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Primary Structures of Ribosomal Protein YS25 from *Saccharomyces cerevisiae* and Its Counterparts from *Schizosaccharomyces pombe* and Rat Liver†

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ABSTRACT: Protein YS25 and its counterparts, SP-S28 and rat S21 [nomenclature according to Sherton, C. C., & Wool, I. G. (1972) *J. Biol. Chem. 247*, 4460–4467], from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and rat liver cytoplasmic ribosomes, respectively, were sequenced by a combination of various enzymatic digestions and/or chemical cleavage. Proteins YS25 and SP-S28 consist of 87 amino acid residues, and rat S21 consists of 83. The amino termini are all N^{α}-acetylated. The amino-terminal halves of the protein molecules are highly conserved (73–85% homologies) in contrast to the carboxy-terminal parts. Overall, rat S21 is 54% homologous to YS25 and 57% to SP-S28, despite a 76% homology between YS25 and SP-S28. Direct comparison with the available prokaryotic ribosomal protein sequences did not reveal any significant homology.

In a previous paper, we presented amino-terminal amino acid sequences of various ribosomal proteins from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and rat liver cytoplasmic ribosomes. In total, those analyses suggested that *Sc. pombe* is relatively distantly related to yeast, *Sa. cerevisiae*, and rather close to rat in phylogenetic distance, in accord with the result deduced from 5S rRNA sequences (Otaka et al., 1983). In order to examine the evolutionary relationships further, we sequenced three homologous proteins, YS25, SP-S28, and rat S21, from the same organisms. Blocked amino termini of the proteins were determined in these eukaryotic ribosomal proteins. Recently, protein YS25 was shown to react with *Escherichia coli* S6 antisera, indicating an ancestral relationship of these eukaryotes and *E. coli* (Chooi & Otaka, 1984). The sequence homologies of the those proteins will be discussed in terms of protein evolution.

Materials and Methods

Main materials used here were purchased from the sources indicated in parentheses: cellulose thin-layer plates (Macherey-Nagel, FRG), polyamide sheets (Schleicher & Schüll,

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FRG), DABITC[1] and fluorescamine (Fluka, Switzerland), TPCK-trypsin and carboxypeptidase B (Worthington Diagnostic Systems), chymotrypsin (Worthington Biochemical Co.), *Staphylococcus aureus* V8 protease (Miles Laboratories), carboxypeptidase Y (Oriental Co., Japan), and carboxypeptidase P (Peptide Institute, Japan). Pyridine (Wako Pure Chemical Co., Japan) was redistilled, first over KOH pellets and then from ninhydrin. Other reagents were of the highest purity commercially available (from Tokyo Kasei Co., Japan, and Wako Pure Chemical Co., Japan) and were used without further purification.

Proteins YS25 and SP-S28 isolated from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* 80S ribosomes according to Otaka et al. (1983) were used. Rat S21 (Sherton & Wool, 1972) was isolated from rat liver 80S ribosomes (Ogata & Terao, 1981) as described for yeast ribosomal proteins.

Ribosomal proteins were reduced, S-carboxymethylated (Waxdal et al., 1968), and subjected to various cleavages. Enzymatic digestion with trypsin, chymotrypsin, and *Staphylococcus aureus* V8 protease, isolation of peptides, and amino acid analyses were performed according to Heiland et al. (1976), Itoh et al. (1982), and Itoh & Higo (1983). Digestion was with carboxypeptidase B in 0.1 M methylmorpholine–acetate buffer (pH 8.1), with carboxypeptidase Y in 0.1 M pyridine–acetate buffer (pH 5.5), and with carboxypeptidase P in 0.1 M acetic acid at an enzyme/substrate molar ratio of about 1:50. Cleavage of protein at methionine by cyanogen bromide was made as described by Steers et al. (1965). Determination of amino acid sequences of peptides with a free amino terminus was by the manual DABITC/PITC double-coupling method (Chang et al., 1978); isoleucine was assigned from the amino acid composition of the peptides or identified by amino acid analysis after conversion of the DABTH derivatives in HI (130 °C).

To obtain the blocked amino-terminal peptides, enzymatic digests were loaded on a cation-exchange column of AG 50W-X2 (0.5 × 1 cm) and the peptides were eluted with 10 mL of H_2O (acidic fraction) (Tsunasawa & Narita, 1981). Detection of the amino-terminal acetyl group was by hydrazinolysis (Schmer & Kreil, 1969) and by chromatography on micro polyamide layers (Takagi & Doolittle, 1974).

Results

Sequence Determination of Protein YS25. Reduced and S-carboxylmethylated protein YS25 was cleaved with trypsin, and all peptides isolated by thin-layer fingerprinting (Figure 1A), except for T1, were sequenced (Figure 2). A blocked amino terminus in YS25 was suggested by the failure to find a DABTH-amino acid by DABITC/PITC degradation of T1 or of YS25. Staining of T1 was positive with starch/iodide solution (Rydon & Smith, 1952) but not with fluorescamine, and peptide T1 (residues 1–15) appeared as two spots due to partial oxidation of methionine to methionine sulfoxide. Hydrazinolysis of the peptides, followed by dansylation at low pH, gave 1-acetyl-2-dansylhydrazide indicating that the amino terminus is blocked by N^{α}-acetylation. T1 cleaved with *St. aureus* V8 protease yielded three subpeptides, T1-SP1, T1-SP2, and T1-SP3. Their amino acid compositions accorded with that of T1 (Table I). The sequence of T1-SP1 was Ac-Met-Glu as judged by the reaction with starch/iodide

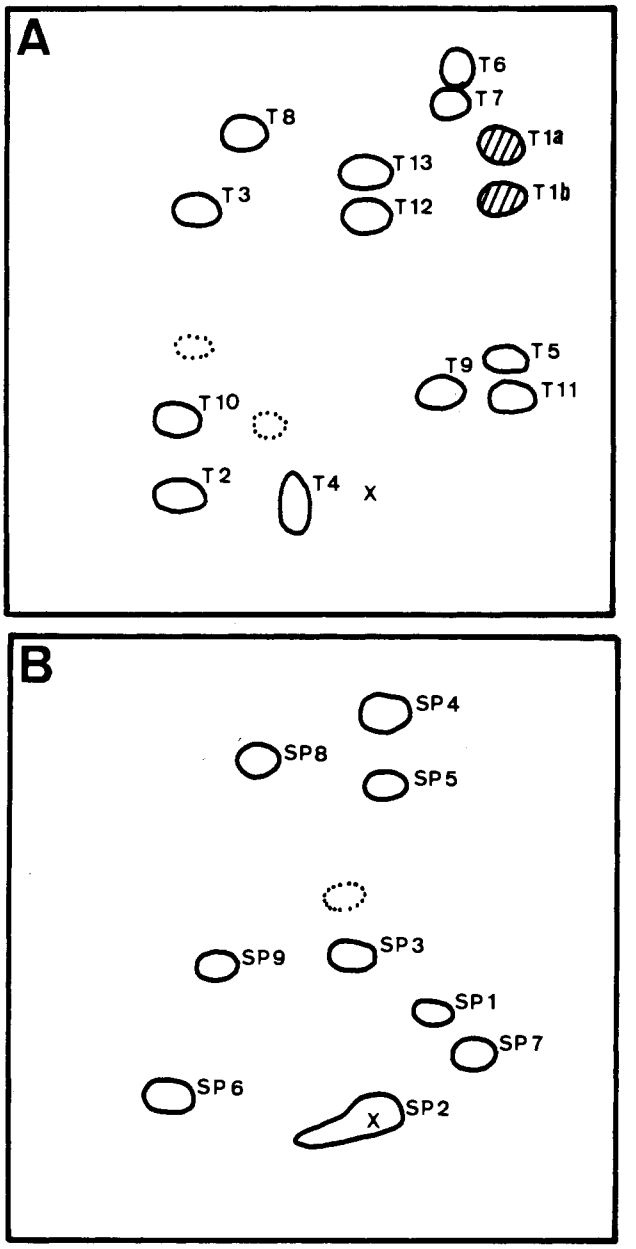

FIGURE 1: Thin-layer fingerprints of enzymatic digests of ribosomal protein YS25 from *Sa. cerevisiae*: (A) tryptic digest; (B) *St. aureus* V8 protease digest. The first dimension (horizontal) was electrophoresis at pH 6.5 in pyridine/acetic acid/water (100:3:900 v/v/v), at 400 V for 80 min [cathode (left), anode (right)]. The second dimension (vertical) was chromatography in a system composed of pyridine/1-butanol/acetic acid/water (10:15:3:12 v/v/v/v) from bottom to top. Peptides with a free amino terminus (open spot) were detected with 0.004% fluorescamine/acetone solution under UV light; peptides with a blocked amino terminus (hatched spots) were detected by the chlorine/starch/iodide method (Rydon & Smith, 1954). Dotted spots indicate minor peptides generated by incomplete or unspecific digestion. X denotes the origin.

solution, the amino acid composition, and the cleavage specificity of *St. aureus* V8 protease. T1-SP3 must be the carboxyl-terminal peptide of T1 as it contains a carboxyl-terminal arginine. Thus, the alignment SP1–SP2–SP3 in T1 was obtained. This was confirmed by the first 13-residue sequence of a cyanogen bromide fragment of YS25 starting at position 2 of the protein. T7 and T8 were derived from the amino- and carboxyl-terminal sequences of T6, respectively, by a chymotrypsin-like cleavage of Tyr-Ala (residues 53 and 54). T9 containing T10 and T11 as its amino- and carboxyl-terminal portions was generated by an incomplete cleavage of

[1] Abbreviations: DABITC, 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate; PITC, phenyl isothiocyanate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene 4′-thiohydantoin; Ac, acetyl.

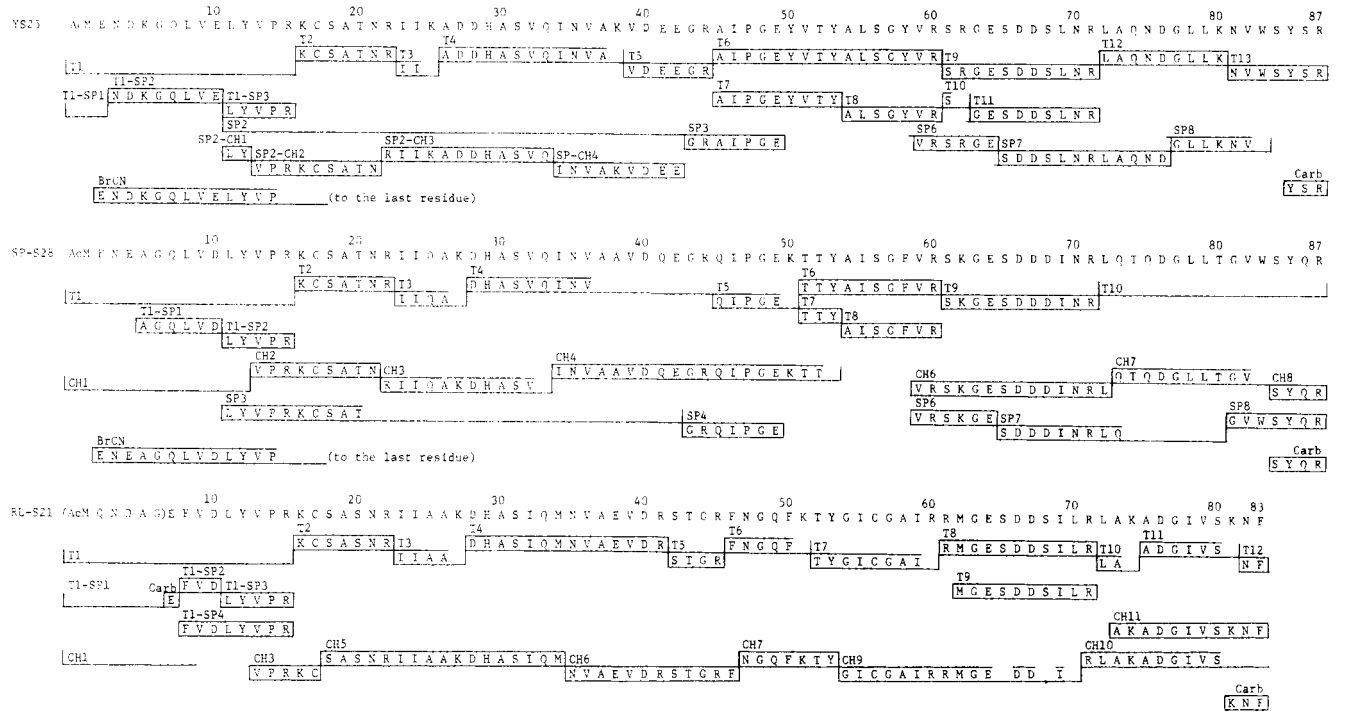

FIGURE 2: Sequence determination of ribosomal proteins YS25, SP-S28, and RL-S21 from *Sa. cerevisiae*, *Sc. pombe*, and rat liver, respectively. The one-letter code within bars designates amino acids identified. The length of each bar indicates the proportional length of that peptide. Enclosure of the top indicates a proven sequence; gaps in the upper enclosure signify portions of that sequence that were not identified. Residues identified by carboxypeptidase digestion (Carb) are also within the enclosure. Tryptic, *St. aureus* V8 protease, chymotryptic, and cyanogen bromide peptides are designated by the prefixes T, SP, CH, and BrCN, respectively. Residues in parentheses, the first six residues of RL-S21, are the most probable alignment deduced from the data available. See the text for further details.

Table I: Amino Acid Compositions of Tryptic Peptides (T) of Protein YS25[a]

amino acid	T1[b]	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
Cmc[c]		0.8 (1)											
Asp	2.2 (2)	1.3 (1)		3.6 (3)	1.3 (1)				2.9 (3)		3.1 (3)	2.1 (2)	1.3 (1)
Thr		1.1 (1)				1.0 (1)	1.0 (1)						
Ser		1.0 (1)		0.9 (1)		0.8 (1)		0.9 (1)	2.4 (3)	0.9 (1)	1.7 (2)		1.8 (2)
Gul	2.8 (3)			1.1 (1)	2.0 (2)	1.1 (1)	1.1 (1)		1.1 (1)		1.3 (1)	1.0 (1)	
Pro	1.0 (1)					0.8 (1)	0.9 (1)						
Gly	1.2 (1)				1.1 (1)	2.2 (2)	1.2 (1)	1.2 (1)	1.0 (1)		1.2 (1)	1.0 (1)	
Ala		1.0 (1)		2.8 (3)		2.3 (2)	1.2 (1)	1.0 (1)				1.0 (1)	
Val	2.0 (2)			2.1 (2)	1.0 (1)	1.8 (2)	0.8 (1)	1.1 (1)					1.1 (1)
Met	0.7 (1)												
Ile			1.5 (2)	1.0 (1)		1.3 (1)	1.3 (1)						
Leu	2.1 (2)					1.0 (1)		1.0 (1)	0.9 (1)		1.0 (1)	2.7 (3)	
Tyr	0.8 (1)					2.7 (3)	1.5 (2)	0.7 (1)					0.8 (1)
Phe													
His				1.0 (1)									
Lys	0.8 (1)	0.6 (1)	1.0 (1)	1.0 (1)								0.9 (1)	
Trp													+ (1)[d]
Arg	1.0 (1)	1.0 (1)			0.9 (1)	1.0 (1)		0.9 (1)	2.1 (2)	1.0 (1)	0.9 (1)		1.0 (1)
tot residues	15	7	3	13	6	16	9	7	11	2	9	9	7

[a] Values are expressed as molar ratio. Numbers of residues determined by sequence studies are in parentheses. [b] The peptide was detected with KI/starch solution and located at two positions (T1a and T1b) within the fingerprint because of aut-oxidation of methionine. [c] (Carboxymethyl)-cysteine. [d] Tryptophan was detected on thin-layer fingerprint by spraying with the Ehrlich reagent.

Arg-Gly (residues 62 and 63). T13 was recognized as follows: YS25 hydrolyzed with carboxypeptidase B released Arg (1.00), Ser (0.16), and Tyr (0.10) during 1 h of incubation; carboxypeptidase B (1-h incubation) and additional carboxypeptidase Y (1-h incubation) treatment released Arg (1.00), Trp (0.90), Tyr (1.00) and Ser (1.94), indicating the carboxyl-terminal peptide of YS25 having Tyr-Ser-Arg in its carboxyl-terminal portion.

The five major peptides drived from *St. aureus* V8 protease digests, except for SP2, were sequenced directly [Figures 2 and 1B; Table II of supplementary material (see paragraph at end of paper regarding supplementary material)]. Chymotryptic digestion of the peptide SP2 yielded subpeptides SP2-CH1, SP2-CH2, SP2-CH3, and SP2-CH4. As SP2-CH2 contained T1-SP3 and the first six residues of T2, T1 and T2 were aligned. Sequences of subpeptides SP2-CH3 and SP2-CH4 served to align T2 to T5, T5–T6 ordered by the sequence of SP3. Sequences of SP6, SP7, and SP8 allowed the alignments of T6–T9, T9–T12, and T12–T13, respectively. In this way, the primary amino acid sequence of YS25 was determined as given in Figure 2.

Sequence Determination of Protein SP-S28. Seven tryptic peptides, T2, T3, T5, T6, T7, T8, and T9, were completely sequenced (Figure 2; Figure 1A, and Table III of supplementary material). T7 and T8 were derived from the amino- and carboxyl-terminal sequences of T6 by a chymotrypsin-like

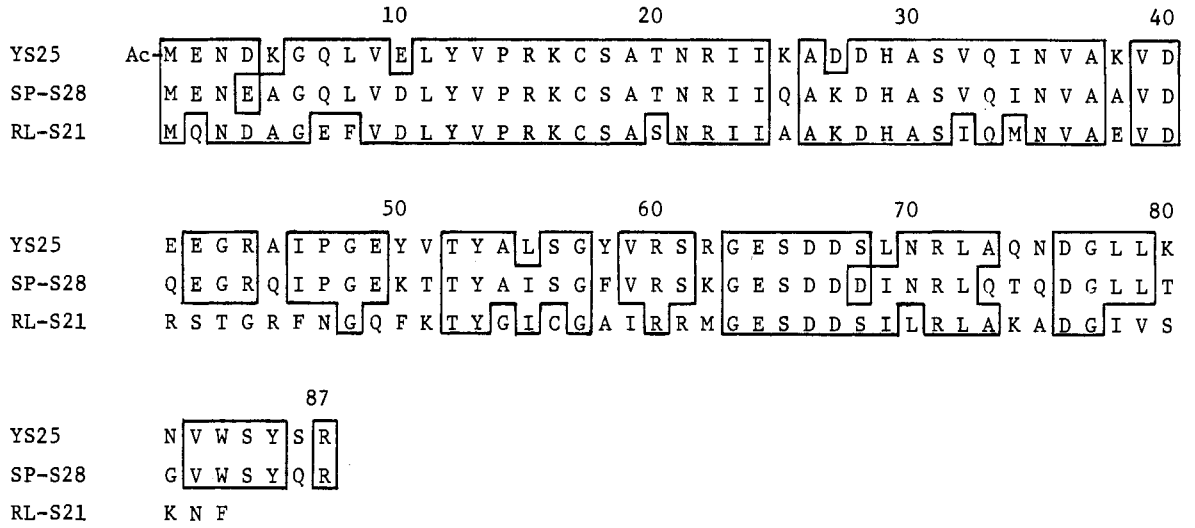

FIGURE 3: Comparison among sequences of ribosomal proteins YS25, SP-S28, and RL-S21 from *Sa. cerevisiae*, *Sc. pombe*, and rat liver, respectively. The solid boxes indicate identical residues.

cleavage (residues 53 and 54) as observed in YS25 (residues 53 and 54). T1 was found to have an amino terminus blocked by N^{α}-acetylation. Subpeptide T1-SP2 by *St. aureus* V8 protease cleavage of T1 was the carboxyl-terminal portion of T1 due to the carboxyl-terminal arginine. The sequence of the blocked amino-terminal portion was done by sequencing of subpeptide T1-SP1 and a cyanogen bromide fragment of protein SP-S28. The first nine residues of T4 were sequenced, and the remainder was obtained by sequencing of CH4 starting at the middle region, residues 34–36, of T4. Determination of T10 was deduced from other peptide analyses because of its low yield from thin-layer sheets.

The chymotryptic peptides, except for CH1, were sequenced directly (Figure 2; Figure 1B and Table IV of supplementary material). CH1 contained the blocked amino-terminal portion of SP-S28. The sequences of CH2, CH3, and CH6 served to align T1–T2, T2–T3, and T6–T9 and of CH4 to align T4 through T6. CH7 covered parts of T10 due to its amino acid composition. CH8 was the carboxyl-terminal peptide of SP-S28 as SP-S28 treatment with carboxylpeptidase B resulted in the release of Arg (1.00), Gln or Ser (0.54), and Tyr (0.50).

The peptides from *St. aureus* V8 protease digests were sequenced either partially or entirely (Figure 2; Figure 1C and Table V of supplementary material). The sequence of the first 10 residues of SP3 provided the alignment of T1–T2; the sequences of SP4 and SP6 provided the alignment of T4–T5 and T6–T9, respectively. SP8 and the first nine residues of SP7 served to align CH6–CH7 and CH7–CH8, respectively. Thus, the sequence of CH6 to CH8 established that of T10 and aligned T9–T10. Finally, the primary amino acid sequence of SP-S28 resulted as given in Figure 2.

Sequence Determination of Protein Rat S21 (RL-S21). All tryptic peptides, except for T1, were sequenced completely (Figure 2; Figure 2A and Table VI of supplementary material). T1 was carrying the N^{α}-acetylated amino terminus of S21. The sequence of the first part of T1 was made likely as follows: hydrolysis with carboxypeptidase P of subpeptide T1-SP1 released mainly Glu during 10 min of incubation and Asp (0.92), Gln (0.86), Asn (1.00), Glu (0.94), Gly (0.90), and Ala (0.89) during 2.5 h of incubation, indicating that the amino terminus of T1-SP1 is acetylmethionine and the carboxyl terminus is glutamic acid. The further sequence of T1-SP1 was deduced from the sequence homologies with YS25 and SP-S28 (Figure 2). The sequence of the carboxyl-terminal portion of T1 was derived from subpeptides T1-SP2, T1-SP3, and T1-SP4. T8 originates from incomplete cleavage of an Arg–Met bond (residues 61–62), caused by adjacent basic residues (residues 60 and 61). T12 without any lysine or arginine at the carboxyl terminus (residues 82 and 83) was placed at the carboxyl-terminal peptide of S21. This was confirmed by release of Phe (1.00), Asn or Ser (0.76), and Lys (0.60) during 1-h incubation of S21 with carboxylpeptidase Y.

The chymotryptic peptides, except for CH1, were sequenced either partially or entirely (Figure 2; Figure 2B and Table VII of supplementary material). The sequence of CH3 served of align T1–T2. The sequences of CH5, CH6, CH7, CH9, CH10, and CH11, in turn, provided alignment of T2 through T12. Thus, the primary amino acid sequence of rat S21 was deduced as given in Figure 2.

Discussion

The amino acid sequences of proteins YS25, SP-S28, and rat S21 are established as shown in Figure 2. The sequences agree with their amino acid compositions (Table II), and that of the rat agrees with the values obtained by Collatz et al. (1977). The amino-terminal sequence up to the sixth residue of rat S21 is represented as the most probable alignment as deduced from the analytical data.

Proteins YS25 and SP-S28 from the two yeast species consist of 87 amino acid residues whereas rat S21 (RL-S21) is shorter by four amino acids. The shorter sequence of rat S21 is the result of a deletion in the carboxyl-terminal region (Figure 3). To compare their sequence homologies, the identical residues were estimated (Table III). In the first half of the molecule, the three sequences show extensive homology; however, in the latter half of the two yeasts are still homologous but vary significantly from rat. The extensive homology over the entire length of the molecules documents the close relationship between the two yeasts. The sequence data indicate that the amino-terminal portion of the three protein species has been conserved highly throughout the course of evolution, indicating its structural or functional importance.

By comparing several amino-terminal sequences of ribosomal proteins from these three organisms, we suggested that *Sc. pombe* is more sequence-related to that of rat rather than to *Sa. cerevisiae* (Otaka et al., 1983). The amino–terminal sequence data together with the data in Table III indicate a similar extent of sequence conservation among the three organisms. Sequence conservation has been shown in three

Table II: Amino Acid Compositions of Three Ribosomal Proteins (A) from Hydrolysate of the Intact Protein[a] and (B) from Sequence Determination

amino acid	*Sa. cerevisiae* YS 25		*Sc. pombe* SP-S28		rat liver S21	
	A	B	A[c]	B	A	B
Cys	ND[b]	1	ND	1	ND	2
Asx	12.6	13	10.6	11	12.2	12
Thr	2.5	2	4.8	5	2.6	2
Ser	6.9	8	5.5	6	6.8	7
Glx	9.7	9	12.8	13	6.3	6
Pro	2.5	2	2.9	2	1.8	1
Gly	6.2	6	7.2	7	7.5	7
Ala	7.3	7	7.2	7	9.3	9
Val	7.5	8	7.2	7	5.2	5
Met	1.0	1	1.0	1	2.0	3
Ile	4.1	4	5.4	6	6.7	7
Leu	7.1	7	5.2	5	3.9	3
Tyr	4.6	5	3.0	3	1.1	2
Phe	0.4	0	1.2	1	4.1	4
His	1.2	1	1.1	1	1.2	1
Lys	5.2	5	4.3	4	5.5	5
Trp	ND	1	ND	1		
Arg	6.4	7	5.8	6	6.8	7
tot residues		87		87		83

[a] Values are given as residues per mole of ribosomal proteins for 24-h hydrolysis. No corrections for incomplete hydrolysis or for decomposition were made. [b] Not determined. [c] Otaka et al. (1984).

Table III: Sequence Homology of Proteins YS25, SP-S28, and Rat S21

protein	region	homology[a] YS25	homology[a] SP-S28
SP-S28	1–40[b]	85	
	1–83	76	
	41–83	67	
RL-S21	1–40	73	78
	1–83	54	57
	41–83	37	37

[a] Percent of identical residues per examined residues. [b] Residue number of sequence.

eukaryote-type ribosomal "A" proteins and in YL35 and rat L37 (Lin et al., 1982, 1983); in contrast, the eukaryotic "A" proteins differ from the prokaryotic "A" proteins (Itoh & Otaka, 1984). The termini of YL2 and its counterparts from *Sc. pombe* and rat liver are another example of this conservation (Otaka et al., 1983).

Otaka et al. (1984) reported that the frequency of the blocked amino termini in yeast small subunit proteins is greater than found in those of *E. coli* (Wittmann-Liebold, 1973; Isono, 1980) and *Bacillus subtilis* (Higo et al., 1982). The small ribosomal subunit proteins that are blocked in *E. coli* are different than those blocked in *B. subtilis* (Higo et al., 1982). However, the first amino-terminal blocked eukaryotic ribosomal proteins determined here for YS25, SP-S28, and rat S21 were found to have N^{α}-acetylmethionine, which is a common prokaryotic modification (Isono, 1980; Higo et al., 1982). The biological significance of those posttranslational modifications is still not clear.

In summary, the evolutionary relationships of the two yeast species and the corresponding rat proteins on the basis of their complete sequences resemble that deduced from 5.8S rRNA sequences, not from the 5S rRNA; i.e., a closer relationship between the two yeasts than between *Sc. pombe* and rat is indicated (Schaak et al., 1982; Hori et al., 1982).

Although YS25 and its equivalent proteins from *Drosophila melanogaster* and rabbit liver have been reported to be related to protein *E. coli* S6 immunologically (Chooi & Otaka, 1984), the direct comparison with the *E. coli* S6 sequence did not show any significant sequence homology. An ancestral relationship among them was shown by means of a computer program that searches for tertiary structural homologies (Kubota et al., 1981, 1982; Otaka et al., 1985).

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SUPPLEMENTARY MATERIAL AVAILABLE

Seven tables showing amino acid compositions of peptides and subpeptides and two figures showing thin-layer fingerprints of enzymatic digests (10 pages). Ordering information is given on any current masthead page.

Registry No. Protein YS25, 98820-51-0; protein SP-S28, 98820-50-9; protein rat S21, 98820-49-6.

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Type IV Collagen Synthesis by Cultured Human Microvascular Endothelial Cells and Its Deposition into the Subendothelial Basement Membrane†

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ABSTRACT: Cultured microvascular endothelial cells isolated from human dermis were examined for the synthesis of basement membrane specific (type IV) collagen and its deposition in subendothelial matrix. Biosynthetically radiolabeled proteins secreted into the culture medium were analyzed by sodium dodecyl sulfate gel electrophoresis after reduction, revealing a single collagenous component with an approximate M_r of 180 000 that could be resolved into two closely migrating polypeptide chains. Prior to reduction, the 180 000 bands migrated as a high molecular weight complex, indicating the presence of intermolecular disulfide bonding. The 180 000 material was identified as type IV procollagen on the basis of its (1) selective degradation by purified bacterial collagenase, (2) moderate sensitivity to pepsin digestion, (3) immunoprecipitation with antibodies to human type IV collagen, and (4) comigration with type IV procollagen purified from human and murine sources. In the basement membrane like matrix elaborated by the microvascular endothelial cells at their basal surface, type IV procollagen was the predominant constituent. This matrix-associated type IV procollagen was present as a highly cross-linked and insoluble complex that was solubilized only after denaturation and reduction of disulfide bonds. In addition, there was evidence of nonreducible dimers and higher molecular weight aggregates of type IV procollagen. These findings support the suggestion that the presence of intermolecular disulfide bonds and other covalent interactions stabilizes the incorporation of the type IV procollagen into the basement membrane matrix. Cultured microvascular endothelial cells therefore appear to deposit a basal lamina-like structure that is biochemically similar to that formed in vivo, providing a unique model system that should be useful for understanding microvascular basement membrane metabolism, especially as it relates to wound healing, tissue remodeling, and disease processes.

The basal lamina underlying most capillaries serves several important functions, such as acting as a selective permeability barrier and providing support and orientation for the endothelium (Vracko, 1974; Caufield & Farquhar, 1978). During wound healing and tissue remodeling, the microvasculature must invade surrounding tissue, a process that necessitates modifications in the basal lamina. The composition of the microvascular basal lamina has been determined to some degree by the use of immunohistochemistry (Kleinman et al., 1982; Timpl et al., 1979; Hassel et al., 1980). Accurate biochemical studies are hampered, however, by difficulties in obtaining sufficient quantities of purified material and by the high insolubility of these structures. The presence of aldehyde-derived and disulfide intermolecular cross-linking in the basement membrane has frustrated attempts to characterize individual components without resorting to proteolysis. However, the availability of cell cultures that retain the correct phenotypic expression of basal lamina production would provide an appropriate system for the detailed analysis of the process. Using such a system, it may be possible to delineate the steps involved in the secretion and assembly of matrix components into the basal lamina and to evaluate alterations that occur in diseases of the microvasculature.

Type IV collagen has been shown to be specific to basal laminae and is believed to act as the major structural protein in this matrix (Timpl et al., 1981; Yurchenco & Furthmayr, 1984). It would be expected, then, that if cultured microvascular endothelial cells maintain their correct differentiated phenotype, they would secrete type IV collagen as the primary collagen type. The present study describes the synthesis of type IV procollagen by cultured human microvascular cells and its deposition into the subendothelial matrix.

Experimental Procedures

Cell Culture. Microvascular endothelial cells were isolated from the dermis of newborn human foreskin as reported (Davison et al., 1980; Kramer et al., 1984, 1985). Cells were

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