown).

Characterization of PEST-deleted Sak Mutants—As shown in Fig. 2A, when expressed alone in 293 cells, only a trace amount of Sak protein is detectable by immunoblotting. Similarly, although expression of endogenous Sak message was identified in BA/F3, 32D, and K562 cells, we could not detect Sak protein in these cells with our polyclonal anti-Sak serum (data not shown). These results are in good agreement with the reported short half-life of the Sak protein (10). Therefore, it was necessary to generate more stable Sak proteins to obtain information on Sak in cells.

There are two clusters of PEST sequence in human Sak (Fig. 1B). Thus, we constructed cDNAs encoding the FLAG-tagged Sak protein that lacks the N-terminal PEST cluster (ΔPEST1) or the C-terminal cluster (ΔPEST2). To test whether these variants have a prolonged half-life in cells, Sak was immunoprecipitated from 293 cells expressing Sak-F, ΔPEST1, or ΔPEST2, and probed with aFLAG Ab (Fig. 4A). In both constructs, internal deletion of the PEST clusters led to the stabilization of Sak proteins. This result supports the idea that Sak is degraded in cells through a PEST-dependent proteolysis.

Since ample expression of ΔPEST1 could be easily achieved in 293 cells even without the co-expression of other kinases such as Tec, we tried to prove the serine-threonine kinase activity of Sak by using this mutant. ΔPEST1 or ΔPEST1⁷⁷⁷⁷ (Lys⁴¹ in the ATP-binding site of ΔPEST1 is replaced with Met) was transiently expressed in 293 cells, immunoprecipitated, and subjected to an in vitro kinase assay without exogenous substrates. As shown in Fig. 4B, abundant phosphorylation of ΔPEST1 protein was observed, and this ³²P incorporation was completely abolished when its kinase activity was canceled. The phosphorylation of ΔPEST1, therefore, should represent the kinase activity of ΔPEST1 itself. This phosphorylated ΔPEST1 protein was then subjected to one-dimensional phosphoamino acid analysis, revealing that autophosphorylation takes place on both serine and threonine residues of ΔPEST1, but not on tyrosine (Fig. 4C). This is the first report proving that Sak protein contains an intrinsic serine-threonine kinase activity, as predicted from its amino acid sequence.

Next, we questioned whether these Sak mutants retain the ability to become phosphorylated by Tec in cells. Sak-F, ΔPEST1, or ΔPEST2 were transiently expressed in 293 cells with or without Tec, immunoprecipitated and blotted with α-Tyr(P) Ab (upper panel of Fig. 4D). In contrast to Sak-F, we could detect a significant level of tyrosine phosphorylation in ΔPEST1 even without the co-expression of Tec (compare lanes 1 and 3). This effect was carried out, probably, by PTKs endogenously expressed in 293 cells. By introduction of Tec, how-
Fig. 4. PEST-deleted Sak in 293 cells. A, an expression plasmid (10 μg each) of Sak-F, PEST1, or PEST2 was introduced into 2 × 10⁶ of 293 cells by the calcium phosphate method. Sak and its variants were immunoprecipitated by αFLAG Ab, separated through 7.5% SDS-PAGE, and probed with the same antibody. The positions of Sak-F (WT), PEST1 (ΔP1), and PEST2 (ΔP2) are indicated by arrows. B, Sak mutants were immunoprecipitated by αFLAG Ab from 293 cells transfected with the blank vector (V) or the expression plasmid for PEST1 (ΔPEST1) or PEST1KM (ΔPEST1KM). The immunoprecipitates were washed by the kinase buffer, incubated with [γ-32P]ATP, and separated through 7.5% SDS-PAGE. The positions of molecular weight standards (×10⁻³) are indicated at the left, and that of ΔPEST1 at the right. C, the phosphorylated PEST1 in B was subjected to acid hydrolysis, and electrophoresed through a TLC plate. The positions of phosphoserine (p-Ser), phosphothreonine (p-Thr), phosphotyrosine (p-Tyr), and the sample loading point (ori) are indicated. D, Sak-F (WT), ΔPEST1 (ΔP1), or ΔPEST2 (ΔP2) was transiently expressed in 293 cells with (+) or without (−) the co-expression of Tec, immunoprecipitated, and subjected to an in vitro kinase assay without exogenous substrates. Autoradiography of the phosphorylated ΔPEST1 is shown (KA). The same set of samples was also immunoblotted with αFLAG Ab (αFLAG). The amounts of total cell lysates for immunoprecipitation were adjusted to normalize the quantity of precipitated ΔPEST1.

However, ΔPEST1 became profoundly tyrosine phosphorylated (lane 4). The protein amount of ΔPEST1 slightly increased in response to the Tec expression (more evident in a film with a shorter exposure). Tyrosine phosphorylation of ΔPEST2 was less significant than that of ΔPEST1 (lane 6), although it was difficult to quantitatively compare the phosphorylation level between the two forms because of the vast difference in their protein amounts. Since both tyrosine phosphorylation and the effect of protein stabilization were more evident in ΔPEST1 than in ΔPEST2, we used the former protein in the following experiments.

From the experiments described so far, we concluded that 1) Tec can phosphorylate and induce the activity of Sak, and 2) Tec can stabilize the Sak protein in mammalian cells. However, we do not yet know whether these two effects are independent or have a causal relationship. In other words, is the Sak activation in an in vitro kinase assay a mere result of the increase in protein amount, or caused by a regulatory effect by Tec (through, for example, tyrosine phosphorylation)? Since ΔPEST1 could escape from proteolysis to some extents, we supposed that ΔPEST1 would be a useful tool to address this question. By using ΔPEST1, it should be possible to dissect the issue of Sak activity from the effect of change in its protein amount.

ΔPEST1 was transiently expressed in 293 cells with or without Tec, immunoprecipitated and subjected to an in vitro kinase assay (upper panel of Fig. 4E). The same samples were also probed with αFLAG Ab to estimate the amounts of Sak proteins precipitated (lower panel). As demonstrated in Fig. 4D, Tec expression may slightly raise the protein level of ΔPEST1. Thus, in Fig. 4E, we adjusted the amounts of total cell lysates for immunoprecipitation so that comparable amounts of ΔPEST1 were precipitated irrespective of the presence of Tec. Autophosphorylation activity of Sak seems to be slightly enhanced by the co-expression of Tec. This observation is consistent with the retarded electrophoretic mobility of Sak in the presence of Tec. These results suggest Tec may have a weak regulatory effect on Sak activity. It should be noted, however, that Tec increase of the kinase activity was far more prominent in wild Sak than in ΔPEST1 (compare Figs. 3C and 4E). Therefore, it is highly possible that the majority of the enhancement effect by Tec resulted from the accumulation of Sak proteins in cells.

To further investigate the Tec regulation of Sak activity, we also synthesized Sak and Tec proteins by an in vitro transcription/translation system and tested if co-translation of Tec affects the Sak activity. Although a significant amount of both proteins was produced in this system, we could not detect kinase activity of Sak irrespective of the presence of Tec protein. In addition, neither tyrosine phosphorylation nor kinase activity could be observed on synthesized Tec. It is, thus, likely that the in vitro transcription/translation system cannot create Tec and Sak with an appropriate protein folding which is indispensable for their kinase activity.

Expression of PEST-deleted Sak in Blood Cells—We have revealed that Sak becomes tyrosine phosphorylated by Tec in the reconstitution system with 293 cells. Thus, we next tested whether this is also the case for Sak proteins stably expressed in blood cells. Since it was difficult to detect Sak proteins endogenously expressed in BA/F3 cells by our serum, we prepared BA/F3 cells and 32D cells stably expressing Sak or its mutants for further analysis.

When characterizing cell clones stably expressing an exogenous gene, it is sometimes difficult to determine whether the phenotype of the transformants arises from the expression of the incorporated gene or from clonal variations. To overcome such uncertainty, a rapid and easy method was invented to obtain a polyclonal cell population expressing a gene of interest. The internal ribosome entry site (ires) elements are known to allow translation of internal cistrons of mRNAs independently from the well characterized capping-mediated ribosome scanning (24). The irs fragment derived from encephalomyocarditis virus was ligated with mouse CD8 cDNA, and this irs-CD8 cassette was inserted into the retroviral pMX vector (25), giving rise to pMX-iresCD8. Further insertion of the ΔPEST1 cDNA generated a bicistronic pMX-ΔPEST1-iresCD8 vector of which transcript simultaneously produces ΔPEST1 and CD8 in the same cells (Fig. 5A). We also constructed the

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pMX-ΔPEST1KM-iresCD8 vector to express ΔPEST1KM instead of ΔPEST1.

BA/F3 cells were infected with the recombinant retrovirus generated from pMX-iresCD8, pMX-ΔPEST1-iresCD8, or pMX-ΔPEST1KM-iresCD8. After 24 h of infection period, cells were cultured for further 24 h, mixed with anti-CD8 antibody and then loaded onto the mini-MACS magnetic separation columns to purify the CD8-high-populations. The Sak variants were immunoprecipitated from BA/F3 cells expressing CD8 alone (V), CD8+ΔPEST1 (ΔP1), or CD8+ΔPEST1KM (ΔP1KM), and probed with either αFLAG Ab (αFLAG) or α-Tyr(P) Ab (α-Tyr). The position of ΔPEST1 is indicated by an asterisk. The molecular weight standards (×10^{-2}) are shown at the left. C, 32D cells expressing ΔPEST1 were cultured without fetal calf serum and IL-3 for 2.5 h at the concentration of 5 × 10^{6} cells/ml, and then for 40 min at 1 × 10^{7} cells/ml. The cells (1 × 10^{7} cells/fraction) were then stimulated with 250 units/ml of mouse IL-3 for the period of 0, 5, 10, 20, or 40 min as indicated at the top. ΔPEST1 was immunoprecipitated from each fraction, and probed with α-Tyr(P) Ab (α-Tyr) or αFLAG Ab (αFLAG).

We then investigated whether PEST-deleted Sak proteins were detectable in these cells, and whether they were tyrosine phosphorylated. The Sak variants were immunoprecipitated from the BA/F3 transfecteds, and probed with αFLAG Ab (upper panel of Fig. 5B). Both ΔPEST1 and ΔPEST1KM were readily identified in these cells by the immunoblot analysis. The same membrane was also probed with α-Tyr(P) Ab to demonstrate that both of the Sak variants were phosphorylated on tyrosine residues (lower panel). We also examined whether tyrosine phosphorylation of ΔPEST1 was affected by the stimulation with cytokines. BA/F3-ΔPEST1 cells were incubated without serum and IL-3, and then stimulated with IL-3. ΔPEST1 was immunoprecipitated from each cell fraction, and probed with α-Tyr(P) Ab. However, we observed that ΔPEST1 was constitutively tyrosine phosphorylated irrespective of IL-3 stimulation (data not shown).

To test whether this constitutive phosphorylation of ΔPEST1 was a phenomenon specific to BA/F3 cells, similar experiments were carried out in 32D cells. By the recombinant retrovirus described above, 32D cells expressing CD8 plus ΔPEST1 were prepared, and were used to investigate the cytokine responsiveness of ΔPEST1 phosphorylation. As shown in Fig. 5C, starvation of serum and IL-3 did not decrease the phosphorylation level of ΔPEST1 in 32D cells, and again the IL-3 stimulation did not further increase the tyrosine phosphorylation in ΔPEST1.

No information has been available for the subcellular localization of Sak, probably, owing to the very low expression level of endogenous Sak proteins in cells. We could, however, express Sak Kinase as an Effector of Tec.
mation) in cytoplasm of BA/F3 cells. We did not find any Sak signals associated with the interzone or mitotic spindles in M-phase. This is in contrast to the reported localization of Plk and Polo, which may suggest a distinct role of Sak from that of the Plk/Polo family kinases. Although it is possible that the deletion of PEST regions affected the localization of Sak protein in cells, we could not detect, in BA/F3 cells, full-length Sak proteins even when introduced exogenously (see the next paragraph).

De-regulation of Sak Activity Induces Cell Death in Blood Cells—During the purification step with the mini-MACS magnetic cell separation system, we noticed that the total cell numbers of BA/F3-ΔPEST1 and BA/F3-ΔPEST1KM cells were significantly smaller than that of control BA/F3-CD8 cells. This is in good agreement with the previous observation that both of forced overexpression and suppression of Sak mRNA cause growth inhibition in CHO cells (1, 10). These results indicate that de-regulation of Sak activity is harmful to cell growth. Actually, as shown in Fig. 7A, growth of BA/F3-ΔPEST1 and BA/F3-ΔPEST1KM cells was retarded compared with that of BA/F3-CD8 cells. Therefore, both of ΔPEST1 (as a hyperactive form) and ΔPEST1KM (probably as a dominant negative form) suppress the proliferation of BA/F3 cells.

In addition to BA/F3 cells expressing ΔPEST1 or ΔPEST1KM, we also tried to prepare BA/F3 cells expressing full-length Sak-F or SakKM-F by using the pMX-iresCD8 system. Although the CD8high fraction of infected cells was obtained and the growth rate of them was comparable to that of ΔPEST1 or ΔPEST1KM-expressing cells, we could not detect the full-length Sak proteins in the infected cells by the immunoblot analysis, probably, due to the rapid degradation of PEST-containing Sak proteins (data not shown).

How does de-regulation of Sak activity inhibit cell growth? Fode et al. (10) reported that forced expression of Sak in CHO cells did not alter the cell cycle distribution in the transfectants. We thus tested the DNA contents of BA/F3-CD8, BA/F3-ΔPEST1, or BA/F3-ΔPEST1KM cells by using flow cytometry. As shown in Table I, no significant accumulation of BA/F3 transfectants in a specific cell cycle phase was observed, although a slight decrease in the cell number for S-phase was noticed in both BA/F3-ΔPEST1 and BA/F3-ΔPEST1KM cells. It is not clear whether this subtle change plays a causative role for the growth suppression.

Next, we tested whether the transfectants were forced to undergo apoptosis. The cells were stained with annexin-V-FLUOS, and subjected to FACS analysis to detect the translocation of phosphatidylserine from the inner part to the outer layer of cell membrane, a characteristic feature of apoptosis. As shown in Fig. 8, the translocation of phosphatidylserine may also occur in the late phase of necrosis, we confirmed the apoptotic change of the transfectants with electron microscopy. In ~30% of BA/F3-ΔPEST1 cells observed was the marked shrinkage and blebbing of cell membrane, and disappearance of microvilli (left panel of Fig. 8). In addition, we could readily identify apoptotic cells with condensed nuclear chromatin and eosinophilic stain-
ing (right panel). Similar changes were also noticed in BA/F3-ΔPEST1/KM cells and IL-3-depleted BA/F3 cells (data not shown). Therefore, an apoptosis pathway should be triggered in BA/F3 cells by the de-regulated Sak kinase activity. In contrast to the report using CHO cells, however, we could not detect multinucleation in any of the transfectants. This discrepancy may result from the different cell lines used for the Sak analysis. It is also possible that the truncated area in ΔPEST1 contains an indispensable region for Sak to regulate mitosis.

**DISCUSSION**

Our experiments have demonstrated an unexpected scenario to place Sak in the signaling system of tyrosine kinases. Tec directly phosphorylates the tyrosine residues in Sak, and the Tec activity regulates the protein amount of Sak in cells. In our transient expression system with 293 cells, the PEST-mediated protein degradation should be the critical regulation point of Sak protein amounts, since deletion of the PEST1 or PEST2 region results in a profound increase of Sak proteins (Fig. 4A). This is in good agreement with the observation by Fode et al. (10) that Sak is multiubiquitinated and has a short half-life (2–3 h) in CHO cells. Co-expression of Tec should efficiently protect Sak from proteolysis, but this effect is only marginal on the PEST-deleted Sak proteins (compare lanes 3 and 4 in the lower panel of Fig. 4D). Therefore, it is most likely that Tec increases the Sak protein level by preventing the PEST-dependent proteolysis. These lines of evidence proposed an intriguing possibility that Tec-mediated tyrosine phosphorylation directly controls the PEST-dependent protein-degradation machinery. This possibility was further supported by the fact that prevention of Sak degradation required the kinase activity of Tec.

A number of studies have also reported that PEST-mediated proteolysis is dependent on the phosphorylation of target proteins. Weil et al. (26) observed that, for efficient PEST-dependent protein degradation, 1kBβ requires both the constitutive phosphorylation within its PEST sequences and the stimulated-induced phosphorylation outside the PEST elements. Similarly, for rapid turnover, G1 cyclins need to be phosphorylated on serine residues in their PEST regions (27). It is presumed that these phosphorylation events are requisite for the PEST-dependent ubiquitination of the target proteins, which is the first step of the PEST-dependent proteolysis cascade.

There are, however, some differences in our observation on Sak proteolysis from theirs. First, Tec activity blocks, not accelerates, the protein degradation. Second, phosphorylation of tyrosine residue(s) in the Sak kinase domain decreases the accessibility of free NH2 groups as the phosphorylation increases (Table 2). In addition, it is possible that the truncated area in ΔPEST1 may result from the different cell lines used for the Sak analysis or that these phosphorylation events are requisite for the PEST-dependent ubiquitination of the target proteins. Weil et al. (10) that Sak contains an indispensable region for Sak to regulate mitosis. It is also possible that the truncated area in ΔPEST1/KM cells and IL-3-depleted BA/F3 cells (data not shown).

One possible scenario to be compatible with these findings is that phosphorylation of tyrosine residue(s) in the Sak kinase domain decreases the accessibility of free NH2 groups as the ubiquitination targets. For instance, all of Tyr27, Tyr49, and Tyr100 in the Sak kinase domain have basic amino acid residues at their neighbor. Thus, if one of these charged amino acids is the target of ubiquitination, phosphorylation of the adjacent tyrosine residue may block the access of binding factors for ubiquitination. Another scenario may be that Sak binds phosphotyrosine-binding proteins which, in turn, block the protein degradation process.

The in vivo signaling pathway in which Sak is involved is still unclear. On the contrary to the cell cycle distribution of yeast cells expressing abnormal Plk or Cdc5, no evidence for cell cycle arrest was observed in the mammalian cells constitutively expressing Sak. Thus, it is likely that de-regulated Sak inhibits cell growth by a mechanism not utilized by the Plk/Polo family members. Our experiments provide an intriguing idea that Sak may be linked to apoptosis pathways in blood cells. The significantly retarded growth rate, increase of phosphatidylserine-positive cells, and the electron microscopic data all support the apoptotic changes of BA/F3 cells expressing ΔPEST1. However, our current data does not clarify whether endogenous Sak proteins work for or against apoptosis of blood cells. Since loss of Sak activity (either by antisense RNA introduction or expression of kinase-inactive Sak) suppresses cell growth, it would be possible that the precisely controlled Sak activity in vivo prevents cells from apoptosis, and that disruption of this control leads to cell death. To elucidate the Sak-dependent control of apoptosis, it would be indispensable to identify the cellular targets of the Sak kinase.

Although there exists a limitation in the interpretation of experiments with the PEST-deleted mutants, they are to date the only Sak proteins which demonstrate a serine-threonine kinase activity. Therefore, these proteins should be useful tools to decipher the regulation mechanism as well as the in vivo role of Sak protein.

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Sak Serine-Threonine Kinase Acts as an Effector of Tec Tyrosine Kinase
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