The Acylation of Megakaryocyte Proteins: Glycoprotein IX Is Primarily Myristoylated While Glycoprotein Ib Is Palmitoylated

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The acylation of megakaryocyte proteins was studied with special emphasis on the myristoylation and palmitoylation of the glycoprotein (GP) Ib complex. Guinea pig megakaryocytes were purified and separated into subpopulations at different phases of maturation. Cells were incubated with 3H-myristate, 3H-palmitate, or 3H-acetate to study endogenous protein acylation. Cycloheximide was used to distinguish between cotranslational and posttranslational acylation and hydroxylamine to distinguish between thioester and amide linkages. After incubations, delipidated proteins or GP Ib complex subunits, immunoprecipitated with PG-1, AN-51, or FMC-25 monoclonal antibody, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and assessed by fluorography. Radiolabeled fatty acids bound to GPIX and GP Ib were also analyzed by high pressure liquid chromatography (HPLC) and scintillation spectrometry. With 3H-myristate and 3H-palmitate, 3H-acetate was found to be a major myristoylated protein in megakaryocytes and CHRF-288 cells. Myristic acid was linked to GPIb by an amide bond, and this process occurred cotranslationally. With 3H-acetate, GPIb was primarily palmitoylated, but with 3H-myristate, GPIb was acylated with about equal amounts of myristic acid and palmitic acids. Both fatty acids were linked to GPIb by thioester bonds, and acylation was posttranslational. The myristoylation of GPIb was found to be most active in mature megakaryocytes, while the palmitoylation of GPIb occurred throughout megakaryocyte maturation. Myristoylation and palmitoylation may have different functions relevant to the assembly of the GPIb complex in megakaryocytes.

Materials and Methods

Radiochemicals, Antibodies, and Reagents

The 3H-myristic acid (33.5 Ci/mmol), 3H-palmitic acid (39 Ci/mmol), 3H-acetate (4.13 Ci/mmol), and ENHANCE were purchased from Dupont/New England Nuclear (Boston, MA). Myristic acid, palmitic acid, stearic acid, and their methyl esters were from NuChek Prep (Elysian, MN). Mouse monoclonal antibody PG-1, directed against the guinea pig von Willebrand receptor, was a gift from Dr Johnathan Miller, and its characterization has been reported.9 AK-1 and SZ-1, both monoclonal antibodies against the human GPIb-IX complex, were available commercially from Dako (Carpinteria, CA) and Immunotech (Westbrook, ME), respectively. AN-51, a monoclonal antibody against GPIb-alpha (Dako), and FMC-25, a monoclonal antibody against GPIX (ICN, Costa Mesa, CA), were also used.

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Isolation of Cells

Guinea pigs were used in most of the experiments in the study because, in our experience, this species is the best available source for the isolation of viable purified megakaryocytes and the preparation of megakaryocyte subpopulations at different phases of maturation. Human platelets were also studied and prepared as previously described. 3 Guinea pig megakaryocytes and platelets were isolated as previously described. 11 Megakaryocytes were isolated to about 85% purity by cell number and greater than 98% by protein content, as megakaryocytes are considerably larger than other bone marrow cells. The viability of the isolated megakaryocytes was about 90%.

Megakaryocyte subpopulations at different phases of maturation were prepared by the Celsep procedure.12 This procedure separates megakaryocytes by size and can be used to prepare megakaryocyte subpopulations that contain primarily mature megakaryocytes and subpopulations that are highly enriched with immature megakaryocytes. The viability of the isolated subpopulations of megakaryocytes is greater than 89%, and purity is greater than 95% by protein content.

CHRF-288 cells13 were obtained from Dr. M. Lieberman (University of Cincinnati, Cincinnati, OH). The cells were grown in Fisher's medium supplemented with 20% (vol/vol) horse serum and penicillin (50 U/mL) streptomycin (50 U/mL). Cultures were seeded at 3 x 10^5 cells in 8 mL of culture medium in a 25-cm2 plastic flask and were grown to 8 x 10^6 cells. All cell cultures were maintained at 37°C in a humidified incubator in the presence of 5% CO2 in air.

Incubation Conditions for the Acylation of Cells

Megakaryocytes. Megakaryocytes (2 x 10^5/mL) were resuspended in Eagle's medium supplemented with 0.22% fatty acid-free bovine serum albumin (BSA) and incubated with [3H]myristic acid (300 μCi/mL, 8.9 μmol/L), [3H]palmitic acid (300 μCi/mL, 7.7 μmol/L), or [3H]acetate (66.7 μCi/mL, 16.3 μmol/L). To distinguish between cotranslational and posttranslational acylation, cycloheximide (10 μg/mL) was added to the incubation medium of some samples before the addition of radiolabeled fatty acids. After the labeling period, the medium was removed by centrifugation, and the cells were washed twice with calcium magnesium-free Hanks' balanced salt solution with adenine and theophylline.

CHRF-288 cells. At 54 hours after the initiation of the culture, cells were transferred into a fresh flask and incubated with 1 mM [3H]myristic acid (3.9 μmol/L) for 18 hours. Cells were seeded to generate at least 8 x 10^6 cells after an 18-hour incubation with the radioisotope. Cells were pelleted and washed twice with fresh medium.

Processing and Purification of Acylated Proteins

Delipidation in preparation for the assessment of total acylated proteins. Washed and pelleted cells were incubated with 6 mL of CHCl3:MeOH (2:1) for 30 minutes at room temperature. The protein was pelleted by centrifugation (4,500 g for 10 minutes at room temperature) and then extensively delipidated by sequential treatment with 6 mL each of CHCl3:MeOH (2:1), CHCl3:MeOH (1:2), CHCl3:MeOH:H2O (1:1:0.3), and acetone.

Immuno precipitation for the assessment of the acylation of GPIb subunits. The cells were first resuspended in 500 μL of calcium magnesium-free Hank's pH 7.4. Protease inhibitors were added to a final concentration of 2.75 μmol/L aprotenin, 1 mM/L phenyl methylsulfonyl fluoride (PMSF), and 1 μmol/L iodoacetate. Nonlabeled P-40 (NP-40) at 0.5% (vol/vol) was added to lyse the cells, which were then incubated at 4°C for 1 hour. Cell lysates were centrifuged at 10,000g for 1 hour before immunoadsorption. Aliquots of NP-40 lysates were incubated with 10 μg of various monoclonal antibodies to the GPIb complex. PG-1, AN-51, and FMC-25 were used for the lysates from guinea pig cells, and both SZ-1 and AK-1 were used on the lysates from the human cell line. The antibodies were precipitated with goat anti-mouse IgG conjugated to agarose beads at 4°C overnight. The beads were then washed extensively with tris-buffered saline containing 0.1% NP-40. The bound material was solubilized at 37°C with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then centrifuged to remove the agarose beads. We compared the recovery of immuno precipitated GPIb and GPIX by the method described above to that in which Triton-100 was used to lyse the cells, as the latter treatment would recover glycoproteins associated with the skeletal membrane.

The results of these experiments revealed that there was comparable recovery by both methods.

SDS-PAGE, Hydroxyaminetreatment, and Fluorography of Gels

Delipidated samples or immunopurified GPIb subunits were placed in treatment buffer for SDS-PAGE. Most samples were analyzed under nonreducing conditions, but some samples were reduced by treatment with 1% beta-mercaptoethanol. Samples were also reduced by 20 mM/L dithiothreitol (DTT) and heated at 80°C for 3 minutes. SDS-PAGE was performed on 5% to 15% gradient gels and 10% gels. Gels were soaked in EN'HANCE and dried, and acylated proteins were detected by fluorography.

To identify a thioester-linkage, gels were washed three times for 10 minutes in D2O. Then, gels were treated with 1 mol/L hydroxylamine, pH 7, or 1 mol/L Tris-HCl, pH 7 (as a control), for 6 hours, stained with Coomassie Blue, and analyzed by fluorography as described above. To identify an O-ester linkage, gels were treated with 1 mol/L hydroxylamine, pH 10, and in other experiments, were subjected to alkaline hydrolysis with 0.2 mol/L KOH in methanol.

Identification of Fatty Acid Linked to Protein by High Pressure Liquid Chromatography

Methanolysis of acylated proteins. Radiolabeled gel slices containing the protein of interest were excised from slab gels after SDS-PAGE, washed four times with 10% MeOH, homogenized in 50 mm/L ammonium bicarbonate containing 0.1% SDS, and incubated with 0.1 mg TPCK-treated trypsin (Sigma, St Louis, MO) for 24 hours. After 6 and 18 hours, fresh trypsin at 0.1 mg/mL was added to the homogenate to completely digest the protein. The gel mass was filtered from the solution using a 0.2-μm membrane, 200 μg of BSA was added as a carrier, and the solution was evaporated to dryness in a Savant Speed Vac Concentrator (Forma Sci, Marietta, OH). Two milliliters of 83% MeOH, 17% HCl was added to each sample, and they were maintained under N2 for 24 hours. Each sample was extracted three times with petroleum ether and dried. Acetone or ethanol containing nonradioactive fatty acid standards palmitate, myristate, stearate, methyl myristate, methyl palmitate, and methyl stearate were added to each sample in preparation for analysis by high pressure liquid chromatography (HPLC).

HPLC. Separation of the fatty acids and fatty acid methyl esters was achieved by reverse-phase HPLC (Waters 600E System, Millford, MA) on a C18 column (Ecosil 5 μm, 25 mm × 4.6 mm; Alltech Scientific Inc, Deerfield, IL) using 84% or 94% acetonitrile: H2O at a flow rate of 1 mL/min. Detection was by ultraviolet monitoring at 205 nm. One-milliliter fractions were collected when the mobile phase was 95% acetonitrile, while 2-mL fractions were collected when the mobile phase was 84% acetonitrile. The radioactivity in the collected fractions was estimated by scintillation spectrometry.

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The retention times of the radiolabeled products were determined by coelution with the unlabeled standards.

RESULTS

After the incubation of megakaryocytes with \(^{3}H\)myristic acid and \(^{3}H\)palmitic acid, delipidated proteins were separated by 10% unreduced SDS-PAGE, and acylated proteins were identified by fluorography. Incubations with palmitic acid demonstrated that the most prominent acylated proteins were 24, 27 to 29, 36 to 38, 61, 87 to 97, and 140 kD proteins. Similar species of proteins were found to be palmitoylated in guinea pig platelets. There is a species difference in the acylation of GPIX in human and guinea pig platelets. Our experiments confirmed that in human platelets, GPIX and GPIb are palmitoylated. GPIX is palmitoylated to a greater extent than GPIb. However, in guinea pig platelets, GPIb but not GPIX is palmitoylated.

With myristic acid, a 19-kD band was the most intensely acylated protein in megakaryocytes, and other prominently acylated proteins were 16, 40 to 48, 55 to 59, 97, and 170 kD. Both 2-hour and overnight incubations were performed, and the results showed that there were no significant differences in the protein species that were acylated at these two time periods. Thus, several megakaryocyte proteins were shown to be acylated. These data are consistent with the demonstration that a number of proteins are acylated in most cells, although fewer proteins are acylated than phosphorylated or glycosylated.

The palmitoylation and myristoylation of megakaryocyte proteins were compared under unreduced and reduced conditions. After the incubation of megakaryocytes with radiolabeled fatty acids, the delipidated proteins were separated on a 5% to 15% SDS-PAGE gradient gel, and acylated proteins were detected by fluorography. Samples were reduced by the addition of 1% beta-mercaptoethanol or 20 mmol/L DTT rather than the standard 5% beta-mercaptoethanol to avoid disruption of thioester bonds. Figure 1 demonstrates that several megakaryocyte proteins had been acylated. One of the most intensely myristoylated proteins is a 19-kD protein that was evident in both unreduced and reduced gels. This behavior is consistent with that of GPI. In experiments with \(^{3}H\)palmitic and \(^{3}H\)myristic acids, a protein of about 23 kD is only evident in the reduced gel, and this band represents GPIb-beta. In the experiment shown in Fig 1, gel samples had been reduced with 1% beta-mercaptoethanol, but those data were similar to those of other experiments in which gels had been reduced with DTT.

To determine whether the GPIb complex and its subunits were acylated, megakaryocytes were incubated with radiolabeled fatty acids, the intact GPIb complex was immunoprecipitated, GPIb subunits were separated by 10% unreduced SDS-PAGE, and acylation was estimated by fluorography. PG-1, a monoclonal antibody specific to intact GPIb complex in guinea pig platelets and megakaryocytes, was used in the experiments shown in Fig 2. Figure 2A demonstrates the palmitoylated megakaryocyte proteins immunoprecipitated with PG-1. GPIb (170 kD) was found to be palmitoylated. The identity of the two fainter bands has not been determined. Cycloheximide, an inhibitor of protein synthesis, did not block the palmitoylation of GPIb, as shown in Fig 2A. Figure 2B demonstrates the myristoylated megakaryocyte proteins immunoprecipitated with PG-1. GPIX (19 kD) and GPIb were found to be myristoylated, but GPIb was considerably more myristoylated than GPIb. After treatment with cycloheximide, myristoylation of GPIX was blocked, while myristoylation of GPIb was unchanged. This suggests that the myristoylation of GPIX is cotranslational, while the myristoylation of GPIb is a posttranslational event.

The acylation of subunits of the GPIb complex in guinea pig megakaryocytes was also studied with AN-51, which recognizes GPIb-alpha, and with FMC-25, which can immunopurify the GPIX subunit. Both of these antibodies reacted with their respective epitopes in guinea pig megakaryocytes. The results of these experiments are not shown but confirmed that GPIX was intensely myristoylated in guinea pig megakaryocytes.

The myristoylation of GPIb subunits was also studied in CHRF-288 cells, a tumor cell line with megakaryocytic char-
Fig 2. Palmitoylation and myristoylation of the GPIX and GPIb complex in guinea pig megakaryocytes: effect of cycloheximide. Guinea pig megakaryocytes in Eagle's medium supplemented with 0.22% FSA (200,000/mL) were incubated with (A) [3H]palmitate or (B) [3H]myristate (300 μCi/mL) for 17 hours in the presence or absence of cycloheximide (10 μg/mL). Cells were lysed and immunoprecipitated with PG-1 antibody. The immunoprecipitated samples were separated by unreduced 10% SDS-PAGE, and acylated proteins were detected by fluorography. CONT, megakaryocytes not treated with cycloheximide; CH, megakaryocytes treated with cycloheximide.

characteristics. CHRF-288 cells were studied to determine whether the myristoylation of GPIX occurs in this cell line and, thus, to provide indirect evidence for this event in human megakaryocytes. Figure 3 is a fluorogram of an experiment in which CHRF-288 cells were incubated with [3H]myristic acid, and the GPIb complex was immunoprecipitated with SZ-1 monoclonal antibody. GPIX was heavily myristoylated, while GPIb was minimally myristoylated.

Endogenous protein acylation was studied by the incubation of megakaryocytes with [3H]acetate, which revealed that GPIX and GPIb were acylated. To study the acylation of GPIb subunits, immunoprecipitated GPIb subunits were separated by SDS-PAGE, and acylated GPIb and GPIX were eluted from the gels and subjected to acid methanolysis to produce fatty acid methyl esters that were analyzed by HPLC. Radioactivity was measured by scintillation spectrometry. These experiments revealed that the principal fatty acid linked to GPIX was myristic acid (Fig 4C), and the main fatty acid linked to GPIb was palmitic acid (Fig 4B). The synthesis of fatty acid species from [3H]acetate is shown in Fig 4A, and the data were derived from the extraction of megakaryocyte total lipids, the production of fatty acid methyl esters, and analysis by HPLC and scintillation spectrometry. The data indicate that with [3H]acetate as a precursor, palmitic acid is the primary radiolabeled fatty acid along with smaller amounts of stearic acid, and only trace amounts of [3H]myristic acid could be detected in megakaryocyte lipids. Although the pool of radiolabeled myristic acid with acetate as a precursor is extremely small, GPIX appears to be selectively acylated by myristic acid.

It was important to identify the fatty acid that acylated GPIb and GPIX after incubation with exogenously supplied [3H]myristic and [3H]palmitic acids because megakaryocytes have the capacity to elongate these precursors. Therefore, after incubation with [3H]myristate or [3H]palmitate, radiolabeled fatty acids linked to GPIX and GPIb were assessed by HPLC and scintillation spectrometry, as described above. After the incubation of megakaryocytes with [3H]palmitic
ACLYATION OF MEGAKARYOCYTE GLYCOPROTEIN IIB-IX

Megakaryocytes at different phases of maturation were prepared by the Celsep procedure, and the acylation of GPIb subunits was studied by incubation with [3H]myristic and [3H]palmitic acids. Three different subpopulations of megakaryocytes were prepared: a mature subpopulation that contained 92% stage III and IV (mature based on cytoplasmic maturation) and 99% 16N and 32N ploidy megakaryocytes; an intermediate maturity subpopulation that contained 71% stage III and IV and 74% 16N megakaryocytes; and an immature subpopulation that contained 67% stage I and II (immature based on cytoplasmic maturation) and 66% 8N megakaryocytes. Equivalent amounts of protein from mature, intermediate maturity, and immature megakaryocyte subpopulations were applied to each lane. There is a considerable difference in the size of mature and immature megakaryocytes, and thus, biochemical activities cannot be compared on the basis of cell number but can be compared on the basis of protein content or cell volume. Figure 5A demonstrates that the myristoylation of GPIb occurred primarily in the mature and intermediate maturity subpopulations. Figure 5B demonstrates that the palmitoylation of GPIb occurred in each of the three subpopulations. After incubation with [3H]myristic acid, acylation of GPIb also occurred similarly in each of the cell subpopulations (data not shown). Densitometry indicated that the ratio of myristoylation of GPIb in mature to immature subpopulations shown in Fig 5A was greater than 100:1, while the ratio of palmitoylation of GPIb in mature to immature subpopulations shown in Fig 5B was about 2.5:1.

Several issues should be considered in interpreting the data from Celsep subpopulations. Immature and mature megakaryocyte subpopulations differed significantly in cytoplasmic maturation and in ploidy. Both of these parameters have been considered to reflect megakaryocyte maturation. While the study did not distinguish between the role of cytoplasmic maturation and ploidy in the differences in acylation, previous studies indicated that arachidonic acid uptake, the expression of surface-exposed sialoglycoproteins, and the expression of P-selectin mRNA are related primarily to cytoplasmic maturation rather than to ploidy. This is consis-

The myristoylation of GPIX was studied by incubation with [3H]myristate. The GPIX complex in megakaryocyte subpopulations was immunoprecipitated with PG-1, proteins were separated by 10% unreduced SDS-PAGE, and acylated GPIX and GPIb were assessed by fluorography. Equivalent amounts of protein from immature, intermediate, and mature subpopulations were applied. (A) Myristoylation of GPIX. (B) Palmitoylation of GPIb. M, mature; IT, intermediate maturity; IM, immature subpopulations.

Fig 5. Myristoylated GPIX and GPIb in guinea pig megakaryocytes of differing maturities. Guinea pig megakaryocyte subpopulations at different phases of maturation prepared by the Celsep procedure were incubated for 17 hours with [3H]myristate or [3H]palmitate in Eagle's medium (300 μCi/mL). The GPIb complex in megakaryocyte subpopulations was immunoprecipitated with PG-1, proteins were separated by 10% unreduced SDS-PAGE, and acylated GPIX and GPIb were assessed by fluorography. Equivalent amounts of protein from immature, intermediate, and mature subpopulations were applied. (A) Myristoylation of GPIX. (B) Palmitoylation of GPIb. M, mature; IT, intermediate maturity; IM, immature subpopulations.
tent with the concept that ploidy is established before cytoplasmic maturation.\textsuperscript{18} It is not surprising that GPIX myristoylation is active in the intermediate maturity subpopulation, as it contains 74% cytoplasmically mature megakaryocytes.\textsuperscript{12} However, the virtual absence of evidence of myristoylation in the immature subpopulation is surprising, because this fraction contains 33% mature megakaryocytes based on morphologic criteria.\textsuperscript{12} Nonviability of the immature fraction does not account for this finding, because this fraction is active in proteoglycan synthesis,\textsuperscript{12} in lipid synthesis,\textsuperscript{15,19,20} and in the expression of mRNA for vWF and GPIb-alpha.\textsuperscript{17} Although the immature subpopulation has been classified on the basis of morphologic evidence of cytoplasmic maturation, other, as yet undefined characteristics may better distinguish the immature from the mature subpopulations.

**DISCUSSION**

The study showed that a 19-kD protein is one of the most prominently myristoylated proteins in guinea pig megakaryocytes. This protein was identified as GPIX using monoclonal antibodies PG-1, AN-51, and FMC-25. The myristoylated 19-kD protein was evident in both reduced and unreduced gels as would be expected for GPIX. GPIb was also found to be acylated after the incubation of megakaryocytes with myristic acid, and reduced SDS-PAGE revealed that GPIb-beta was the site of acylation. The study indicated that megakaryocyte GPIX was considerably more acylated than GPIb when megakaryocytes had been incubated with myristic acid.

GPIX was also found to be myristoylated in CHRF-288 cells, a tumor cell line with megakaryocytic characteristics.\textsuperscript{13} Because the SZ-1 monoclonal antibody that recognizes the intact GPIb complex was used for immunoprecipitation, the demonstration of acylated GPIb suggests that the intact GPIb complex exists in CHRF cells. This information indicates that the myristoylation of GPIX is not limited to guinea pig megakaryocytes but may also occur in human megakaryocytes.

The study showed that myristic acid is covalently linked to GPIX by an amide bond, because hydroxylamine at pH 7 and pH 10 did not result in the loss of myristic acid from GPIX. This agent at pH 7 hydrolyzes thioester bonds and at pH 10 hydrolyzes O-ester bonds but not amide bonds. Amide-linked myristoylation has been shown to usually occur at an N-terminal glycine in viruses, yeasts, and mammalian cells.\textsuperscript{16} However, GPIX appears to be linked to myristic acid at a different site, because it does not have an N-terminal glycine.\textsuperscript{21} There are several exceptions to the absolute need for an N-terminal glycine as the site for amide linkage of myristoylated proteins.\textsuperscript{2} The insulin receptor, immunoglobulin heavy chain, the interleukin (IL)-1-alpha and IL-1-beta precursors, and tumor necrosis factor lack glycine residues correctly positioned for classical myristoylation.\textsuperscript{22-24} In these exceptions, myristic acid was considered to be amide-linked to an internal lysine. Myristoylation at a specific internal lysine residue in IL-3-alpha and -beta precursors has been demonstrated using an in vitro myristoylation assay.\textsuperscript{25} A specific peptide-lysine amino myristoyltransferase most likely is involved in these exceptions, as myristoyl-CoA:protein N-myristoyltransferase has no activity against lysine.\textsuperscript{27} GPIX has one lysine side chain near the N-terminus that may be the site of amide-linked myristoylation.\textsuperscript{21}

The myristoylation of GPIX in megakaryocytes is cotranslational and tightly coupled with protein synthesis, as it was inhibited by cycloheximide. Myristoylation is usually cotranslational in cases of linkage of myristic acid to N-terminal glycine\textsuperscript{26,27} and in the linkage of myristic acid to a lysine residue in the insulin receptor.\textsuperscript{22}

The investigation of palmitoylation revealed that similar proteins were palmitoylated in guinea pig platelets and megakaryocytes as had been reported in human platelets with the exception of GPIX.\textsuperscript{28,29} Palmitoylation of GPIX occurs in human platelets but not in guinea pig platelets. GPIb-beta subunit is the site of palmitoylation of GPIb in both human and guinea pig platelets and megakaryocytes.

Our study has demonstrated that megakaryocyte GPIb can be palmitoylated after incubation with [\textsuperscript{3H}]palmitic acid or [\textsuperscript{14}C]myristic acid. When megakaryocytes were incubated with exogenous [\textsuperscript{3H}]myristic acid, 48% of the fatty acids bound to GPIb was myristic acid, and 52% was palmitic acid. Both of these fatty acids were bound to GPIb by a thioester linkage and were posttranslational events. The demonstration of thioester-linked myristic acid to GPIb in megakaryocytes is consistent with similar observations in human platelets.\textsuperscript{7} This most likely accounts for the finding that GPIb-beta was the site of acylation in megakaryocytes that had been incubated with [\textsuperscript{3H}]myristic acid (Fig 1). In human platelets, myristic and palmitic acids bind to the same set of platelet proteins, but palmitic acid is preferred over myristic acid. Also, the rate and intensity of the binding of fatty acids to proteins was considerably greater with palmitic acid than with myristic acid in human platelets.\textsuperscript{7}

It was important to study endogenous protein acylation, because differences in the acylation of proteins have been noted in other cells when the fatty acid had been derived from acetate versus exogenously supplied myristic or palmitic acids.\textsuperscript{30} The study of endogenous acylation of GPIX using acetate confirmed the data from exogenously supplied myristic acid to study protein acylation. With [\textsuperscript{3H}]acetate as a precursor, GPIb was acylated with primarily myristic acid. Megakaryocytes can synthesize palmitic and stearic acid with acetate as a precursor. However, with [\textsuperscript{3H}]acetate as a precursor, radiolabeled palmitic acid was the primary fatty acid, while only trace amounts of radiolabeled myristic acid were found in megakaryocytes. Thus, there appears to be a selective demand for myristic acid for the acylation of GPIX.

With acetate as a precursor, GPIb was mainly acylated with palmitic acid. However, as noted above with exogenous myristic acid, GPIb is acylated with both myristic and palmitic acids. In the experiments with exogenous myristic acid, myristic and palmitic acids are ester-linked at similar sites, and the linkage of myristic acid to GPIb may be related to high concentrations of the precursor. The results of the acetate experiments most likely reflect in vivo protein acylation of GPIb. The information from the acetate and exogenous fatty acid experiments are, nevertheless, consistent in the
conclusion that GPIX is primarily myristoylated and GPIb is palmitoylated in megakaryocytes.

The study indicates that the palmitoylation of GPIb is established in immature megakaryocytes, but GPIX is primarily myristoylated in mature megakaryocytes. The possibility that GPIX is expressed during later phases of megakaryocyte maturation has been postulated and may explain why the myristoylation of GPIX was not observed in immature megakaryocytes.

Differences in other biochemical activities have been observed in mature and immature megakaryocytes. For example, mRNAs for fibronectin, vWF, and GPIb-alpha are expressed in immature megakaryocytes, and most aspects of lipid synthesis are established in immature megakaryocytes. However, de novo fatty acid synthesis occurs primarily in mature megakaryocytes. Surface-exposed si-glycoproteins and the expression of mRNA for P-selectin are evident mainly in mature megakaryocytes. Platelets are most likely assembled during the terminal phases of megakaryocyte maturation. Therefore, the myristoylation of GPIX as well as other biochemical activities mentioned above appears to be characteristics and markers of the terminal phases of megakaryocyte maturation and may be important for platelet production.

Myristoylated proteins may be responsible for the mediation of protein-protein interactions and the assembly of large protein complexes. For example, many myristoylated proteins are subunits of large protein complexes, such as protein kinase A or alpha subunits of G proteins. In addition, myristoylation can stabilize the interaction between capsid proteins in picorna viruses. The myristoylation of GPIX may be important for the interaction of GPIb subunits and the formation of a functional complex in mature megakaryocytes.

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