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Primary Structure of Mammalian Ribosomal Protein S6†

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ABSTRACT: Ribosomal protein S6 was isolated from rat liver ribosomes by reversed-phase high-performance liquid chromatography (HPLC) and subjected to cyanogen bromide and proteolytic cleavages. The cleavage fragments were resolved by HPLC and sequenced by automated Edman degradation. The overall amino acid sequence of S6 (249 residues) was determined by alignment of the overlapping sequences of selected cyanogen bromide, chymotryptic, tryptic, and clostripain cleavage fragments. The only protein found to exhibit close homology with the S6 sequence is yeast ribosomal protein S10 (61% sequence identity). Previously, characterized phosphopeptide derivatives of S6 containing phosphorylation sites for adenosine 3′,5′-cyclic phosphate dependent and protease-activated protein kinases originate from the carboxy-terminal region of S6 encompassing residues 233-249.

Mammalian ribosomal S6 is the only ribosomal protein to be extensively phosphorylated at multiple sites in response to mitogenic and other polypeptide growth factors (Wool, 1979; Leader, 1980; Traugh, 1981; Thomas et al., 1982; Lastick & McConkey, 1981; Wettenhall et al., 1982, 1983; Perisic & Traugh, 1983; Tabarini et al., 1985; Martin-Perez & Thomas, 1983; Wettenhall & Howlett, 1979). This phenomenon is of interest because of the possibility that phosphorylation leads to increased protein synthetic activity in stimulated cells (Wettenhall & Howlett, 1979; Thomas et al., 1982; Duncan & McConkey, 1982; Traugh & Pendergast, 1986). S6 phosphorylation is catalyzed by various protein kinases, including adenosine 3′,5′-cyclic phosphate (cAMP)[1] dependent (Traugh, 1981; Wettenhall & Cohen, 1982), cGMP-dependent (Traugh, 1981), and Ca^{2+}/calmodulin-dependent (Padel & Soling, 1985) protein kinases, protein kinase C (Le Peuch et al., 1983; Parker et al., 1985), protease-activated protein kinases (Traugh, 1981; Perisic & Traugh, 1983; Donahue &

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[1] Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; Pth, 3-phenyl-2-thiohydantoin; GdmCl, guanidinium chloride; S2 and S4, solvents diethyl acetate and acetonitrile, respectively, used in the automated sequenator; TFA, trifluoroacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; cAMP, adenosine 3′,5′-cyclic phosphate; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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Masaracchia, 1984; Gabrielli et al., 1984), and the highly specific S6 kinase whose activity is elevated following growth stimulation (Novak-Hofer & Thomas, 1984; Erikson & Maller, 1985; Blenis & Erikson, 1986).

The preferred S6 phosphorylation sites for the cAMP-dependent protein kinase, protease-activated kinase, and protein kinase C are clustered within a serine-rich region having the structure Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-Lys (Wettenhall & Morgan, 1984; Gabrielli et al., 1984; House et al., 1987).[2] Tryptic map analyses have suggested that the growth factor regulated S6 phosphorylation sites are also clustered within this region (Wettenhall et al., 1982, 1983; Wettenhall & Morgan, 1984). The only other reports of S6 sequence data are on the NH_2-terminal segment of 33 residues (Wittmann-Liebold et al., 1979; Nick et al., 1985). The possible location of the known phosphorylation sites within the COOH-terminal region of S6 has been suggested by the finding of a related sequence at the COOH terminus of yeast ribosomal protein S10, which displays considerable sequence identity with S6 in the NH_2-terminal region and is considered to be the homologue of mammalian S6 (Leer et al., 1982; Otaka et al., 1983).

In this report, we describe an investigation of the primary structure of S6 using microsequencing techniques. The determination of the structures of chemically and proteolytically derived fragments has enabled the elucidation of the overall sequences of S6 by alignment of the overlapping sequences and by comparison with the closely related sequence of yeast ribosomal S10 (Leer et al., 1982, 1985). The analyses confirm that the previously described tryptic phosphopeptide derivatives of S6 originate from the COOH-terminal region of the protein.

Experimental Procedures

Materials. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, clostripain, and *N*-(*p*-tosyl)lysine chloromethyl ketone treated chymotrypsin were obtained from Worthington; cyanogen bromide was from Sigma Chemical Co.; protein sequencer reagents were from Applied Biosystems and Pth–amino acid standards from Pierce Chemical Co.

Isolation of S6. S6 was isolated directly from rat liver ribosomes (Wettenhall & Cohen, 1982) or 40S ribosomal subunits (Wettenhall & Wool, 1972) by reversed-phase HPLC using a 0.46 × 25 cm wide-pore C_8 column (Bakerbond, J. T. Baker, RP 7105-0) eluted with a gradient of acetonitrile in aqueous 0.1% trifluoroacetic acid (v/v) as described previously (Nick et al., 1985). The preparations of S6 used for fragmentation experiments were greater than 95% pure as judged by SDS–polyacrylamide gel electrophoresis and amino acid analysis (Nick et al., 1985). The HPLC fractions containing 10–20-μg portions of S6, estimated by the Coomassie Blue method (Sedmak & Grossberg, 1977), were dried in Eppendorf tubes in a Savant vacuum centrifuge (without heating) in preparation for cyanogen bromide and proteolytic cleavage.

Proteolytic Digestion. Portions of HPLC-purified S6 (10–20 μg) were dissolved in 0.2 M NH_4HCO_3/1 mM EDTA buffer, pH 7.8, containing 0.5 μg of either trypsin or chymotrypsin per 20 μg of S6 and incubated at 37 °C for the times indicated. Digestions with clostripain (0.13 unit/20 μg of S6) were performed for 6 h at 35 °C in 100 μL of 50 mM sodium phosphate buffer, pH 7.6, containing 7 mM dithiothreitol and 0.5 mM $CaCl_2$.

Cyanogen Bromide Cleavage. Lyophilized portions of S6 (ca. 20 μg) were dissolved in 100 μL of aqueous 70% trifluoroacetic acid (v/v) containing 200 μg of cyanogen bromide. After 20 h at 20 °C, the mixture was diluted with 900 μL of H_2O and injected directly onto the Bakerbond wide-pore C_8 column equilibrated in aqueous 0.1% trifluoroacetic acid (v/v), and the peptides were eluted with a linear gradient of acetonitrile as described below.

Separation of Peptides by Reversed-Phase HPLC. Proteolytic digests in 0.2 M NH_4HCO_3 were usually applied directly to the Bakerbond C_8 column or a 5-μm 0.46 × 25 cm C_{18} column (Vydac type 201 TP54) equilibrated with aqueous 0.1% trifluoroacetic acid (v/v). The peptides were eluted with a linear gradient of acetonitrile increasing from 0 to 50% in 90 min at a flow rate of 1 mL/min and monitored at 215 nm using a Waters system. In cases of heterogeneous peak fractions, individual peptides were further resolved by rechromatography on the Vydac C_{18} column using a modified gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Usually, separation was achieved when the acetonitrile concentration was increased at 0.5%/min and continued isocratically at an acetonitrile concentration 5% below the concentration at which the peptide eluted in the original HPLC step. Fractions containing peptides were usually dried in borosilicate glass tubes in a Savant vacuum centrifuge (without heating). The peptides for sequencing were dissolved in aqueous 50% trifluoroacetic acid (v/v) or in water containing a trace of trimethylamine.

Amino Acid Analysis. Portions of S6 and peptides purified by HPLC were hydrolyzed in 6 N HCl containing 10 mM phenol for 24 h at 110 °C, and amino acid analyses were performed on a Beckman 6300 analyzer; 10% correction was made for serine destruction.

Amino Acid Sequence Determination. Automated amino acid sequence determinations were performed in duplicate on an Applied Biosystems Model 470A gas–liquid-phase sequencer using trifluoroacetic acid conversion chemistry (Hewick et al., 1981). Washout of peptides from the reaction cartridge was minimized by reducing the flow rate of S2 by 30%, and the recoveries of Pth–serine derivatives were improved by reducing the conversion flask dry-down time and by including additional dithiothreitol in S4 (10 mg/200 mL of S4). Pth–amino acids recovered at each cycle of the Edman degradation were dissolved in 10 mM sodium acetate buffer (pH 5.0) containing 30% acetonitrile and analyzed on a Zorbax ODS reversed-phase column (Du Pont) eluted with a discontinuous acetonitrile gradient (Zimmerman et al., 1977). The chromatograms were simultaneously scanned at 254 and 313 nm (for detection of dehydroserine and -threonine derivatives) using a dual-channel Waters 440 monitor. Quantitation of Pth–amino acid derivatives was achieved by measuring peak heights and comparing with known standards. Nonideal chromatography of Pth–arginine and –histidine and instability of the Pth–threonine, –serine, and –lysine derivatives meant that the detection and quantitation of these residues were sometimes more difficult in high-sensitivity analyses.

Results and Discussion

Sequencing Strategy. An RP-HPLC procedure for isolating microgram quantities of S6 from rat liver ribosomes (Nick et al., 1985) was used to obtain material for sequencing. Cleavage products of S6, generated by proteolytic and cyanogen bromide procedures, were resolved by RP-HPLC using a wide-pore Bakerbond C_8 column eluted with gradients of acetonitrile in aqueous 0.1% (v/v) TFA buffer (Figure 1). The structures of cyanogen bromide, tryptic, chymotryptic,

[2] R. E. H. Wettenhall, B. Gabrielli, L. Bozinova, and B. E. Kemp, submitted for publication.

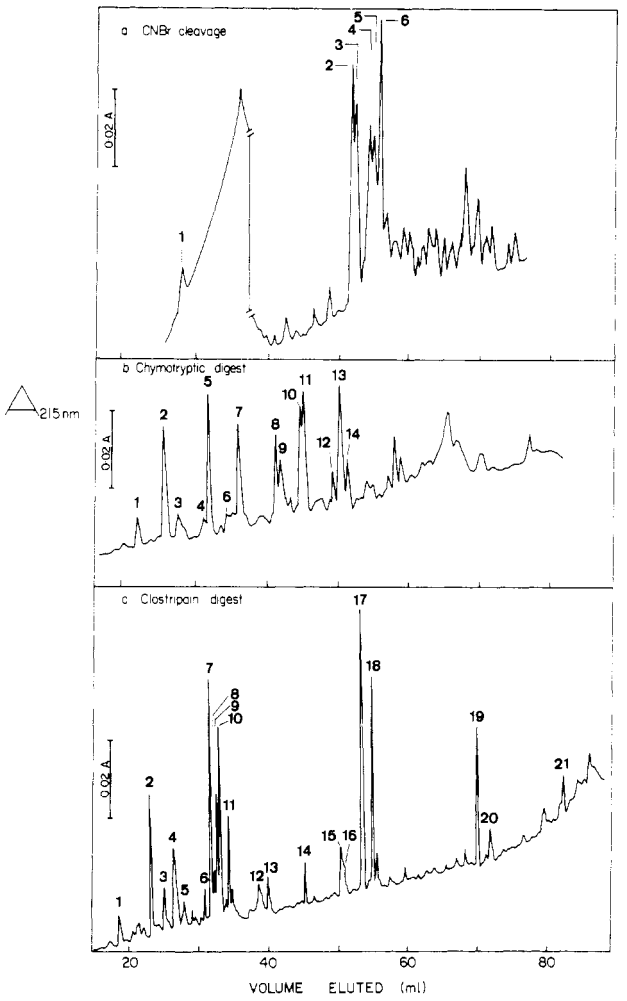

FIGURE 1: Cyanogen bromide cleavage and proteolytic fragments of S6 resolved by reversed-phase HPLC. Fragments of S6 were resolved by HPLC using either a Bakerbond C_8 column (a and b) or a Bakerbond diphenyl column (c) eluted with a linear gradient of acetonitrile as described under Experimental Procedures. The major CNBr and proteolytic fragments referred to in the text are numbered in accordance with peak numbers. (a) Cyanogen bromide cleavage fragments: The various minor peaks included species originating from partial or nonideal cleavages, together with species which gave no sequence. The high background in the early region of the chromatogram necessitated adjustment to the base line after elution of 37 mL. (b) 1.5-min chymotryptic digestion. (c) 6-h clostripain digest.

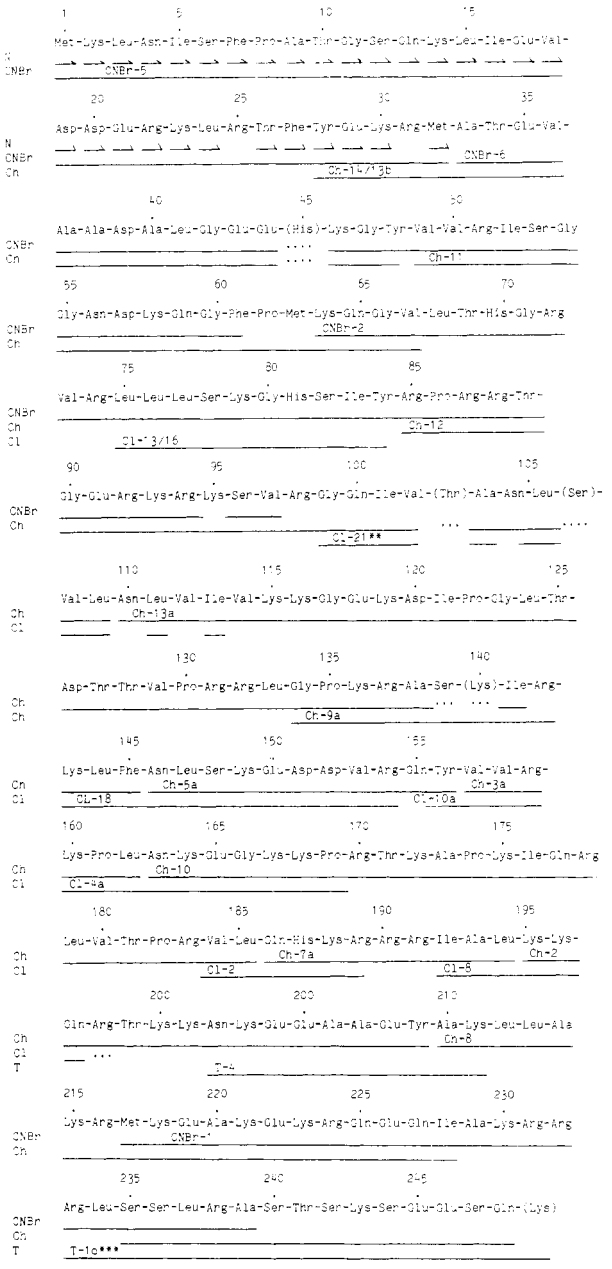

FIGURE 2: Amino acid sequence of rat liver ribosomal S6. The residues identified during NH_2-terminal analyses of whole S6 (Nick et al., 1985) are marked with arrows. The residues identified in cyanogen bromide (CNBr), chymotryptic (Ch), clostripain (Cl), and tryptic (T) fragments are indicated by the solid lines (residues in parentheses and marked with dotted lines indicate tentative identifications). Species T4 was generated during tryptic digestion of S6 for 1.5 min (Table IV). The sequence of the tryptic derivative T-1c was determined previously (Wettenhall & Morgan, 1984).

and clostripain cleavage fragments were analyzed by automated Edman degradation. The primary structure of S6 was determined by alignment of the NH_2-terminal 33-residue sequence (Wittmann-Liebold et al., 1979; Nick et al., 1985) and the overlapping sequences of the various fragments of S6 (Figure 2).

Cyanogen Bromide Peptides. Six cyanogen bromide cleavage fragments were resolved by RP-HPLC (Figure 1a). Unambiguous NH_2-terminal sequences were obtained for each of these fragments (Table I). In the case of the partially resolved CNBr-2/CNBr-3 and CNBr-4/CNBr-5 peaks, cross-contamination was evident, but the main sequences were clearly identifiable from the quantitative Pth–amino acid data. The six CNBr fragments isolated included variants of the S6.33–63 (CNBr-4 and -6) and the S6.1–32 (CNBr-3 and -5) sequences. The CNBr-4 and CNBr-6 fragments had identical NH_2 termini. The earlier eluting and presumably shorter CNBr-4 fragment must have been generated through nonideal cleavage. The first 20 residues were identified in the sequence analyses of CNBr-4, suggesting that the cleavage occurred somewhere between Ile-53 and Met-63, possibly involving acid cleavage at Asp-57. Overall, the analyses of the CNBr fragments accounted for 116 residues in S6 (Table I). The remaining residues appeared to reside in CNBr-2 of which only 34 residues were identified by NH_2-terminal sequence analysis (Table I). Subsequent analyses of proteolytically generated fragments showed the absence of methionine residues between positions 63 and 217 (Figure 2).

Chymotryptic Peptides. Unambiguous sequences obtained for 22 of the chymotryptic derivatives, resolved by RP-HPLC (Figure 1b; Table II) and where necessary purified by further HPLC steps (not illustrated), accounted for a total of 194 residues in S6. Twelve of these sequences were required for establishing the structure of S6 (Table III). The S6.110-141 peptide could not be resolved from the S6.29–48 derivative

Table I: Automated Amino Acid Sequence Analyses of Cyanogen Bromide Cleavage Fragments of S6

cycle no.	recoveries of Pth–amino acids (pmol)[a]			
	CNBr-1	CNBr-2	CNBr-5	CNBr-6
1	K, 122 (8)	K, 119 (4)	M, 65 (9)	A, 75 (4)
2	E, 122 (22)	Q, 87 (4)	K, 47 (2)	T, 21 (3)
3	A, 106 (17)	G, 113 (4)	L, 76 (6)	E, 72 (10)
4	K, 92 (21)	V, 100 (15)	N, 51 (3)	V, 60 (18)
5	E, 63 (15)	L, 101 (18)	I, 54 (7)	A, 61 (73)
6	K, 23 (10)	T, 36 (9)	S, 9 (1)	A, 73 (18)
7	R, 50 (20)	H, 37 (10)	F, 41 (4)	D, 38 (15)
8	Q, 49 (19)	G, 52 (27)	P, 40 (7)	A, 42 (17)
9	E, 44 (13)	R, 32 (13)	A, 35 (6)	L, 35 (20)
10	Q, 34 (24)	V, 29 (12)	T, 12 (2)	G, 39 (22)
11	I, 30 (21)	R, 37 (14)	G, 33 (7)	E, 23 (42)
12	A, 15 (10)	L, 36 (40)	S, 5 (2)[c]	E, 42 (29)
13	K, 10 (6)	L, 40 (47)	Q, 24 (3)	(H), +
14	R, 14 (16)	L, 47 (28)	K, 18 (6)	K, 10.6 (8.2)
15	R, 16 (12)	S, 10 (5)	L, 23 (6)	G, 10.1 (7.8)
16	R, 17 (13)	K, 24 (16)	I, 16 (4)	Y, 7.8 (6.8)
17	L, 14 (10)	G, 17 (11)	E, 13 (3)	V, 9.2 (15.4)
18	S, +	H, 6 (3)	V, 25 (6)	V, 15.4 (10.3)
19	S, +	S, 4 (3)	D, 21 (17)	R, +
20	L, 7 (5)	I, 5.1 (3.4)	D, 17 (6)	I, 6.3 (4.7)
21	R, 3 (2)	Y, 8.7 (6)	E, 20 (6)	S, +
22	A, 6 (5)	R, 3.1 (2.3)	R, 10.7 (5.1)	G, 7.4 (11.8)
23		P, 5.8 (5.0)	K, 21 (4)	G, 11.8 (9.3)
24		R, 2.0 (2.5)	L, 23 (6)	N, 3.2 (2.7)
25		R, 2.5 (2.3)	R, 15.2 (7.1)	-
26		T, 1.9 (1.6)	T, 7.3 (1.5)	K, 3.5 (3.2)
27		G, 3.0 (2.6)	F, 18.8 (8.6)	Q, 2.8 (2.6)
28		E, 2.8 (2.2)	Y, 16.7 (6.2)	G, 2.2 (2.0)
29		R, +	E, 15.8 (8.4)	F, 1.4 (1.3)
30		K, 2.3 (1.8)	K, 12.4 (6.5)	
31		R, +	R, 4.7 (3.5)[b]	
32		- -		
33		S, +		
34		V, 1.0 (0.9)		

[a] Cyanogen bromide cleavage fragments were resolved by HPLC (Figure 1a). Yields of individual Pth–amino acids attributed to the sequence at each cycle (n) are recorded; the yield of the same Pth–amino acid at the following cycle ($n + 1$) is given in parentheses. The (+) symbol is to indicate that the residue was detected but not quantitated. The one-letter amino acid notation is used. Data for the CNBr-3 (KLNI...) and CNBr-4 (sequence identified ATEVAADALGEEHKGYVVRI...) fragments have not been recorded as these peptides were variants of the CNBr-5 and CNBr-6 sequences, respectively. In the case of CNBr-4, the site of nonideal cleavage was not determined. [b] Analysis of CNBr-5 was terminated after 31 cycles. [c] Serine identification was based solely on a signal in position of elution of the dithiothreitol adduct of Pth–serine.

Table II: Chymotryptic Fragments of S6 Resolved by Reversed-Phase HPLC

HPLC peak no.	sequence identified (single-letter amino acid code)[a]	initial yield (pmol)	residue no. in S6
1	SSLRASTSKSEESQ	100	235–248
2	KKQRTKKNKEEAAEY	200	195–209
3a	VVRKPL	210	157–162
3b	NKEGKKP-TKA	35	163–173
4	THGRVRL	100	69–75
5a	NLSKEDDVRQY	250	146–156
5b	AKLLA	105	210–214
6a	NKEGKKPRTKAPKI	140	163–176
6b	VTPRVL	120	180–185
7a	QHKRRRI	160	186–192
7b	THGRVRLL	50	69–76
8	AKLLAKRMKEAKEKRQEQI	130	210–228
9a	GPKRAS(K)IRKLF	70	134–145
9b	RLVTPRVL	100	178–185
9c	VVRISGGNDKQGF	60	49–61
9d	EKRMAT	30	29–34
9e	---NIG(V)--P-	25	-
10	NKEGKKPRTKAP-KIQRLVTPRVL	190	163–185
11	VVRISGGNDKQGFPMKQGV	80	49–67
12	RPRRTGERKRKSVRGQIV-TANL(S)VL	60	85–109
13a	NLVIVKKGEKDIP-GLTDTTVPRRLGPKRA--I	155	110–141
13b	EKRMATEVAADALGEE(H)-KGY	93	29–48
14	YEKRMATEVAADALGEE-KGY	15	28–48

[a] The data in all cases except for HPLC peaks 6 and 9 were obtained from the analyses of 1.5-min chymotryptic digests (Figure 1b). The data for peaks 6 and 9 were obtained with peptides recovered from 15-min chymotryptic digests. The individual species in peaks 5, 6, 7, and 9 were further resolved by rechromatography on a Vydac C_{18} column (see Experimenal Procedures). Species 9e did not originate from S6. Tentative identifications are given in parentheses.

by reversed-phase HPLC (peak 13, Figure 1b). The S6.110–141 sequence (termed Ch-13a) was clearly evident as the major sequence (initial yields 155 versus 93 pmol) in the analysis of the peak 13 material obtained when chymotryptic digestion was performed for only 1.5 min (Figure 1b), whereas the S6.29–48 (termed Ch-13b) fragment contributed the major peak 13 sequence (initial yields 160 versus 75 pmol) in the analyses of 15-min digests (not illustrated). The S6.110–141 sequence was confirmed by subtractive Pth analysis after taking into account the sequence information from the analysis of the S6.28–48 species (Ch-14, Table II). The S6.133–145 sequence was obtained from the analysis of Ch-9a, which required further purification by HPLC using a 5-μm Vydac C_{18} column (not illustrated; see Experimental Procedures). The analyses of the purified Ch-9a gave a major sequence, Gly-Pro-Lys-Arg-Ala-Ser-(Lys)-Ile-Arg-Lys-Leu-Phe, and a minor sequence unrelated to S6, X-X-X-Asn-Ile-Gly-(Val)-X-Pro-. There was an ambiguity at cycle 7 because of the similar yields of the two derivatives Pth–valine and Pth–lysine. Lysine was assigned to the main sequence at this position after taking into account the instability of Pth–lysine, which often results in low yields under microsequencing conditions. This conclusion was supported by the fact that Pth–valine was not observed at cycle 31 in the analysis of Ch-13a (Table III).

Clostripain Peptides. Alignment of most of the cyanogen bromide and chymotryptic fragments (Figure 2) was achieved with the overlapping sequences of seven of the clostripain cleavage products (Table IV), resolved by RP-HPLC (Figure 1c). The clostripain peptide Cl–4a, required for establishing the overlap at the chymotryptic site Leu-162 (Figure 2), was resolved from Cl–4b (Table V) by rechromatography on the Vydac C_{18} column (not illustrated; see Experimental Procedures). Only partial sequence data were obtained for the Cl–21 derivative which was recovered in very low yields despite extensive attempts to improve the recoveries (initial yield ca. 5 pmol).

Overall, a total of 27 amino acid sequences were identified in the clostripain digests, accounting for 197 of the 249 residues in the overall sequence of S6 (Table V). Most of the unaccounted for sequences were in regions rich in basic residues.

Tryptic Peptides. The digestion of S6 (5 μg) for 1.5 min with trypsin generated several partial tryptic fragments which were resolved by RP-HPLC (not illustrated). Of these, the sequence of the T-4 peptide, eluting at 12% acetonitrile (Table IV), corresponded to the S6.202–211 sequence, which established the required overlap at the chymotryptic site Tyr-209.

Overall Structure of S6. Overlaps between the sequences of the various fragments of S6 were established throughout the sequence (Figure 2). Difficulty was encountered with the overlap for the chymotryptic site Leu-109. Only traces of Cl–21 were obtained (Table IV). A related tryptic peptide has not been found. It is presumed that the nonpolar nature

Table III: Automated Amino Acid Sequence Analyses of Chymotryptic Peptides Required for the Determination of the Overall Structure of S6

Part A
recoveries of Pth–amino acids (pmol)[a]

cycle no.	Ch-1	Ch-2	Ch-3a	Ch-5a	Ch-7a	Ch-8
1	S, 23 (21)	K, 94 (97)	V, 220 (227)	N, 115 (8)	Q, 196 (3)	A, 108 (13)
2	S, 21 (5)	K, 97 (11)	V, 227 (12)	L, 120 (9)	H, 112 (12)	K, 133 (12)
3	L, 83 17)	Q, 78 (8)	R, 165 (30)	S, 21 (2)	K, 200 (23)	L, 124 (129)
4	R, 66 (16)	R, 61 (3)	K, 156 (18)	K, 98 (18)	R, 125 (162)	L, 129 (19)
5	A, 99 (35)	T, 23 (4)	P, 68 (8)	E, 87 (26)	R, 162 (122)	A, 90 (14)
6	S, 8 (2)	K, 41 (48)	L, 38 (16)	D, 65 (86)	R, 122 (21)	K, 79 (23)
7	T, 27 (16)	K, 48 (6)		D, 86 (41)	I, 102 (8)	R, 58 (18)
8	S, 7 (4)	N, 55 (12)		V, 45 (22)	A, 52 (10)	M, 42 (15)
9	K, 16 (14)	K, 33 (4)		R, 25 (11)	L, 30 (11)	K, 52 (15)
10	S, 5 (3)	E, 33 (43)		Q, 41 (17)		E, 61 (25)
11	E, 12 (16)	E, 43 (14)		Y, 24 (14)		A, 38 (12)
12	E, 16 (13)	A, 22 (31)				K, 39 (19)
13	S, +	A, 31 (11)				E, 36 (20)
14	Q, 3 (2)	E, 21 (12)				K, 33 (21)
15		Y, 7 (4)				R, 10 (4)
16						Q, 20 (10)
17						E, 12 (7)
18						Q, 12 (8)
19						I, 4 (3)

Part B
recoveries of Pth–amino acids (pmol)[a]

cycle no.	Ch-9a	Ch-10	Ch-11	Ch-12	Ch-13a[b]	Ch-13b[c]
1	G, 48 (4)	N, 156 (8)	V, 92 (93)	R, 40 (9)	N, 155 (7)	E, 160 (30)
2	P, 34 (3)	K, 162 (5)	V, 93 (9)	P, 44 (9)	L, 149 (14)	K, 98 (16)
3	K, 22 (3)	E, 131 (5)	R, 94 (13)	R, 42 (12)	V, 109 (9)	R, 94 (8)
4	R, 25 (8)	G, 150 (11)	I, 82 (8)	R, 50 (16)	I, 87 (6)	M, 111 (20)
5	A, 22 (7)	K, 139 (142)	S, 12 (3)	T, 9 (6)	V, 98 (11)	A, 124 (14)
6	S, 10 (4)	K, 142 (10)	G, 49 (51)	G, 38 (14)	K, 45 (52)	T, 29 (3)
7	K, 7.8 (6)	P, 76 (16)	G, 51 (12)	E, 22 (4)	K, 52 (5)	E, 105 (18)
8	I, 8.4 (5)	R, 60 (10)	N, 38 (7)	R, 15 (8)	G, 74 (9)	V, 96 (20)
9	R, 4 (2)	T, 25 (9)	D, 24 (7)	K, 11 (5)	E, 77 (11)	A, 102 (110)
10	K, 3.2 (1.2)	K, 37 (13)	K, 20 (6)	R, 16 (7)	K, 33 (6)	A, 110 (21)
11	L, 2.1 (1.3)	L, 60 (24)	Q, 14 (11)	K, 12 (5)	D, 65 (15)	D, 90 (25)
12	F, 1.0 (0.6)	P, 37 (9)	G, 11 (2)	S, 4 (2)	I, 53 (9)	A, 67 (22)
13		K, 24 (11)	F, 8.1 (3)	V, 10 (6)	P, 56 (12)	L, 63 (19)
14		I, 31 (22)	P, 6.2 (3)	R, 3 (2)	G, 47 (17)	G, 60 (11)
15		Q, 24 (12)	M, 3.3 (2.2)	G, 10 (4)	L, 57 (18)	E, 48 (55)
16		R, 10 (6)	K, 4.0 (3.0)	Q, 7 (5)	T, 17 (4)	E, 55 (14)
17		L, 27 (21)	Q, 1.2 (0.8)	I, 3.5 (1)	D, 42 (18)	(H), 2 (1)
18		V, 23 (18)	G, 2.0 (1.2)	V, 4 (2.3)	T, 14 (16)	K, 10 (3)
19		T, 8 (6)	V, 0.8 (0.6)	T, 2 (1)	T, 16 (5)	G, 19 (10)
20		P, 7 (6)		A, 4.1 (2.2)	V, 39 (18)	Y, 17 (11)
21		R, 5 (4)		N, 2.1 (1.5)	P, 15 (6)	
22		V, 8 (6)		L, 2.5 (1.8)	R, 10 (18)	
23		L, 3 (2.5)		(S), +	R, 18 (9)	
24				V, 1.5 (1.4)	L, 22 (8)	
25				L, 1.0 (0.7)	G, 16 (6)	
26					P, 10 (5)	
27					K, 3.1 (2)	
28					R, 5.0 (3)	
29					A, 10.1 (6.2)	
30					S, -	
31					K, +	
32					I, 4 (3)	

[a] Chymotryptic species were resolved by HPLC (Figure 1b) and, in the case of Ch-9a, further purified by HPLC using modified conditions (see text). Yields of individual Pth–amino acids attributed to the sequence at each cycle (n) are recorded; the yields of the same amino acid at the following cycle ($n + 1$) are given in parentheses. Single-letter notation for amino acids is used. [b] Major sequence in the analyses of peak 13 from 1.5-min chymotryptic digestion. [c] Major sequence in the analyses of peak 13 from 15-min digestion.

of the sequence in this region confers poor solubility and hence low recoveries of the Cl–21 and related fragments. The incomplete data on Cl–21 leave open the possibility that additional sequence may exist in this region of the structure. However, support for the overlap suggested by the Cl–21 data is the appreciable identity between the regions flanking Leu-109 and the corresponding region of yeast S10 (Figure 3).

The COOH terminus of S6 appeared to be contained in CNBr-1, Ch-1, and the previously described tryptic peptide termed T-1c (Wettenhall & Morgan, 1984). The final residue unequivocally identified in the Ch-1 and T-1c analyses corresponded to Gln-248. However, the amino acid compositions (moles per mole of peptide) of the Ch-1 ($Thr_{1.07}Ser_{5.80}Glx_{2.62}Ala_{1.40}$, $Leu_{1.25}Lys_{2.00}$, $Arg_{1.26}$) and T-1c (Wettenhall & Morgan, 1984) fragments indicated the presence of an additional lysine which was assigned to position 249 (Figure 2).

Assignments at four positions in the sequence were only tentative. The apparent histidine at position 45 was encountered at late stages in the analyses (position 45: Cl–19, Ch-13b and Ch-14, CNBr-4, and CNBr-6). Under these circumstances, the assignment of histidine was usually equivocal

Table IV: Automated Amino Acid Sequence Analyses of Clostripain and Tryptic Cleavage Products of S6 Required for Establishing Overlaps in the S6 Sequence

	recoveries of Pth–amino acid (pmol)[a]							
cycle no.	Cl-2	Cl-4a	Cl-8	Cl-10	Cl-13	Cl-18	Cl-21	T-4
1	V, 201 (10)	K, 122 (24)	I, 156 (2)	Q, 110 (4)[b]	L, 62 (69)	K, 160 (11)	G, 5 (2)	N, 55 (7)
2	L, 200 (8)	P, 79 (8)	A, 164 (9)	Y, 137 (14)	L, 69 (59)	L, 196 (11)	Q, 5 (3)	K, 49 (8)
3	Q, 125 (11)	L, 76 (10)	L, 169 (17)	V, 152 (156)	L, 59 (9)	F, 174 (7)	I, 4 (2)	E, 54 (57)
4	H, 47 (8)	N, 50 (14)	K, 110 (29)	V, 136 (17)	S, 5 (1)	N, 156 (8)	V, 4 (3)	E, 57 (25)
5	K, 92 (27)	K, 66 (21)	K, 135 (33)	R, 24 (9)	K, 29 (15)	L, 174 (15)	–	A, 31 (40)
6	R, 29 (9)	E, 33 (17)	Q, 60 (38)		G, 16 (9)	S, 24 (3)	A, 3.2 (2)	A, 40 (19)
7		G, 45 (23)	(R), +		H, 12 (7)	K, 159 (22)	–	E, 42 (17)
8		K, 23 (32)			S, +	E, 107 (26)	L, 2.1 (1.5)	Y, 21 (13)
9		K, 32 (15)			I, 3 (2)	D, 72 (98)	–	A, 22 (19)
10		P, 14 (8)			Y, 2.0 (1.5)	D, 98 (46)	V, 2.2 (2.0)	K, 9 (7)
11					R, +	V, 63 (32)	L, 1.8 (1.5)	
12						R, 5 (4)	–	
13							L, 1.5 (1.1)	
14							–	
15							I, 1.1 (0.6)	

[a] Peptides in clostripain digests of S6 were resolved by HPLC as described for Figure 1c. Tryptic peptides (1.5-min digestion at 37 °C) were applied directly to a Vydac C_{18} column and eluted with a linear gradient of acetonitrile increasing from 0 to 50% in 90 min as described under Experimental Procedures. The T4 species eluted at 12% acetonitrile (22 min). In the case of Cl-4a, Cl-8, and Cl-13, the peptides were further purified by HPLC using modified conditions (see Experimental Procedures). Yields of individual Pth-amino acids attributed to the sequence at each cycle (n) are recorded; in addition, the yields of the same amino acid at the following cycle (n + 1) are given in parentheses. The single-letter notation for amino acids is used. [b] In the analyses of Cl-10, Pth-glycine (30 pmol) was also detected at cycle 1, reflecting the presence of some GYVVR peptide.

Table V: Clostripain Cleavage Products of S6 Resolved by Reversed-Phase HPLC

HPLC peak no.	sequence identified (single-letter amino acid notation)[a]	initial yield (pmol)	residue no. in S6
1a	KPLNKEGK	80	160–167
1b	MKEAKEKR	140	217–224
2	VLQHKR	201	184–189
3	QEqIAKR	100	225–231
4a	KPLNKEGKKP	122	160–169
4b	LGPKR	180	133–137
5	APKIQR	120	173–178
6	QGVLTHGR	180	65–72
7a	LVTPR	220	179–183
7b	TKAPKIQ(R)	150	171–178
7c	LSSLR	75	234–238
8	IALKKQ(R)	170	192–198
9	MKEAKEKRQEQIA	140	217–229
10a[b]	QYVVR	110	155–159
10b	GYVVR	30	47–51
11	TFYEKR	180	26–31
12	ISGGNDKQGFPMK	80	52–64
13	LLLSKGHSIYR	62	75–85
14	KLRTFYEKR	50	23–31
15	ISGGNDKQGFPMKQGVL	20	52–68
16	LLLSKG(H)SIY-P-	35	75–86
17[c]	KGEKDIPGLTDTTV	170	116–129
18	KLFNLSKEDDVR	190	143–154
19	MATEVAADALGEE-	120	32–45
20a	MATEVAADALGEE-KGYVVR	30	32–51
20b	LNISFPATGSQKLIEVDDE-(R)	90	3–22
21	GQIV-A-L-VL-LV	5	99–115

[a] The data were obtained from the analyses of clostripain digestion products of S6 resolved by HPLC (Figure 1c). Individual species in peaks 1, 4, 7, 15/16, and 20 were further resolved by rechromatography on a Vydac C_{18} column (see Experimental Procedures). Tentative identifications are given in parentheses. [b] The presence of two peptides was deduced from the yields of Pth–glutamine and Pth–glycine at the first cycle. The presence of the GYVVR sequence was more apparent in analyses of more extensively digested S6. [c] Analysis of Cl-17 was terminated after 14 cycles.

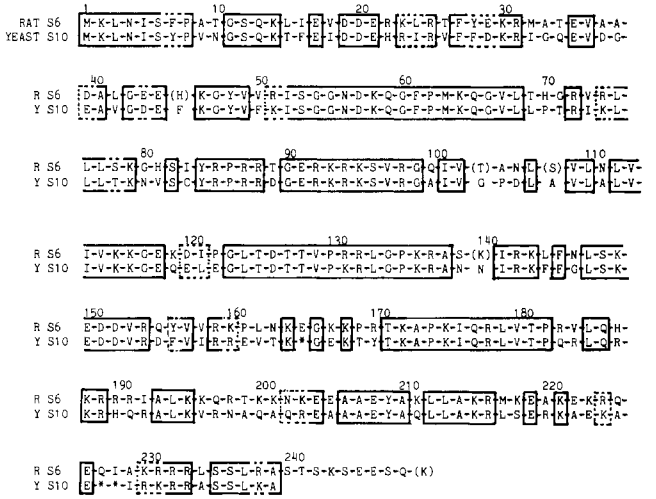

FIGURE 3: Alignment of amino acid sequences of rat liver S6 and yeast S10. The sequence of S6 (Figure 2) is compared with the sequence of yeast (*Saccharomyces cerevisae* and *Saccharomyces carlsbergensis*) ribosomal S10 deduced from the sequence of the S10-1 (Leer et al., 1983) and S10-2 (Leer et al., 1985) genes. The two sequences are aligned from the NH_2 terminus except where the presence of additional residues in the S6 sequence (positions 165, 227, and 228) is marked with an asterisk. The single-letter amino acid notation is used. Positions of identity and similarity between the two sequences are boxed in solid and dashed lines, respectively.

because of the low recoveries of Pth–histidine from the sequencer. The assignments of Thr-103 and Ser-107, which were encountered at a late stage of the sequence analyses of Ch-12 (Table III), were also based on weak signals. Finally, the assignment of lysine at position 140 was tentative because of the ambiguity of the analysis of the Ch-9a peptide.

Overall, S6 was determined to have 249 residues. The molecular weight calculated from the numbers of individual amino acid residues was 28 643, which was lower than our estimate of 34 000 from SDS–polyacrylamide gel analyses (Wettenhall & Howlett, 1979) but which was closer to the value of 31 000 obtained from sedimentation equilibrium analysis (Collatz et al., 1976). The total numbers of individual amino acid residues identified by sequence analysis were in general agreement with the amino acid composition of the protein determined on acid hydrolysates except for small discrepancies between the values for valine, phenylalanine, and glycine and for the presence of some cysteic acid in the hydrolysates, which was unaccounted for in the sequence analyses[3] (Table VI).

Table VI: Amino Acid Composition of S6

amino acid	composition of S6 (mol %)[a]	composition predicted from sequence analysis[b]
Asp	7.0	6.0 (8)
Asn		(7)
Thr	5.0	5.2 (13)
Ser	5.5	6.0 (15)
Glu	10.8	11.7 (18)
Gln		(11)
Pro	4.0	4.0 (10)
Gly	7.3	6.0 (15)
Ala	6.4	6.4 (16)
Val	5.8	6.8 (17)
Met	1.7	1.6 (4)
Ile	4.3	4.4 (11)
Leu	8.6	9.2 (23)
Tyr	2.3	2.0 (5)
Phe	2.2	1.6 (4)
His	1.8	1.6 (4)
Lys	13.4	14.9 (37)
Arg	12.2	12.4 (31)

[a] Composition was determined on acid hydrolysates of RP-HPLC-purified S6 [see Nick et al. (1985)]; acid hydrolysates of performic acid treated S6 contained 0.7 mol % of cysteic acid; tryptophan was not detected in either acid or alkaline hydrolysates of S6. [b] Number of residues identified by Pth–amino acid analysis shown in parentheses. The total number of residues assumed in the calculation of composition (249) includes Lys-249 which was inferred from the amino acid composition of the tryptic and chymotryptic COOH-terminal fragments (see text).

Conclusions

The alignment of overlapping fragments of S6 established an overall sequence of 249 residues (Figure 2). Further support for the alignment of the S6 fragments was provided by the sequence of yeast ribosomal protein S10 which displayed considerable homology throughout the sequence, with the overall sequence identity being 61% (Figure 3). A comparison of the two sequences (Figure 3) shows that S6 contains internal insertions corresponding to Glu-165, Gln-227, and Ile-228, plus a COOH-terminal extension of 10 residues (S6.240–249), thus explaining the slightly slower migration of S6 during SDS–polyacrylamide gel electrophoresis. The COOH extension in S6 contained several potential phosphorylation sites which may be seen as an important evolutionary difference, given the possible role of phosphorylation of this class of proteins in growth regulation (Kruse et al., 1985; Traugh & Pendergast, 1986). Close homology with other ribosomal proteins has not been found by direct comparisons with the available prokaryotic and eukaryotic ribosomal protein sequences (NBRF data base), although some localized regions of sequence similarities with other rat ribosomal proteins are evident.

The determination of the primary structure of S6 establishes the location of the phosphorylation sites previously identified in tryptic peptides. The preferred site for cAMP-dependent protein kinase corresponds to Ser-235 (Wettenhall & Cohen, 1982; Wettenhall & Morgan, 1984). Ser-236 constitutes the preferred site for major forms of protease-activated protein kinase although these enzymes can also phosphorylate Ser-235 (Gabrielli et al., 1984).[2]

A similar site preference (Ser-236) for protein kinase C was apparent from analyses of tryptic derivatives of ribosomal S6[2] as well as synthetic peptide analogues of S6 (House et al., 1987) phosphorylated by the purified enzyme.

[3] Cysteine residues can give rise to a similar signal to the dithiothreitol adduct of Pth–serine. Thus, an ambiguity exists at position 12 in the S6 sequence where serine identification was based solely on this adduct.

The possible existence of phosphorylation sites in the COOH-terminal region, in addition to the Ser-235 and Ser-236 sites, is suggested by the properties of highly acidic tryptic phosphopeptide derivatives of S6 from insulin- and platelet-derived growth factor (PDGF)-stimulated cells (Wettenhall et al., 1982, 1983; Wettenhall & Morgan, 1984). These peptides coeluted during Sephadex G-25 filtration with one or the other of the tryptic phosphopeptides termed T-1a (S6.233–243) and T-1c (S6.233–249) which were derived from S6 phosphorylated with cAMP-dependent protein kinase (Wettenhall et al., 1982; Wettenhall & Morgan, 1984). Further, the isoelectric focusing and HPLC characteristics of the derivatives from the insulin- and PDGF-treated cells were consistent with highly phosphorylated forms of the S6.233–243 and S6.233–249 sequences. The incomplete tryptic digestion of this region can be attributed to inhibitory effects of phosphorylated residues on neighboring tryptic cleavage sites (Wettenhall & Morgan, 1984). On inspection of the overall S6 sequence in the vicinities of the 15 serine residues, it is difficult to envisage how the tryptic phosphopeptides with the particular properties referred to above could have originated from regions of S6 other than the COOH-terminal region. However, until the individual phosphopeptides have been structurally defined, the existence of sites at other locations remains a possibility, particularly given the multiplicity of tryptic phosphopeptides in digests of S6 from growth factor stimulated cells.

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Registry No. Phosphoprotein (rat liver ribosome S6 protein moiety), 110874-13-0.

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Identification of 2-Enolbutyrate as the Product of the Reaction of Maize Leaf Phosphoenolpyruvate Carboxylase with (*Z*)- and (*E*)-2-Phosphoenolbutyrate: Evidence from NMR and Kinetic Measurements†

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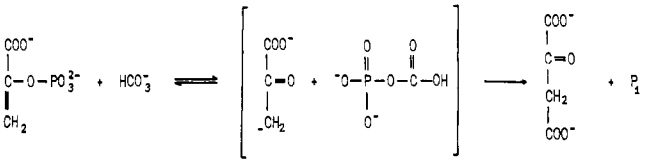
ABSTRACT: (*Z*)- and (*E*)-2-phosphoenolbutyrates were dephosphorylated at similar rates by phosphoenolpyruvate carboxylase purified from maize leaves, as determined from proton nuclear magnetic resonance measurements. The product of the reaction in D_2O was a mixture of 60–70% 2-oxo[3-H,D]butyrate, 25–30% 2-oxo[3-D_2]butyrate, and 5–10% 2-oxo[3-H_2]butyrate. The amounts of (*R*)- and (*S*)-2-oxo[3-H,D]butyrate in this mixture were determined by exchange at C-3 in D_2O catalyzed by pyruvate kinase as described by Hoving et al. [Hoving, H., Nowak, T., & Robillard, G. T. (1983) *Biochemistry 22*, 2832–2838]. Forty-five minutes after the addition of pyruvate kinase, the proportions of 2-oxo[3-H,D]butyrate and 2-oxo[3-D_2]-butyrate were 36–39% and 61–64%, respectively, indicating that the original mixture contained equal amounts of *R* and *S* enantiomers. In addition, a compound with properties similar to those of enolpyruvate was detected in solution during the action of phosphoenolpyruvate carboxylase on 2-phosphoenolbutyrate. This compound, most likely 2-enolbutyrate, presented maximum light absorption at 220–230 nm and was ketonized in a solution containing 80% D_2O and 20% H_2O (pH 7) with a rate constant of 1.33 min^{-1}. From these results, it is concluded that the actual product released from the active site of phosphoenolpyruvate carboxylase during the reaction with 2-phosphoenolbutyrate is the enolic form of 2-oxobutyrate and that protonation of this form takes place at random in solution.

Phosphoenolpyruvate (PEP)[1] carboxylase [orthophosphate: oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate [for reviews, see O'Leary (1982) and Andreo et al. (1987)]. This enzyme is especially important in the so-called C_4 plants, where it participates in photosynthetic CO_2 fixation (Edwards et al., 1985), but is also present in all other types of plants, algae, and bacteria, with different functions and properties depending on the source (Utter & Kolen-

Scheme I: Two-Step Mechanism Postulated for PEP Carboxylase (O'Leary et al., 1981)



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[1] Abbreviations: PEP, phosphoenolpyruvate; PEB, 2-phosphoenolbutyrate; PEIV, 2-phosphoenolisovalerate; LDH, lactic dehydrogenase.

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