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Immunology

Marked hydrophobicity of the NH_2-terminal extra piece of immunoglobulin light-chain precursors: Possible physiological functions of the extra piece

(duplication of a gene segment/antigen-recognizing receptor/secretory proteins/translation of immunoglobulin light chain mRNA/amino acid sequence of cell-free product)

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ABSTRACT mRNAs coding for mouse immunoglobulin light chains direct the cell-free synthesis of precursors in which extra peptide segments precede the NH_2-termini of the mature proteins. The abundance (18–30%) of leucine residues in the extra piece indicates that it is quite hydrophobic [Schechter and Burstein (1976) *Biochem. Biophys. Res. Commun.* 68, 489]. Accordingly, we have determined the positions of all hydrophobic residues by sequencing two κ-type light (L)-chain precursors that were labeled with: [^{3}H]Ala, [^{3}H]Val, [^{3}H]Leu, [^{3}H]Ile, [^{3}H]Thr, [^{3}H]Pro, [^{3}H]Phe, [^{3}H]Tyr, [^{3}H]Trp, [^{35}S]Met, and [^{35}S]Cys. The partial sequences (and sizes) of the extra pieces obtained are: in MOPC-321 precursor, Met-X-Thr-X-Thr-Leu-Leu-Leu-Trp-Val-Leu-Leu-Leu-Trp-Val-Pro-X-X-Thr-X- (20 residues; X is unknown); in MOPC-41 precursor, Met-X-Met-X-Ala-Pro-Ala-X-Ile-Phe-X[b]Phe-Leu-Leu-Leu-Leu-Phe-Pro-X-Thr-X-Cys- (22 residues). Despite the fact that these extra pieces differ extensively in sequence (68%), both of them are highly enriched with hydrophobic residues (75% in MOPC-321, 73% in MOPC-41). This marked hydrophobicity suggests that the extra piece favors interaction of the precursor with cell membranes, in a manner similar to the function of the "hydrophobic domain" of membrane-bound proteins (e.g., glycophorin). We propose that the hydrophobic extra piece directs most precursor molecules to the endoplasmic reticulum, where they are cleaved to yield mature L chain destined for secretion; a few precursor molecules escape cleavage and are embedded in the cell surface to serve as the antigen-recognizing receptor. The probability that the Leu-Leu-Leu-Trp-Val sequence occurs by chance is 1.6×10^{-8}. Therefore, the data provide evidence for duplication of a short DNA segment in the structural gene coding for the MOPC-321 precursor. Duplication with inversion is also indicated from inverted repetition of the Phe-Leu-Leu sequence in the extra piece of the MOPC-41 precursor.

It has been shown previously that a variety of Ig light (L)-chain mRNAs direct the synthesis of precursors (reviewed in ref. 1) in which extra peptide segments (19–22 residues in length) precede the NH_2-termini of the mature L chains (1–4). Earlier experiments have indicated that the extra piece might be remarkably hydrophobic, since it was found to contain large (and seldom observed) clusters of leucine residues. Partial amino acid sequence analyses of the precursors of MOPC-321 and MOPC-63 L chains showed two triplets of leucines in the extra piece (5, 6); in MOPC-41 precursor the extra piece contains a quadruplet of leucines (3); five closely gathered leucines were found in the extra piece of MOPC-104E L-chain precursor (4). These primary structural features of the extra piece (i.e., marked hydrophobicity) suggest that the role of the extra piece is to favor interaction of the precursor with the endoplasmic membranes, with the cell surface, or both (1, 5). We here report the sequence of all hydrophobic residues (7) in the extra pieces of the precursors of M-321 and M-41 κ-type L chains, which are of different subgroups (8). Despite the fact that these extra pieces differ extensively in sequence, both of them are found to be highly enriched (73–75%) with hydrophobic residues. Furthermore, the data provide evidence for duplication of a short DNA segment in the structural gene coding for the M-321 L-chain precursor.

Abbreviations: L, light chain of immunoglobulin; V, variable region of immunoglobulin chain. MOPC-321, MOPC-104E, MOPC-63, and MOPC-41 myelomas are abbreviated to M-321, M-104E, M-63, and M-41, respectively.

MATERIALS AND METHODS

The M-321 and M-41 myeloma tumors were kindly donated by Dr. M. Potter (National Institutes of Health, Bethesda, Md.), and were maintained as solid tumors in female BALB/c mice (9). Commercial wheat germ was supplied by the Bar-Rav Mill, Tel Aviv. [^{3}H]Thr (2.1 Ci/mmol), [^{3}H]Phe (9.1 Ci/mmol), [^{3}H]Trp (20 Ci/mmol), and [^{35}S]cystine (58 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. [^{3}H]Val (15.3 Ci/mmol) and [^{3}H]Tyr (22 Ci/mmol) were obtained from the Radiochemical Centre, Amersham Bucks, U.K. [^{35}S]Cystine was reduced to [^{35}S]cysteine in 80 mM Tris·HCl (pH 7.6), 20 mM dithiothreitol, at 36° for 1 hr.

Immunoglobulin L-chain mRNAs. The mRNAs coding for the L chains were prepared from myeloma polysomes specifically precipitated by anti-L-chain antibodies (10). The M-321 (2) and M-41 (3) L-chain mRNAs have been previously characterized.

Cell-Free Synthesis of L-Chain Precursors. Translation of the L-chain mRNAs was carried out in the wheat germ cell-free system (11) at 25° for 4 hr, as described (1).

Amino Acid Sequence Analyses. The total cell-free products that were labeled by one radioactive amino acid at a time were analyzed in the Beckman model 890C Automatic Sequencer as detailed elsewhere (1), with the exception of [^{35}S]Cys-labeled products that were completely reduced and alkylated prior to sequence analyses. To the [^{35}S]Cys-labeled cell-free reaction mixture was added 1 volume of 1 M Tris base (to release nascent chains, ref. 1), and after 30 min at 36° the mixture was adjusted to pH 8.2 by adding 0.5 volume of 1 M acetic acid. Concentrated guanidine·HCl and Tris·HCl (pH 8.2) were added to final concentrations of 6.0 M and 0.5 M, respectively. The proteins were reduced in 0.02 M dithiothreitol for 1 hr at 36°, and alkylated with 0.16 M ethyleneimine for 1 hr. After exhaustive dialysis against 0.01 M Tris·HCl (pH 7.6), the proteins were precipitated by cold 10% trichloroacetic acid. The precipitate was washed twice with cold 5% trichloroacetic acid, twice with acetone:water (95:5, vol/vol); it was then dissolved in 40% acetic acid and applied to the sequencer (1). The radioactive samples were supplemented with 5 mg of sperm whale apomyoglobin carrier. Repetitive yields of the protein carrier and of the ra-

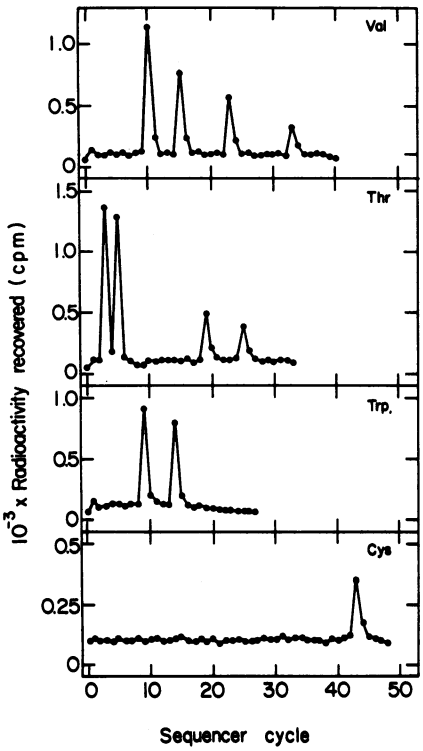

FIG. 1. Radioactivity recovered at each sequencer cycle from the total cell-free products programmed by M-321 L-chain mRNA. The products were labeled with the following radioactive amino acids (numbers in parentheses represent cpm in the sample analyzed): [^{3}H]Val (31,200), [^{3}H]Thr (39,500), [^{3}H]Trp (13,500), [^{35}S]Cys (34,600). Background radioactivity obtained from sequencer run of the control sample was subtracted. The control sample consisted of wheat germ extract containing each of the labeled amino acids, but without added mRNA (1). Cycle zero represents a blank cycle (without phenyl isothiocyanate) which was used to wash out potential radioactive contaminants.

dioactively labeled materials ranged between 92 and 95%. All samples were sequenced twice. In the duplicate the pattern of radioactive peaks was identical. The absolute yields were corrected for background and "out of step" radioactivity (12), and for endogenous activity of the cell-free extract (1). The absolute yields for the M-321 labeled precursors ranged between 95 and 106%, with the exception of the [^{3}H]Trp-labeled precursor, where the yield was 81%. The absolute yields for the M-41 labeled precursors ranged between 63 and 98%.

RESULTS

Sequence analyses of the precursor of M-321 L chain

Analyses of M-321 precursor labeled with [^{35}S]Met, [^{3}H]Leu, [^{3}H]Pro, [^{3}H]Ile (1, 3), and [^{3}H]Ala (6), have been reported. These analyses showed that the NH_2-terminal extra piece of the M-321 precursor is 20 residues in length and that it contains one methionine at the NH_2-terminus; six leucines at positions 6, 7, 8, 11, 12, 13; one proline at position 16; and no isoleucine or alanine. In order to determine the sequence of all hydrophobic residues (7), we have synthesized and sequenced M-321 precursor molecules that were labeled with [^{3}H]Val, [^{3}H]Thr, [^{3}H]Phe, [^{3}H]Tyr, [^{3}H]Trp, and [^{35}S]Cys. From Fig. 1 it is seen that sequence analyses of [^{3}H]Val-labeled precursor yielded radioactive peaks at cycles 10, 15, 23, and 33; [^{3}H]Thr-labeled precursor yielded peaks at cycles 3, 5, 19, and 25; [^{3}H]Trp-labeled precursor yielded peaks at cycles 9 and 14; with the [^{35}S]Cys-labeled precursor one radioactive peak at cycle 43 was obtained. As expected from the size of the extra piece, after 20 degradative cycles the positions of amino acid residues in the cell-free product show perfect homology with the positions of these residues in the mature L chain. That is Val^{23}, Thr^{25}, Val^{33}, and Cys^{43} in the precursor match with Val^3, Thr^5, Val^{13}, and Cys^{23} in the mature M-321 L chain (8). It is also evident that the extra piece contains valine at positions 10 and 15; threonine at positions 3, 5, and 19; tryptophan at positions 9 and 14; and no cysteine. Flat and low backgrounds of radioactivity were obtained from sequencer runs (25 cycles) of [^{3}H]Phe- and [^{3}H]Tyr-labeled precursors, thus showing that this extra piece does not contain phenylalanine or tyrosine residues. The positions of hydrophobic residues in the extra piece of M-321 precursor determined here and in previous studies are given in Fig. 3.

Sequence analyses of the precursor of M-41 L chain

Analyses of the M-41 precursor labeled with [^{35}S]Met, [^{3}H]Leu, [^{3}H]Pro, [^{3}H]Ile (3), and [^{3}H]Ala (6), have been reported. These studies showed that the M-41 mRNA directs the synthesis of two precursors in which 22 or 20 amino acid residues precede the NH_2-terminus of the mature L chain. About 75% of the molecules have a long extra piece (22 residues); the remaining molecules have a shorter extra piece (20 residues). The two NH_2-terminal residues of the long extra piece are missing in the short extra piece; in the other 20 positions both extra pieces have identical sequences. The long extra piece contains two methionines at positions 1 and 3; a leucine quadruplet at positions 13 through 16; two prolines at positions 6 and 18; one isoleucine at position 9; and two alanines at positions 5 and 7. To gain information on the positions of all hydrophobic residues we have synthesized and sequenced M-41 precursors that were labeled with [^{35}S]Cys, [^{3}H]Thr, [^{3}H]Phe, [^{3}H]Val, [^{3}H]Tyr, and [^{3}H]Trp.

Sequencer runs (30 cycles) of [^{3}H]Val-, [^{3}H]Tyr-, and [^{3}H]Trp-labeled precursors yielded only background radioactivity, thus showing that these amino acids are not present in the extra piece of the M-41 precursor. Analyses of precursors labeled with other amino acids (Fig. 2) show that minor radioactive peaks precede the major peaks by two sequencer cycles (the minor peak of Phe^{12} is included in the peak of Phe^{10}). These results confirm our previous observations on the occurrence of two M-41 precursors, where the major component is longer by two residues than the minor component (3). The data of [^{3}H]Phe- and [^{3}H]Thr-labeled precursors show that the long extra piece contains three phenylalanines at positions 10, 12, and 17, and one threonine at position 20. As expected, Thr^{27} (major peak) and Thr^{25} (minor peak) in the long and short precursors (see Fig. 2) match with Thr^5 in the mature M-41 L-chain (8). Analyses of the [^{35}S]Cys-labeled precursor show a small radioactive peak at cycle 11, a prominent peak at cycle 22 with a minor peak at cycle 20, and another peak at cycle 45. The peak obtained at cycle 45 matches with Cys^{23} in the mature M-41 L chain, since the longer extra piece has 22 residues (the minor peak expected at cycle 43 from a precursor with 20 residues in the extra piece was not detected, probably because this species is present in small amounts and the sequencer efficiency is decreased at long runs). The minor and major peaks at cycles 20 and 22 correspond to Cys^{20} and Cys^{22}, in the short and long extra pieces, respectively. These cysteine residues were obtained in 98% yield. The radioactivity recovered at cycle 11 would have corresponded only to 5.7% of the expected amount had this position been occupied by a cysteine residue. The origin of this small peak at cycle 11 is not yet clear, since preliminary experiments have shown that this position is occupied by a

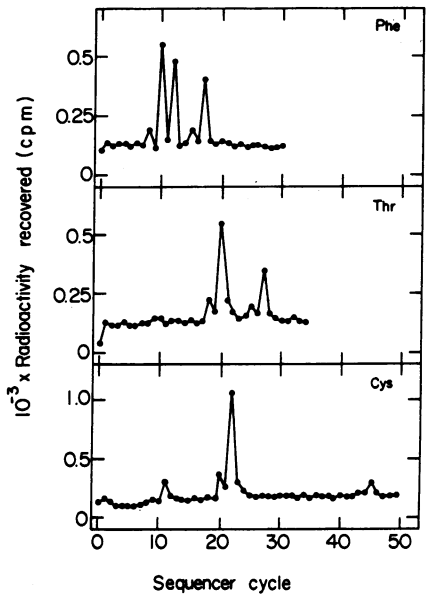

FIG. 2. Radioactivity recovered at each sequencer cycle from the total cell-free products programmed by M-41 L-chain mRNA. The products were labeled with the following radioactive amino acids (numbers in parentheses represent cpm in the sample analyzed): [^{3}H]Phe (17,600), [^{3}H]Thr (51,000), [^{35}S]Cys (27,500). Data were corrected and are presented as detailed in the legend to Fig. 1.

glycine residue (Gly^9 in short precursor and Gly^{11} in long precursor; Burstein and Schechter, unpublished data).

The positions of all hydrophobic residues in the long and short extra pieces of M-41 precursor molecules, determined here and in previous studies, are given in Fig. 3.

DISCUSSION

In the L-chain precursor the extra piece is linked to the NH_2-terminus of the mature protein, i.e., to the variable (V) region. The κ-type M-321 and M-41 L chains belong to different subgroups (8); accordingly, they share the same constant region but differ at the V region. Although the sequence data are incomplete, it is evident that the extra pieces of these precursors differ in size and sequence (Fig. 3). These findings indicate strongly that the NH_2-terminal extra piece is part of the V region; the implication at the genome level is that the V region gene may be larger than hitherto known (3).

The sequence data are limited to two precursors. Bearing this reservation in mind, it is still interesting to note that sequence comparisons indicate that the gene-segment coding for the extra piece may be a "hot spot" with accelerated mutation rate, similar to the DNA regions coding for the "hypervariable regions" (13). The results summarized in Table 1 show that the extent of variability in the extra piece (minimal value 65–68%) is larger than in the entire V-region domain (48%), and is comparable to the variability found between the "hypervariable regions" of the M-321 and M-41 L chains (65%).

The evolutionary tree of L chains shows that the branching point for divergence of the M-321 and M-41 *V* genes (14) is probably the oldest one (about 100 million years) in κ-type *V*-gene evolution (15). The ancestral κ-type *V* gene probably contained the genetic information for the extra piece, since it is present in both branches (one leading to M-321 the other to M-41) emanating from the earliest known κ-chain divergence, and since expression of the *V* gene is presumably contingent on the presence of the extra piece (see below).

The extra piece of M-321 contains repetition of the Leu-Leu-Leu-Trp-Val sequence (the probability that this sequence occurs by chance is 1.6×10^{-8}), suggesting duplication of a short DNA segment in the structural gene coding for this precursor. It is likely that this event occurred recently in evolutionary time, as the repeating sequence retained complete homology despite the indication for accelerated mutation rate in this region. Duplication with inversion is also indicated from inverted repetition of the Phe-Leu-Leu sequence in the extra piece of the M-41 precursor (Fig. 3). It is generally believed that Ig chains originated from duplication of a single primordial gene coding for about 110 residues, and that diversity was generated by point mutations, insertions, and deletions. However, evidence for duplication of a short DNA segment in the evolution of Ig chains is scarce. To our knowledge there is only one clear example reported by Smith (14), showing repetition of a sequence of six amino acids at positions 1–6 and 13–18 in the mature MPC-11 L chain.

The identification of NH_2-terminal methionine in the four precursor molecules investigated so far (M-321 and M-41 reported here; M-63; ref. 6; M-104E L-chain precursor, ref. 4) suggests that the precursors represent the direct product of L-chain mRNA translation within the cell. This is based on the fact that in every precursor analyzed methionine has not been found at the position preceding the NH_2-terminal residue of the mature L chain (e.g., Fig. 3), and that unblocked methionine is the initiator residue for protein synthesis in eukaryotes (reviewed in ref. 1). It is therefore expected that translation of the L-chain mRNA should be contingent on the integrity of the nucleotide sequence coding for the NH_2-terminal extra piece. This segment is located at the 5′ end of the mRNA, where mRNA translation is initiated. In agreement, it was shown that L-chain mRNA molecules that are deficient at the 5′ end are untranslatable in a cell-free system (16). The finding that myeloma polysomes incubated *in vitro* synthesize precursors (17, 18) also indicates that the precursor is formed within the cell. The vast majority of precursor molecules should be short lived within the cell and processed to the mature protein (cleavage of the extra piece) rather quickly, because only mature L chains have been isolated from myeloma tumors or body fluids (2, 19). Therefore, the cleavage step(s) converting the

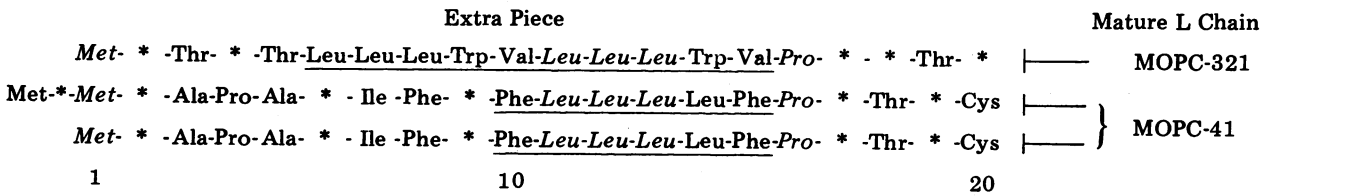

FIG. 3. Comparison of the size and sequence of hydrophobic residues in the NH_2-terminal extra pieces of the precursors of M-321 and M-41 L chains. Sequences are lined up to give maximal homology with the NH_2-terminal extra piece of the M-321 precursor. The extra pieces of the two M-41 precursor molecules are given; in the short extra piece (20 residues) two residues from the NH_2-terminus of the long extra piece (22 residues) are missing (3). Homologous residues in M-321 and M-41 precursors are typed in italics. Sequence repetitions of the same (M-321) and opposite (M-41) polarity are underlined. Unidentified residues are indicated by *.

Table 1. Sequence heterogeneity at the NH_2-terminal extra piece of κ-type L-chain precursors

Precursors	Residues in extra piece	Amino acid substitutions† in Extra piece‡ No.	%	Variable region§ No.	%	Hypervariable regions§ No.	%
MOPC-321	20						
MOPC-41(a)*	22	15/22	68	53/111	48	20/31	65
MOPC-41(b)*	20	13/20	65	53/111	48	20/31	65

* Data refer to the long and short extra pieces in the two M-41 precursors (3).
† Amino acid substitutions are compared to the M-321 L-chain precursor.
‡ Numbers represent minimal values since they are based on partial sequences given in Fig. 3.
§ Numbers for the entire variable region and for the hypervariable regions (13) are based on sequences of the mature L chains (8).

precursor to the mature protein may provide a point of metabolic control to regulate L-chain secretion.

It is generally thought that secretory proteins are synthesized on microsomes. The "signal hypothesis" postulates that signal peptides located at the NH_2-termini of secretory proteins direct polysomes synthesizing these proteins to the endoplasmic membranes, the growing nascent chains are vectorially discharged across the microsome membrane, and the signal peptides are then cleaved (18). It has been proposed that the modified NH_2-terminus of L-chain precursors may serve as the signaling device (17, 18). The marked hydrophobicity of the NH_2-terminal extra piece reported here (Table 2) supports these proposals. It should be realized, however, that attempts to obtain mature L chain from precursor by incubating it *in vitro* with microsomes have not yet been successful. Presumably cleavage of the extra piece occurs during (rather than after) synthesis of the precursor (17, 20). Blobel and collaborators proposed that the signal peptides of secretory proteins may be identical or have considerable sequence homology (21). This may be limited to one family of proteins (e.g., pancreatic enzymes, ref. 21), but not to all of them, since in L-chain precursors the extra pieces exhibit a high degree of sequence heterogeneity (Table 1).

Despite the fact that the extra pieces differ extensively in sequence, both of them were found to be highly enriched with hydrophobic residues (75% in M-321, 73–75% in M-41). The percentage of apolar residues in the extra pieces is considerably larger than their percentage in the mature L chains (46–49%) or in other secretory proteins (48–49%) (Table 2). The clustering of hydrophobic residues in a distinct region (i.e., the extra piece) of the precursor is reminiscent of the molecular topography of membrane proteins. These proteins contain a distinct hydrophobic domain which is embedded in the lipid portion of the membrane, and a hydrophilic portion which is exposed to the surrounding environment (22). For example, in glycophorin the hydrophobic domain embedded in the reticulocyte membrane is 23 residues in length and is located close to the carboxy-terminus of the molecule (22, 23); in cytochrome b_5 the hydrophobic domain (40 residues in length) is at the NH_2-terminus of the molecule (24). The abundance of hydrophobic residues in the extra piece and mature L-chain "portions" of the precursor is comparable to that found in the hydrophobic domain and exposed portion of membrane proteins (Table 2). These findings suggest that the role of the hydrophobic extra piece is to favor interaction of the precursor with cell membranes (1, 5) and have led us to propose the following hypothesis. The Ig precursor, which is the immediate product of mRNA translation, is the common intermediate for secreted Ig and antigen-recognizing receptor. In plasma cells that synthesize large amounts of antibodies most of the precursor molecules are directed to the endoplasmic reticulum, where they undergo the maturation process (cleavage of the extra piece) to produce mature Ig destined for secretion; a few precursor molecules escape cleavage and are anchored in the surface membrane to serve as the antigen receptor. In immature plasma cells and lymphocytes most of the precursor molecules remain as such and are embedded in the cell surface. A few lines of evidence supporting this hypothesis are listed below. Myeloma tumors with few membrane-bound polysomes have large amounts of surface Ig and secrete little Ig, whereas tumors rich in membrane-bound polysomes have little surface Ig and se-

Table 2. Distribution of hydrophobic residues in immunoglobulin L-chain precursors and membrane-bound proteins

Protein	Residues Hydrophobic/total	%	Residues Hydrophobic/total	%
	Extra piece		*Mature L chain**	
MOPC-321 precursor	15/20	75	107/218	49
MOPC-41 precursor(a)	16/22	73	97/213	46
MOPC-41 precursor(b)	15/20	75	97/213	46
	Hydrophobic domain		*"Residual" protein*	
Glycophorin*	17/23	74	60/124	48
Cytochrome b_5*	29/40	72	40/97	41
Lactalbumin (bovine)*			60/123	49
Trypsinogen (bovine)*			110/229	48

* Numbers are calculated from data in: ref. 8 for the mature L chains; refs. 22 and 23 for glycophorin; ref. 24 for cytochrome b_5; and ref. 15 for lactalbumin and trypsinogen.

crete copious amounts of Ig (25). Surface Ig is present in most (if not all) plasmacytomas (26). Under appropriate conditions the surface Ig of plasma cells can produce polar caps routinely produced by the presumed antigen receptor of bone-marrow-derived (B)-lymphocytes (27). Of particular interest is the recent report on the penicillinases of *Bacillus licheniformis*. This bacterium produces two types of penicillinases, a highly soluble exoenzyme (secreted into the medium) and a hydrophobic membrane-bound form, while containing only a single penicillinase structural gene. The membrane-bound enzyme differs from the exoenzyme in carrying at the NH_2-terminus an additional peptide (25 residues in length) containing phosphatidylserine. The latter grouping (containing esterified fatty acids) is responsible for anchoring the hydrophobic form of the enzyme to the cell membrane (28).

We realize that much work is required to prove this hypothesis: identification of heavy (H)-chain precursor, demonstration that H- and L-chain precursors can associate to generate molecules with antigen-binding activity, search for precursor molecules in the cell surface, etc. Nonetheless, experimental evaluation of this hypothesis should be greatly facilitated since membrane bound Ig can be isolated, and the NH_2-terminal sequence of this Ig can be determined and compared with known sequences of the mature Ig and of the precursor.

Finally, it has not escaped our attention that by virtue of its structural variability the NH_2-terminal extra piece can also function as a new recognition system. Yet, speculations and experiments aimed at exploring this possibility should be deferred until precursor molecules have been identified on the surface membrane of B and/or thymus-derived (T) lymphocytes.

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