Intracellular Transport of Rat Serum Albumin Is Altered by a Genetically Engineered Deletion of the Propeptide

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Many secreted proteins are synthesized with amino-terminal propeptides which are removed prior to secretion. Like many secretory proteins, serum albumin is synthesized in precursor form with pro- and propeptides which are removed during biosynthesis and transport through the secretion pathway (Peters, 1985). The propeptide (18 amino acids) functions to target poly-somes synthesizing RSA to the ER. However, a function has not been previously ascribed to the albumin propeptide (6 amino acids). Various suggestions for a propeptide function have been made and refuted by experimental testing. For example, Peters and Reed (1980) questioned the role of the propeptide with studies on the composition and properties of proalbumin compared with mature albumin. They showed that denatured albumin and proalbumin regained native structure equally rapidly and to the same extent with proper disulfide bond alignment, demonstrating that the propeptide was not controlling or advantageous for polypeptide folding, as is the C-peptide of insulin (Steiner and Clark, 1968). They also showed that, based on binding to both polyclonal and the monoclonal antibodies, the antigenic determinants of albumin are fully exhibited in proalbumin and that proalbumin binds palmitate and bilirubin as effectively as does albumin. These results indicated that physiologically important binding sites are fully functional and thus the propeptide does not function to block an important binding site on proalbumin as does the propeptide of trypsinogen, where cleavage activates proteolytic activity (Walah and Neurath, 1964). Furthermore, cleavage of the propeptide to form mature albumin is not required for normal secretion as some individuals have circulating proalbumin (Brennan and Carrell, 1978).

Studies to probe the role of prosequences of other proteins have revealed functional activities that are directly related to the secretion pathway. For example, the propeptide of the coagulation Factor IX participates in defining a recognition site for γ-carboxylation of specific glutamic acid-rich domains, a post-translational processing event of the mature Factor IX (Jorgensen et al., 1987). The von Willebrand factor propeptide serves to direct the assembly of disulfide-linked factor multimers (Wise et al., 1988), a post-Golgi intracellular event which is important in promoting the blood-clotting activity (Wagner et al., 1987). The yeast vacuolar targeting signal of carboxypeptidase Y and proteinase A appears to reside in the propeptide of these proteins (Johnson et al., 1987; Klionsky et al., 1988). Furthermore, although the propeptides of trypsinogen and insulin do not appear to be required for sorting into the regulated secretion pathway (Burgess et al., 1987; Powell et al., 1987), recent data have shown that the proregion of somatostatin is an important mediator for intracellular transport and sorting to the regulated secretion pathway (Stoller and Shields, 1989).

In light of the observations above, it seemed reasonable to propose that the serum albumin propeptide may have a role in the secretion process. To investigate this possibility, we have analyzed the secretion of recombinant RSA produced in transfected monkey kidney (COS) cells. We have previously shown that these cells, which do not normally produce RSA, have the requisite machinery for synthesis and secretion of this protein (McCracken and Fishman, 1986). We now report experiments in which RSA cDNA was altered by site-directed mutagenesis to remove the propeptide and present data that suggest a role for the propeptide in the intracellular transport of serum albumin.

EXPERIMENTAL PROCEDURES

Methods—The application of standard procedures for cell culture, DNA transfection, and ELISA (McCracken and Fishman, 1986), oligonucleotide mutagenesis (McCracken et al., 1988), immunofluorescence, immunoadsorbance, gel electrophoresis, and quantification by densitometry (McCracken et al., 1989) have been previously described.

Amino-terminal Nickel 63 Binding—Recombinant RSA was affin-
ity-purified from culture medium of transfected cells using affinity-purified antibodies raised against RSA (McCracken and Fishman, 1986). Anti-RSA was added to culture medium samples (700 ng/ml) which were then mixed at 4 °C overnight. The entire medium plus antibody sample was then put through a protein A acti-disk (FMC, Rockland, ME) to collect the antibody-antigen complexes. The immunoreactive RSA was eluted from the protein A acti-disk with 0.1 M glycine (pH 2.8), neutralized with 1.0 M Tris (pH 9.6), and concentrated by vacuum centrifugation. The RSA was resuspended in 20 μl of borate-buffered saline (pH 7.5). One μCi of nickel 63 was mixed with 5 μl of the purified RSA and was loaded onto a 0.7% agarose gel. Electrophoresis was carried out in Tris barbiturate buffer (pH 8.6) to separate the RSA from the unbound nickel 63. Conditions of electrophoresis have been previously described (Emmett et al., 1984). The gel was dried, autoradiographed, and then stained with Coomassie Blue and photographed.

RESULTS

Construction of pSV2rsaApro—To construct an albumin gene with a deletion of the nucleotides which encode the propeptide, oligonucleotide-directed mutagenesis on gapped heteroduplex DNA was used as described previously (McCracken et al., 1988). The shuttle vector carrying the RSA cDNA gene (pSV rsa, McCracken and Fishman, 1986) was digested with restriction endonucleases in three separate experiments: 1) to remove the plasmid β-lactamase gene which confers ampicillin resistance; 2) to remove the region of the RSA gene where the mutation was planned; and 3) to produce a 526-base pair fragment required to inhibit secondary structure formation in the single-stranded region of the heteroduplex. These three fragments were pooled along with the mutagenic oligonucleotide (21 mer designed to delete the 18 base pairs which encode the propeptide), denatured by heat, and allowed to renature. After annealing and heteroduplex formation, the remaining single-stranded regions of the DNAs were filled in using the activity of DNA polymerase (Klenow), and the entire reaction mixture was used to transform Escherichia coli. The ampicillin-resistant colonies were screened, and the resulting prodeletion mutants (pSV rsaApro) were confirmed by restriction endonuclease mapping and DNA sequencing.

Transfected COS Cells Secrete Less RSAΔpro—When the mutant construction (pSV rsaApro) was used to transfact COS cells, RSA was synthesized and secreted into the medium. To assess the secretion of RSA by cells transfected with either the wild type gene, pSV rsa, or the mutant gene, pSV rsaApro, the culture medium was collected 72 h post-transfection and immediately assayed by ELISA. The results of seven separate transfections showed reproducible decreases (36% ± 10) in the amount of RSAΔpro, relative to the amount of RSA secreted by the transfected cells. For example, in two experiments culture medium from cells transfected with pSV rsaApro contained 26.8 and 22.0 ng of RSA/ml while medium from cells transfected with pSV rsa contained 38.8 and 39.9 ng of RSA/ml, respectively.

Deletion of the Propeptide Affects the Rate of RSA Secretion—To further study the inhibition to secretion caused by the deletion of the RSA propeptide, the kinetics of RSA transport through the secretory pathway were determined by pulse-chase protein-labeling experiments. Cell lysates and media samples collected at various time points during the chase were immunoadsorbed and the immunoreactive proteins resolved on SDS-PAGE (Fig. 1). These results show that approximately equal amounts of protein were synthesized by cells transfected with pSV rsa and pSV rsaApro but that the rate of secretion was very different. To quantify these results, the autoradiogram was measured by a scanning densitometer and the area of the peaks determined as the amount of radioactive protein in the sample. From these data we calculated the total amount of RSA and the percent released into the medium as a function of the chase duration (Table I). By 45 min, 53% of the RSA but only 6% of the RSAΔpro had been secreted, indicating that the absence of the propeptide greatly inhibits the transport rate of RSA through the secretion pathway.

Accumulation of Intracellular RSAΔpro in the ER—To begin to study the intracellular site for accumulation of RSAΔpro and therefore identify the intracellular location of the defective transport, transfected cells were studied by

![FIG. 1. Secretion time course for RSA and RSAΔpro. Transfected COS cells were pulse-labeled with [35S]methionine for 30 min and chased in nonradioactive methionine for 15, 30, 45, 60, and 120 min. Cell lysates and medium samples from 100-mm culture dishes of cells were immunoadsorbed with rabbit anti-RSA. The immunoreactive proteins were separated by gel electrophoresis on 7% SDS-polyacrylamide, and the labeled proteins were visualized by radioautography. neo, cells transfected with a control plasmid, pSV neo; rsa, cells transfected with pSV rsa; pro, cells transfected with pSV rsaApro. S, origin and molecular weight standards.

<table>
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<th>Time (min)</th>
<th>RSA area units</th>
<th>RSAΔpro area units</th>
<th>% total</th>
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*Calculated from densitometric analysis using a Hoefer GS900 scanning densitometer under conditions in which the response of the film and the instrument was in the linear range. Computer-assisted integrations of the area under the peaks were used to derive a relative area/density unit for each band.

**Determined as RSA in media divided by total RSA in media plus lysate.
double indirect immunofluorescence (Fig. 2). Cells transfected with pSV₄RSA showed intracellular concentrations of RSA in the Golgi (panels A and B), while cells transfected with pSV₄RSAΔpro showed intracellular concentrations of RSA in the endoplasmic reticulum and the Golgi (panels C and D). Localization of wild type RSA to the Golgi was expected as COS cells efficiently secreting proteins are known to demonstrate an observable concentration of the secretory proteins in the Golgi (McCracken et al., 1989). However, the ER localization of fluorescence seen in cells transfected with pSV₄RSAΔpro indicated that RSAΔpro was accumulating in the ER and suggested that the slow rate of secretion of RSAΔpro resulted from a decreased efficiency of normal transit through and/or out of the ER.

The Prepeptide Is Not Retained on RSAΔpro—RSA is synthesized in precursor form with an 18-amino acid signal sequence (prepeptide) that is removed upon translocation through the ER membrane (Peters, 1985). It was possible that the mutagenesis to remove the prepeptide had also altered the prepeptide cleavage site. An uncleaved prepeptide on RSAΔpro would add 2480 to its molecular weight. However, both intracellular and secreted RSA and RSAΔpro had the same migration rate on SDS-PAGE (see Fig. 1), indicating that the prepeptide had been removed. To substantiate this conclusion and determine whether or not the prepeptide had been properly cleaved, we carried out an NH₂-terminal specific binding reaction. Mature albumin binds nickel(II) and copper(II) when the NH₂-terminal amino acid sequence is X-X-His (Peters, 1985). Furthermore, the prepeptides had been removed (resulting in an NH₂ terminus of Met-Lys-Trp) or if it was incorrectly cleaved. RSA was affinity-purified from the culture medium of transfected cells, reacted with nickel 63, or if it was incorrectly cleaved. RSA was affinity-purified from the culture medium of transfected cells, reacted with nickel 63, and analyzed by autoradiography (Fig. 3). The results show that both RSA and RSAΔpro bound nickel 63, indicating that the prepeptides had been properly removed from both proteins. Control lanes 1, 2, and 6 of Fig. 3 show that nickel 63 binding is specific for X-X-His at the amino terminus. Neither ovalbumin (lane 1) nor dog albumin (lane 2) have histidine in the third position and did not bind nickel in this reaction. The sample in lane 6 was 5 μg of RSA reacted with copper(II) prior to addition of the nickel 63. The lack of nickel binding in this sample demonstrated the specificity of the binding reaction, since Cu(II) is known to have a greater binding affinity to the amino-termini X-X-His than Ni(II) (Peters, 1985). Furthermore, the immunopurification of protein from culture medium of mock transfected cells did not yield a protein which binds nickel 63 (lane 3).

**DISCUSSION**

Despite previous attempts to elucidate the function of the albumin propeptide, its role had remained elusive. These studies focus on the possible role of the RSA propeptide in...
intracellular transport and secretion of serum albumin. Expression and secretion studies with the deletion mutant, pSV2RSApro, showed that the total amount of immunoreactive RSA secreted by transfected cells was only 64% of that secreted by cells transfected with pSV2rsa and that the deletion of the propeptide had an inhibitory effect on the rate of secretion of RSA. In addition, we found that RSApro accumulated in the ER of transfected cells. These results suggest a role for the propeptide as a positive factor in the intracellular transport of RSA.

An alternative explanation for the results seen in our studies is that a genetically engineered deletion of the propeptide may have modified some bulk property of the protein which alters surface charge or solubility. Such physical changes could affect transport of RSApro through the ER, leading to the results seen in our studies. Based on the physical studies of Peters and Reed (1980), this is clearly not the case. Furthermore, since we determined that the propeptide was properly cleaved, the possibility that an altered NH2 terminus was retarding transport of RSApro is unlikely.

Thus, our results add to a growing list of observations that implicate prosequences as mediators for post-translational processing and transport through the secretory pathway. However, most serum proteins synthesized and secreted by liver cells do not contain a prosequence and thus may carry transport information elsewhere in the protein. Relative to this concept, it is interesting that: 1) albumin is one of the few secreted proteins which is not glycosylated; 2) that some secreted proteins require oligosaccharides for normal secretion (Lodish and Kong, 1984); and 3) that two other secreted nonglycosylated proteins studied, proparathormone and proapolipoprotein, have propeptides (Peters, 1985). These observations lead to the idea that carbohydrate moieties may sometimes carry information similar to that in propeptides which facilitates secretion; however, this premise has not been substantiated.

What has been demonstrated is that propeptides of some proteins carry information required for normal transport, while others do not. A relevant example is the role of the propeptides of proteins which are secreted by the regulated secretion pathway. Both insulin and trypsinogen have been studied, and the propeptides of these proteins appear to be unnecessary for sorting into the regulated pathway (Powell et al., 1987; Burgess et al., 1987). However, the somatostatin propeptide, when attached to chimpanzee a-globin, functions both to protect globin from degradation and thus facilitate transport from the ER to the Golgi and to target globin to the regulated secretion pathway (Stoller and Shields, 1989). Furthermore, studies on the secretion of heterologous proteins from yeast, using various yeast pre- and prepro-secretion signals, support the idea that transport information carried in a prosequence of one protein may be located elsewhere in other proteins (Zesbo et al., 1986; MacKay, 1987; Smith et al., 1985). In particular, these studies have shown that the prosequence of prepro-a mating factor enhances transport of some but not all heterologous proteins through the yeast secretory pathway.

Our results indicate that for RSA essential transport information is located in the serum albumin propeptide. Although the data are not complete enough to define a model for the role of the RSA propeptide, we can envision a possible mode by which propeptide might act to promote efficient transport of albumin. Likely candidates for the information content of the propeptide are the basic residues which could form ionic interactions with accessory proteins of the cellular transport machinery, allowing for selective protein transport. The ability of liver cells to selectively package and transport specific secretory proteins would be a useful mechanism for response to physiological demands on protein concentrations in the circulatory system. We propose that our data support, and can be explained by, a transport mechanism which allows selective export of soluble proteins from the ER, a mechanism in which the albumin propeptide contains information required for its efficient transport. Results obtained from further experiments designed to test this hypothesis should be useful in elucidating the selective transport of soluble proteins from the lumen of the ER.

REFERENCES