Palmitoylation of Cysteine 69 from the COOH-terminal of Band 3 Protein in the Human Erythrocyte Membrane

ACYLATION OCCURS IN THE MIDDLE OF THE CONSENSUS SEQUENCE OF F--I-I-IICLAVL FOUND IN BAND 3 PROTEIN AND G2 PROTEIN OF RIFT VALLEY FEVER VIRUS*

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One of the major physiologic functions of erythrocytes is the mediation of chloride-bicarbonate exchange in the transport of carbon dioxide from the tissues to the lungs. The anion exchange is mediated by a typical polytopic transmembrane protein in the cell membrane, designated Band 3. A carboxyl-terminal peptide of Band 3 was affinity-labeled with pyridoxal phosphate, a substrate for the anion transport system, and then sequenced (Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S., and Hamasaki, N. (1988) J. Biol. Chem. 263, 8232–8238). The 10th amino acid residue of the peptide could not be determined, suggesting post-translational modification of the residue. In the present communication, we have investigated the molecular structure of human Band 3 and the COOH-terminal 8500-dalton peptide using gas-liquid chromatography-mass spectrometry. Band 3 was modified covalently by fatty acids and these acids were released from Band 3 by hydroxylamine treatment at either pH 7 or 11, indicating that the linkage between Band 3 and the fatty acid is a thio ester bond. 1 mol of Band 3 interacted with 1 mol of fatty acid at a cysteine residue located 69 residues from the COOH terminus of Band 3. The fatty acids used in the modification were myristate, palmitate, oleate, and stearate, with palmitate being the major component. The esterified site is close to the site affinity-labeled with pyridoxal phosphate (Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S., and Hamasaki, N. (1988) J. Biol. Chem. 263, 8232–8238).

The amino acid sequence including the acylation site was Phe-Thr-Gly-Ile-Gln-Ile-Ile-Cys-Leu-Ala-Val-Leu, which is conserved in the G2 protein of Rift Valley fever virus as Phe-Ser-Ser-Ile-Ala-Ile-Ile-Cys-Leu-Ala-Val-Leu. The G2 protein, like Band 3, is a polytopic transmembrane protein. Although acylation of the cysteine residue of G2 protein has not been examined, the Phe-X-X-Ile-X-Ile-Cys-Leu-Ala-Val-Leu sequence could be a common motif for fatty acylation of certain membrane proteins.

Recently, a variety of viral and cellular membrane proteins have been reported to be modified by fatty acid acylation or farnesylation. Those modifications may make the proteins more hydrophobic and might play a role in protein targeting and/or a role in signal transduction systems concerned with cellular function (Towler et al., 1988; Olson, 1988; Schultz et al., 1988; Schmidt et al., 1989; James and Olson, 1990). One of the most common types of fatty acylation, myristoylation, occurs cotranslationally or shortly after protein synthesis; myristic acid is linked through a amide bond to an NH₂ terminal glycine. Another common type of acylation, palmitoylation, occurs when palmitic acid is linked posttranslationally to a cysteine by a thioester bond. Palmitoylation is found in many diverse membrane proteins, including the transferrin receptor (Omary and Trowbridge, 1981), ras proteins (Finkel et al., 1984), major histocompatibility complex antigen (Kaufman et al., 1984), several viral glycoproteins (Schmidt et al., 1979; Schmidt, 1982; Gebhardt et al., 1984), the sodium channel (Schmidt and Catterall, 1987), the G₂ adrenergic receptor (O'Dowd et al., 1989), the acetylcholine receptor (Olson et al., 1984), and rhodopsin (O'Brien and Zatz, 1984; O'Brien et al., 1987; Ochinnikov et al., 1988). Although many palmitoylated proteins are known, detailed analyses of acylation sites are limited. In myelin lipopphilin, Stoffel et al. (1983) show by amino acid sequencing of the protein that the fatty acid is bound to a threonine residue within an extracellular loop.

In chicken, rabbit, rat, and human erythrocytes, evidence for fatty acylation of several membrane proteins has been reported. Incorporation of [³H]palmitic acid revealed that: arkyrin; Band 3 (Marinetti and Cattieu, 1982; Keenan et al., 1982); Band 4.1 (Keenan et al., 1982); a protein in the Band 4.5 region of SDS-PAGE† (Marinetti and Cattieu, 1982; Martzki et al., 1990); and sialoglycoproteins (Dolci and Palade, 1985) are acceptors of fatty acylation. The fatty acylation and turnover of ankyrin are particularly well studied (Stoffel and Lazarides, 1986; Staufenbiel, 1987, 1988). It has also been demonstrated that Band 3 is palmitoylated by way of a thio ester bond at a molar ratio of about one (Zdebiska et al., 1989).

We have recently found that a peptide containing the COOH terminal of human erythrocyte Band 3 is affinity-labeled with pyridoxal phosphate, and that this region constitutes a part of the active center for anion transport (Hamasaki et al., 1983; Hamasaki and Kawano, 1987; Kawano et al., 1988). The 10th amino acid residue of this peptide could not be determined by a gas-phase sequencer or by amino acid analysis; the residue, however, was deduced to be cysteine from the cDNA clone of human erythrocyte Band 3 (Tanner et al., 1988; Lux et al., 1989). The difficulty in chemical...
determination of this cysteine residue suggested that the residue may be modified by lipids, and we have investigated this possibility in the present communication.

MATERIALS AND METHODS

Purification of Band 3—The erythrocytes were stored in a citrate/phosphate/dextrose buffer at 4°C for less than 3 weeks before being used. The cells were treated with trypsin to digest glycoporphins and then hemolyzed with an ice-cold phosphate buffer (5 mM sodium phosphate, pH 8.0). Erythrocyte membranes (white ghosts) were prepared from the hemolyzate by centrifugation and were extracted with 0.1 M sodium hydroxide. Band 3 was purified from the alkali-extracted membranes as described previously (Kawano et al., 1988).

Purification and S-Pyridylethylidation of the 8500-Dalton Peptide from Band 3 CNBr Fragments—Following preparation of CNBr fragments from S-pyridylethylidated Band 3, the 8500-dalton peptide was purified as described previously (Kawano et al., 1988).

Acid Methanolysis of Fatty Acids Esterified to Band 3 and to the 8500-Dalton Peptide—Analysis of fatty acids esterified to amino acids was carried out according to Zdebska et al. (1989) with minor modifications. In brief, the isolated protein or peptide was successively treated with chloroform/methanol solutions at various proportions (2/1, 1/1, 1/2, v/v) and then treated three times with butan-1-ol/0.02 M phosphate buffer, pH 7.5 (1/1, v/v) to remove tightly, but noncovalently, bound fatty acids from the protein or peptide. After these samples were dried under nitrogen, the lipids were extracted with chloroform/methanol as well as fatty acid standards, were hydrolyzed with 5% (v/v) HCl-methanol at 80°C for 20 h (Slomiany et al., 1984). For quantitative analysis, authentic heptadecanoic acid was added as an internal standard to the samples and fatty acid standards. Fatty acid methyl esters were extracted with hexane three times. The organic phase was pooled, washed with distilled water three times, and evaporated under nitrogen. The residues were dissolved in a minimal volume of hexane and analyzed on a liquid chromatography-mass spectrometer.

Hydroxylamine Treatment of Band 3 and the 8500-Dalton Peptide—The ester linkages in Band 3 and the 8500-dalton peptide were analyzed by hydroxylamine cleavage according to Cockie et al. (1980). The lipophilized protein or peptide was suspended in 1.0 ml of isopropanol-acidified methanol and incubated for 3 h at room temperature with 1.0 ml of 2 M NH₂OH·HCl, which was previously adjusted with NaOH to pH 7 or 11. The reaction was terminated by addition of 0.5 ml of 6 M HCl. After the released hydroxamic acids were extracted three times with chloroform/methanol (2/1, v/v) the organic phase was pooled and evaporated under nitrogen. The residues were subjected to methanolysis with 5% (v/v) HCl-methanol at 80°C for 20 h or with 14% BF₃ in methanol at 100°C for 5 min (Bazan, 1970). The fatty acid methyl esters were analyzed and quantified as described previously.

Gas Liquid Chromatography-Mass Spectrometry Analysis—Electron ionization mass spectra were acquired on a Shimadzu QP-2000 instrument (Shimadzu Corp., Kyoto, Japan) with an ionizing energy of 50 eV, an accelerating potential of 8 kV, and ion source temperature of 250°C. A Shimadzu GC-14A gas chromatograph, interfaced directly with the mass spectrometer, was used for the mass spectrometric analysis. Chromatography was performed on a fused silica DB-5 capillary column (12 m × 0.25 mm, film thickness 0.25 μm, J & W Scientific, CA). The injector temperature was 250°C. The column was initially set at 50°C for sample injection (spitless) and held for 2 min; it was then programmed to 270°C at a rate of 30°C per min. Selected ion monitoring was carried out for quantitative determination of fatty acid methyl esters by calculating the peak area at m/z 74 for saturated and m/z 55 for unsaturated fatty acids.

Determination of the Fatty Acylation Site in the 8500-Dalton Peptide—The purified 8500-dalton peptide was treated with hydroxylamine as described above and applied to a Chromosorb 300-7Dph column (4.5 × 150 mm) to isolate the peptide. The lipophilized 8500-dalton peptide was dissolved in a solution containing 4% SDS, 8 M urea, and 20 mM Tris phosphate (pH 6.8) and used for S-pyridylethylidation. S-Pyridylethylidation was carried out according to the method of Heinrikson and Meredith (1984) in 4-vinylpyridine solution containing tri-n-butylphosphine and triethylamine. The materials were incubated at 37°C for 30 min after which the reaction was stopped by addition of trifluoroacetic acid. The reaction mixture was directly applied on a Polybrene-activated glass fiber membrane for sequencing. For amino acid analysis of the hydroxylamine-treated peptide, the 8500-dalton peptide was reisolated using the Chromosorb 300-7Dph column. Amino acid analyses of the peptide was performed by a PICO TAG system (Waters, Millipore Corp.) according to the method of Heinrikson and Meredith (1984). Peptides were sequenced on a gas phase sequencer (Applied Biosystems, Model 470A), and the phenylthiobutyldantoin were identified by an Applied Biosystems 120 A phenylthiodyantoin analyzer on-line system.

Analytical Procedures—Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Protein analysis was carried out using SDS-PAGE according to the method of Lasemml (1970), and peptides were analyzed by SDS-urea-PAGE according to the method of Kawano and Hamasaki (1986). Gels were stained with silver or Coomassie Brilliant Blue.

Materials—Fatty acid standards were purchased from Nu-Check Prep., Inc., MN. Other reagents were of analytical reagent grade. Chromosorb 300-7Dph and Superose 12 columns were from Pharmacia LKB Biotechnology Inc., respectively.

RESULTS

Characterization and Quantitation of Fatty Acids Covalently Bound to Band 3 and the 8500-Dalton Peptide—Treatment of Band 3 with chloroform/methanol and butan-1-ol/phosphate buffer was used to remove detergents and noncovalently bound lipids, after which lipids covalently bound to Band 3 were hydrolyzed with 5% (v/v) HCl-methanol. The ester linkages in Band 3 were characterized by hydroxylamine cleavage at pH 7.0 and pH 11.0. The methanolyzed fatty acids were analyzed by gas liquid chromatography-mass spectrometry, and for further quantitation, selected ion spectra were used. A constant amount of fatty acids, 2.8 μg, was liberated from 1 mg of Band 3 by the acid methanolysis, suggesting that approximately 1 mol of fatty acid is covalently linked to 1 mol of Band 3. Palmitate was bound to Band 3 at a mole ratio of 0.63 mol of palmitate per mol of Band 3 (Table I); therefore palmitic acid was the predominant component of the liberated fatty acids. Although in less quantity, myristate, oleate, and stearate were also bound to Band 3 with molar ratios of 0.01, 0.08, and 0.25, respectively (Table I). Palmitoleate could not be detected. Hydroxylamine treatment of Band 3 at either pH 7 or 11 gave essentially the same results as methanolysis (Table I), indicating that Band 3 is acylated mainly with palmitic acid through a thioester bond at one fatty acylation site; this is consistent with the results of Zdebska et al. (1989).

The HCl-methanol and hydroxylamine treatments were also performed on the 8500-dalton peptide. As shown in Table I, the amount of fatty acids liberated from the peptide by methanolysis was similar to that liberated from Band 3; 0.69 mol of palmitic acid and 0.37 mol of stearic acid were freed per mol of peptide. Hydroxylamine cleaved the fatty acid ester bond at either pH 7 or 11 (Table I), indicating that the fatty acylation site of Band 3 was located within the 8500-dalton peptide. There was little difference between the palmitic acid liberated by the two hydrolysis procedures (0.42–0.69), although the total amount of fatty acids liberated was relatively constant, 0.94–1.16 mol/mol of Band 3 or the peptide (Table I). It was not clear whether or not this was due to the molecular divergence.

Amino Acid and Sequencing of the 8500-Dalton Peptide Treated with Hydroxylamine—The 8500-dalton peptide purified from S-pyridylethylated Band 3 was treated with 1 mol hydroxylamine at pH 7 under the same conditions for lipid analysis as described above. Some peptide was not treated with the hydroxylamine as a control. Following S-pyridylethylation of the treated 8500-dalton peptide, amino acid sequencing of the peptide was carried out. During these procedures, the cysteine modified with the hydroxylamine-labile fatty acid should be converted to pyridylethylated cysteine. A new peak of pyridylethylated cysteine appeared at the retention time of 20.88 min between the peaks of valine and diphenylthioureia at the
proteolipids, a number of proteins is ubiquitous in viral membrane proteins as well as is the acylation site of Band 3 cysteine appears at the retention time of valine (20.2 min) and diphenylthiourea treated with hydroxylamine. The results confirm that the 10th residue of pyridylethylated 8500-dalton peptide not hydroxylamine-treated. Pyridylethylated 8500-dalton peptide treated (Fig. 1a), but not in the untreated peptide (Fig. 1b). The actual amino acid sequences of the treated and untreated peptides are shown in Table II. The amount of Pe-Cys was about 50% of the amount of the 9th or 11th amino acids of the peptide. The results confirm that the 10th residue is the acylation site of Band 3 suggested in Table I.

**DISCUSSION**

Since the pioneering work of Folch and Lees (1951) on proteolipids, a number of proteins have been shown to be linked to long chain fatty acids. Acylation of specific cellular proteins is ubiquitous in viral membrane proteins as well as in proteins of yeast and animal cells (see reviews, Schlesinger, 1981; Towler et al., 1988). In this report, we have demonstrated that Band 3 is acylated with palmitic (or stearic) acid at the 69th cysteine residue from its COOH terminus, corresponding to Cys^{695} of the sequence deduced from its cDNA (Tanner et al., 1988; Lux et al., 1989). This evidence is

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**Table I**

<table>
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<tr>
<th>Methyl esters</th>
<th>Band 3 protein</th>
<th>The 8500-dalton peptide</th>
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<tr>
<td></td>
<td>Methanolyysis (n = 3)</td>
<td>NH$_2$OH treatment</td>
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<tr>
<td></td>
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<td>pH 11 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>mol/mol</td>
<td>mol/mol</td>
</tr>
<tr>
<td>Myristate</td>
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<td>&lt;0.01</td>
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<tr>
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<td>0.05 ± 0.01</td>
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<tr>
<td>Stearate</td>
<td>0.25 ± 0.02</td>
<td>0.21 ± 0.01</td>
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<tr>
<td>Total</td>
<td>0.97 ± 0.02</td>
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* Average of two experiments (mean ± S.D.).

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**Table II**

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<tr>
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<tr>
<td>11</td>
<td>Leu</td>
<td>75</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Gas-phase sequencing patterns of the 8500-dalton peptide (a) with or (b) without hydroxylamine treatment. a, hydroxylamine-treated. Pyridylethylated 8500-dalton peptide treated with hydroxylamine. At the 10th cycle, a new peak of pyridylethylated cysteine appears at the retention time of 20.88 min between the peaks of valine (20.2 min) and diphenylthiourea (DPTU) (21.7 min). b, control (nontreated). Pyridylethylated 8500-dalton peptide not treated with hydroxylamine. The arrowheads indicate the peak of DPTU.

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**Fig. 2.** Consensus sequence found in Band 3-related proteins (AE 1, AE 2, and AE 3), G2 protein of Rift Valley fever virus (G2-RVFV), and GTPase activating protein (GAP). a, consensus sequence found in erythroid Band 3 (AE 1), G2-RVFV, and GAP. b, the consensus sequence in the Band 3-related proteins. The amino acid sequence near the acylation site of Band 3, Phe-Thr-Gly-Ile-Gln-Ile-Cys-Leu-Ala-Val-Leu (FTGIQIICLAVL), is conserved in the G2 protein of Rift Valley fever virus as Phe-Ser-Ser-Ile-Ala-Ile-Cys-Leu-Ala-Val-Leu (FFSIAIICLAVL). The similar sequence is also found in GTPase-activating protein whose sequence is partially replaced Ile and Val by Leu and Ile, respectively.
consistent with a previous report by Zdebiska et al. (1989) that although the acylation site was not determined, Band 3 is acylated with palmitate and stearate as the major components. The β2-adrenergic receptor has been reported to be palmitoylated by a thioester bond on the cytoplasmic side near the COOH-terminal region, and the acylation site is proposed to be Cys341 by site-directed mutagenesis (O’Dowd et al., 1989). The cysteine residue is conserved in G protein-coupled receptors (O’Dowd et al., 1989), suggesting that fatty acid acylation near the COOH terminus is a common feature for certain transmembrane proteins and may have a functional role.

The topological situation for the acylation site in Band 3 appears to be different than that of the G protein-coupled receptors. The cysteines of G protein-coupled receptors are located in hydrophilic loops, 12–14 residues downstream from the carboxyl portion of the last putative transmembrane-spanning domain, but the palmitoylated cysteine of Band 3 is located at position 843 in the deduced sequence, which is the beginning of the last transmembrane domain deduced from the hydropathy plot (Passow, 1986; Lux et al., 1989). The amino acid sequence near the acylation site of Band 3, Phe-Thr-Gly-Ile-Gln-Ile-Ile-Cys-Leu-Ala-Val-Leu (FTGQII-CLAVL), is almost conserved in the G2 protein of Rift Valley fever virus (Collett et al., 1985), and the sequence of F-I-ICLAVL is completely conserved in both proteins as well as in mouse erythroid Band 3 (Kopito and Lodish, 1985) (Fig. 2). The conserved sequence, therefore, may be a common motif for the palmitoylation of some transmembrane proteins, although acylation of the cysteine residue in the consensus sequence of the G2 protein and mouse Band 3 have not yet been proven. The similar sequence was also found in the sequence of the G2 protein of Rift Valley fever virus (Collett et al., 1985) (Fig. 2a). Residues of Ile and Val were partially replaced by the similar hydrophobic amino acids of Leu and Ile as shown in Fig. 2a. GTPase-activating protein (GAP) is not a membrane protein, and the next leucine to the cysteine residue is replaced by proline in GAP, suggesting that the cysteine residue in GAP might not be acylated. The acylated cysteine residue and its surrounding amino acid sequence were conserved in the Band 3-related proteins, AE 1 (erythroid Band 3), AE 2, and AE 3 (Fig. 2b). The acylation of the cysteine residue and its functional role remain to be examined in these anion exchanger (AE) proteins as well as the G2 protein and GAP. The additional point to be mentioned is Gln36 in the deduced sequence of erythroid Band 3, which was conserved in the anion exchangers of AE 1, AE 2, and AE 3, but not in the G2 protein and GAP. The glutamine residue might have a particular functional role to anion exchange.

The thioester bond of rhodopsin is readily displaced by mercaptoethanol (O’Brien et al., 1987; Ovchinnikov et al., 1988), but this is not the case with Band 3. The thioester bond of the 8500-dalton peptide is strong enough to withstand treatment with mercaptoethanol or CNBr digestion. The chymotryptic 35,000-dalton domain of Band 3 contains 2 cysteine residues which do not react with N-ethylmaleimide, but do become reactive by reduction with mercaptoethanol (Rao, 1979). According to the deduced sequence (Tanner et al., 1988; Lux et al., 1989), the 2 cysteine residues in the 8500-dalton peptide are the only cysteine residues present in the 35,000-dalton domain. The present study demonstrates that one of these is fatty acylated and is resistant to mercaptoethanol treatment. Characterization of reactive cysteine residues in Band 3 should be reexamined in detail.

We have shown that the 8500-dalton peptide containing the acylation site is affinity-labeled with pyridoxal phosphate, a substrate of the anion transport system (Kawano et al., 1988). The transport activity diminishes in proportion to the labeling of the peptide with pyridoxal phosphate (Hamasaki et al., 1983), indicating that the labeled residue must be at least a part of the active center of the transport system (Hamasaki et al., 1983; Nanri et al., 1983; Matsuyma et al., 1983; Kawano and Hamasaki, 1986). The affinity labeled site is the 61st lysine residue from the COOH terminus, corresponding to Lys681 in the deduced sequence. The acylated cysteine and affinity labeled lysine are very close together, only 8 amino acid residues apart. According to the hydropathy plot (Passow, 1986; Lux et al., 1989), the portion from Cys642 to Lys681 is expected to form an α-helix in the lipid bilayer of the membrane. Thus, the acylated cysteine residue should be located opposite side to the lysine residue side in the helix, indicating that the acylated cysteine is facing the hydrophobic lipid bilayer, while the affinity labeled lysine should be a significant part of a hydrophilic anion binding center. The functional roles of palmitoylation at this site and of the consensus sequence, F–I–ICLAVL, remain to be resolved.

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