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Cell-free synthesis of fish preproinsulin, and processing by heterologous mammalian microsomal membranes

(mRNA from islets of Langerhans/wheat germ system/canine pancreatic microsomal membranes/amino-terminal sequences/sequence homologies)

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ABSTRACT **Poly(A)-containing mRNA isolated from the islets of Langerhans obtained from two species of fish, angler fish (*Lophius americanus*) and sea raven (*Hemitripterus americanus*), stimulated protein synthesis 16-fold in a wheat germ cell-free system. Characterization of the translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed a major polypeptide weighing 11,500 daltons that was specifically precipitated by an antibody against angler fish insulin. Partial sequence analysis of the amino terminal revealed that this polypeptide is preproinsulin, in which the amino terminus of proinsulin is preceded by either 23 (angler fish) or 25 (sea raven) amino acid residues. Translation of fish islet mRNA in a wheat germ cell-free system in the presence of dog pancreas microsomal membranes led to the correct cleavage of the nascent preproinsulin, resulting in the synthesis of authentic fish proinsulin, as verified by partial sequence analysis. Moreover, the synthesized fish proinsulin was segregated, presumably into the luminal space of the dog pancreas microsomal vesicles, because it was found to be resistant to proteolysis by added trypsin and chymotrypsin. Our data thus suggest that the mechanisms and information for the transfer of secretory proteins across the microsomal membrane are highly conserved during evolution.**

It has been shown that a number of mammalian secretory proteins are synthesized as larger molecules with an amino-terminal extension of 16–25 amino acid residues (1–5) when their mRNAs are translated in a cell-free system devoid of microsomal membranes. In the recently formulated signal hypothesis (6), this sequence extension ("signal" peptide) was postulated to constitute an essential, albeit short-lived, feature of all nascent secretory polypeptide chains.

The aim of the present study was to determine if the predictions made in the signal hypothesis (6) could be substantiated for the synthesis and segregation of a secretory protein, proinsulin, obtained from animals evolutionarily far removed from mammals, e.g., fish. Two laboratories (7, 8) have reported the synthesis of molecules larger than fish proinsulin when total mRNA from the islets of Langerhans was translated in a wheat germ cell-free system. Immunoprecipitation was used for the identification of insulin-related molecules from the other translation products. With a similar approach, the synthesis of molecules larger than fish proinsulin is also confirmed here by immunoprecipitation and by partial sequence analysis. Moreover, we show that in the two species of fish investigated, these larger molecules contain amino-terminal sequence extensions (signal peptides) of 23 or 25 amino acids. However, most significantly, our results demonstrate a functional equivalence of fish signal peptides with their mammalian counterparts. Thus, fish signal peptides were recognized by mammalian microsomal membranes, leading to segregation of the fish proinsulin into dog pancreas microsomal vesicles. Furthermore, the fish signal peptide was removed correctly by the mammalian signal peptidase, resulting in the synthesis of proinsulin rather than preproinsulin.

METHODS

Extraction of mRNA from Islet Tissue. Approximately 1 g of islet tissue was homogenized in a Waring Blendor for 1 min at room temperature in 30–40 volumes of 150 mM NaCl/50 mM Tris·HCl, pH 7.4/5 mM EDTA/1% sodium dodecyl sulfate. Proteinase K was added to a concentration of 100 μg/ml and the solution was incubated at room temperature for 10 min. Nucleic acids were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). Approximately 300 A_{260} units of nucleic acid were obtained from 1 g of islet tissue. (One A_{260} unit is the amount that has an A_{260} of 1 when dissolved in 1 ml and the light path is 1 cm.) Poly(A)-containing mRNA was separated from total nucleic acid by affinity chromatography on oligo(dT)-cellulose columns using the procedure of Aviv and Leder (9). The yield of mRNA was between 1.5 and 2% of the total nucleic acid applied to the column.

Cell-Free Protein Synthesis. A wheat germ S-23 supernate was prepared by the method of Roman *et al.* (10). Typical incubations were performed in a final volume of 100 μl as described elsewhere (11), and contained 4 μCi of a reconstituted protein hydrolysate (algal profile), and 0.1 A_{260} unit of mRNA. Incubations were at 27° for 90 min. Dog pancreas microsomal membranes were prepared from rough microsomes using EDTA for the removal of ribosomes (12), and were used at a final concentration of 4.5 A_{260} units/ml. Translation products were analyzed by polyacrylamide gradient gel electrophoresis in sodium dodecyl sulfate as previously described (6) except that the resolving gels were composed of gradients of 10–17% acrylamide, and the spacer gel was 4% acrylamide. The slab gels were stained, destained, dried, and subjected to autoradiography as described previously (6).

Antibody Precipitation. After incubation, a 50 μl aliquot of the cell-free system was centrifuged for 30 min at 100,000 $\times$ *g*. Rabbit antiserum (1 μl) directed against angler fish insulin was added to 50 μl of the supernate, which had been adjusted to 800 mM NaCl, 1% Triton X-100, and 50 mM Tris·HCl at pH 7.4. Samples were incubated for 1 hr at 37°, followed by 14 hr at 4°; 10 μl of sheep antiserum to rabbit IgG was then added and the samples were allowed to stand for a further 6 hr at 4°. The antibody precipitate was collected by centrifugation and washed three times in phosphate-buffered saline prior to analysis by polyacrylamide gradient gel electrophoresis in sodium dodecyl sulfate (6).

Partial Sequence Determination. Incubation volumes for protein synthesis were scaled up and contained one tritiated

amino acid and [^{35}S]methionine (adjusted to approximately the same specific activity as the tritiated amino acid). The translation products were subjected to electrophoresis (see above); however, the slab gels were dried directly after conclusion of electrophoresis without prior staining. After autoradiography, the desired band was excised from the dried gel and re-hydrated in electrophoresis buffer containing 0.1% sodium dodecyl sulfate (6), and the protein was electrophoretically eluted into dialysis tubing for 16 hr at a current of 8 mA per tube. No further radioactivity was recovered by increasing the duration of elution. Electrophoretic elution was judged as complete because the eluted radioactivity amounted to 15–20% of the total acid-precipitable radioactivity applied to the original gel. This value is in close agreement with data obtained from densitometric tracings of the autoradiographs (data not shown), which had demonstrated that the 11.5 kilodalton band corresponded to approximately 15–20% of the translation products. Whale apomyoglobin (3 mg) was added to the eluted sample (3 ml), which was then dialyzed extensively against water. After dialysis the sample was adjusted to 15 mM NaCl and nine volumes of −20° acetone was added to extract sodium dodecyl sulfate and to precipitate the protein. Greater than 85% of the eluted radioactivity was recovered by this procedure. The purity of the eluted samples was routinely checked by re-electrophoresis of a small aliquot of the eluate—only one radioactively labeled band was observed (data not shown). Samples were dried under nitrogen, dissolved in 0.6 ml of 60% (vol/vol) heptafluorobutyric acid, applied to the Beckman 890 C sequencer, and subjected to up to 50 cycles of Edman degradation using an *N,N*-dimethylallylamine (DMAA) program (no. 102974). The recovered thiazolinones were dried under nitrogen and their radioactivity was measured directly in 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole/xylene scintillant using a Beckman LS 350 scintillation counter setup for double label counting.

Source of Materials. [^{3}H]Leucine at 57.4 Ci/mmol, [^{3}H]alanine at 18.3 Ci/mmol, [^{3}H]phenylalanine at 40 Ci/mmol, [^{3}H]valine at 12.5 Ci/mmol, and [^{3}H]proline at 60 Ci/mmol were obtained from New England Nuclear, Boston, MA. [^{35}S]Methionine at 480 Ci/mmol was purchased from Amersham/Searle, Arlington Heights, IL, and ^{14}C-labeled reconstituted protein hydrolysate, algal profile, was from Schwarz/Mann, Orangeburg, NY. Sperm whale apomyoglobin was obtained from Beckman Instruments, Inc., Palo Alto, CA. Sea raven (*Hemitripterus americanus*) islets were kindly provided by L. Garibaldi of the New England Aquarium, Boston, MA; angler fish (*Lophius americanus*) islets and rabbit antiserum against angler fish insulin were generous gifts of E. Bauer and B. Noe (Marine Biological Laboratories, Woods Hole, MA). Porcine proinsulin was a gift of R. Chance of Lilly Research Laboratories, Indianapolis, IN. Wheat germ was obtained from the Pillsbury Co., Minneapolis, MN.

RESULTS

Our initial experiments were performed to determine if mRNA extracted from fish islets of Langerhans could stimulate protein synthesis in a wheat germ cell-free system. Polyadenylylated mRNA extracted from the islets of Langerhans of two species of fish (sea raven and angler fish) produced a 16-fold increase over the endogenous levels of protein synthesis in the wheat germ system (data not shown). Analysis of the translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and subsequent autoradiography (Fig. 1) revealed several major polypeptides ranging from 11.5 to 18 kilodaltons (tracks 1 and 8). On the basis of its molecular weight, the 11.5 kilodalton band appeared to be a most likely candidate for being preproinsulin, because a precursor to proinsulin with a signal sequence of 20–25 amino acids would be expected to have a molecular weight in this range. In addition, Chan *et al.* (4) demonstrated the synthesis of a polypeptide of this molecular weight using mRNA isolated from rat islets of Langerhans and identified it as rat preproinsulin. Furthermore, incubation of the wheat germ cell-free products synthesized in response to sea raven mRNA, with an antiserum directed against angler fish insulin, precipitated only one band, that migrating with an apparent molecular weight of 11,500 (Fig. 1, track 2). However, it should be noted that only approximately 2–3% of the total acid precipitable radioactivity was precipitated by this antiserum, equivalent to about 10–20% of the radioactivity in the 11.5 kilodalton band. It could be argued, therefore, that the insulin-like material in the 11.5 kilodalton band is only a minor component; however, the sequence data (see below) suggest rather that the antibody precipitation was incomplete and that the polypeptide was most likely homogeneous.

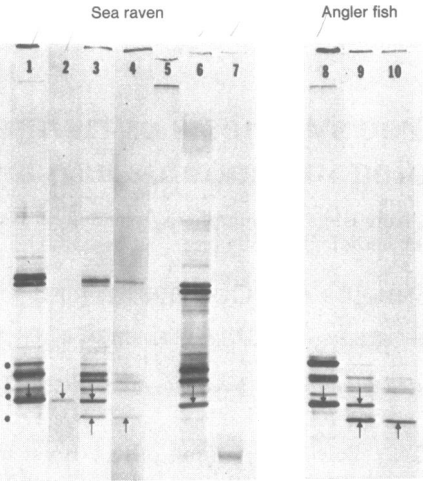

FIG. 1. Cleavage and segregation of nascent fish preproinsulin by dog pancreas microsomal membranes. Analysis was by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and subsequent autoradiography of dried slab gels. mRNA isolated from the islets of Langerhans of two species of fish (sea raven: tracks 1–7; and angler fish: tracks 8–10) was translated in a wheat germ cell-free system containing 4 μCi of reconstituted algal hydrolysate, in the absence (tracks 1, 2, 6, 7, and 8) or presence (tracks 3, 4, 5, 9, and 10), of dog pancreas microsomal membranes. Following completion of translation, 20 μl aliquots were processed for electrophoresis either directly (tracks 1, 3, 8, and 9) or after they had been subjected to various posttranslational incubations: with proteolytic enzymes alone (tracks 4 and 10) or together with 1% Triton X-100 (track 5); or first for 90 min at 27° with dog pancreas microsomal membranes alone (track 6), followed by proteolytic enzymes (track 7); or with rabbit antiserum to angler fish insulin (track 2). Incubation with proteolytic enzymes (10 μg each of trypsin and chymotrypsin) was for 30 min at 27°. Downward- and upward-pointing arrows indicate preproinsulin and proinsulin, respectively. Dots to the left of track 1 indicate position of molecular weight markers; these are, in descending order: whale apomyoglobin, rabbit globin, horse cytochrome *c*, and porcine proinsulin, weighing 17, 15, 11.7, and 9.1 kilodaltons, respectively.

To test the assumption that the 11.5 kilodalton band was preproinsulin, we used sequential Edman degradations of the radioactively labeled protein to determine its partial amino acid sequence (see *Methods*). It can be seen (Figs. 2 and 3) that discrete peaks of radioactivity were associated with certain of the 29–46 cycles of Edman degradation. For the 11.5 kilodalton polypeptide of angler fish (Fig. 2) this enabled us to identify leucine as the amino acid at positions 3, 5, 10, 11, 13, 14, 30, 35, 39, and 41; valine at positions 12, 15, 16, and 24; alanine at

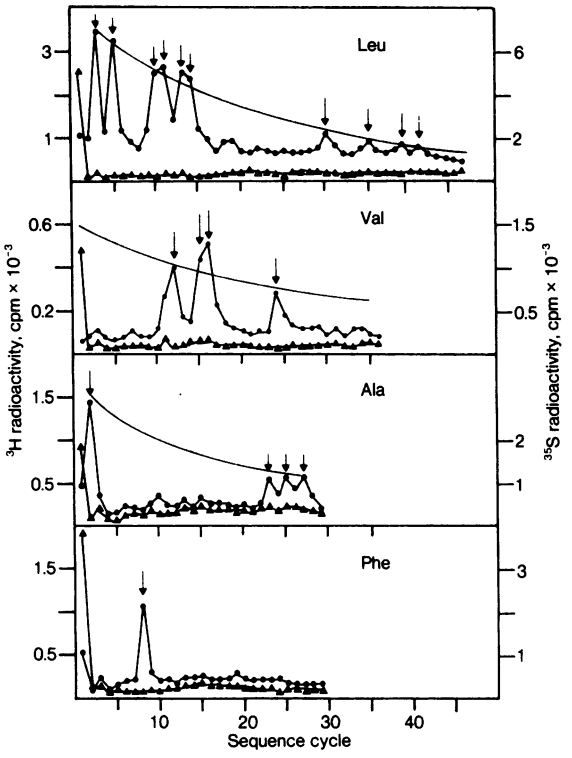

FIG. 2. Partial amino-terminal sequence analysis of angler fish preproinsulin. mRNA from angler fish islets was translated in the wheat germ cell-free system containing: Leu, 6 μCi of [^{3}H]leucine and 37.2 μCi of [^{35}S]methionine (50 Ci/mmol) in a final volume of 250 μl; Val, 12 μCi of [^{3}H]valine and 36 μCi of [^{35}S]methionine (14.9 Ci/mmol) in a final volume of 500 μl; Ala, 12 μCi of [^{3}H]alanine and 69.6 μCi of [^{35}S]methionine (19.3 Ci/mmol) in a final volume of 500 μl; Phe, 6 μCi of [^{3}H]phenylalanine and 34.8 μCi of [^{35}S]methionine (38.7 Ci/mmol) in a final volume of 250 μl; specific activities of the tritiated amino acids are given in *Methods*. The preproinsulin band (indicated by the downward-pointing arrow in track 6 of Fig. 1) was eluted and taken through 30–50 cycles of Edman degradation. The curved line indicates the theoretical yield of radioactivity based on the repetitive yield normalized to the first radioactive peak; calculated repetitive yields (15) were Leu, 94.8%; Val, 95.9%; Ala, 94.4%; Phe, not determined. Total input radioactivity: Leu, 300,000 cpm; Val, 210,000 cpm; Ala, 320,000 cpm; Phe, 152,000 cpm. ●, ^{3}H radioactivity; ▲, ^{35}S radioactivity. Sequence positions assigned to various amino acid residues are indicated by arrows.

positions 2, 23, 24, and 27; and phenylalanine at position 8. The alignment of this sequence with the known amino terminal sequence of angler fish proinsulin (13) provided strong evidence that the 11.5 kilodalton band consisted of predominantly angler fish preproinsulin in which the amino terminus of proinsulin is preceded by 23 amino acid residues (see Fig. 4). Likewise, that the 11.5 kilodalton band of sea raven (Fig. 1, track 1) contained preproinsulin is strongly suggested not only by the antibody precipitation (Fig. 1, track 2) but also by the distribution of leucine (Fig. 3) at positions 5, 12, 13, 15, 16, 18, 32, 37, 41, and 43. The latter four residues can be aligned with the four invariant leucine residues common to the amino-terminal region of proinsulin from 18 different species (13). It should be noted that the ^{35}S radioactivity at residue 1 was approximately 6-fold higher in preproinsulin of sea raven than in that of angler fish. For this reason, methionine was not identified as the amino-terminal residue of angler fish preproinsulin; it is conceivable that it originated from contaminants, such as prematurely terminated polypeptides that comigrated with preproinsulin. It is noteworthy that Met-Met, which we found to occur at positions 1 and 2 of sea raven preproinsulin, has also

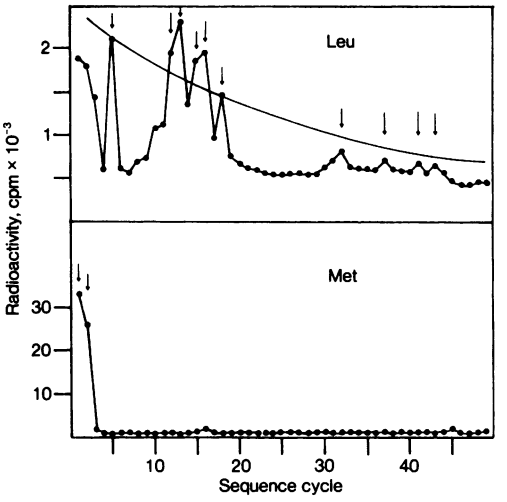

FIG. 3. Partial amino-terminal sequence analysis of sea raven preproinsulin. mRNA from sea raven islets was translated in the wheat germ cell-free system containing 61.5 μCi of [^{35}S]methionine (50 Ci/mmol) and 12 μCi of [^{3}H]leucine in a final volume of 250 μl. The eluted preproinsulin was taken through 50 cycles of Edman degradation. Total input radioactivity was approximately 400,000 cpm. The curved line indicates the theoretical yield of ^{3}H radioactivity based on a repetitive yield of 94.0% normalized to the first radioactive peak.

been found to be the amino terminal of bovine preproparathyroid hormone (3). Leucine has not been assigned to positions 1 or 2 of sea raven preproinsulin (Fig. 3) because the observed radioactivity was below that expected from the repetitive yield curve. That this radioactivity might also originate from contaminants is suggested from Fig. 1, which shows the 11.5 kilodalton band of sea raven (lane 1) to be considerably less sharp than that of angler fish (lane 8).

Evidence having been provided that proinsulin from two species of fish is synthesized as a larger molecule with an amino-terminal sequence extension analogous to that of mammalian presecretory proteins (1–5), it was of interest to investigate any functional homology of the signal sequence, i.e., whether it would be possible to synthesize proinsulin by translating fish islet mRNA in the presence of dog pancreas microsomal membranes. For mammalian presecretory proteins, *in vitro* processing has been shown to occur when their mRNAs are translated in systems that are derived entirely from animal tissue (12, 14) or that were made up of components of animal (dog pancreas microsomal membranes) as well as plant (wheat germ ribosomes and factors) tissues (11). It can be seen from Fig. 1 that translation of fish islet mRNA in the wheat germ system in the presence of dog pancreas microsomal membranes yielded a number of new bands (tracks 3 and 9) distinctly different from those synthesized in the absence of membranes (tracks 1 and 8). Prominent among these was a band of approximately 9 kilodaltons that comigrated with porcine proinsulin. Concomitant with the appearance of the 9 kilodalton band was an apparent reduction in the intensity of the 11.5 kilodalton band, suggesting a precursor–product relationship. Moreover, the 9 kilodalton band was among those few bands that were largely resistant to posttranslational proteolysis (tracks 4 and 10). As previously noted (12), the extent of protection of the segregated protein was not complete, probably due to the leakiness of some microsomal vesicles. Resistance, however, was abolished when posttranslational proteolysis was performed in the presence of the detergent Triton X-100 (track 5). It should be noted that the appearance of the 9 kilodalton band was dependent on *co-translational* incubation with dog pancreas microsomal

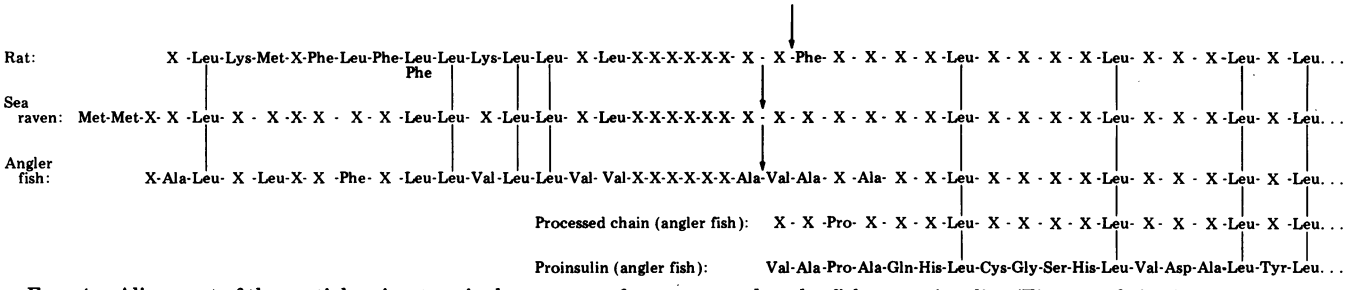

FIG. 4. Alignment of the partial amino-terminal sequences of sea raven and angler fish preproinsulins (Figs. 2 and 3), of rat preproinsulin (4), of angler fish proinsulin (processed chain) synthesized *in vitro* (Fig. 5), and of authentic angler fish proinsulin (13). Alignment was with respect to four invariant leucine residues present in the amino-terminal region of proinsulin in 18 different species (13). Xs indicate residues either unknown or not established by radiosequencing methods. Vertical lines indicate positions of homology. Arrows designate the start of authentic proinsulin within the corresponding preproinsulin.

membranes, because *posttranslation* incubation (track 6) yielded a banding pattern identical to that obtained when translation occurred in the absence of membranes (track 1). Likewise, only *cotranslational* (tracks 4 and 10) and not *posttranslational* (track 7) incubation with membranes yielded resistance of some of the translation products to proteolysis. These data suggested that the 9 kilodalton band represented fish proinsulin that had been synthesized via nascent preproinsulin and that was cleaved and transferred to the luminal space of the dog pancreas microsomal vesicles, where it was resistant to proteolysis by exogenously added enzymes. It should be noted (tracks 4 and 10) that some additional proteins appear to be segregated by the dog pancreas microsomal membranes, suggesting that they are secretory proteins. However, their identities remain to be determined.

In an attempt to establish the identity of the 9 kilodalton protein as proinsulin and to determine the fidelity of cotranslational cleavage, we subjected the 9 kilodalton band, labeled with either [^{3}H]proline and [^{35}S]methionine or [^{3}H]leucine and [^{35}S]methionine, to sequential Edman degradations. From the peaks of radioactivity (Fig. 5) it was possible to identify proline as the amino acid at position 3 and leucine at positions 7, 12, 16, and 18. Alignment of this partial sequence with the known amino-terminal sequence of angler fish proinsulin (Fig. 4) strongly suggested that the 9 kilodalton band was indeed proinsulin and, moreover, that cleavage of the signal peptide from nascent fish preproinsulin by dog pancreas microsomal membranes had occurred at the correct site. The [^{35}S]methionine radioactivity found in residue one in this experiment is thought to have arisen from use of [^{35}S]methionine at a specific activity 10 times greater than previously used (Fig. 2, Leu, and Fig. 3). This radioactivity is therefore considered to have resulted from the amplification of the presence of premature chain termination products generated in the wheat germ system. That this assumption could be correct is borne out by comparison of the data of Fig. 2 (Leu) and Fig. 5; for despite a 10-fold difference in specific activity, the methionine radioactivity in position 1 of the processed proinsulin (Fig. 5) was less than 2-fold greater than that of the precursor (Fig. 2, Leu)—for this reason methionine was not assigned to position 1.

DISCUSSION

Our data are in agreement with previous reports (7, 8), which demonstrated that mRNA extracted from fish islets of Langerhans could be translated *in vitro* in a wheat germ cell-free system to yield a major polypeptide that was immunologically related to but larger by 2000–4000 daltons than proinsulin. By partial amino-terminal sequence analysis of this *in vitro* synthesized radioactively labeled polypeptide, we have demonstrated that it contains 23–25 amino acids residues that precede the amino terminus of fish proinsulin. This sequence extension (signal peptide) is analogous to that found in rat preproinsulin (4) as well as in other mammalian presecretory polypeptides recently synthesized *in vitro* (1–3, 5). Alignment of the partially determined amino-terminal sequence of preproinsulin of angler

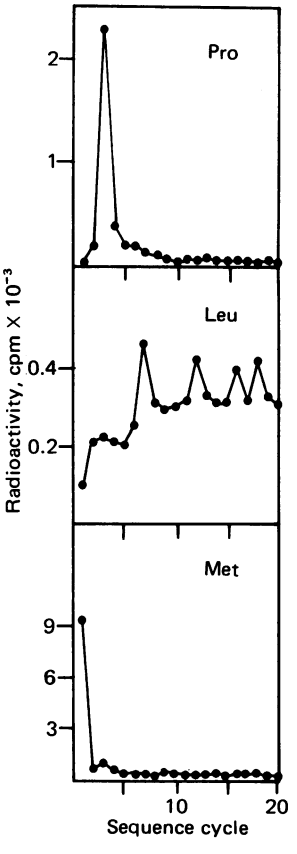

FIG. 5. Partial amino-terminal sequence determination of angler fish proinsulin, synthesized *in vitro* via nascent preproinsulin that was cleaved by dog pancreas microsomal membranes. mRNA from angler fish islets was translated in the wheat germ cell-free system in the presence of dog pancreas microsomal membranes; a final volume of 500 μl contained: 18 μCi of [^{3}H]proline and 125 μCi of [^{35}S]-methionine (480 Ci/mmol) or 18 μCi of [^{3}H]leucine and 125 μCi of [^{35}S]methionine (480 Ci/mmol). It should be noted that in these experiments the specific activity of [^{35}S]methionine was not adjusted to that of the tritiated amino acids. The proinsulin band (upward-pointing arrow in track 9 of Fig. 1) was eluted and taken through 20 cycles of Edman degradation. Input radioactivity was approximately 50,000 cpm for [^{3}H]proline, 60,000 cpm for [^{3}H]leucine, and 120,000 cpm for [^{35}S]methionine in both the [^{3}H]proline- and [^{3}H]leucine-labeled proinsulin bands.

fish and sea raven with each other and with that previously reported for rat preproinsulin (4) revealed considerable homology in the signal peptide region, at least with respect to the position of the leucine residues, although other residues appear to differ. The degree of homology between the two species cannot be determined until the complete signal sequences are known. However, it is becoming increasingly evident that the signal peptides of a variety of mammlian presecretory proteins differ significantly both in length and sequence. Yet despite this diversity in the primary structure of various signal peptides, a common feature, e.g., in their secondary structure, has to be involved in order to explain the results of the "reconstitution" experiments described here. In light of the signal hypothesis (6), these results imply that the signal peptide of *nascent* fish preproinsulin as synthesized by a wheat germ ribosome was recognized by dog pancreas microsomal membranes to establish a functional heterologous ribosome–membrane junction. This interaction provided the topological conditions for a unidirectional and cotranslational transfer of *nascent* fish preproinsulin into the luminal space of a heterologous microsomal vesicle. Most significantly, however, the cleavage site for the conversion of *nascent* fish preproinsulin to *nascent* proinsulin was recognized by the dog pancreas microsomal enzyme correctly, so that cleavage yielded a molecule with the same amino-terminal sequence as authentic proinsulin. Thus, our data indicate that the structural information, characteristic of signal peptides and responsible for ribosome attachment to microsomal membranes, as well as subsequent cleavage of nascent presecretory proteins, has been conserved throughout vertebrate evolution.

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