Cloning and Expression of Two Human p70 S6 Kinase Polypeptides Differing Only at Their Amino Termini

J. RUSSELL GROVE,[1] PAPIA BANERJEE,[1] ASHOK BALASUBRAMANYAM,[1] PAUL J. COFFER,[2]
DANIEL J. PRICE,[1] JOSEPH AVRUCH,[1]* AND JAMES R. WOODGETT[2]

*Medical Services and Diabetes Unit, Massachusetts General Hospital, and Department of
Medicine, Harvard Medical School, Boston, Massachusetts 02114,[1] and Ludwig
Institute for Cancer Research, London WIP 8BT, England[2]*

Two classes of human cDNA encoding the insulin/mitogen-activated p70 S6 kinase have been isolated; the two classes differ only in the 5' region, such that the longer polypeptide (p70 S6 kinase αI; calculated M_r 58,946) consists of 525 amino acids, of which the last 502 residues are identical in sequence to the entire polypeptide encoded by the second cDNA (p70 S6 kinase αII; calculated M_r 56,153). Both p70 S6 kinase polypeptides predicted by these cDNAs are present in p70 S6 kinase purified from rat liver, and each is thus expressed in vivo. Moreover, both polypeptides are expressed from a single mRNA transcribed from the (longer) p70 S6 kinase αI cDNA through the utilization of different translational start sites. Although the two p70 S6 kinase polypeptides differ by only 23 amino acid residues, the slightly longer αI polypeptide exhibits anomalously slow mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), migrating at an apparent M_r of 90,000 probably because of the presence of six consecutive Arg residues immediately following the initiator methionine. Transient expression of p70 αI and αII S6 kinase cDNA in COS cells results in a 2.5- to 4-fold increase in overall S6 kinase activity. Upon immunoblotting, the recombinant p70 polypeptides appear as a closely spaced ladder of four to five bands between 65 and 70 kDa (αII) and 85 and 90 kDa (αI). Transfection with the αII cDNA yields only the smaller set of bands, while transfection with the αI cDNA generates both sets of bands. Mutation of Met-24 in the αI cDNA to Leu or Thr suppresses synthesis of the αII polypeptides. Only the p70 αI and αII polypeptides of slowest mobility on SDS-PAGE comigrate with the 70- and 90-kDa proteins observed in purified rat liver S6 kinase. Moreover, it is the recombinant p70 polypeptides of slowest mobility that coelute with S6 kinase activity on anion-exchange chromatography. The slower mobility and higher enzymatic activity of these p70 proteins is due to Ser/Thr phosphorylation, inasmuch as treatment with phosphatase 2A inactivates kinase activity and increases the mobility of the bands on SDS-PAGE in an okadaic acid-sensitive manner. Thus, the recombinant p70 S6 kinase undergoes multiple phosphorylation and partial activation in COS cells. Acquisition of S6 protein kinase catalytic function, however, is apparently restricted to the most extensively phosphorylated recombinant polypeptides.

Polypeptide mitogens, upon binding their cell surface receptors, elicit a sequence of intracellular effects culminating in cell division. An early effect is stimulation of protein synthesis, which is an obligate precursor for cell replication (30). Several proteins involved in protein translation undergo changes in their phosphorylation state upon stimulation (16). One of these, ribosomal protein S6, is rapidly phosphorylated on multiple serine residues in response to insulin, epidermal growth factor, platelet-derived growth factor, serum, phorbol esters, and several other artificial perturbants such as cycloheximide (14). Although the specific role of ribosomal S6 phosphorylation in mitogen-enhanced protein synthesis is still unknown, S6 phosphorylation has been used as a model to address the intracellular signal transduction mechanisms utilized by these mitogens.

Agonist-induced increases in S6 phosphorylation are mediated by rapid transient activation of protein-serine kinases. Although many protein kinases are capable of phosphorylating S6 in vitro, two types of mitogen-activated S6 protein kinases have been characterized at a molecular level; these kinases are distinguished by a very high rate of S6 phosphorylation combined with a relative specificity for S6 in comparison with other known substrates and the ability to

phosphorylate in vitro all of the multiple sites on S6 phosphorylated in situ in response to mitogen. The first of these S6 kinases to be purified and cloned were from *Xenopus laevis*; extracts prepared from *Xenopus* eggs exhibit two peaks of S6 kinase activity on elution from DEAE, S6 kinase I and II (10). Tryptic peptides corresponding to S6 kinase II were used to isolate a cDNA called S6 kinase α (18), which may correspond to S6 kinase I inasmuch as certain monoclonal antibodies to recombinant S6 kinase α are reactive with S6 kinase I but not S6 kinase II (9). S6 kinase α exhibits a feature unique among Ser/Thr kinases, namely, the presence of two protein kinase catalytic domains. The more amino-terminal catalytic domain is most closely related to the kinase C family, whereas the second carboxy-terminal catalytic domain is most closely related to the catalytic (γ) subunit of phosphorylase *b* kinase. Protein kinases of homologous structure have recently been cloned from avian, mouse (1), and rat (4) sources. Polyclonal antisera to recombinant S6 kinase α immunoprecipitate *Xenopus* S6 kinase I and II (12), mitogen-stimulated S6 kinase activity, and 85- to 90-kDa polypeptides from chicken embryo fibroblasts and cultured mammalian cells (6, 33). This family will be referred to henceforth as the p85 S6 kinases.

Despite fairly ubiquitous expression (1), the p85 kinases are not the quantitatively dominant mitogen-stimulated kinases toward 40S S6 in mammalian cells. Purification of

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* Corresponding author.

activated S6 kinases from cultured mammalian cells (17, 27) and from rat liver during regeneration (25) or after cyclohex-imide injection (28) has consistently yielded 65- to 70-kDa polypeptides that are not reactive with the antisera to recombinant p85 S6 kinase α. The p70 and p85 S6 kinases do share several important properties, including substrate spec-ificity, which overlaps substantially but is not identical (11, 24, 28). Moreover, both the p70 and p85 kinases are acti-vated by the phosphorylation of multiple Ser/Thr residues on the enzyme polypeptide (3, 12, 27), although the upstream protein kinases that mediate this activating phosphorylation may be different, at least in part. An insulin-stimulated 42-kDa MAP-2 kinase phosphorylates and partially (30%) reactivates the Xenopus S6 kinase II (32) but does not modify the rat liver p70 S6 kinase (27).

The recent isolation of rat cDNAs corresponding to the p70 S6 kinases (4, 21) provides a basis for understanding the similarities and differences in the substrate specificities and regulatory mechanisms of the two S6 kinase families: the rat p70 S6 kinases exhibit a single, centrally located catalytic domain that is most homologous to the more amino-terminal kinase C-like catalytic domain of the p85^{rsk} kinases (57% identity). By contrast, the noncatalytic sequences of p70 S6 kinase are completely divergent from those of the p85^{rsk} kinase. Two rat clones encoding the p70 S6 kinase were independently isolated and are identical except in the 5' region, which results in distinct predicted amino termini. One cDNA predicts a polypeptide of 502 amino acid residues (21); the second cDNA encodes 525 amino acid residues, of which the last 502 are identical in sequence to the other p70 S6 kinase polypeptide (4). To evaluate the physiologic sig-nificance of this difference, we have isolated human p70 S6 kinase cDNAs. Here we show that two forms of human p70 cDNA analogous in structure to the two types of rat cDNAs are present in human libraries. Recombinant polypeptides derived from both forms have been expressed by in vitro translation and transient transfection of COS cells. Such transfectants exhibit elevated S6 kinase activity as a result of multiple phosphorylation of the recombinant polypeptides and thus provide a system in which we are able to investigate the molecular mechanism of activation of the p70 S6 kinase.

MATERIALS AND METHODS

Isolation of S6 kinase cDNAs. A human AG1518 diploid fibroblast cDNA library in λgt10 was kindly provided by L. Claesson-Welsh (7). A human HepG2 cell cDNA library in λZAP was from Stratagene (La Jolla, Calif.). Libraries (10^6 plaques) were screened with a randomly primed polymerase chain reaction fragment (5 × 10^8 cpm/μg) derived from the rat p70 S6 kinase corresponding to nucleotides 651 to 1056 (4) in 5× SSPE–1% sodium dodecyl sulfate (SDS)–5× Den-hardt's solution–30% formamide–100 μg of sonicated DNA per ml at 42°C for 18 h. Filters were washed with 0.5× SSPE–0.1% SDS at 55°C twice for 30 min each time and subjected to autoradiography with intensifying screens for 48 h at −70°C. One λgt10 positive plaque was purified; the insert was excised by digestion with EcoRI and subcloned into pBluescript SK (Stratagene). Ten λZAP plaques were purified, and the cDNA inserts were isolated as plasmids by superinfection with R408 helper phage. All cDNAs were sequenced at their 5' and 3' ends with Taq polymerase and an Applied Biosystems 373 DNA sequenator. Two appar-ently full-length λZAP clones corresponding to p70 S6 kinase αII and the λgt10 clone corresponding to p70 S6 kinase αI were subsequently entirely sequenced by using a combina-tion of oligonucleotides, restriction fragments, and exonu-clease III-derived deletions, using fluorescence-labeled primers or dideoxynucleotides (DyeTerminators) supplied by Applied Biosystems (Foster City, Calif.). The p70 S6 kinase αI cDNA contained an unspliced intron and an apparent reverse transcription error that inserted an extra nucleotide. Although having a novel 5' region, the nucleotide sequence of this cDNA was identical to that of the p70 S6 kinase αII cDNAs downstream of a unique BstUI site (nucleotide [nt] 141) with the exception of the intron and extra base. To generate an expressible clone, the sequence of the p70 S6 kinase αI cDNA upstream of the BstUI site was fused to the sequence of one of the HepG2 cDNAs down-stream of this site.

The ATG codon corresponding to residue 24 (the initiating codon of p70 αII protein) was mutated to either CTG (leucine, referred to as M24L) or ACG (threonine, referred to as M24T) by site-directed mutagenesis using the pSelect vector system (Promega). All mutants were verified by DNA sequencing.

In vitro transcription and translation. Plasmids were lin-earized at the 3' ends of the cDNA insert, RNA was synthesized by using either T7 or T3 RNA polymerase, and capping was carried out for some samples (see legend to Fig. 2) according to the Promega protocol. Following DNase treatment, RNA was ethanol precipitated and 1 μg was used to program an RNA-dependent reticulocyte lysate in the presence of [^{35}S]methionine for 60 min at 30°C as recom-mended by the supplier (Promega). The samples were dena-tured by boiling in the presence of 1% SDS and resolved on a 12.5% SDS-polyacrylamide gel. Newly synthesized pro-teins were detected by fluorography. Samples were matched for equivalent representation of the 84- and 62-kDa bands. Amersham rainbow markers were used in Fig. 2b; the molecular weights indicated in the figure are those calculated from comparison with unmodified markers.

Generation of p70 S6 kinase antipeptide antiserum. A polyclonal antiserum to a synthetic peptide corresponding to p70 residues 337 to 352 (4), with the addition of a carboxy-terminal cysteine, was generated in rabbits. The peptide was cross-linked to rabbit serum albumin by the amino terminus, using glutaraldehyde, and via the carboxy-terminal cysteine SH, using maleimidobenzoyl-N-hydroxysuccinimide (23). The amino-terminal-versus-cysteine-coupled peptides were mixed in a 2:1 molar ratio, and 930 μg of conjugate of coupled peptide was mixed in complete Freund's adjuvant and inoculated intradermally and into the popliteal lymph nodes of rabbits. Boosts were given intradermally at 1 month and at 3-week intervals thereafter. Serum was screened by immunoblot of purified rat liver p70 S6 kinase, carried out after SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretic transfer to a polyvinyl difluoride (PVDF) membrane (Millipore). Peptide linked to Affi-Gel 10 was found to adsorb all specific immunoblotting activity toward purified rat liver p70 S6 kinase and was used for antibody purification (23).

Transfection of COS-7 cells. The human cDNA inserts were excised as EcoRI fragments and cloned into the EcoRI site of the mammalian expression vector pMT2. The rat p70 cDNA was cloned into the BstXI site of pCDM8, using synthetic oligonucleotide adapters (2). DNA (20 μg) was introduced into COS-7 cells by calcium phosphate- or DEAE-dextran-mediated transfection (31). Cells were har-vested after 48 to 60 h and extracted in homogenization buffer (27), which contains 10 mM KP$_i$ (pH 6.5), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl$_2$, 2 mM dithiothreitol, 1

mM vanadate, 50 mM β-glycerophosphate, 10 kallikrein inhibitory units of Trasylol per ml, 0.2 mM diisopropylfluorophosphate, and 0.2% Triton X-100. A portion of the extract was subjected to Mono Q anion-exchange chromatography as described previously (28), and aliquots of each fraction as well as aliquots of the original extract were taken for immunoblot and assay of S6 kinase activity. The S6 kinase assay was carried out as previously described (28). For immunoblotting, proteins from SDS-polyacrylamide gels were transferred to PVDF membranes and probed with the affinity-purified antipeptide antibody described above. Following blocking with 5% dried milk in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (BLOTTO), the membrane was incubated for 60 min with anti-S6 kinase antibody at a 1:500- or 1:1,000-fold dilution in BLOTTO or BLOTTO-PBS (1:1). After removal of unbound antibody, the blot was incubated for 30 to 60 min with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (1:5,000 to 1:10,000). Antigens were detected by ECL chemiluminescence (Amersham). To demonstrate the specificity of the antibody, a peptide competition was performed (see Fig. 3g): the antipeptide antiserum was incubated in BLOTTO with peptide at 10 times the indicated concentrations for 30 min; this preincubated antiserum was then diluted 1:10 (to the indicated concentration) with BLOTTO and used to probe the blots. All transfection-dependent signals detected with this antipeptide antibody are competed for by peptide in a concentration-dependent fashion at peptide concentrations as low as 5 μM (Fig. 3g). Nevertheless, variable nonspecific binding of antibody to non-p70 polypeptides, e.g., the molecular weight markers, is occasionally observed, and conditions that reliably prevent such binding have not been identified. Thus, in the leftmost set of lanes in Fig. 3g there is no binding to the molecular weight markers, but in the second and third sets of lanes there is significant binding. However, this obviously nonspecific binding is not displaced by peptide under conditions in which a strong transfection-dependent signal is completely displaced.

RESULTS

Cloning of two p70 S6 protein kinase cDNAs. The two rat p70 S6 kinase cDNAs recently reported differed only in their 5' regions, such that one of the deduced polypeptides extends amino terminal of the other by 23 amino acids; the two polypeptides are otherwise identical in sequence (4, 21). To determine whether one of these cDNAs was an artifact of cloning or whether both are physiologically relevant, we probed human fibroblast and HepG2 cell cDNA libraries for clones of this protein kinase. Two distinct classes of cDNA were found, differing only at their 5' ends in a manner corresponding to the two rat cDNAs previously reported and thus confirming the existence of two distinct types of the p70 S6 kinase mRNA (Fig. 1). As with the two rat cDNAs reported earlier, the two human cDNAs encode polypeptides of 525 and 502 amino acids. The first methionine of the cDNA with the shorter open reading frame corresponds to the second methionine of the longer sequence, and the amino acid sequences encoded by the two human cDNAs are identical carboxy terminal to this methionine. Inasmuch as this extensive sequence identity strongly suggests origination from a single gene, the p70 S6 kinase cDNAs will be designated αI (525 amino acids) and αII, by analogy to protein kinase C βI and βII (26). Inspection of the cDNA sequence 5' to the initiator ATG of αII shows that the two

human cDNAs remain closely related in sequence for 36 nt and then diverge completely (Fig. 1b). Just 8 nt upstream of this divergence, a stop codon occurs in the αII DNA sequence, inframe with the downstream ATG. By contrast, the αI open reading frame continues 5' beyond the divergence, to encode 23 amino acid residues upstream of the initiator methionine of the αII sequence.

Comparison of the rat and human p70 cDNA sequences shows that the human αI cDNA encodes 27 nt of 5' untranslated sequence that, when optimally aligned, extends 6 nt beyond the available rat αI cDNA sequence. The overlapping 21 nt in the 5' untranslated sequences differ at only one base. This extensive homology continues into the coding sequences, which differ at only two amino acids (99.6% identity). The molecular basis for the structural variation at the 5' end of the αI and αII cDNAs will require isolation and sequence analysis of the gene for the p70 S6 kinase.

In vitro translation. Another feature common to the human and rat αI cDNAs is the presence of a very weak translational initiation sequence surrounding the most 5' ATG, with a C at −3 and an A at +4 (20). By contrast, the sequence surrounding the second methionine in rat and human αI, which is the initiator methionine for the rat and human αII cDNAs, exhibits (in all four cDNAs) the much stronger motif of a G at −3 and +4. It was therefore of interest to determine whether p70 S6 kinase αI cDNA directed the synthesis of an elongated protein with respect to the p70 S6 kinase αII cDNA. RNA synthesized in vitro was translated in a reticulocyte lysate, and the proteins were detected by fluorography following SDS-PAGE. The αI and αII open reading frames predict proteins of 58,946 and 56,153 Da, respectively. Translation of human p70 S6 kinase αI-derived RNA resulted in the synthesis of numerous [^{35}S]methionine-labeled polypeptides, with the two largest corresponding to relative molecular masses of ~84 and ~62 kDa (Fig. 2a). Many smaller polypeptides were also formed, presumably as a result of internal initiation or premature termination (see below). The human p70 S6 kinase αII cDNA generated a virtually identical pattern except that none of the 84-kDa polypeptide was formed (Fig. 2a). Translation of the rat form of the p70 S6 kinase αI cDNA gave a pattern essentially indistinguishable from that of human αI mRNA, with the synthesis of proteins of 84 and 62 kDa (Fig. 2a). The virtual identity between the translation products of the αI and αII mRNAs, except for the presence of the 84-kDa polypeptide with both the human and rat αI mRNAs, implies that this 84-kDa polypeptide is initiated at the first AUG in the αI mRNA, a translational start site not present in the αII mRNA. To investigate this point further, we mutated Met-24 of the human αI cDNA to Leu or Thr (yielding cDNAs called M24L and M24T, respectively) and generated synthetic RNA from the resulting constructs. Following their synthesis, these RNAs were tested by in vitro translation with or without the addition of a 5' cap structure (see Materials and Methods). The wild-type human αI and the M24L and M24T mutants all give virtually identical patterns of translation products except that the 62-kDa band, which represents initiation at Met-24, is absent from the mutants (compare lane 3 with lanes 5 and 7 in Fig. 2b).

The experiment depicted in Fig. 2b also addresses the question of the origin of the smaller bands seen as translation products of all three cDNAs used in Fig. 2a. To determine whether these smaller products resulted from premature termination of translation or aberrant initiation at internal methionines, we translated RNAs corresponding to αI and αII cDNAs either with or without the addition of a 5' cap,

a

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                                    M  R  R  R  R  R  R  D  G  F  Y    11
  1-GCACGAGGCTGCGGCGGGTCCGGGCCCATGAGGCGACGAAGGAGGCGGGACGGCTTTTAC
      P  A  P  D  F  R  D  R  E  A  E  D  M  A  G  V  F  D  I  D    31
 61-CCAGCCCCGGACTTCCGAGACAGGGAAGCTGAGGACATGGCAGGAGTGTTTGACATAGAC
      L  D  Q  P  E  D  A  G  S  E  D  E  L  E  E  G  G  Q  L  N    51
121-CTGGACCAGCCAGAGGACGCGGGCTCTGAGGATGAGCTGGAGGAGGGGGGTCAGTTAAAT
      E  S  M  D  H  G  G  V  G  P  Y  E  L  G  M  E  H  C  E  K    71
181-GAAAGCATGGACCATGGGGGAGTTGGACCATATGAACTTGGCATGGAACATTGTGAGAAA
      F  E  I  S  E  T  S  V  N  R  G  P  E  K  I  R  P  E  C  F    91
241-TTTGAAATCTCAGAAACTAGTGTGAACAGAGGGCCAGAAAAAATCAGACCAGAATGTTTT
      E  L  L  R  V  L  G  K  G  G  Y  G  K  V  F  Q  V  R  K  V   111
301-GAGCTACTTCGGGTACTTGGTAAAGGGGGCTATGGAAAGGTTTTTCAAGTACGAAAAGTA
      T  G  A  N  T  G  K  I  F  A  M  K  V  L  K  K  A  M  I  V   131
361-ACAGGAGCAAATACTGGGAAAATATTTGCCATGAAGGTGCTTAAAAAGGCAATGATAGTA
      R  N  A  K  D  T  A  H  T  K  A  E  R  N  I  L  E  E  V  K   151
421-AGAAATGCTAAAGATACAGCTCATACAAAAGCAGAACGGAATATTCTGGAGGAAGTAAAG
      H  P  F  I  V  D  L  I  Y  A  F  Q  T  G  G  K  L  Y  L  I   171
481-CATCCCTTCATCGTGGATTTAATTTATGCCTTTCAGACTGGTGGAAAACTCTACCTCATC
      L  E  Y  L  S  G  G  E  L  F  M  Q  L  E  R  E  G  I  F  M   191
541-CTTGAGTATCTCAGTGGAGGAGAACTATTTATGCAGTTAGAAAGAGAGGGAATATTTATG
      E  D  T  A  C  F  Y  L  A  E  I  S  M  A  L  G  H  L  H  Q   211
601-GAAGACACTGCCTGCTTTTACTTGGCAGAAATCTCCATGGCTTTGGGGCATTTACATCAA
      K  G  I  I  Y  R  D  L  K  P  E  N  I  M  L  N  H  Q  G  H   231
661-AAGGGGATCATCTACAGAGACCTGAAGCCGGAGAATATCATGCTTAATCACCAAGGTCAT
      V  K  L  T  D  F  G  L  C  K  E  S  I  H  D  G  T  V  T  H   251
721-GTGAAACTAACAGACTTTGGACTATGCAAAGAATCTATTCATGATGGAACAGTCACACAC
      T  F  C  G  T  I  E  Y  M  A  P  E  I  L  M  R  S  G  H  N   271
781-ACATTTTGTGGAACAATAGAATACATGGCCCCTGAAATCTTGATGAGAAGTGGCCACAAT
      R  A  V  D  W  W  S  L  G  A  L  M  Y  D  M  L  T  G  A  P   291
841-CGTGCTGTGGATTGGTGGAGTTTGGGAGCATTAATGTATGACATGCTGACTGGAGCACCC
      P  F  T  G  E  N  R  K  K  T  I  D  K  I  L  K  C  K  L  N   311
901-CCATTCACTGGGGAGAATAGAAAGAAAACAATTGACAAAATCCTCAAATGTAAACTCAAT
      L  P  P  Y  L  T  Q  E  A  R  D  L  L  K  K  L  L  K  R  N   331
961-TTGCCTCCCTACCTCACACAAGAAGCCAGAGATCTGCTTAAAAAGCTGCTGAAAAGAAAT
      A  A  S  R  L  G  A  G  P  G  D  A  G  E  V  Q  A  H  P  F   351
1021-GCTGCTTCTCGTCTGGGAGCTGGTCCTGGGGACGCTGGAGAAGTTCAAGCTCATCCATTC
      F  R  H  I  N  W  E  E  L  L  A  R  K  V  E  P  P  F  K  P   371
1081-TTTAGACACATTAACTGGGAAGAACTTCTGGCTCGAAAGGTGGAGCCCCCCTTTAAACCT
      L  L  Q  S  E  E  D  V  S  Q  F  D  S  K  F  T  R  Q  T  P   391
1141-CTGTTGCAATCTGAAGAGGATGTAAGTCAGTTTGATTCCAAGTTTACACGTCAGACACCT
      V  D  S  P  D  D  S  T  L  S  E  S  A  N  Q  V  F  L  G  F   411
1201-GTCGACAGCCCAGATGACTCAACTCTCAGTGAAAGTGCCAATCAGGTCTTTCTGGGTTTT
      T  Y  V  A  P  S  V  L  E  S  V  K  E  K  F  S  F  E  P  K   431
1261-ACATATGTGGCTCCATCTGTACTTGAAAGTGTGAAAGAAAAGTTTTCCTTTGAACCAAAA
      I  R  S  P  R  R  F  I  G  S  P  R  T  P  V  S  P  V  K  F   451
1321-ATCCGATCACCTCGAAGATTTATTGGCAGCCCACGAACACCTGTCAGCCCAGTCAAATTT
      S  P  G  D  F  W  G  R  G  A  S  A  S  T  A  N  P  Q  T  P   471
1381-TCTCCTGGGGATTTCTGGGGAAGAGGTGCTTCGGCCAGCACAGCAAATCCTCAGACACCT
      V  E  Y  P  M  E  T  S  G  I  E  Q  M  D  V  T  M  S  G  E   491
1441-GTGGAATACCCAATGGAAACAAGTGGCATAGAGCAGATGGATGTGACAATGAGTGGGGAA
      A  S  A  P  L  P  I  R  Q  P  N  S  G  P  Y  K  K  Q  A  F   511
1501-GCATCGGCACCACTTCCAATACGACAGCCGAACTCTGGGCCATACAAAAAACAAGCTTTT
      P  M  I  S  K  R  P  E  H  L  R  M  N  L                    525
1561-CCCATGATCTCCAAACGGCCAGAGCACCTGCGTATGAATCTATGACAGAGCAATGCTTTT
1621-AATGAATTTAAGGCAAAAAGGTGGAGAGGGAGATGTGTGAGCATCCTGCAAGGTGAAACA
1681-AGACTCAAAATGACAGTTTCAGAGAGTCAATGTCATTACATAGAACACTTCGGACACAGG
1741-AAAAATAAACGTGGATTTTAAAAAATCAATCAATGGTGCAAAAAAAAACTTAAAGCAAAA
1801-TAGTATTGCTGAACTCTTAGGCACATCAATTAATTGATTCCTCGCGACATCTTTCTCAAC
1861-CTTATCAAGGATTTTCATGTTGATGACTCGAAACTGACAGTATTAAGGGTAGGATGTTGC
1921-TCTGAATCACTGTGAGTCTGATGTGTGAAGAAGGGTATCCTTTCATTAGGCAAGTACAAA
1981-TTGCCTATAATACTTGCAACTAAGGACAAATTAGCATGCAAGCTTGGTCAAACTTTTCCC
2041-AGGCAAAATGGGAAGGCAAAGACAAAAGAAACTTACCAATTGATGTTTTACGTGCAAACA
2101-ACCTGAATCTTTTTTTTTATATAAATATATATTTTTCAAATAGATTTTTGATTCAGCTCAT
2161-TATGAAAAACATCCCAAACTTTAAAAATGCGAAATTATTGGTTGGTGTGAAGAAAGCCAGA
2221-CAACTTCTGTTTCTTCTCTTGGTGAAATAATAAAATGCAAATGAATCATTGTTAACACAG
2281-CTGTGGCTCGTTTGAGGGATTGGGGTGGACCTGGGGTTTATTTTTCAGTAACCCAGCTGCG
2341-GAGCCT
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FIG. 1. Nucleotide and protein sequence of the two different human ribosomal S6 kinase cDNA clones. (a) Nucleotide sequence of the cDNA for the αI form of the 70-kDa S6 kinase. Nucleotides are numbered on the left; amino acids (single-letter code) are numbered on the right, starting at the initiating methionine as +1. (b) Direct comparison of 5' nucleotide and amino acid sequences of the long (αI) and short (αII) forms of human and rat p70 S6 kinase cDNAs.

b

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Rat αII          AGT ACA ACA GCA GCA GTG GAG GTG GTG GAG GGG GCG GTG GAG GCA ACT ATG

Human αII     AC CAT GAT ACA CAG CAG AAG GAG GGG GGA AAA ACA CGT AGA AAT TGC CCT TCG

Human αI         GCA CGA GGC TGC GGC GGG TCC GGG CCC ATG AGG CGA CGA AGG AGG CGG GAC
                                                          M   R   R   R   R   R   R   D

Rat αI               GTC TGC GGC GGG TCC GGG CCC ATG AGG CGA CGA CGG AGG CGG GAC
                                                      M   R   R   R   R   R   R   D


Rat αII        GCC AAG ATC AGT CCT CTA TGA GTG GCG GCG GCG GTG GTG GTG GTT ATG GCA GGA
                                                                               M   A   G

Human αII      GCT TAA AAT CCA GTC CCG GAC TTC CGA GAC AGG GAA GCT GAG GAC ATG GCA GGA
                                                                               M   A   G

Human αI       GGC TTT TAC CCA GCC CCG GAC TTC CGA GAC AGG GAA GCT GAG GAC ATG GCA GGA
               G   F   Y   P   A   P   D   F   R   D   R   E   A   E   D   M   A   G

Rat αI         GGC TTT TAC CCA GCG CCT GAC TTC CGA CAC AGG GAA GCT GAG GAC ATG GCA GGA
               G   F   Y   P   A   P   D   F   R   H   R   E   A   E   D   M   A   G
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FIG. 1—*Continued.*

the presence of which increases the efficiency of translation initiation (22). Although the full-length products at 84 and 62 kDa were more abundant when the RNA was capped, the same pattern of translation products appeared, suggesting that the smaller products result primarily from internal initiation at Met residues present on both αI and αII RNAs.

The apparently large molecular size of the full-length αI polypeptide, over 20 kDa more with the addition of only 23 amino acids, is an unexpected result and appears to reflect anomalous electrophoretic retardation of this polypeptide on SDS-PAGE. This is likely due to the unusually long polybasic stretch at the amino terminus. Thus, in vitro, the p70 S6 kinase αI mRNAs can direct synthesis of protein from the first ATG, although these results suggest that initiation at the second ATG in the αI mRNAs occurs with at least similar efficiency.

Transient expression. The initiation specificity of reticulo-
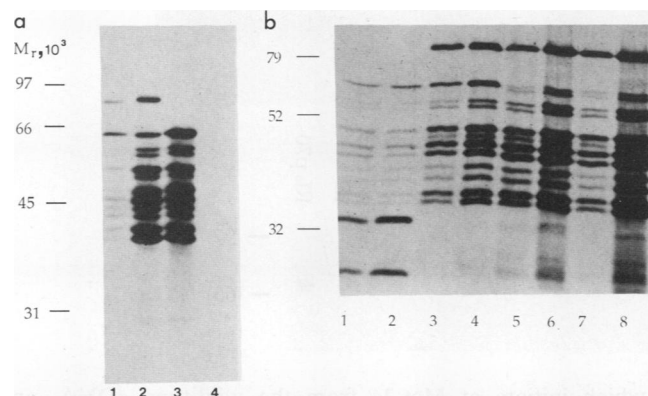
FIG. 2. In vitro translation of S6 kinase cDNAs. (a) RNA synthesized from rat p70 S6 kinase αI (lane 1), human p70 αI (lane 2), human p70 αII (lane 3), or water (lane 4) was used to program a message-dependent reticulocyte lysate in the presence of [^{35}S]methionine. Proteins were resolved by SDS-PAGE and visualized by fluorography. Molecular weights are indicated. (b) Translation was performed as for panel a, using RNA that was either uncapped (lanes 2, 4, 6, and 8) or capped (lanes 1, 3, 5, and 7) and was derived from human p70 S6 kinase αII (lanes 1 and 2), human p70 S6 kinase αI (lanes 3 and 4), human αI M24L (lanes 5 and 6), and human αI M24T (lanes 7 and 8).

cyte lysates is often more relaxed than occurs in vivo. To determine the nature of the polypeptides synthesized in intact cells from p70 αI and αII mRNAs, the two human cDNAs were cloned into the expression plasmid pMT2 and the rat p70 S6 kinase αI cDNA was cloned into pCDM8 for transfection of COS-7 cells. Extracts were prepared 48 to 60 h following introduction of DNA; a portion of the extracts was separated by Mono Q anion-exchange chromatography, and the initial extracts and column fractions were examined by immunoblotting using affinity-purified antibody to a synthetic peptide based upon amino acids 337 to 352 of the p70 S6 kinase (Fig. 3). Immunoblots of unfractionated extracts prepared after transfection with vector DNA exhibited a number of immunoreactive polypeptides as a result of nonspecific cross-reactivity; however, no dominant signal at 60 to 70 kDa was detected (Fig. 3a). In contrast, extracts of cells transfected with p70 S6 kinase αII cDNA show a prominent new signal consisting of a ladder of closely spaced polypeptide bands between 65 and 70 kDa (Fig. 3c). Transfection of both rat (Fig. 3b and e) and human (Fig. 3h to j) p70 S6 kinase αI cDNAs resulted in the synthesis of two sets of immunoreactive polypeptides, one corresponding to the 65- to 70-kDa array observed with the αII cDNA and the other a cluster of immunoreactive polypeptides migrating at 85 to 90 kDa. These two polypeptide ladders at 65 to 70 and 85 to 90 kDa essentially coelute over three fractions on Mono Q chromatography (Fig. 3b and i). Moreover, the successive Mono Q fractions present a steplike appearance because the bands within each ladder that have lower mobility on SDS-PAGE elute progressively later upon anion-exchange chromatography; consequently, each successive Mono Q fraction includes immunoreactive polypeptides of progressively slower mobility on SDS-PAGE (see especially Fig. 3a to f). Transfection with the human p70 S6 kinase αI cDNA also led to the production of two sets of S6 kinase peptides, although for this construct the αI forms were much more abundant that the αII forms (Fig. 3i).

Examination of a longer autoradiographic exposure of another immunoblot of the same extracts shown in Fig. 3a to c (see Fig. 3d to f) shows that an array of polypeptide bands at 65 to 70 kDa is visualized from the vector-only transfections, predominantly in fraction 12, corresponding to the peak fraction of endogenous S6 kinase activity (see Fig. 4a) as well as a peak fraction containing the recombinant p70
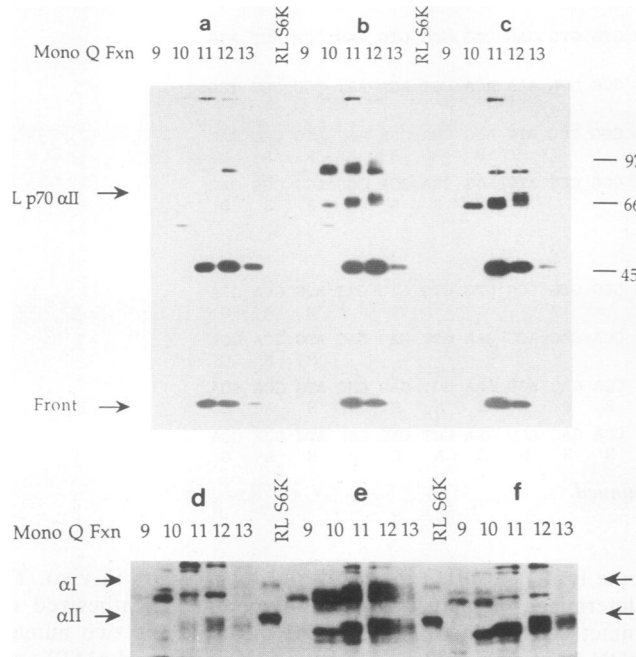
FIG. 3. Expression of S6 kinase cDNAs in COS-7 cells. (a to f) Cells were transfected with pCDM8 (a and d), the rat αI in pCDM8 (b and e), or the human p70 αII in pMT2 (c and f). Extracts were prepared 60 h later and applied to Mono Q anion-exchange chromatography (28); elution was carried out with a linear gradient of NaCl to 400 mM; 1-ml fractions were collected. One aliquot of each fraction was taken for assay of S6 kinase (see Fig. 4), and another aliquot was subjected to SDS-PAGE, transferred to PVDF membranes, and probed with affinity-purified antipeptide antibody (see Methods). Recombinant polypeptides were visualized only in the fractions surrounding the major peak of S6 kinase activity (see Fig. 4). The immunoblot of these fractions is displayed. Panels a to c show the entire gel so as to permit comparison of the p70 cDNA-dependent immunoreactive bands with the endogenous, nonspecific bands; panels d to f show a close-up and higher exposure of another blot of these same fractions, which permits visualization of the endogenous COS p70 αII polypeptides, as well as both the p90 (αI) and p70 (αII) polypeptides in the purified rat liver S6 kinase (S6K). For panel g, antibody was preincubated with peptide prior to incubation with the blot (see Materials and Methods). Lanes: 1, crude extract from vector-transfected COS cells; lane 2, crude extract from human αI-transfected cells; lane 3, purified rat liver S6 kinase; lanes 4 and 5, Mono Q fractions 14 and 15 from the separation of extract from human αI-transfected cells used for Fig. 3i; lane 6, molecular weight markers. For panels h to j, cells were transfected with pMT2 vector (h) human p70 S6 kinase αI (i), or the M24L mutant of human αI (j), harvested, fractionated on Mono Q (except that fractions were 0.75 ml), and blotted as for panel a. In all panels, molecular weights are indicated in thousands.
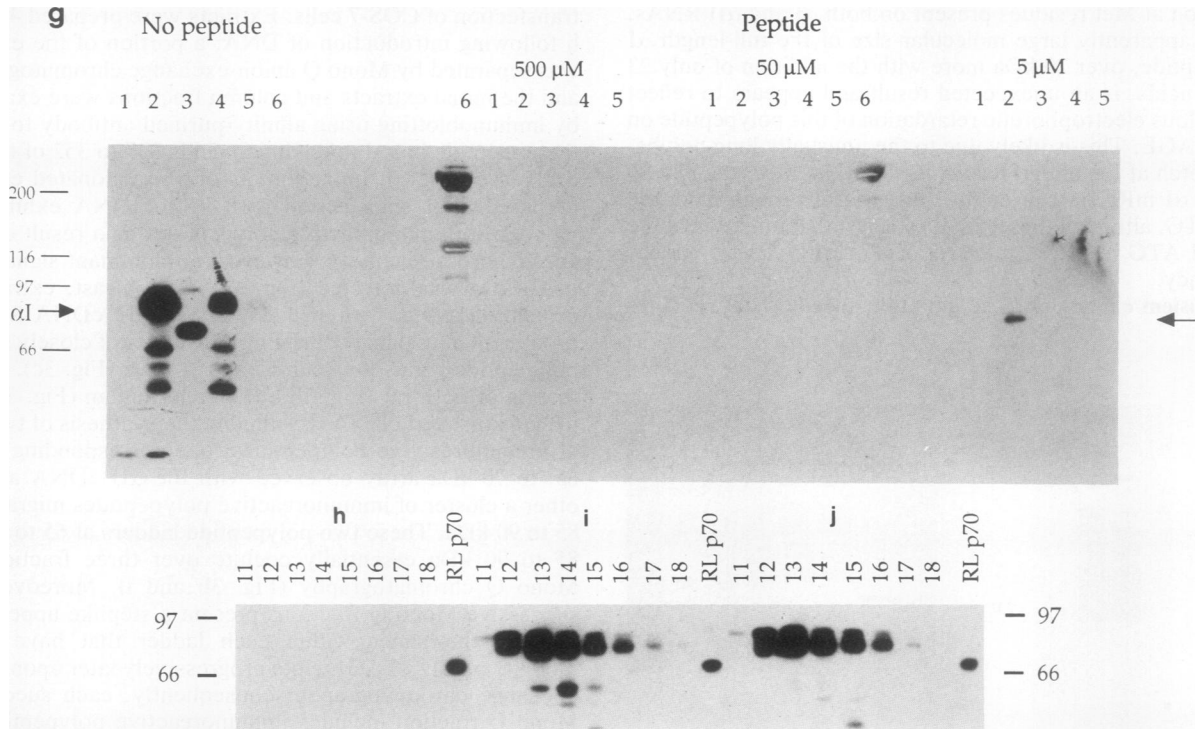
polypeptides. We infer this set of endogenous COS cell polypeptides to be the COS p70 S6 kinase αII polypeptides; endogenous COS polypeptides corresponding to the p70 S6 kinase αI polypeptides (i.e., at ~85 to 90 kDa) are not detected.

To confirm the origin of the αI and αII protein products, we transfected the human Met-24 mutants, M24L and M24T, into COS cells. As was the case for the in vitro translation, expression in COS cells of these mutants yielded the αI protein that comigrated with the dominant product of the human wild-type αI cDNA; however, the αII polypeptides, which initiate at Met-24 from the wild-type cDNA, are absent from the products of transfection with M24L (Compare Fig. 3i and j) and M24T (data not shown).

Immunoblotting of purified rat liver p70 S6 kinase (28) reveals a dominant 70-kDa band and an additional minor but easily visualized polypeptide band at 90 kDa (Fig. 3d to f). The copurification of 70- and 90-kDa polypeptides with S6 kinase activity from rat liver was noted previously, although the identity of the 90-kDa polypeptide was not established; the 90-kDa polypeptide was observed to undergo autophosphorylation in vitro and to coelute on gel filtration with the

70-kDa polypeptide. The reactivity of the 90-kDa polypeptide with the p70 antipeptide antibodies now demonstrates its identity as an isoform of the p70 S6 kinase; on the basis of its mobility on SDS-PAGE and behavior on gel filtration, we conclude that the rat liver 90-kDa band represents the full-length p70 S6 kinase αI translation product, as expressed in vivo.

In summary, the results obtained by translation of αI mRNA in reticulocyte lysates, together with the transient expression of p70 αI cDNA in COS cells, indicates that initiation of translation of the αI mRNA occurs from both the first and second AUG codons in intact cells. The product initiated at the first AUG exhibits an anomalously slow mobility on SDS-PAGE at ~90 kDa, and a polypeptide with precisely these properties copurifies together with the dominant 70-kDa polypeptide upon isolation of the cycloheximide-stimulated S6 kinase activity from rat liver.

Analysis of S6 kinase activity. Protein S6 kinase activity toward exogenous 40S ribosomal subunits was assayed in lysates of the transfected cells. Significant activity was present in extracts from the vector-only or untransfected (not shown) cells and was largely unaffected by omission or reintroduction of fetal calf serum (data not shown). Lysates prepared from the αI and αII cDNA transfectants exhibited a 2.5- to 4-fold increase in total kinase activity toward 40S S6 compared with lysates from cells transfected with vector DNA, when normalized for extract protein. Mono Q anion-exchange column chromatography of the COS lysates resolved two peaks of S6 kinase activity. The minor, earlier-eluting peak corresponds to the endogenous p85^{rsk} S6 kinase, as determined by immunoblotting with antibodies to the *Xenopus* and rat counterparts of this enzyme (data not shown); the S6 kinase activity in this peak was unaltered by transfection of p70 cDNA (Fig. 4). When identical amounts of extract protein were subjected to Mono Q chromatography, the later-eluting peak of activity, which is the quantitatively dominant peak of endogenous COS cell 40S S6 kinase activity, was elevated three- to fivefold in chromatograms from cells transfected with either the p70 αI or αII cDNA compared with the same fractions from chromatograms of control cell extracts (Fig. 4). Significantly, transfection with M24L, which gives rise to only the αI protein, yields a comparable increase in 40S phosphorylating activity, indicating that the 90-kDa isoform is an active S6 kinase (Fig. 4b). Thus, because transfection with the αII cDNA, which produces only the 70-kDa polypeptides, also yields a boost in S6 kinase activity, we have demonstrated that both isoforms of p70 S6 kinase are active enzymes.

Comparison of the anti-p70 immunoblot with the profiles of S6 kinase activity in these Mono Q fractions reveals several interesting features. The fraction that contains the major late-eluting peak of endogenous COS p70 S6 kinase activity (i.e., the vector-only transfectants) corresponds to the fraction (fraction 12) that exhibits the faint ladder of endogenous COS cell 65- to 70-kDa immunoreactive polypeptides (Fig. 3d to f). In contrast to the three- to fourfold increase in S6 kinase activity in this peak after transfection, the increase in p70 polypeptides observed after transfection appears far in excess of the gain in S6 kinase activity, suggesting that most of the recombinant p70 polypeptides lack S6 kinase activity. Moreover, although all fractions with S6 kinase activity exhibit p70 immunoreactivity, the majority of the recombinant p70 polypeptides elute one or two fractions earlier than the peak of S6 kinase activity, in fractions that exhibit little or no S6 kinase enzymatic activity. Examination of the pattern of p70 polypeptide bands in
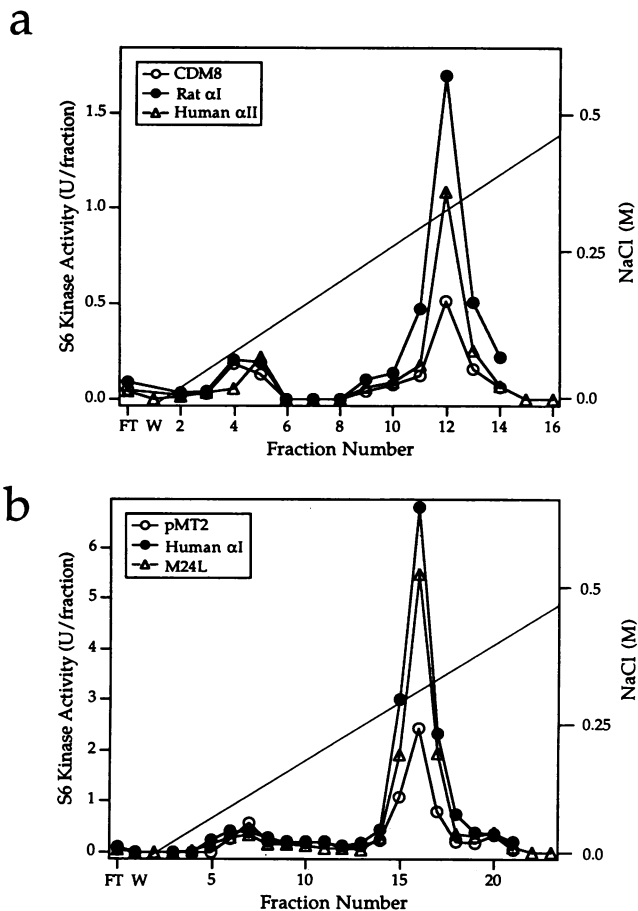
FIG. 4. Partial purification of S6 kinase activity from transfected cells. (a) Lysates of COS cells transfected with pMT2 vector only, rat p70 αI in pCDM8, or human p70 αII in pMT2 were applied to a Mono Q anion-exchange column and eluted with a linear gradient of NaCl from 0 to 400 mM. Kinase activity was assayed by using 40S ribosomes as described previously (28). (b) Results of a similar experiment in which pMT2 vector only, human αI cDNA, and the M24L mutant were transfected into COS cells. The fraction size for the experiment in panel b was 0.75 ml instead of 1.0 ml; therefore, the peak activity, which eluted at the same NaCl concentration, was in fraction 16 instead of fraction 12.

each of these fractions, shows that although the 70- and 90-kDa ladders essentially coelute, the bands of faster mobility on SDS-PAGE in each array elute earliest from Mono Q, whereas the slowest-migrating polypeptides elute latest. It is the elution of these slowest-moving SDS-PAGE immunoreactive bands that correlates best with elution of S6 kinase activity, implying that the active enzyme actually corresponds only to the slowest-migrating polypeptides. Consistent with this deduction is the fact that the 70- and 90-kDa immunoreactive polypeptides visualized in the purified rat liver S6 kinase preparation comigrate upon SDS-PAGE with the slowest-moving polypeptide in each ladder of recombinant p70 polypeptides (Fig. 3d to f).

We demonstrated previously that the activity of the p70 S6 kinase is totally dependent on endogenous phosphorylated Ser and Thr residues, inasmuch as treatment with purified phosphatase 2A completely and specifically inactivates the kinase activity (27). As shown in Fig. 5, partial inactivation of the purified rat liver S6 kinase with phosphatase 2A is
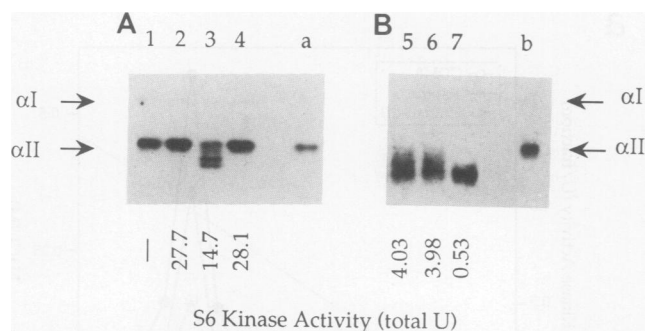
FIG. 5. Phosphatase 2A treatment of rat liver and recombinant COS cell S6 kinase. Purified rat liver S6 kinase (A) and a portion of a Mono Q fraction (corresponding to the peak of S6 kinase activity; see Fig. 4) of COS cells transfected with human p70 S6 kinase αII (B) were treated at 30°C for 90 min with carrier (lane 1), 200 nM okadaic acid (lanes 2 and 5), 10 U of phosphatase per ml (lanes 3 and 7), or both okadaic acid and phosphatase (lanes 4 and 6). Reactions were terminated by addition of okadaic acid to 300 nM. A portion of each incubation was assayed for S6 kinase activity in duplicate; the average value is shown. The remainder of the sample was subjected to immunoblot analysis with anti-p70 S6 kinase peptide antibody. Untreated rat liver S6 kinase is shown in lanes a and b.

concomitant with an increase in the mobility of the 70-kDa polypeptide on SDS-PAGE and the generation of a ladder of polypeptide-bands between 65 and 70 kDa; the appearance of these new bands is completely prevented by okadaic acid and therefore attributable exclusively to dephosphorylation. Similarly, the ladder of recombinant p70 polypeptides that coelute with S6 kinase activity exhibits a progressive increase in mobility on SDS-PAGE with phosphatase treatment, approaching in mobility that of the fastest-moving polypeptide observed in the untreated array of recombinant p70 polypeptides or in the phosphatase 2A-treated, rat liver p70 S6 kinase (Fig. 5). This dephosphorylation of the recombinant p70 protein is also accompanied by inactivation. The data shown in Fig. 5 argue strongly that the molecular basis of this ladder array of S6 kinase polypeptides is posttranslational modification of the kinase polypeptide by phosphorylation at multiple sites. Moreover, the restriction of p70 S6 kinase activity to Mono Q fractions that contain the p70 polypeptides exhibiting the slowest migration on SDS-PAGE, corresponding to the mobility exhibited by the p70 polypeptides found in purified, active rat liver p70 S6 kinase, implies that only the most highly phosphorylated, slowest-migrating p70 polypeptides exhibit S6 kinase activity.

DISCUSSION

Two families of S6 kinase have now been characterized at a molecular level, one corresponding to the *Xenopus* S6 kinase first cloned by Jones et al. (18) and a second corresponding to the enzymes studied in this investigation, first cloned by Banerjee et al. (4) and Kozma et al. (21). Both classes of S6 kinase have been shown to phosphorylate in vitro proteins other than S6 at rates that suggest that their actual substrate specificity in vivo is broader than 40S subunits (11, 24, 28). Consequently, it would be more accurate to replace the identification of these kinases by a single substrate with a nondescriptive name (as done for protein kinase C) or one describing an aspect of their regulation. Nevertheless, the extensive literature that has accumulated on these two classes of mitogen-activated pro-

tein kinases is united primarily by the use of 40S subunits as the identifying substrate together with the use of the name S6 kinase, arguing strongly for retention of the S6 kinase designation. It is now necessary, however, to identify when possible the two known families of S6 protein kinase, using available cDNA or antibody probes. We suggest that the modifiers p85 and p70 S6 kinase can serve as a useful discriminatory designation. We suggest the use of p85 instead of p90 to avoid confusion between the p90 *Xenopus* homolog and the p90 αI isoform of the p70 S6 kinase. This work demonstrates that these numerical modifiers are no more inclusive or intrinsically accurate as a description of enzyme relative mass than is the term S6 as a description of substrate specificity; nevertheless, these numerical modifiers serve to effectively distinguish the two molecular families of protein kinase while retaining the familiar name.

This work demonstrates the existence of two closely related p70 protein S6 kinases in human cDNA libraries corresponding to the rat cDNAs previously reported separately. While this report was in preparation, the molecular cloning of a rabbit liver protein-serine kinase related to the p85 S6 kinase was reported (15). Although this clone was isolated by using low-stringency hybridization to a phosphorylase kinase γ-subunit probe, comparison of this cDNA with those reported here clearly shows it to represent the rabbit αI p70 S6 kinase with a solitary amino acid difference out of a total of 525.

The most likely explanation for the origin of the two isoforms of the p70 S6 kinase is the use of alternative promotors to generate distinct 5' exons from the same gene or alternative mRNA splicing. There are several precedents for such organization (5, 29), for example, the c-*abl* protein-tyrosine kinase and the differentiation factor LIF. In the case of the c-*abl* protein-tyrosine kinase, two polypeptides are synthesized from the c-*abl* locus, termed type I and IV, which differ only in their amino-terminal regions.

A second rather novel feature of the p70 S6 kinases is the demonstration that a single (p70 αI) mRNA is translated in the intact cell from two alternative start sites, separated by 69 nt. This phenomenon appears to reflect relaxed or leaky scanning of the message facilitated by the weak ribosomal recognition sequences surrounding the first ATG (C at −3, A at +4) and is not due to termination-reinitiation, inasmuch as the first and second ATG are in frame with no intervening termination codons (20). Unless initiation from the two sites is regulatable or cell specific, cells expressing the αI form will, by default, also synthesize αII product. The anomalously slow mobility on SDS-PAGE of the slightly (23 amino acids) longer αI polypeptide significantly facilitates detection of both of the two isoforms. The presence of the αI polypeptide in our isolates of p70 S6 kinase from rat liver establishes its expression in vivo. We do not yet know whether the cellular localization or functional properties of the αI and αII S6 kinase polypeptides differ.

Although the rat and human αI cDNAs produced the same set of products in in vitro translation experiments, there was a difference in the relative amounts of the αI and αII polypeptides produced following transfection of COS cells. A difference in the stability of the rat and human proteins is an unlikely explanation, because the proteins differ by only two amino acids, one in the amino-terminal extension and one in the body of the protein. Similarly, the cDNAs are identical in sequences surrounding both the Met-1 and Met-24 initiation sites, suggesting that translation initiation probably does not differ for the two constructs. The reason for this quantitative difference is currently unclear; however,

it is true that both rat and human cDNAs are capable of detectable initiation from both methionines both in vitro and in vivo.

The generation of smaller translation products in the in vitro translations deserves some comment. Both rat and human αI cDNAs as well as the human αII cDNA produce virtually indistinguishable patterns of bands in the range of 30 to 60 kDa (Fig. 2). Because these clones initiate translation at two different sites (Met-1 and Met-24 for αI but only Met-24 for αII), the virtual identity of translation products is inconsistent with premature termination being the cause. This leaves as the probable cause aberrant internal initiation at Met residues that are, of course, present in both forms of the cDNA (which are, within a species, identical except at the 5' termini). The data shown in Fig. 2b support the conclusion that the majority of the smaller translation products result from internal initiation: capping only increased the efficiency of synthesis of the full-length polypeptides, but the same set of smaller products was also observed.

The most striking feature of the short amino-terminal extension of the αI isoform is the presence of six consecutive arginine residues at the amino terminus. Such sequences are found in proteins that bind to polynucleotides and are reminiscent of a number of nuclear localization signals (8, 19, 34). We have previously shown that rat liver S6 kinase phosphorylates many nonhistone proteins present in nuclear extracts (28). Recently, the hormonally regulated phosphorylation of a pool of nuclear S6 has been reported (13). It is tempting to speculate that the highly basic nature of the amino-terminal region of p70 S6 kinase αI is analogous to the nuclear localization sequence of simian virus 40 T antigen (19) and c-abl (34) and directs the protein to a specific subcellular compartment, perhaps the nucleolus where ribosomal proteins are assembled with rRNA into ribosomal subunits. Alternatively, the two forms of the protein may interact differentially with the different populations of ribosomes within cells. Experiments to test these hypotheses are in progress.

Insulin/mitogen stimulation of S6 kinase activity is due to the phosphorylation of the S6 kinase polypeptide on serine and threonine residues; treatment of the purified enzyme with (Ser/Thr) protein phosphatase causes inactivation. The present results demonstrate that the S6 kinase cDNAs that we have isolated direct the expression of 40S S6 kinase activity when introduced into COS cells. It appears, however, that only a small fraction of the synthesized protein is catalytically active, and this active component correlates with the most electrophoretically retarded polypeptide bands. These observations can be explained by the known properties of the major 70-kDa liver S6 kinase if the multiple electrophoretic bands observed in the recombinant p70 polypeptides are caused by phosphorylation. In support of this idea, phosphatase 2A treatment of the purified rat liver p70 S6 kinase, concomitant with extensive inactivation of kinase activity, converts the single band migrating at 70 kDa to a ladder of immunoreactive polypeptides migrating between 65 and 70 kDa, very similar in appearance to the recombinant p70 polypeptide ladder (Fig. 5). Both inactivation and the altered pattern of mobility are completely blocked by okadaic acid. In addition, phosphatase 2A treatment of the recombinant p70 S6 kinase produces a further okadaic acid-sensitive increase in electrophoretic mobility of the immunoreactive polypeptides. At least four or five electrophoretically variant recombinant p70 αI and αII polypeptides are discernible; only the most slowly migrating (SDS-PAGE) band in each ladder comigrates on detergent

gel electrophoresis with the αI and αII polypeptides found in the active enzyme purified from rat liver. In addition, only the recombinant p70 polypeptide band migrating most slowly on SDS-PAGE coelutes with the transfection-induced 40S S6 kinase activity on Mono Q chromatography. We have no information relating the actual extent of phosphorylation with the shift in electrophoretic mobility. Each band may differ from the adjacent bands by a constant number of phosphate groups; conversely, phosphorylation at certain sites may exert disproportionate effects on polypeptide conformation and SDS binding as observed for protein kinase C (35). Nevertheless, it appears that multiple phosphorylation is required to generate active S6 kinase, and significant phosphorylation of the p70 polypeptide can occur without production of any measurable S6 kinase activity. Whether this multiple phosphorylation is sequential and catalyzed by one or several protein kinases is not yet known. Inasmuch as the electrophoretic mobilities of the proteins translated in vitro correspond to those of the fastest-migrating species in the transfected cells, it would appear that the S6 kinase kinase(s) is not active in the reticulocyte lysate.

We have tried to activate a greater proportion of the recombinant p70 kinase in transfected COS cells by addition of agents such as tetradecanoylphorbol acetate and have tried to reduce activity by serum deprivation. Thus far these manipulations have failed to modulate the level of p70 S6 kinase activity, although significant alterations in the activity of the earlier-eluting endogenous peak of p85^{rsk} S6 kinase have been observed. This observation tends to underscore the likelihood that the upstream regulators of those two families are, at least in part, independent. Moreover, this independence suggests that neither S6 kinase is capable of directly phosphorylating and regulating the other. COS-7 cells appear unable to regulate the endogenous or recombinant p70 enzyme in response to external mitogens; a fraction of the COS cell p70 S6 kinase is activated under all conditions examined thus far. This behavior may reflect the immortalized state of these cells, which cannot be made quiescent. We are constructing cell lines expressing the two p70 S6 kinase cDNAs in an effort to mimic more closely the physiological environment of this enzyme. Such cell lines may allow further study of the mechanisms by which this kinase is regulated.

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