Myristoylation and the post-translational acquisition of hydrophobicity by the membrane immunoglobulin heavy-chain polypeptide in B lymphocytes

(Membrane immunoglobulin/acylation)

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ABSTRACT Membrane immunoglobulin heavy chain in pre-B and in B cells is initially synthesized as a relatively hydrophilic protein that is nonetheless stably anchored in the endoplasmic reticulum membrane. In B cells, but not in pre-B cells, the membrane immunoglobulin heavy chain is posttranslationally converted to a relatively hydrophobic form that partitions into the oil phase when solubilized with the phase-separating detergent Triton X-114. Covalent myristoylation of the membrane and secretory forms of immunoglobulin heavy chains as well as of light chains was observed in B cells. Myristoylation of the membrane immunoglobulin heavy chain correlates with its transport to the cell surface and its posttranslational conversion to a relatively hydrophobic form. This post-translational modification is hydroxylamine resistant and may be responsible for the assembly and transport of membrane immunoglobulin to the cell surface in B cells.

In pre-B cells only the μm1 form of membrane immunoglobulin was observed. From the kinetics of μm2 appearance in B cells it appears likely that the conversion of μm1 to μm2 is the result of a post-translational modification. The hydrophobic nature of μm2 suggests that it might be modified by fatty acid acylation. Although a number of proteins that traverse the secretory pathway are acylated, previous attempts to metabolically label immunoglobulin in B cells with palmitate were unsuccessful (6). We show here that intracellular immunoglobulin in B cells but not in pre-B cells can be labeled with myristic acid. The relatively hydrophilic nonacylated μm1 form in pre-B cells is nonetheless stably anchored in microsomal membranes.

MATERIALS AND METHODS

Cell Lines. 230-37 (7) and 54.3 (8) are mouse lymphoid cell lines that represent the pre-B cell stage of immunodifferentiation. WEHI 231 (9) expresses surface immunoglobulin and represents the virgin B-cell stage.

Antisera. A commercial μ-chain-specific, anti-IgM antibody (anti-μ, Southern Biotechnology Associates, Birmingham, AL), was used to precipitate both μm and μL. A μm-specific anti-peptide antibody was raised against the 12 extracellular amino acids encoded by the μm1 exon. A synthetic peptide (Glulys-Glu-Val-Asn-Ala-Glu-Glu-Gly-Phe-Glu-Tyr) was obtained commercially (Children’s Hospital, Boston) and was conjugated to bovine serum albumin (BSA) using bis-diazotized benzidine (10). Rabbits were immunized at monthly intervals (1 mg of conjugate initially emulsified with complete Freund’s adjuvant; all booster injections were with incomplete Freund’s adjuvant).

Pepptide-BSA and BSA were separately coupled to Sepharose 4B using vinyl sulfone (11). The anti-peptide antibody was affinity purified by depleting the antiserum of anti-BSA antibodies using BSA-Sepharose and then isolating anti-peptide antibodies using a peptide-BSA immunoabsorbent. A rabbit antiserum specific for the gag proteins of the Moloney murine leukemia virus and capable of immunoprecipitating the p16[pre-abl] protein of the Abelson virus (12) was obtained from Bernard Mathey-Prevot (Whitehead Institute).

Phase Separation and Immunoprecipitation of μ Chains from Metabolically Labeled Pre-B and B Cells at Various Chase Points. 54.3, a pre-B cell line, and WEHI 231, a B cell line, were pulse labeled with [35S]methionine (5 × 10⁶ cells, 100 μCi of 700 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear; labeling period, 2 min). Cells were chased with an excess of unlabeled methionine in culture medium for various periods of time and were lysed with Triton X-114 essentially as described by Bordier (13). Detergent and aqueous frac-

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tions were immunoprecipitated with anti-\( \mu \), and the immunoprecipitates were analyzed by electrophoresis through a 5–15% polyacrylamide/NaDodSO\(_4\) gradient gel.

**Cell Surface Iodination of B Cells and Immunoprecipitation of Phase-Separated Lysates.** WEHI 231 cells (5 × 10\(^6\) cells) were surface iodinated using the lactoperoxidase–glucose oxidase method (14). Cells were lysed with Triton X-114, and detergent and aqueous fractions were separately immunoprecipitated with anti-\( \mu \). Immunoprecipitated proteins were analyzed as described above.

**Metabolic Labeling and Immunoprecipitation of \( \mu \) Chains and p160\(^{\text{polCD}}\).** [\(^{3}H\)]Myristate. Cells were labeled for 90 min at 37°C in serum-free RPMI 1640 at a density of 5 × 10\(^6\) cells per ml. [9,10-\(^{3}H\)]Myristic acid (22.4 Ci/mmol, New England Nuclear) was dissolved in dimethyl sulfoxide and was added to the culture medium at a final concentration of 0.5 mCi/ml.

[\(^{35}S\)]Methionine. Cells were labeled for 90 min at 37°C in methionine-free Dulbecco’s modified Eagle’s medium at a density of 5 × 10\(^6\) cells per ml with 100 \( \mu \)Ci of [\(^{35}S\)]methionine (700 Ci/mmol; New England Nuclear) per ml of medium. At the end of the labeling period, cells were washed with 50 mM sodium phosphate, pH 7.4/150 mM NaCl and were lysed in 0.5% Nonidet P-40 (Shell)/10 mM Tris-HCl, pH 7.4/3 mM MgCl\(_2\)/10 mM NaCl/1 mM phenylmethylsulfonil fluoride. After immunoprecipitation, protein A-Sepharose pellets were washed four times with 50 mM Tris-HCl, pH 8.0/0.5% Nonidet P-40/0.1% NaDodSO\(_4\)/0.5% sodium deoxycholate/0.5 M NaCl and were extracted twice with chloroform/methanol, 2:1 (vol/vol), to remove noncovalently attached lipids. Protein complexes were dissociated by boiling in a 2% (wt/vol) NaDodSO\(_4\) sample buffer (14) for 5 min.

**Phase Separation with Triton X-114 and Resistance of Acyl Moiety to Hydroxylamine.** WEHI 231 cells (5 × 10\(^6\) cells) were separately labeled with [\(^{35}S\)]methionine and \([H]\)myristate as described above. Cells were lysed with a 1% Triton X-114 (Rohm and Haas) solution, and detergent and aqueous phases were separated as described by Bordier (13). The detergent pellet was taken up in 10 mM Tris-HCl, pH 7.4/100 mM NaCl. The final volume of the aqueous phase was 2 ml. Immunoprecipitation with anti-\( \mu \) was carried out as described above, and 10% polyacrylamide/NaDodSO\(_4\) gels and samples were prepared and electrophoresed essentially according to the procedure described by Laemmli (14). To determine whether the [\(^{3}H\)]myristate label was resistant to cleavage by hydroxylamine (15), we analyzed samples from the detergent and aqueous fractions described above were split into two equal portions and electrophoresed through two gels run in parallel. After a 30-min fixation period, one gel was left in fixative overnight, whereas the other gel was exposed to 4 M hydroxylamine (pH 6.9) with constant shaking at 25°C for 1 hr.

**Preparation of Microsomes and Extraction of Luminal and Anchored Immunoglobulin Species.** Pre-B cells (line 54.3) (10\(^7\) cells) were metabolically labeled with [\(^{35}S\)]methionine for 30 min. Cells were washed and suspended for 5 min in 3.3 mM Tris-HCl, pH 7.4/1 mM MgCl\(_2\)/3.3 mM NaCl/1 mM phenylmethylsulfonil fluoride and were homogenized using a hand-held Potter-Elvejhem homogenizer. The lysate was layered over an equal volume of 10% (wt/vol) sucrose in reticulocyte saline buffer (RSB); 10 mM Tris-HCl, pH 7.4/3 mM MgCl\(_2\)/10 mM NaCl. Nuclei were spun out at 700 × g for 10 min, and a crude microsomal supernatant was obtained by centrifugation at 10,000 × g for 7 min. Microsomes were pelletized by centrifugation at 100,000 × g for 45 min and were washed with RSB. Microsomes were extracted with 0.02% saponin in RSB, and the 100,000 × g supernatant was set aside for immunoprecipitation of \( \mu \) chains. A second saponin extraction was performed before extracting the pellet with 0.5% Nonidet P-40/RSB. Immunoprecipitates from all three detergent extracts were analyzed by electrophoresis on polyacrylamide/NaDodSO\(_4\) gels as described above.

**RESULTS**

Post-translational Acquisition of Hydrophobicity by \( \mu_m \) in B Cells but Not in Pre-B Cells. To compare the biosynthesis and fate of \( \mu_m \) in pre-B cells and in B cells, we attempted to separate and distinguish \( \mu_m \) from \( \mu_s \) in lysates from these cell types. We initially planned to use Triton X-114, a phase-separating detergent that can separate integral membrane proteins from soluble nonanchored proteins (13). We lysed lymphocytes with this detergent expecting \( \mu_m \) to partition in the oil or detergent phase and \( \mu_s \) to remain in the aqueous phase. To confirm the identity of the species so separated we immunoprecipitated immunoglobulin with a \( \mu \)-chain-specific antiserum and also with an anti-peptide antibody specific for \( \mu_m \).

Short pulse labeling revealed that soon after synthesis, in both Abelson virus-transformed pre-B and in WEHI 231 B cells, \( \mu_m \) and \( \mu_s \) unexpectedly partitioned into the aqueous phase of Triton X-114 lysates (Fig. 1, lanes 1, 2, 7, and 8). Chase experiments showed that in B cells, but not in pre-B cells, starting 20–30 min after synthesis, \( \mu_m \) was converted

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**Fig. 1.** Analysis of \( \mu \)-chain immunoprecipitates from a pre-B Abelson cell line and from a B-cell line following phase separation with Triton X-114. A pre-B-cell line, 54.3 (lanes 1–6), and a B-cell line, WEHI 231 (lanes 7–14), were metabolically labeled with [\(^{35}S\)]methionine. After various chase periods (indicated in minutes at the top of the gel), cells were lysed with Triton X-114, and lysates were separated into detergent (D) and aqueous (A) phases and immunoprecipitated with a total anti-\( \mu \) antibody. Cell-surface iodinated (LAC, lactoperoxidase iodinated) WEHI 231 cells were also lysed and phase separated using Triton X-114, and the detergent and aqueous fractions were immunoprecipitated with anti-\( \mu \) (lanes 15 and 16). m, \( \mu_m \); s, \( \mu_s \).
from an aqueous, relatively hydrophilic form to a detergent-soluble, hydrophobic form (Fig. 1, lane 13; band also evident at 20 min but not reproduced in lane 9). For ease of identification we refer to the initial hydrophilic form of \( \mu_m \) as \( \mu_m^1 \) and the subsequent relatively hydrophobic form that migrates with a similar mobility on polyacrylamide/NaDodSO\(_4\) gels as \( \mu_m^2 \). It has been observed in previous work that after longer chase periods \( \mu_m \) is converted to a slower-migrating terminally glycosylated form \( \mu_m^3 \). After 210 min of chase (Fig. 1, lanes 11 and 12), \( \mu_m \) in a B-cell line was totally converted to the hydrophobic \( \mu_m^2 \) form that in turn was in part converted to a slower-migrating terminally glycosylated form, which we term \( \mu_m^3 \). Cell-surface iodination confirmed that \( \mu_m \) on the surface almost entirely partitioned in the detergent phase of Triton X-114 and corresponded to the \( \mu_m^3 \) form (Fig. 1, lanes 15 and 16).

The identity of the \( \mu_m^1, \mu_m^2, \) and \( \mu_m^3 \) forms was confirmed using an anti-peptide antibody specific for \( \mu_m \). Lysates from metabolically labeled B cells made after 30 and 210 min of chase were immunoprecipitated with a total anti-\( \mu \) antibody and with an antibody raised against a \( \mu_m \)-specific peptide. \( \mu_m^1 \) (Fig. 2, lanes 3 and 4), \( \mu_m^2 \) (Fig. 2, lanes 1, 2, 5, and 6), and \( \mu_m^3 \) (Fig. 2, lanes 5 and 6) species were brought down by anti-\( \mu \) and by the anti-peptide antibody, whereas \( \mu_m \) was precipitated only by the total anti-\( \mu \) antibody (Fig. 2, lanes 3, 4, 7, and 8). In pre-B cells, pulse-chase studies revealed the initial synthesis of the relatively hydrophilic \( \mu_m^1 \) form just as seen in B cells. In the pre-B cell line, however, no subsequent conversion to the \( \mu_m^2 \) and \( \mu_m^3 \) forms was observed.

**\( \mu_m, \mu_s, \) and Light Chain Are Myristoylated in B Cells.** To examine whether the B-cell-specific conversion of \( \mu_m \) to a relatively hydrophobic form might be due to the post-translational addition of myristic acid, we metabolically labeled WEHI 231, a B-cell line, separately with \([^{35}\text{S}]\)methionine and with \([^{3}\text{H}]\)myristate. Immunoprecipitation revealed that both immunoglobulin heavy and light chains were labeled with myristate (Fig. 3, lane 2). We were unable to detect myristoylated immunoglobulin in 230-37 and in 54.3 cells, both Abelson virus-derived pre-B cell lines, although the \( p160_{\text{myr-abl}} \) protein in those lines could be readily labeled with myristate (data not shown).

To determine whether \( \mu_m \) and \( \mu_s \) in B cells are labeled with myristate and to investigate whether the conversion of the \( \mu_m^1 \) form to the \( \mu_m^2 \) form could be explained by this post-translational modification, we phase-separated B-cell lysates made with Triton X-114 after a 90-min pulse period with \([^{35}\text{S}]\)methionine or with \([^{3}\text{H}]\)myristate. Methionine-labeled \( \mu_m^1 \) and \( \mu_m \) were partitioned into the aqueous phase,

![Fig. 2. Analysis of \( \mu \)-chain immunoprecipitates using a total (Tot) anti-\( \mu \) antibody and an antibody directed against a \( \mu_m \)-specific peptide (Pep). WEHI 231 cells were pulse labeled with \([^{35}\text{S}]\)methionine, and after 30 and 210 min of chase, respectively, cells were lysed and phase separated into detergent (Det) and aqueous fractions. Each fraction was divided into two portions, one portion was immunoprecipitated with a total anti-\( \mu \) antibody and the other with a \( \mu_m \)-specific antibody. \( \mu_m, \mu_m^1, \mu_s, \mu_s^1 \).](image2)

![Fig. 3. Analysis of immunoprecipitates from \([^{35}\text{S}]\)methionine (lane 1)- and \([^{3}\text{H}]\)myristate (lane 2)-labeled B cells (using anti-\( \mu \)).](image3)

![Fig. 4. Correlation of detergent solubility and myristoylation. (A) Analysis of \( \mu \)-chain immunoprecipitates from WEHI 231 cells pulse labeled with \([^{35}\text{S}]\)methionine. Cells were pulse labeled for 90 min and fractionated using Triton X-114 into detergent (D) and aqueous (A) fractions that were separately immunoprecipitated with total anti-\( \mu \) antibody. (B) Analysis of \( \mu \)-chain immunoprecipitates from WEHI 231 cells pulse labeled (90 min) with \([^{3}\text{H}]\)myristate and fractionated onto detergent (D) and aqueous (A) fractions. In lanes 1 and 2 the gel was immersed in fixative prior to exposure; in lanes 3 and 4 the gel was treated with hydroxylamine prior to exposure. \( \mu_m^1, \mu_m \); \( \mu_m^2, \mu_m^3 \); \( \mu_s, \mu_s^1 \).](image4)
whereas $\mu_m$ was detected in the detergent phase (Fig. 4A). [$^3$H]Myristate labeling revealed preferential labeling of $\mu_s$ and $\mu_m$ but not of $\mu_r$ (Fig. 4B, lanes 1 and 2). (A barely detectable amount of $\mu_m$ is seen in lanes 2 and 4 of Fig. 4B; this probably represents an unavoidable, but minimal, contamination of the aqueous phase with some detergent-soluble material.) The results indicate that whereas $\mu_m$, $\mu_s$, and $\kappa$ are all labeled with myristate in B cells, acylation also coincides with the conversion of $\mu_m$ to the more hydrophobic $\mu_m$ form.

Comparison of a hydroxyxylamine-treated gel (Fig. 4B, lanes 3 and 4) with its nontreated counterpart (lanes 1 and 2) shows that the myristoyl group on immunoglobulin is hydroxyxylamine resistant.

$\mu_m$ is Stably Anchored in Pre-B Cells. To investigate whether the relatively hydrophilic and presumably nonacylated form of $\mu_m$ in pre-B cells is stably anchored in microsomal membranes, we metabolically labeled cells and prepared a crude microsomal fraction. Treatment of microsomes with low concentrations of saponin is known to create "pores" that facilitate the release of luminal proteins but not of integral membrane proteins (16). Saponin extraction of microsomes released $\mu_s$ but not $\mu_m$, suggesting that even in pre-B cells $\mu_m$ is stably anchored in the endoplasmic reticulum membrane (Fig. 5). $\mu_m$ and residual $\mu_s$ were released from membranes by extraction with Nonidet P-40 (Fig. 5).

**DISCUSSION**

We studied the biosynthesis and fate of intracellular immunoglobulin in pre-B cells and in B cells using a phase-separating detergent that we believed would separate $\mu_m$ and $\mu_s$. Studies with this detergent, Triton X-114, revealed that, in pre-B cells as well as in B cells, $\mu_m$ is initially synthesized as a relatively hydrophilic protein. In B cells, but not in pre-B cells, this protein is post-transcriptionally converted to a relatively hydrophobic form. This hydrophobic form is subsequently terminally glycosylated and transported to the cell surface. Metabolic labeling with [$^3$H]myristic acid revealed that in B cells, $\mu_m$, $\mu_s$, and light chains are covalently acylated. The myristate moiety was resistant to cleavage with hydroxyxylamine, suggesting that it is amide-linked, probably to a lysine side chain. Phase separation of myristate-labeled B-cell lysates revealed that the hydrophobic form of $\mu_m$ (as compared with the hydrophilic $\mu_m$ form) was preferentially labeled; myristoylation may account for the post-translation al conversion of $\mu_m$ from a relatively hydrophilic form to a hydrophobic form. Our inability to detect myristate-labeled immunoglobulin in two pre-B cell lines might suggest that at this stage of differentiation intracellular immunoglobulin is not acylated or that it does not reach the intracellular compartment in which acylation takes place. However, in the light of the knowledge (ref. 17; unpublished data) that intracellular immunoglobulin in pre-B cells is rapidly turned over, it is possible that immunoglobulin is myristoylated in pre-B cells, but that its rapid degradation soon after acylation precludes detection of fatty acid labeled immunoglobulin at this stage of differentiation.

The predicted membrane anchor sequence of $\mu_m$ contains 26 amino acids; 11 of these residues are polar and of these, 9 are serines and threonines (18). As compared with the transmembrane anchors of most membrane proteins this sequence is remarkably hydrophilic (19). Considering the amphiphilic nature of this anchor sequence it is, in retrospect, easy to explain why this protein initially migrates with $\mu_s$ in the aqueous phase of Triton X-114 extracts. Although this predicted transmembrane domain is relatively hydrophilic, it is probably of sufficient hydrophobicity to function as a membrane anchor. Other workers have used this anchor sequence in recombinant plasmids and have demonstrated that for proteins synthesized in *in vitro* transcription-linked translation systems, this sequence serves effectively as a stop-transfer and anchor signal (20). Our experiments on microsomal membranes confirm that even in pre-B cells the relatively hydrophilic $\mu_m$ form is stably anchored in the endoplasmic reticulum membrane.

There are four major classes of acylated proteins. Two of these classes are cytosolic proteins and the others are secreted or surface membrane-associated proteins. N-terminal myristoylation (21–23) involves the acylation of certain cytosolic proteins that have a glycine residue in position 2; palmitoylation (24, 25) of certain cytosolic proteins occurs by thioesterification of cysteines that fit the consensus Cys-Aaa-Aaa-Xaa, where Xaa is the C terminus and Aaa is an aliphatic residue. Both of these classes of acylation may be responsible for directing cytosolic proteins to the inner face of the plasma membrane. Some proteins that are directed into the endoplasmic reticulum are modified by a C-terminal "glypiation" that is involved in anchoring these proteins to the cell surface. Glypiated proteins are initially synthesized with a short C-terminal hydrophobic tail that is cleaved immediately after translation in the course of a transpeptidation reaction that results in the addition to the new C terminus of a complex glycosyl-inositolphospholipid (26).

The category of acylation into which the myristoylation of immunoglobulin fits involves the side-chain acylation of proteins along the pathway to the cell surface or secretion. Acylation occurs after a lag phase, presumably in the cis regions of the Golgi. Integral membrane and secretory proteins are acylated through this pathway, and the acyl moiety is usually palmitate though it may also be stearate or myristate. No function has been established for this category of acylation, though as has been pointed out by Schekman (27), it does not appear to serve an anchor function; acylated transmembrane proteins that fall into this group retain their anchors and in the case of a number of yeast cell surface proteins palmitate is attached during Golgi transit and is removed before the protein reaches the cell surface. Exam-
of proteins that fall into this category include the transferrin receptor (28) and the insulin receptor (29).

The conversion of a relatively hydrophobic transmembrane protein (as assessed by Triton X-114 solubilization) to a hydrophobic form has a precedent. The hemagglutinin of influenza is a trimeric transmembrane protein with a relatively hydrophilic anchor sequence. The post-translational trimerization of the hemagglutinin protein has been correlated with its conversion from a Triton X-114 aqueous phase protein to a relatively hydrophobic oil-phase soluble form (30). Although this protein is known to be palmitoylated, no attempt to correlate acylation with conversion to a hydrophobic trimeric form has yet been reported. The conversion of $\mu_\alpha$ or of the influenza hemagglutinin from a relatively hydrophilic form to a hydrophobic form (as assessed by Triton X-114 solubility) may result from the intermolecular packing of the hydrophilic faces of the amphipathic transmembrane domains and the subsequent presentation only of the hydrophobic faces of these domains to the surrounding detergent. The acyl groups may facilitate this packing and may, therefore, play either a direct or an indirect role in causing detergent solubility. We have noted that $\mu_\alpha$, which lacks a transmembrane anchor, is myristoylated in B cells but is not converted to a Triton X-114 soluble form. Thus, myristoylation per se does not cause detergent solubility, suggesting that its effect on $\mu_\alpha$ may well be an indirect one of facilitating intermolecular interactions (acting as a hydrophobic glue). Such a role for myristoylation is also suggested by the observations that the viral protein 4 (VP4) of picornaviruses (31) and the viral protein 2 (VP2) of simian virus 40 and polyomaviruses (32) are myristoylated. For these viruses, which lack envelopes, the acyl group must interact solely with protein side chains as has been shown directly for picornaviruses (31). We suggest that the myristoylation of $\mu_\alpha$, $\mu_\beta$, and light chain may be important for intermolecular packing of immunoglobulin chains. This packing increases the hydrophobicity of the transmembrane domain and may also be a prerequisite for the transport of membrane immunoglobulin to the cell surface.

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