Molecular Cloning and Characterization of a Novel p70 S6 Kinase, p70 S6 Kinase β Containing a Proline-rich Region*

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A novel ribosomal S6 kinase, termed p70 S6 kinase β (p70β), which has a highly conserved amino acid sequence compared with that of p70/p85 S6 kinase (p70α) within the catalytic, kinase extension, and autoinhibitory pseudosubstrate domains, was identified. However, the amino acid sequence of p70β differs from that of p70α in the noncatalytic amino-terminal region and in the carboxyl-terminal tail, which contains a proline-rich region. The majority of the regulatory phosphorylation sites identified in p70α are conserved in p70β. Two isoforms of p70β, referred to as β1 (495 amino acids) and β2 (482 amino acids), could be expressed from the same mRNA either by alternative mRNA splicing or by the use of alternative start codons. Here we report the characterization of p70β2. Similarly to p70α, the catalytic activity of p70β toward ribosomal protein S6 could be rapidly activated by serum, insulin, and phorbol ester in transiently transfected cells. The p70β kinase was found to be significantly less sensitive to wortmannin and rapamycin than p70α. These results indicate that p70β has the potential to participate in the regulation of protein synthesis and the cell cycle.

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p70 S6 kinase was identified as an insulin/mitogen-activated protein kinase in mammalian cells, whose major known substrates is the 40 S ribosomal subunit protein S6 (1–4). Two isoforms of the enzyme, denoted p70α1 and p70α2 (p70 S6 kinases α1 and α2), are known to be generated from a single gene by alternative mRNA splicing and the use of alternative translational start sites (5). The 525-amino acid p70α1 isoform differs from the 502-amino acid p70α2 isoform only at the amino terminus (5). p70α1 is known as p85 S6 kinase because of its reduced mobility when analyzed by SDS-PAGE.1 The 23-amino acid extension at the amino terminus in p70α1 contains a nuclear localization signal that constitutively targets this isoform to the nucleus, whereas p70α2 appears to be expressed exclusively in the cytoplasm (6, 7).

In addition to the role of p70α in protein synthesis, it has been shown that p70 S6 kinase is required during the G1 phase of the cell cycle (6, 8). In these experiments, neutralizing antibodies against p70α were shown to prevent the serum-induced entry of cells into S phase. However, in a recent report on targeted disruption of the p70α gene in murine embryonic stem cells, it was demonstrated that p70α−/− cells still proliferate at a rate slower than the parental cells (9). These results suggest that p70α has a positive influence on cell proliferation but that the disruption of this gene is not lethal. In the present study, using immunoblot analysis with the anti-phosphopeptide antibody against the (Ser/Thr)-Pro motif in the autoinhibitory pseudosubstrate domain of p70α, several novel immunoreactive bands were found in the fractions of HEK293 cells separated by an anion exchange column chromatography. These observations suggested to us that isoforms of p70 S6 kinase, other than p70α, exist and prompted a search of the expressed sequence tag (EST) data base that revealed potentially novel isoforms of p70 S6 kinase. Here we report the identification and characterization of a novel isoform of p70 S6 kinase, designated p70 S6 kinase β (p70β).

EXPERIMENTAL PROCEDURES

Construction and Screening of a HEK293 Uni-ZAP Library and DNA Sequencing Analysis—Total RNA was isolated from HEK293 cells as described (10), and poly(A)+ mRNA was selected by using the Dyna-beads mRNA purification kit (Dynal). An oligo(dT)-primed library was constructed in UNI-ZAP XR vector from 5 μg of HEK293 mRNA, using the Uni-ZAP cDNA synthesis kit (Stratagen). Packing into phages was carried out by using Gigapack III Gold Packaging extracts (Stratagen). The human EST cDNA clones were obtained from the UK HGMP Resource Center. cDNA encoding p70β1 was isolated by screening of 1 × 106 primary phages from the HEK293 Uni-ZAP library with a 24P-labeled 0.65-kb EcoRl/NorI fragment derived from the human EST clone AA410355. Positive cDNA clones were isolated and rescued as Bluescript plasmids by in vivo excision (Stratagen). Sequencing analysis of selected clones was performed on an Applied Biosystem 373A DNA sequencer. As a result, we obtained a cDNA clone, designated clone 53, which contains a full-length coding sequence of human p70β.

Northern Blot Analysis—Membranes containing poly(A)+ RNA samples from various human tissues and human β-actin CDNA probe were purchased from CLONTECH. The following probes were used for the detection of p70α and p70β messages: (i) a 476-bp HindIII fragment spanning 56 bp of the 3′ end coding region and 420 bp of the 3′ noncoding region of the human p70α (EST clone, AA425599) and (ii) a 650-bp fragment spanning 518 bp upstream of the stop codon and about

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag; GST, glutathione S-transferase; Ab, antibody; PI, phosphoinositide; kb, kilobase pair(s); bp, base pair(s); CHO, Chinese hamster ovary.
130 bp of noncoding region of the human p70\(\beta\) (EST clone, AA410355). Construction of Plasmids and Expression of GST Fusion Protein—The full-length coding sequence corresponding to p70\(\beta2\) (amino acids 14–495 of p70\(\beta1\)) was amplified by polymerase chain reaction using the clone 53 as a template and cloned into the pCDNA vector (Invitrogen) in-frame with the amino-terminal FLAG epitope. The expression vector of rat p70\(\alpha1\), pcDNA1 FLAG p70\(\alpha1\), was constructed previously (11). A DNA fragment encoding the amino acids 443–495 of p70\(\alpha1\) was amplified by polymerase chain reaction and cloned into the pGEX-4T expression vector (Amersham Pharmacia Biotech). Expression and purification of a GST fusion protein containing amino acids 443–495 of p70\(\beta1\) (GST/p70\(\alpha1\) fusion protein) were carried out according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Cell Cultures and Transient Expression Analysis—CHO cells stably overexpressing human insulin receptors (CHO-IR) and HEK293 cells were maintained and cultured as described earlier (11) in Ham’s F-12 medium or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, respectively. Cells were transfected with plasmids containing p70\(\alpha0\) and p70\(\alpha2\) inserts using LipofectAMINE under conditions recommended by the manufacturer (Life Technologies, Inc.). Antibodies, Immunoprecipitation, Immunoblot, and p70 S6 Kinase Assay—The anti-phosphopeptide antibody against proline-directed site Ser\(^{434}\) of p70\(\alpha1\) (anti-p70\(\alpha\)CA b) was purchased from New England Biolabs. The anti-peptide antibody against the carboxyl-terminal end of p70\(\alpha1\) (anti-p70\(\alpha\)C Ab) was from Eastman Kodak. A polyclonal antibody against p70\(\alpha1\) carboxyl-terminal end (anti-p70\(\alpha\)C Ab) was raised by immunizing rabbits with the GST/p70\(\alpha\)C fusion protein as an antigen. Immunoreactive sera were affinity-purified on an Affi-Gel matrix containing the GST/p70\(\alpha\)C fusion protein. Cell lysis and immunoprecipitation were carried out as described previously (11). Immunoblotting was performed using the ECL method according to the manufacturer’s protocol (Amersham Pharmacia Biotech). p70 S6 kinase activity was determined in the immunoprecipitates by using 40 S ribosomal subunit as substrate as described earlier (11).

RESULTS AND DISCUSSION

To detect possible p70 S6 kinase isoforms, we carried out immunoblot analysis of chromatographic fractions of lysates of serum-treated HEK293 cells that had been treated with or without rapamycin. We employed for immunoblotting the anti-pS434 Ab, because Ser\(^{434}\) and its surrounding amino acid residues are highly conserved both in mammalian p70\(\alpha\)S6 kinase and in Drosophila p70\(\alpha\) (12, 13). Among fractions of serum-treated HEK293 cells separated using an anion exchange column, we detected several immunoreactive bands that were recognized by immunoblotting with the anti-pS434 Ab in a rapamycin-sensitive manner but were not detected with the anti-p70\(\alpha\)CA Ab (data not shown). These data indicated that p70 S6 kinase isoforms may exist in which the Ser\(^{434}\) site is conserved but that do not have a sequence homologous to the carboxyl-terminal end of p70\(\alpha0\). These results prompted us to search for sequences that could encode mammalian isoforms of p70 S6 kinase in the EST data bases.

Peptide sequences surrounding the proline-directed site Ser\(^{434}\) of human p70\(\alpha1\) were used to search the EST data bases. Extensive analysis of EST clones revealed two nearly identical human EST cDNA clones (AA284234 and AA410355) that were highly homologous but not identical to the p70\(\alpha\) sequences. Therefore, the full-length cDNA clone corresponding to the identified EST clones was isolated from a library of HEK293 cells using the insert of EST clone AA410355 as a probe. Among 12 isolated overlapping clones, one clone (clone 53) was found to contain an in-frame starting frame of 495 amino acids. The carboxyl-terminal sequence of the clone was identical to the sequence of EST clone AA410355. The protein encoded by this clone was named p70\(\beta\), because it encodes a protein that is homologous to but distinct from p70\(\alpha\).

It is known that the two isoforms of the p70\(\alpha0\), namely p70\(\alpha01\) and p70\(\alpha02\), are expressed from a single gene through alternative mRNA splicing and the use of alternative translational start sites (5). Inspection of the sequence of p70\(\beta\) revealed an in-frame AUG codon close to the 5′ end of the cDNA, but this may not be the sole or preferred translational start site, because the sequence preceding this AUG lacks a purine at position −3, which is present just before the second AUG (starting at amino acid 14) (data not shown). Hence, by analogy to p70\(\alpha0\), the novel cDNA may encode two proteins, designated p70\(\beta1\) and p70\(\beta2\). If this is the case, the 495-amino acid sequence of p70\(\beta1\) would differ from the 482-amino acid p70\(\beta2\) only at the amino terminus (Fig. 1A). A 13-amino acid extension of the p70\(\beta1\) contains a putative nuclear localization sequence, RGRRAAP, which is similar to that found within the 23-amino acid extension of p70\(\alpha0\). The overall sequence of p70\(\beta\) is very close to that of p70\(\alpha0\) with 70% identity and 85% similarity at the protein level and consists of the amino-terminal noncatalytic region, the
catalytic domain, the kinase extension domain, the autoinhibitory pseudosubstrate domain, and the carboxyl-terminal tail. The amino acid identity to corresponding domains of p70α is 28, 83, 80, 73, and 25%, respectively (Fig. 1B). It has been observed that p70α undergoes a multisite phosphorylation in response to stimulation by insulin or mitogens (3, 4). These multiple phosphorylation sites are also well conserved in p70β and include: (i) a set of (Ser/Thr)-Pro motifs clustering in the autoinhibitory pseudosubstrate domain (Ser1231, Ser1233, Ser1236, and Ser1241 in p70β1, which correspond to Ser1230, Ser1234, Ser1241, and Ser1245 in p70α1) (14, 15); (ii) Ser1236 and Thr1237 located in the kinase extension domain, which correspond to Ser1236 and Thr1242 in p70α1 (16, 17). The major differences in the amino acid sequence between p70β and p70α are located in the amino-terminal noncatalytic region (28% identity and 45% similarity) and in the carboxyl-terminal tail (25% identity and 38% similarity). Within the amino-terminal noncatalytic region, however, acidic residues are well conserved between amino acids 19–36 of p70β1 and the corresponding amino acids 29–46 of p70α1 (hence, this is called the “acidic region”). A unique feature of the carboxyl-terminal tail of p70β is the existence of a proline-rich region, which might be involved in interactions with SH3 domain-containing molecules.

Northern blot analysis of human tissues revealed a single
2.2-kb transcript for p70b, whereas p70a probe specifically hybridized to two transcripts of 3.4 and 7.4 kb (Fig. 2). The expression patterns of p70a and p70b transcripts are remarkably similar, showing ubiquitous expression in all tissues examined. We have made a mammalian expression construct (FLAG-tag/p70b2) that allows us to examine the expression and subsequent characterization of p70b kinase in vivo and in vitro. As shown in the left panel of Fig. 3A, FLAG-tagged constructs of p70b2 (lane 3) and p70a1 (lane 2) were expressed in HEK293 cells as a 60- and a 85-kDa protein, respectively. Both polypeptides were effectively immunoprecipitated with the anti-FLAG antibody (data not shown). To facilitate the characterization of the p70b, two types of polyclonal antibodies against the carboxyl-terminal peptide and the GST/p70bC fusion protein were generated. As shown in the right panel of Fig. 3A, immunoprecipitation with the p70bC Ab revealed specific recognition of p70b2 but not p70a1. The polyclonal antibody against the carboxyl-terminal peptide recognized p70b2 but did not exhibit cross-reactivity toward p70a1, either (data not shown).

To study the S6 kinase activity of p70b2 and p70a1 in response to various stimuli, both proteins were transiently expressed in CHO-IR cells. After various treatments of the transfected cells, p70b2 and p70a1 were immunoprecipitated with the anti-FLAG antibody, and in vitro kinase activities toward S6 protein of 40S subunit were measured. Almost equal amounts of p70a1 and p70b2 were found to be expressed in transfected cells (Fig. 3B, lower panel). Results shown in Fig. 3B (upper panel) demonstrated that p70a1 kinase activity is activated 3.5-fold by $10^{-7}$ M insulin for 10 min (lane 3), whereas the kinase activity of p70b2 is activated 2.8-fold by the same insulin treatment (lane 5). In addition, serum, 12-O-tetradecanoylphorbol-13-acetate (Fig. 3B, lanes 6 and 7), and platelet-derived growth factor (data not shown) were all able to activate p70b2 kinase activities toward S6 protein.

Because the kinase activity of p70a has been shown to be sensitive to wortmannin (16, 19) and rapamycin (20, 21) in vivo, the effects of those inhibitors on the p70b2 kinase activity were examined. p70a1 and p70b2 were expressed transiently in HEK293 cells, which were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and then treated with various concentrations of rapamycin and wortmannin. Both serum-activated p70a1 and p70b2 kinase activities were inhibited by rapamycin and wortmannin in a dose-dependent manner (Fig. 4, A and B, upper panel). However, it appears that p70b2 activity is less sensitive to rapamycin and wortmannin than p70a1. The extent of inhibition of p70a1 compared with that of p70b2 were as follows: 92% versus 46% by 20 nM rapamycin; 98% versus 62% by 200 nM rapamycin; 86% versus 62% by 100 nM wortmannin; and 97% versus 75% by 1000 nM wortmannin.

The present results suggest that p70b2 kinase may be activated in response to mitogens in vivo through a multisite phosphorylation mechanism similar to that of p70a, which is regulated by upstream signals dependent on PI 3-kinase and mTOR (mammalian target of rapamycin). However, unexpectedly, the potency of inhibition by wortmannin and rapamycin for p70b2 kinase was significantly lower than that for p70a kinase. This suggests that some mechanisms other than PI 3-kinase- and mTOR-dependent inputs may regulate p70b2 activity. For example, there may be unique phosphorylation sites on p70b2, which are regulated by unknown mechanisms. Alternatively, other mechanisms independent of phosphorylation, such as those based on protein-protein interactions, may exist. One possible mechanism is the regulation of p70b2 kinase by the protein-protein interaction via its proline-rich region in the carboxy-terminal tail, which may interact with SH3 domain(s). Thus, we constructed and expressed a p70b mutant that lacks the carboxy-terminal tail (amino acids 442–495 of p70b) containing the proline-rich region. However, the potency of inhibition by wortmannin and rapamycin for the mutant p70b2 kinase activity was almost equal to that for the wild-type p70b2 kinase activity (data not shown). These results indicated that the proline-rich region is not sufficient for the difference in the drug sensitivities between p70a and p70b. Despite these results, the binding of proteins containing SH3 domain(s) to the proline-rich region of p70b2 may play a role in transmitting signals in the p70b2-mediated signaling pathway. Experiments to determine whether SH3 domain-containing proteins bind to p70b2 are currently in progress.

In conclusion, we have identified a novel isoform of p70 S6 kinase, named p70b. Further studies are necessary to clarify the regulation and the role of p70b2 in the control of protein synthesis and the cell cycle.

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