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Initiation by Methionine of Mouse Immunoglobulin Light Chain Containing NH_2-terminal Pyroglutamic Acid

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SUMMARY

The mechanism of biosynthesis of NH_2-terminal pyroglutamic acid has been studied in a mouse plasmacytoma (RPC-20) which produces an immunoglobulin light (λ) chain containing NH_2-terminal pyroglutamic acid. To this end, initiation of λ chain synthesis in plasmacytoma cell suspensions has been investigated. The analysis of radioactive λ chain synthesis by these cells was accomplished with an antibody preparation specific for the precipitation of λ chain protein from total plasmacytoma protein. NH_2-terminal analysis of plasmacytoma cells labeled with [^{35}S]methionine showed that the ratio of radioactivity in NH_2-terminal methionine to total incorporation in λ chain was greater at 2 min of labeling than at 60 min. However, such a pattern of transient labeling of the NH_2 terminus of the λ chain was not obtained when cells were incubated with tritiated leucine, arginine, or tryptophan. The data indicate that methionine is the initiator amino acid for the synthesis of λ chain containing NH_2-terminal pyroglutamic acid.

Pyroglutamic acid (pyrrolidonecarboxylic acid) occurs at the NH_2 terminus of a number of naturally occurring peptides and proteins (1) including immunoglobulin heavy chains (1) and mouse light (κ (2) and λ (3)) chains.

Studies on the initiation of protein synthesis in eucaryotic cytoplasm (4–7) have indicated that methionine is the initiating amino acid. However, the initiation of synthesis of proteins with blocked NH_2-terminal amino acids (*e.g. N*-acetyl-amino acids and pyroglutamic acid) is not well understood.

Recent studies in our laboratory have been concerned with the mechanism of biosynthesis of pGlu[1] in the λ chain produced by the RPC-20 mouse plasmacytoma. We showed earlier (8) that the precursor of pGlu in plasmacytoma protein was glutamic acid, rather than glutamine.

In this communication, we show that methionine transiently labels the amino terminus of the light chain. These data support the model that methionine is the initiator amino acid for the synthesis of mouse plasmacytoma light chain containing NH_2-terminal pGlu.

[1] The abbreviation used is: pGlu, pyroglutamic acid.

METHODS

Preparation of Plasmacytoma Cell Suspension—Plasmacytoma cell suspensions were prepared as described earlier (8) except that cells were washed three times in chilled Eagle's minimal essential medium deficient in glutamine and one or more other amino acids.

Purification of Light Chain (λ)—Light chain was isolated from the urine of female BALB/c mice bearing subcutaneous RPC-20 tumors. *Concentrated urine, 10 ml (about 100 mg of protein),* was dialyzed extensively against 50 mM Tris-acetate (pH 5.5) buffer and then passed through a DEAE-cellulose column (1.5 × 20 cm) equilibrated with the above buffer (9). The column was eluted with a linear gradient (0 to 1 M) of 500 ml of NaCl in 50 mM Tris-acetate (pH 5.5) at room temperature. Fractions eluting close to the void volume were pooled, dialyzed against water, and lyophilized. The purified protein was examined by electrophoresis on 1.5% Agarose (50 mM Tris-acetate, pH 8.0, 300 volts for 50 min). Staining with Coomassie blue (0.004% in 5% trichloroacetic acid) indicated only one band migrating anodally.

Antibody Preparation—Adult goats were injected subcutaneously with 2 mg of purified λ chain in 1 ml of Buffer A (0.1 M sodium phosphate buffer, pH 7.3, in 0.14 M NaCl) after homogenization with 1 ml of Freund's complete adjuvant. Three weeks later a booster injection with 5 mg of light chain homogenized in 1 ml of Buffer A and 1 ml of Freund's incomplete adjuvant was given. Two weeks after the booster injection, the animals were bled and a crude γ-globulin fraction was prepared by Na_2SO_4 precipitation (10).

Preparation of Labeled Plasmacytoma Proteins—In a typical labeling experiment with [^{35}S]methionine or an ^{3}H-labeled amino acid, washed cells (8) from 10 g of tumor were suspended in 100 ml of Eagle's minimal essential medium (devoid of glutamine and the radioactive amino acid to be used). The cells were gently swirled and any large cell aggregates were discarded. The cell suspension was brought to 37° and then equilibrated with 5% CO_2 in air. Protein synthesis was initiated by addition of glutamine (10 μmol) and [^{35}S]methionine (3 μmol, 1300 μCi per μmol), [^{3}H]-methionine (0.52 μmol, 2300 μCi per μmol), [^{3}H]leucine (9.5 μmol. 3000 μCi per μmol), [^{3}H]arginine (0.6 μmol, 1600 μCi per μmol), or [^{3}H]tryptophan (12 μmol, 1250 μCi per μmol). At different time intervals, samples were withdrawn and immediately frozen in a CO_2-alcohol bath and then lyophilyzed. The lyophilyzed material was suspended in 1 to 3 ml of 10 mM Tris-HCl (pH 7.2) containing 0.14 M NaCl, sonicated briefly to break aggregates, and then dialyzed extensively (until greater than 90% of the radioactivity was precipitable by hot 5% trichloroacetic acid) against the same buffer.

NH_2-terminal Analysis—The sample was suspended in 5% trichloroacetic acid, boiled for 10 min, and cooled to room temperature. The precipitated protein was collected on a glass fiber filter (2.5 cm in diameter, Millipore Corp.), washed twice with 25 ml of 5% trichloroacetic acid, and dried by washing twice with 25 ml of ether-ethanol (1:1) and once with 25 ml of ether. The filter containing the dried protein sample was oxidized with performic acid (11) for 2½ hours at 0°. The dissolved proteins were

precipitated by addition of 5 ml of 5% trichloroacetic acid followed by 15 ml of 10% and 0.35 ml of 50% trichloroacetic acid. The precipitated proteins were collected on a glass fiber filter and washed twice with 25 ml of 5% trichloroacetic acid followed by 25 ml of ether-ethanol (1:1) and 25 ml of 66% (v/v) ethanol. Reaction of the performic acid-oxidized protein with dinitrofluorobenzene and acid hydrolysis of the derivatized protein were performed as described elsewhere (12). Protein hydrolysates (in 6 ml of 6 N HCl) were diluted with water to 1 N, then extracted twice with 25 ml of ether. The ether fraction was re-extracted with 50 ml of 1 N HCl, then evaporated to dryness. Samples containing [^{35}S]methionine were counted directly in scintillation fluid (Triton X-100-toluene-liquifluor, 6:12:1) and corrected for quenching by internal standards. Samples containing ^{3}H-labeled amino acids were oxidized to $^{3}H_2O$ in a Packard sample oxidizer, then counted by scintillation counting in Aquasol (New England Nuclear). Portions of the fractions not extracted into ether were prepared similarly for counting. The percentage of NH_2-terminal amino acid was calculated as the part of total radioactivity recovered in the ether fraction.

Immunodiffusion—Ouchterlony agar diffusion tests were performed as described by Ouchterlony (13) on plates (5 cm in diameter) obtained from Miles Laboratories. Samples, 10 μl, were applied to each well.

RESULTS

Amino Acid Incorporation by Cell Suspension of Mouse Plasmacytoma—Cell suspensions of mouse plasmacytoma, when incubated in Eagle's minimal essential medium, actively incorporate radioactive amino acids into protein (Fig. 1). The 2- to 3-fold greater apparent incorporation of arginine compared to methionine, leucine, or tryptophan is probably due to differences in uptake rates or pool sizes in the cells. A similar observation with respect to glutamine has been reported earlier (8).

Immunoprecipitation of Light Chain—In order to study the biosynthesis of the specific light chain protein containing NH_2-terminal pGlu, an antiserum specific for the protein was prepared (see "Methods"). Fig. 2 shows that the immune serum could be used as a specific reagent for precipitation of light chain from a cell extract of RPC-20 plasmacytoma. Diffusion of labeled plasmacytoma samples prepared for NH_2-terminal analysis against the goat antiserum showed only one precipitin line which exhibited a reaction of identity with that of the purified RPC-20 λ chain.

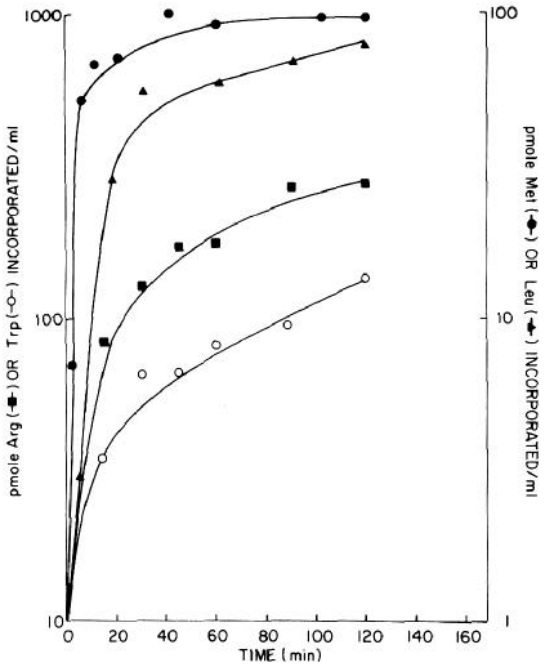

FIG. 1. Amino acid incorporation into plasmacytoma cells. Plasmacytoma cell suspensions were prepared as described under "Methods" and incubated at 37° with the designated amino acids. Aliquots (0.5 ml) were removed at different times, mixed with 1.0 ml of 0.5 M KCl, then proteins precipitated by addition of 1.5 ml of 10% trichloroacetic acid. The samples were then immersed in a boiling water bath for 5 min. After cooling, the precipitates were collected on glass fiber filters and washed with 5% trichloroacetic acid. Radioactivity on the filters was determined by scintillation counting in 10 ml of Triton X-100-toluene-liquifluor (6:12:1).

In order to establish the optimal concentration of immune serum required for maximum precipitation of the RPC-20 λ chain, different amounts of serum were incubated overnight at 4–5° with a fixed amount of sample in a total volume of 1.0 ml (made up with Buffer A). The precipitate was collected by centrifugation in a Microfuge (Eppendorf) for 5 min at 4° and washed twice with 1 ml of Buffer A. The washed precipitate was dissolved in 3% ammonia and the radioactivity was determined by scintillation counting in 10 ml of Triton X-100-toluene-liquifluor (6:12:1). A control experiment with preimmunization serum showed that less than 0.2% of the total radioactivity was precipitated (Fig. 3).

NH_2-terminal Analysis of Light Chain Proteins—Studies on the biosynthesis of hemoglobin have indicated that while the completed protein contains NH_2-terminal valine, there is a precursor form containing NH_2-terminal methionine (4, 14, 15). The existence of a methionine-containing precursor has been established by kinetic labeling experiments in which a species containing NH_2-terminal methionine is found in early labeling experiments but disappears with time (transient labeling). We used a similar approach to explore whether the RPC-20 λ chain which contains NH_2-terminal pGlu exists in a precursor form containing an NH_2-terminal amino acid other than glutamic acid. Table I shows the results of such a study. In the case of labeling with leucine, arginine, or tryptophan, there was no evidence for the transient appearance of species of proteins (in the immune precipitate) containing these amino acids at the NH_2 terminus. However, labeling with methionine produced a

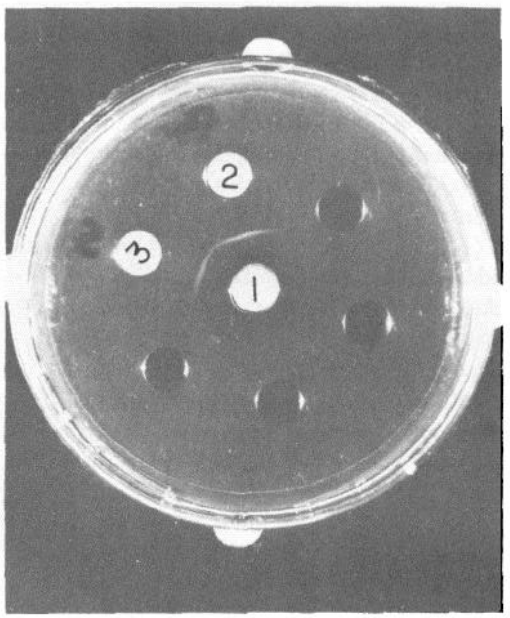

FIG. 2. Immunodiffusion analysis of goat anti-mouse λ chain. Immunodiffusion was performed as described elsewhere (13). The contents of the wells were as follows: (*1*) 10 μl of goat anti-mouse λ chain, (*2*) 10 μl of purified mouse λ chain (10 mg per ml), (*3*) 10 μl of plasmacytoma protein sample used for NH_2-terminal analysis.

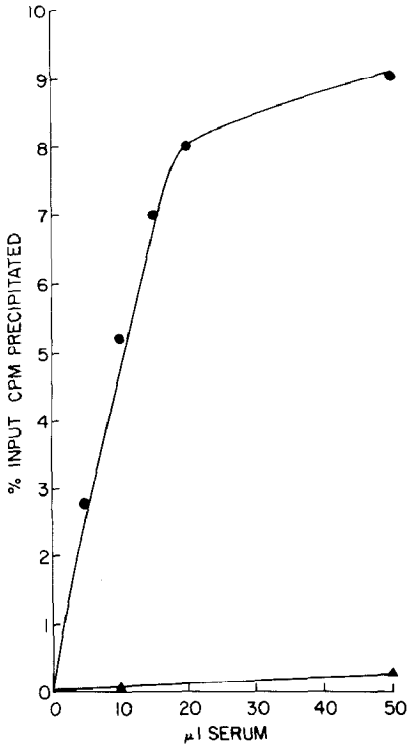

FIG. 3. Immunoprecipitation of radioactive light chain from total plasmacytoma protein. The details of the procedure are as described in the text. A 0.2-ml sample containing 100,000 cpm (equivalent to 4 to 5 ml of plasmacytoma cell suspension) was used per determination.

TABLE I

NH_2-terminal analysis of labeled plasmacytoma protein precipitated by goat antiserum to mouse λ chain

Samples of labeled plasmacytoma protein were prepared and precipitated with immune serum as described under "Methods." NH_2-terminal analyses on immune precipitates were performed as described under "Methods." The samples for analysis of immune precipitates were derived from between 10 and 100 ml of plasmacytoma cell suspension.

^{3}H-Amino acid	Radioactivity in immune precipitate		Radioactivity at the NH_2 terminus after	
	2 min	60 min	2 min	60 min
	cpm		%	
Leucine	120,453	192,134	0.33	0.18
Arginine	66,797	59,507	0.13	0.06
Tryptophan	42,646	10,922	2.08	2.17
Methionine	90,916	14,768	8.72	1.20

unique result. In this case, the NH_2-terminal labeling was greater after 2 min (8.7%) than after 60 min of labeling (1.2%). For this reason, the kinetics of labeling with methionine was explored further. Fig. 4 details the kinetics of appearance of label from [^{35}S]methionine into the NH_2 terminus of the total protein synthesized by the RPC-20 tumor cells (*first panel*). We also show the pattern of labeling in the protein precipitated by anti-λ chain immune serum (*second panel*) as well as the non-precipitated proteins (*third panel*). It can be clearly seen that the protein precipitated by antiserum shows the same type of transient labeling with methionine as do the other proteins synthesized by the tumor cells. We therefore conclude that methionine initiates the synthesis of the RPC-20 λ chain.

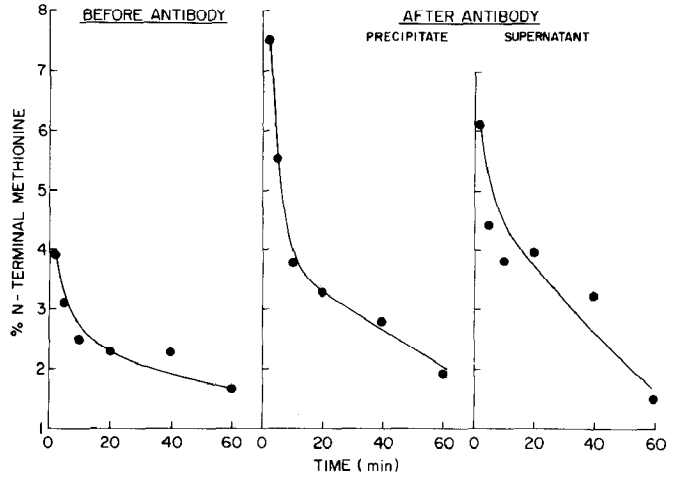

FIG. 4. NH_2-terminal analysis of [^{35}S]methionine-labeled plasmacytoma protein. Samples of methionine-labeled plasmacytoma proteins were prepared as described under "Methods." NH_2-terminal analysis of total protein (*first panel*), protein precipitated by goat anti-mouse λ chain (*second panel*), and protein not precipitated by goat antiserum (*third panel*) was performed as described under "Methods." Samples for analysis of total protein (*first panel*) were derived from between 2 and 40 ml of cell suspension and contained between 4,000 and 16,000 cpm. Samples for analysis of protein precipitated by antiserum (*second panel*) were derived from between 20 and 150 ml of cell suspension and contained between 2,000 and 16,000 cpm. Samples for analysis of protein not precipitated by goat antiserum (*third panel*) were derived from between 10 and 150 ml of cell suspension and contained between 15,000 and 60,000 cpm.

DISCUSSION

The structure of pGlu has a similarity to that of formylmethionine in the sense that both compounds have a blocked amino group. This similarity stimulated studies designed to show a function for pGlu in the initiation of synthesis of proteins containing NH_2-terminal pGlu in a manner analogous to that shown for formylmethionine in bacterial (16–18), chloroplast (19), and mitochondrial (20) protein synthesis. Several lines of evidence have accumulated that make the involvement of pGlu in initiation unlikely. pGlu does not become enzymatically attached to tRNA (21). While glutaminyl-tRNA can be enzymatically cyclized to pGlu-tRNA (22), this reaction appears not to be important in protein synthesis initiation because the biological precursor of pGlu seems to be glutamic acid rather than glutamine (8). Studies designed to show the enzymatic conversion of glutamyl-tRNA to pGlu-tRNA have been unsuccessful.[2] A search for a species of $tRNA^{Glu}$ specific for pGlu formation was also unsuccessful (23).

The background of information suggesting that pGlu is not directly involved in the initiation of synthesis of proteins containing NH_2-terminal pGlu has led us to explore alternate pathways for the initiation of RPC-20 λ chain synthesis. The data presented indicate that methionine is the amino acid that initiates the synthesis of the pGlu-containing protein. These data are consistent with the observations made in other systems indicating that methionine initiates protein synthesis in eucaryotes.

Early studies on hemoglobin synthesis (4, 14, 15) indicated that while methionine initiated the synthesis of hemoglobin, the protein was rapidly processed to the species containing NH_2-terminal valine. Kinetic studies in other systems have re-

[2] Unpublished observations.

peatedly shown this type of "transient labeling" with methionine. Studies in HeLa cells (7, 24) have provided evidence for methionine initiation in total protein by techniques somewhat similar to those used here. In the above studies, the preparations were enriched for early products of synthesis by synchronizing cells for initiation by NaF treatment and analyses were performed on polysome fractions. Our studies, designed to determine labeling patterns in the unique protein containing NH_2-terminal pGlu, depended on isolation using an immune serum raised against the purified λ chain. The results presented here indicate that this protein is also initiated with methionine.

Several investigators (25, 26) have studied the *in vitro* synthesis of proteins by mRNA isolated from plasmacytomas producing κ-type light chains with NH_2-terminal asparagine (25) or aspartic acid (26). In these experiments, exogenous mRNA stimulated the incorporation of radioactive amino acids into protein electrophoretically similar to the authentic κ chain protein and also into a protein interpreted to be a precursor. Electrophoretic analysis of the "precursor" protein suggested that it might have about 8 to 10 extra amino acids. The studies reported here are the first suggestions that a similar biosynthetic pathway for protein containing NH_2-terminal pGlu may exist; a precursor protein containing NH_2-terminal methionine may be processed to yield a protein containing NH_2-terminal pGlu. The mechanism of this conversion is not known. It might involve the cleavage of the NH_2-terminal peptide coupled to cyclization of glutamate to pGlu or may involve the stepwise hydrolysis of a specific peptide bond followed by cyclization of NH_2-terminal glutamic acid to NH_2-terminal pGlu.

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