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Cell-free synthesis and segregation of β_2-microglobulin

(signal hypothesis/histocompatibility antigens/radiochemical sequence analysis/peripheral membrane proteins)

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ABSTRACT **β_2-Microglobulin has been synthesized *in vitro* by using a rabbit reticulocyte lysate system and mRNA from the mouse tumor cell line EL4. The molecule is synthesized as a precursor with an NH_2-terminal extension of 19 amino acids: Ser-X-Ser-Val-X-Leu-Val-Phe-Leu-Val-Leu-Val-Ser-Leu-X-Gly-Leu-Tyr-X. The processing and segregation of this peripheral membrane protein are directly comparable to those of secretory proteins and integral membrane proteins: addition of dog pancreas microsomal membranes during translation caused conversion to the processed chain, but addition of membranes after synthesis did not; only the processed chain sedimented with the membrane vesicles and was protected from proteolysis by the vesicles; and processing of nascent β_2-microglobulin was blocked by competitive inhibitors that prevent processing and segregation of secretory and integral membrane proteins. These results suggest that the signal sequences of secretory proteins, integral membrane proteins, and peripheral membrane proteins have a common function and a common receptor on the cytoplasmic face of dog pancreas microsomal membranes. This system also provides a means for studying *in vitro* the expression and function of the major histocompatibility antigens that are associated with β_2-microglobulin on cell surfaces.**

β_2-Microglobulin is a low molecular weight protein (1) that is found on the surfaces of nearly all cells where it is associated with the major histocompatibility antigens, such as the H-2 antigens in mice and the HLA antigens in man (2–5), and related molecules (6–8). It is not an integral membrane protein in that it does not span the membrane and is not inserted into the lipid bilayer. It is closely, but noncovalently, associated with integral membrane proteins and can be removed by agents such as 3 M thiocyanate. By these criteria, it is one type of peripheral membrane protein (9). The molecule is also found free in various biological fluids including urine, serum, amniotic fluid, milk, and seminal fluid (10). Initially discovered in man, β_2-microglobulin is probably present in nearly all vertebrate species (10, 11), and one such molecule from cow's milk has recently been crystallized (12). A distinguishing feature of this protein is that it resembles the constant region domains (CL, CH1, CH2, and CH3) of immunoglobulins in overall structure (13), in amino acid sequence (14–16), and in the arrangement of its disulfide bond (16). The functions of β_2-microglobulin and the cell surface antigens with which it is associated are unknown. One approach to elucidating the functions of these antigens might be to construct cell-free systems in which molecular interactions could be dissected.

The "signal hypothesis" for the segregation of certain classes of newly synthesized proteins (17) has been confirmed in the case of a large number of secretory proteins (18–21) and several integral membrane proteins (22–24). For both classes, it has been demonstrated that common early events mediate insertion and chain transfer across the microsomal membrane via a ribosome–membrane junction (23). Integral membrane proteins, however, have been proposed to contain a "stop transfer" sequence whose function is to abrogate the ribosome–membrane junction, dissolve the tunnel, and deposit the nascent chain in a correct transmembrane orientation (23, 25).

Cell surface proteins whose interactions with the membrane do not involve integration within the hydrophobic regions of the lipid bilayer would be expected to follow the general biosynthetic plan of secretory proteins. β_2-Microglobulin offers a suitable system for *in vitro* studies of the biosynthesis of this class of membrane proteins.

As a first step toward construction of a cell-free system for the study of the coordinate synthesis of β_2-microglobulin and its associated integral membrane protein (H-2 or HLA antigens), we present here data on the biosynthesis of β_2-microglobulin including its synthesis, segregation, and proteolytic processing. Our results demonstrate that β_2-microglobulin follows a biosynthetic plan indistinguishable in its earliest stages from that of secretory proteins. It is synthesized as a nascent precursor containing a signal sequence of 19 amino acids that is cleaved during the process of cotranslational segregation within microsomal vesicles. Processing of nascent β_2-microglobulin is blocked by a competitive inhibitor previously shown to block the processing and segregation of both secretory and integral membrane proteins, suggesting a common function for the signal sequences of all of these classes of proteins and a common receptor on the cytoplasmic face of microsomal membranes.

MATERIALS AND METHODS

mRNA was isolated from EL4 tumor cells passaged in ascites form in C57BL/6 mice. Anti-mouse β_2-microglobulin was prepared in rabbits by using β_2-microglobulin prepared from liver membranes (26) as antigen; anti-human β_2-microglobulin was also prepared in rabbits by using, as antigen, urinary β_2-microglobulin kindly supplied by Ingemar Berggård. Preparation of mRNA (18), cell-free synthesis in the reticulocyte lysate (27, 28), polyacrylamide gel electrophoresis in sodium dodecyl sulfate (17), and immunoprecipitation and preparation of samples for sequence analysis (23) were carried out as described. Automated Edman degradation of products synthesized *in vitro* was carried out in a Beckman model 890C sequencer equipped with a Sequemat P-6 automatic converter

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Abbreviation: OV_s, tryptic peptide of chicken ovalbumin that includes its internal uncleaved signal sequence.

and using a 0.28 M Quadrol program. Sperm whale myoglobin and ovalbumin (1 mg each) were added as carriers. Sequencer fractions were dried in scintillation vials and 10 ml of Liquifluor was added; ^{35}S and ^{3}H radioactivities were measured with a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Translation of total RNA from EL4 cells in a nuclease-treated reticulocyte lysate system yielded [^{35}S]methionine-labeled polypeptide products ranging from 5000 to 70,000 in M_r (Fig. 1, lane 1). Immunoprecipitation with a rabbit antibody to mouse β_2-microglobulin (Fig. 1, lane 3) yielded one major and one minor component in the region of the gel corresponding to proteins of M_r 10,000–20,000 whereas immunoprecipitation with rabbit antibody against human β_2-microglobulin (Fig. 1, lane 4) gave only the major component. In contrast, no radioactive components were precipitated by a nonimmune serum (Fig. 1, lane 2).

From previous studies of the *in vitro* synthesis system (20), we assumed that the major component in the immunoprecipitates (Fig. 1, lanes 3 and 4) was a precursor of mouse β_2-microglobulin (pre-β_2-microglobulin). Its mobility in the gel suggested that it was about 2000 daltons larger than authentic β_2-microglobulin (M_r 11,800) as expected for such a precursor, and addition of excess unlabeled human β_2-microglobulin inhibited precipitation of this material by both antisera (data not shown). When microsomal membranes from dog pancreas were added during translation (Fig. 1, lanes 7–9), the amount of this component decreased and a new component with a molecular weight corresponding to that of native β_2-microglobulin (Fig. 1, lane 5) appeared. We assume that this material is processed

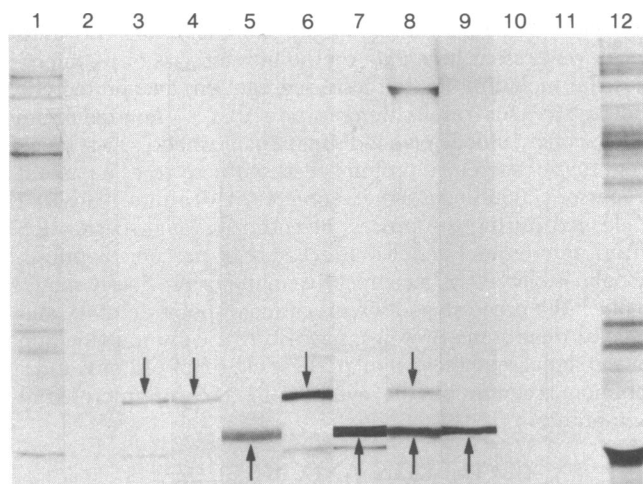

FIG. 1. Synthesis and processing of pre-β_2-microglobulin. Translations were carried out in rabbit reticulocyte lysate programmed with EL4 RNA in the presence of [^{35}S]methionine. Dog pancreas microsomal membranes, pretreated with nuclease, were present during translation in some cases. The products of translation were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis either directly or after precipitation with the appropriate antiserum. Labeled proteins were visualized by autoradiography. Lanes: 1–4, no microsomal membranes present; 5, β_2-microglobulin standard; 6, microsomal membranes added after translation; 7–12, translation in the presence of microsomal membranes (lanes 7, 9, 10, 11, and 12, 2.0 A_{260} units of membranes per ml; lane 8, 1.0 A_{260} unit of membranes per ml). Lanes 1 and 12, total translation products; lanes 2 and 11, precipitation with nonimmune serum; lanes 3, 6, and 7, precipitation with rabbit anti-mouse β_2-microglobulin; lanes 4, 8, 9, and 10, precipitation with rabbit anti-human β_2-microglobulin (lane 10, excess unlabeled human β_2-microglobulin present during precipitation).

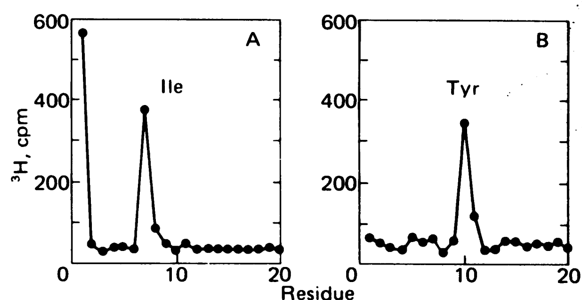

FIG. 2. Sequence analysis of processed β_2-microglobulin. The radioactivity in the fraction obtained in each cycle of degradation was determined by liquid scintillation spectrometry. The processed β_2-microglobulin was synthesized *in vitro* in the presence of microsomal membranes with [^{35}S]methionine and the indicated tritiated amino acid present; after immunoprecipitation it was purified by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Sequence results have been corrected for overlap of ^{35}S into the ^{3}H channel.

β_2-microglobulin. This component, like the putative precursor molecule, was immunoprecipitated with both anti-mouse and anti-human β_2-microglobulin (Fig. 1, lanes 7 and 9) but was not precipitated with nonimmune serum (Fig. 1, lane 11) or with the anti-human serum in the presence of excess unlabeled human β_2-microglobulin (Fig. 1, lane 10). The extent of decrease in the amount of the putative precursor and the increase in the amount of the apparent processed molecule was dependent upon the concentration of microsomal membranes (Fig. 1, lanes 8 and 9) and did not occur when membranes were added after completion of translation (Fig. 1, lane 6). These results are similar to those previously obtained for secretory and integral membrane proteins (18–21, 23, 29).

To establish that the two components described above are in fact the precursor of β_2-microglobulin and processed β_2-microglobulin, radiosequence analyses were performed on both products (Figs. 2 and 3). Sequence analysis of the smaller

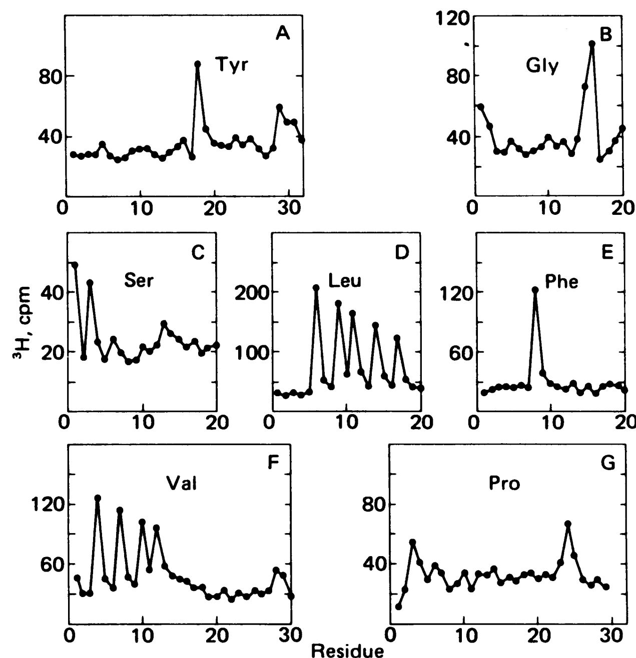

FIG. 3. Amino acid sequence analysis of pre-β_2-microglobulin. Radioactive pre-β_2-microglobulin was prepared for sequencing as described in Fig. 2, except that microsomal membranes were absent from the translation mixture.

	1				5					10					15					↓20					25				
PRE-β_2-MICROGLOBULIN:	SER	X	SER	VAL	X	LEU	VAL	PHE	LEU	VAL	LEU	VAL	SER	LEU	X	GLY	LEU	TYR	X	X	X	X	X	PRO	X	X	X	VAL	TYR
PROCESSED CHAIN:																				ILE	X	X	X	X	X	ILE	X	X	TYR
AUTHENTIC MOLECULE:																				ILE	GLN	LYS	THR	PRO	GLN	ILE	GLN	VAL	TYR
																				1				5					10

FIG. 4. Sequences of *in vitro*-synthesized pre-β_2-microglobulin, processed β_2-microglobulin, and authentic mouse β_2-microglobulin.

component obtained in the presence of microsomal membranes (Fig. 2) gave isoleucine and tyrosine at the same positions as in the published sequence of murine β_2-microglobulin (30). This result, together with the immunoprecipitation and molecular weight data, is consistent with the hypothesis that this component is processed β_2-microglobulin; it suggests further that the dog pancreas microsomal membranes contain enzymes that correctly cleave the precursor to generate authentic β_2-microglobulin.

The putative precursor (Fig. 3) contains proline at position 24, valine at position 38, and tyrosine at position 29. These results are consistent with the assumption that the sequence of β_2-microglobulin begins at position 20 of the precursor (Fig. 4). This alignment suggests that pre-β_2-microglobulin has an NH_2-terminal extension of 19 amino acid residues.

The residues at positions 2, 5, 15, and 19 as yet have not been unequivocally identified. Sequence analysis using [^{3}H]glycine suggested that there was some glycine at position 15, but the level was low; higher yields were obtained at this position when the protein was labeled with [^{3}H]tryptophan, suggesting that position 15 is a tryptophanyl residue. Preliminary studies suggest that the residues at positions 2 and 19 are most likely alanine.

The role of the NH_2-terminal extensions (signal sequences) of secretory and integral membrane proteins is presumably (17, 23) to catalyze formation of a ribosome–membrane junction and a tunnel for transfer of the growing chain across the membrane. Consistent with this hypothesis, only the processed form of the β_2-microglobulin molecule was protected by microsomal vesicles from proteinase K proteolysis (Fig. 5, lanes 1–4). This protection was abolished by solubilization of the membrane with the nonionic detergent Triton X-100 (Fig. 5, lane 5). In addition, only the processed form (Fig. 5, lanes 6 and 7) cosedimented with the membrane vesicles through a high salt/sucrose cushion.

Other studies have shown that both nascent chains of secretory and integral membrane proteins (23, 31) and a peptide derived from the uncleaved internal signal sequence of chicken ovalbumin (32) can competitively inhibit chain translocation, suggesting that the signal sequences of integral membrane proteins and secretory proteins recognize common receptors on the cytoplasmic face of the rough endoplasmic reticulum membrane. Similar experiments were therefore carried out in the presence of the competitive inhibitor (32) derived from chicken ovalbumin (OV_s) to see whether β_2-microglobulin shared the same receptors. This competitive inhibitor blocked processing and segregation of β_2-microglobulin (Fig. 6). The inhibition was reversed by an increase in membrane concentration, indicating that nascent pre-β_2-microglobulin recognizes the same receptors as do the other classes of proteins that are transferred across microsomal membranes.

DISCUSSION

We have synthesized β_2-microglobulin *in vitro* by using a rabbit reticulocyte lysate and mRNA obtained from the murine tumor cell line EL4. These cells express β_2-microglobulin on their surfaces in association with the major histocompatibility antigens H-2K^b and H-2D^b (33). The data indicate that β_2-microglobulin is synthesized as a nascent precursor containing an NH_2-terminal signal sequence of 19 amino acids. The NH_2-terminal amino acid sequences of the processed chain and the precursor (Fig. 4) are in accord with the published amino acid sequence of authentic β_2-microglobulin, and immunoprecipitation of both species is competively inhibited by authentic β_2-microglobulin.

In every respect, the biosynthesis and segregation of β_2-microglobulin appear to follow the same path as that outlined for secretory proteins in the signal hypothesis (17). The NH_2-terminal extension of the precursor (Fig. 4) closely resembles that seen for both secretory and integral membrane proteins, both in size and in having a preponderance of hydrophobic amino acids (four valines, five leucines, one phenylalanine, one tyrosine). The absence of methionine at position 1 indicates that the precursor is already partially processed, most likely with removal of the initial methionine by a ribosome-associated peptidase as has been observed in other cases (34).

In the absence of added membranes, the precursor was the predominant species seen in immunoprecipitation (Fig. 1). Addition of dog pancreas membrane caused conversion of the nascent precursor to the processed molecule, and the amount converted increased with membrane concentration. As seen previously for the secretory proteins, processing and segregation of β_2-microglobulin appear to be closely coupled to translation in that, after synthesis is complete, addition of dog pancreas membranes to the translation mixture does not cause conversion

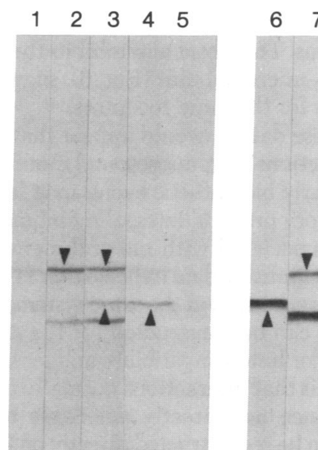

FIG. 5. Segregation of nascent β_2-microglobulin. Translation, proteinase digestion, immunoprecipitation, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis were carried out as described (17, 31). Lanes: 1 and 2, translation in the absence of membranes; 3–7, translation in the presence of membranes (0.5 A_{260} unit/ml). Lanes 1 and 4, proteinase K (100 μg/ml) added after translation; lane 5, after translation membranes were solubilized with Triton X-100 and proteinase K (100 μg/ml) was added; lanes 6 and 7, after translation membranes were sedimented through a sucrose cushion in 0.5 M NaCl/10 mM $CaCl_2$ (lane 6 is the pellet and lane 7 is the supernatant).

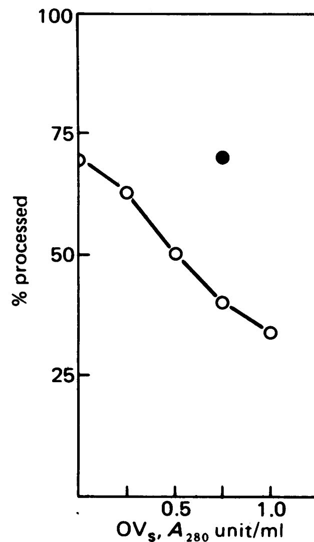

FIG. 6. Inhibition of processing of β_2-microglobulin by the signal-containing peptide from chicken ovalbumin (OV_s). Various amounts of OV_s were present during translation (○) in the presence of microsomal membranes (1.0 A_{260} unit/ml). Excess of microsomal membrane (2.0 A_{260} units/ml) (●) was added to reverse the inhibition. The translation mixtures were subjected to immunoprecipitation and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The bands containing [^{35}S]methionine-labeled pre-β_2-microglobulin were cut out, and radioactivity was determined by liquid scintillation spectrometry.

of the precursor to the processed molecule. Moreover, only the processed chain appears to be fully enclosed in the rough endoplasmic reticulum: the processed chain, but not the precursor, sediments with the microsomal membranes, and the processed chain is protected from proteolysis by the microsomal vesicles whereas the precursor is not.

Various data indicate that a number of secretory proteins and integral membrane proteins compete for the same receptors on microsomal membranes. A convincing demonstration of this phenomenon is the ability of a peptide derived from ovalbumin to inhibit the translocation of secretory (32) and integral membrane proteins. This agent also inhibits the processing and segregation of β_2-microglobulin (Fig. 6), suggesting that this protein competes for the same receptors.

From all of these data, it would appear that there is a common set of interactions with microsomal membrane receptors that control the early biosynthetic events in at least three classes of proteins: secretory proteins, integral membrane proteins, and proteins closely associated with integral membrane proteins. Moreover, our preliminary data indicate that H-$2K^b$ and H-$2D^b$ antigens can be synthesized in the same system, suggesting that cell-free systems can be constructed for the study of the biogenesis of the major histocompatibility antigens. The advantage of this approach is that interactions normally occurring inside the cell, and hence not directly amenable to experimental manipulation, can be reconstructed directly on the topologically equivalent external face of the microsomal vesicle. For example, data from cellular studies of human (35) and mouse (36) tumor lines that do not express HLA or H-2 antigens indicate that expression of β_2-microglobulin is essential for the expression of the major histocompatibility antigens (37–38). It should be possible to examine the need for coexpression of H-2 antigens and β_2-microglobulin and the relative rates of synthesis of the integral membrane proteins and β_2-microglobulin in detail in this system.

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