A number of transmembrane proteins have been recently reported to be modified by the covalent addition of saturated fatty acids which may contribute to membrane targeting and specific protein-lipid interactions. Such modifications have not been reported in cell-associated heparan sulfate proteoglycans, although these macromolecules are known to be hydrophobic. Here, we report that a cell surface heparan sulfate proteoglycan is acylated with both myristate and palmitate, two long-chain saturated fatty acids. When colon carcinoma cells were labeled with [3H]myristic acid, a significant proportion of the label was shown to be specifically incorporated into the protein core of the proteoglycan. Characterization of fatty acyl moieties in the purified proteoglycan by reverse-phase high pressure liquid chromatography revealed that ~60% of the covalently bound fatty acids was myristate. We further show that this relatively rare 14-carbon fatty acid was bound to the protein core via a hydroxylamine-sensitive ester bond. Palmitate appeared to be added post-translationally and derived in part from intracellular elongation of myristate, a process that occurred within the first two hours and was insensitive to inhibition of protein synthesis. Acylation of heparan sulfate proteoglycan represents a novel modification of this gene product and could play a role in a number of biological functions including specific interactions with membrane receptors and ligand stabilization.

A selected number of viral and eukaryotic proteins contain covalently bound, long-chain fatty acyls (for recent reviews, see Refs. 1-3). Although the precise functions of protein acylation have not been clearly elucidated, several biological roles have been assigned to the acyl moiety. For instance, it has been postulated that acylation may increase hydrophobicity which in turn facilitates protein-membrane interactions (1-3), while evidence has been provided that acyl moiety can function as a specific marker for membrane receptors (4, 5). Acylation may play a role in intracellular sorting (6) and glycosylation of certain proteins (7), is involved in modulating the protease sensitivity of gastric mucous (8), and may represent a potential marker for cellular differentiation and development of sea urchin embryos (9).

The addition of lipids to proteins occurs through one of two major pathways that give rise to two general classes of acyl proteins containing 16- or 14-carbon saturated fatty acids, palmitate or myristate, respectively (10, 11). Palmitoylated proteins comprise by far the larger class, since palmitate has been found to be covalently linked to a variety of polypeptides including the transferrin (12) and insulin receptors (13, 14), ankryrin (15), actin (16), various HLA-associated proteins (7, 17, 18), gastric mucus glycoproteins (8), and platelet glycoproteins (19, 20). Palmitate is linked to proteins post-translationally through a hydroxylamine-sensitive ester or thioester bond to serine (threonine) or cysteine, respectively (1-3, 10, 11). Generally, palmitoylated proteins are localized to the plasma membrane, although human apoprotein A-I, the principal protein associated with circulating high density lipoproteins, is palmitoylated (21). The second class of acyl proteins contains covalently linked myristate, a relatively rare fatty acid (1-3). Only a few well characterized eukaryotic proteins have been shown to be modified with myristate. These include the insulin receptor (13), immunoglobulin heavy chain (22), the transforming tyrosine kinase of Rous sarcoma virus (23), the catalytic subunit of cyclic AMP-dependent protein kinase (24), calcineurin B (25), and vinculin (26). In contrast to palmitate, the addition of myristate occurs co-translationally (27) through a hydroxylamine-resistant amide bond to the amino-terminal glycine (1-3). Myristate-containing proteins have been shown to be both soluble and membrane-bound (1-3, 11, 24, 25). The selective nature of protein acylation, together with the recent discovery of high affinity receptors for myristoylated proteins (4, 5), suggest a fundamental role for the addition of fatty acids to a subset of cellular proteins and that this modification may be a general feature of membrane proteins.

The main objective of the present study was to investigate whether the heparan sulfate proteoglycan (HS-PG)1 of human colon carcinoma cells contains covalently bound fatty acids. The rationale for this study is based on the following observations: (i) this proteoglycan is localized at the cell surface (28) where it intercalates into the lipid bilayer presumably via a hydrophobic domain (29); (ii) like many acylated pro-

1The abbreviations used are: HS-PG, heparan sulfate proteoglycan; DMEM/BSA, Dulbecco's modified Eagle's medium, high glucose, containing 1 mg/ml bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
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In the case of double-labeled HS-PG samples, received an additional 400 µg of carrier standard before filtration and analysis by HPLC. The samples were evaporated to dryness under nitrogen and redissolved in 50–100 µl of HPLC-grade methanol. About 30 µl were analyzed by reverse-phase HPLC using a 4.6-mm x 25-cm ALCOSORB ODS 5-µm column (Beckman Instruments, San Ramon, CA) eluted with 0.1% trifluoroacetic acid, 0.62% triethylamine (v/v) at a flow rate of 1-1.2 ml/min (38). One-minute fractions were collected, dried under vacuum, resuspended in 5 ml of Formula-953 (Du Pont-New England Nuclear), and counted in a Formula-953 (Du Pont-New England Nuclear) liquid scintillation counter. The elution position of the added carrier fatty acids and their methyl esters was monitored by UV absorbance at 214 nm and used to identify radioactive methanolic products eluted from acylated HS-PG or protein bands (37).

RESULTS

Co-purification of Lipids and Proteoglycans—In initial experiments we noticed that a significant amount of [3H]myristate-labeled lipids co-eluted from the DAE-TRISacryl gradients with the [35S]sulfate-labeled HS-PG and a significant contamination occurred throughout the gradient. Aliquots of the double-labeled HS-PG peaks were precipitated in ethanol:1.3% potassium acetate (v/v), and the pellets were analyzed by 1% agarose gel electrophoresis before or after three consecutive extractions with chloroform:methanol (1:1, v/v). The results (Fig. 1), showed a single band, the major HS-PG synthesized by human colon carcinoma cells, that migrated slightly faster after chloroform:methanol extraction. To determine whether there was any 3H activity in the HS-PG bands (the autoradiographic bands in Fig. 1 are generated primarily by the 35S activity), the corresponding bands from the dried gels were cut, homogenized, and counted in total.

The results showed that a significant amount of 3H activity was present in the ethanol-precipitated HS-PG, and that the 3H activity decreased following chloroform:methanol extraction (not shown). Taken together, these results indicate that significant amount of 3H activity is associated with the proteoglycan even after DAE-TRISacryl ethanol precipitation, and agarose gel electrophoresis, and suggest that the HS-PG may contain covalently bound lipids since [3H]myristate-labeled material was still associated with HS-PG after these procedures.

Efficient Delipidation of Cellular Proteins and Proteoglycans—To circumvent the lipid contamination discussed above, we first delipidated the cellular pellets before further purification of the proteoglycan. The results (Table I) show that >99% of 3H activity present in the crude cellular pellet was extractable in organic solvents. This indicates that, as expected, most of the radiolabeled fatty acids were either free or esterified to phospholipids or neutral lipids, while only

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0.42% of $^3$H activity was recovered in the delipidated pellet. An additional control was done to exclude the possibility that some of the $^3$H activity in the delipidated pellet was noncovalently, yet tightly bound in the form of free fatty acids or phospholipids. This possibility was tested by adding ["H]myristic acid (about 10$^6$ cpm) to a crude unlabeled cellular pellet and then incubating this mixture for 1 h. The pellet was subsequently extracted as in Table I and recovery of radioactivity was done by scintillation counting. In agreement with previous studies (9), no radioactivity was recovered in the delipidated pellet indicating that any free myristic acid would have been removed by the procedure outlined above.

Cycloheximide Inhibits Protein Acylation but Not Synthesis of Fatty Acids—It has been previously shown (27) that covalent attachment of myristate is a very early event in protein synthesis which occurs co-translationally, at least in certain cell types (3). Therefore, protein inhibitors such as cycloheximide would have profound effects on the incorporation of ["H]myristic acid. To determine whether the $^3$H activity recovered in the delipidated cellular pellet was indeed incorporated through a cycloheximide-sensitive pathway, triplicate confluent dishes of cells were exposed to 0, 30, 60, and 120 min to 100 μg/ml cycloheximide. During the last 30 min of each incubation, the cells were labeled with 100 μCi/ml ["H]myristic acid. The results showed that cycloheximide did not have any effects on the incorporation of ["H]myristic acid into total lipids (Fig. 2A), nor on the intracellular pool of free myristic acid (not shown). However, cycloheximide significantly inhibited the incorporation of ["H]myristic acid into cellular protein ($t_{1/2}$ ~ 45 min), with >90% inhibition at 120 min (Fig. 2B). Thus, the inhibition of protein myristoylation cannot be attributed to the lack of fatty acid uptake, but, in agreement with previous experiments (40), is likely due to the lack of suitable protein acceptor for myristoylation. Interestingly, the inhibitory effect of cycloheximide after a short exposure was less than expected (only 35% inhibition at 30 min (Fig. 2B) for a co-translational process). The results thus raise the possibility that some of the myristate may be added post-translationally or converted into palmitate before being covalently linked to protein, a process known to occur post-translationally (1-3).

Association of Fatty Acids with Heparan Sulfate Proteoglycan—To investigate further the nature of ["H]myristate-labeled material that co-migrated with the HS-PG on agarose gel, delipidated cellular pellets were solubilized in 8 M urea containing 0.2% Triton X-100 and 0.1 M NaCl and analyzed by DEAE-Trisacryl with a linear (0.1-0.9 M) NaCl gradient (34). The double-labeled HS-PG pool (peak c, Fig. 3A) eluting at ~0.4 M NaCl was clearly resolved from two, less anionic peaks, a and b, respectively. The former contained weakly charged acylated proteins, while the latter contained primarily free precursors still remaining after delipidation (not shown). Peak c was pooled, dialyzed, lyophilized, and further studied by analytical Sepharose CL-4B chromatography (34). The results (Fig. 3B) showed two major $^{35}$S-labeled HS-PG products (peak two and peak three) eluting at ~0.4 M NaCl with a $K_v$ of 0.25 which corresponds to the intact HS-PG and a small peak with a $K_v$ of 0.8 which contains heparan sulfate glycopeptides derived from the intracellular catabolism.
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Total Incorporation

B Delipidated Protein

Incubation Time with Cycloheximide (min)

FIG. 2. Effects of cycloheximide on the incorporation of \([3\text{H}]\) myristate into total lipids (A) and delipidated proteins (B) of human colon carcinoma cells. Triplicate 35-mm confluent dishes of colon carcinoma cells were incubated for the designated time intervals in DMEM/BSA supplemented with 100 \(\mu\text{g/ml}\) cycloheximide and 5 \(\text{mM}\) sodium pyruvate. The cells were labeled with 100 \(\mu\text{Ci/ml}\) \([3\text{H}]\)myristic acid during the last 30 min of each incubation, after which, the medium was removed, the cells were extensively washed with ice-cold DMEM/BSA and subsequently extracted with 4 M guanidine HCl, 2\% Triton X-100 in the presence of various protease inhibitors (31). The incorporation of \([3\text{H}]\)myristic acid into total cellular lipids (A) was estimated by Sephadex G-50 chromatography. The excluded volumes from the triplicate cultures were pooled, frozen at \(-70^\circ\text{C}\), lyophilized and delipidated as in Table I. The values in A represent the mean \(\pm\) S.D. The values in B represent the total radioactivity recovered in the delipidated cellular pellets. The included volumes of the Sephadex G-50 columns showed similar levels of intracellular free \(\text{H}^\text{2}\) activity (not shown), suggesting no significant change in the intracellular pool of fatty acid precursors.

The results thus far present strong evidence that \([3\text{H}]\)myristate-labeled peak coeluted with the major HS-PG (31). Of note, a \([3\text{H}]\)myristate-labeled peak could be dissociated from either the major HS-PG or the glycopeptides (Fig. 3B). Trypsin treatment (Fig. 3C) slightly moved the HS-PG to smaller \(M_c\) position (from \(K_{av}\) of 0.25 to \(K_{av}\) of 0.29) without generating any new \(\text{H}^\text{2}\)labeled peak not affecting the elution position of the other peaks. However, when peak c was treated with alkaline borohydride, all of the \(\text{H}^\text{3}\) activity was recovered in the total column volume (not shown) indicating that it was linked to a protein moiety. Treatment of \([3\text{H}]\)myristic acid-labeled HS-PG with heparitinase in the presence of protease inhibitors (33) shifted the whole HS-PG peak to \(K_{av}\) of 0.5–0.6, without generating any \(\text{H}^\text{3}\)-labeled material of smaller \(M_c\) (not shown).

The results thus far present strong evidence that \([3\text{H}]\)myristate-labeled material was tightly bound to the HS-PG since \(\text{H}^\text{3}\) activity was still present after extensive delipidation, anion exchange chromatography, and size exclusion chromatography in high guanidine concentration. Additionally, the \(\text{H}^\text{3}\) activity appeared to be associated primarily with the core protein since it was sensitive to alkali but remained associated with the heparitinase-treated proteoglycan. In a broad sense, the fact that our delipidation procedure allowed recovery of the \(\text{H}^\text{3}\)-labeled intracellular degradation product, in a proportion similar to that observed using more conventional approaches (31), indicates that the purification scheme adopted here may be useful for investigating proteoglycan acylation in other cellular systems.

Covalent Linkage of Fatty Acids to Heparan Sulfate Proteoglycan—To characterize in more details the nature of the
fatty acyl moiety, experiments were performed in which cells were labeled with either [3H]myristic acid alone or in combination with trace amounts of [35S]sulfate. The labeled products were delipidated as in Table I, subjected to DEAE-Trisacryl and Sepharose CL-4B chromatography, and further analyzed by 5% SDS-PAGE or 1% agarose gel electrophoresis. The initial delipidated material gave rise to five distinct fatty-acylated proteins (Fig. 4A, lane 1). A high molecular mass material recovered on the top of the stacking and separating gel (the completed HS-PG, see below), and four major acylated proteins of approximately 87, 43, 33, and 18 kDa. Determination of the apparent M, of smaller bands was obtained using SDS-PAGE of higher acrylamide concentration (cf. Fig. 9, lane 3). Also evident was that contaminant free lipids, detectable as an intense and diffuse band (asterisk, Fig. 4A), migrated faster than the dye front. Using the purification procedure outlined above, the high M, HS-PG (fractions 20–40 in Fig. 3B) could be clearly separated (Fig. 4A, lane 2) from the other acylated proteins (Fig. 4A, lane 3) recovered in the more retarded fractions of the Sepharose CL-4B column (fractions 42–62 in Fig. 3B). Notice also that the purification scheme used here removed the contaminating lipids from the proteoglycan fraction (Fig. 4, lane 2) since no radioactive band migrating ahead of the dye front was observed. In contrast, the lower M, fractions of the Sepharose CL-4B contained the acylated proteins and the free lipids (Fig. 4, lane 3). When [3H]myristate-labeled HS-PG was analyzed by agarose gel electrophoresis (Fig. 4B, lane 1), it co-migrated with [35S]sulfate-labeled HS-PG purified in parallel (Fig. 4B, lane 2).

Taken together, the results indicate that the 3H activity is covalently bound to the HS-PG as evidenced by the electrophoretic experiments in which the HS-PG is subjected to reduction and heating at 100°C.

Nature of Fatty Acyl Linkage to Heparan Sulfate Proteoglycan—The results with cycloheximide have raised the possibility that the 14-carbon myristate may be elongated to the 16-carbon palmitate, an interconversion that has been shown in other cellular systems (1–3). To assess the nature of the fatty acyl linkage, quadruplicate samples of [3H]myristate-labeled HS-PG were subjected to 5% SDS-PAGE; before fluorography, one-half of the gel was incubated for 16 h with 1 M Tris-HCl, pH 7.0 (control), and one-half with 1 M NH2OH, pH 10.0. The latter procedure cleaves ester or thioester bonds, typical of the bond between palmitate and proteins, but does not affect amide bonds, typical of myristoylated proteins. The results show that, following NH2OH treatment, the intensity of HS-PG bands (Fig. 5A, lanes 3 and 4) was markedly reduced as compared with that of samples treated with neutral Tris buffer (Fig. 5A, lanes 1 and 2). Quantitation of the fluorograms by scanning densitometry (Fig. 5B) or recovery of the 3H activity from SDS-PAGE (Fig. 5C) revealed that about 40% of the 3H activity was displaceable by NH2OH, consistent with an ester or thioester bond, while the remaining 60% was in a hydroxylamine-resistant amide linkage.

Characterization by HPLC of Fatty Acids Bound to Heparan Sulfate Proteoglycan—To characterize the nature of 3H activ-

![Fig. 4. Electrophoretic analysis of acylated proteins and proteoglycans of human colon carcinoma cells. A, a fluorogram of a 5% SDS-PAGE of a total delipidated pellet (lane 1), proteoglycan peak (lane 2), and acylated lower M, proteins (lane 3). Notice the presence of free fatty acids (asterisk) that migrate ahead of the dye front (DF); in contrast, the HS-PG pool does not penetrate the stacking gel or barely penetrates the separating gel. O, origin. The migration and apparent molecular mass in kilodaltons of prestained molecular mass markers (Bethesda Research Laboratories) are indicated at the left margin. This gel was exposed for 11 days. B, the autoradiographic profiles of 1% agarose gel electrophoresis of [3H]myristate/[35S]sulfate-labeled HS-PG (lane 1) in which the sulfate was present in only trace amounts and of [35S]sulfate-labeled HS-PG (lane 2). The gel was exposed for 5 days.](http://www.jbc.org/)

![Fig. 5. Hydroxylamine sensitivity of fatty acids in the heparan sulfate proteoglycan of human colon carcinoma cells. Replicate samples of purified HS-PG preparations labeled with [3H] myristic acid were resuspended in equal volumes of SDS-PAGE buffer, boiled for 2 min, and analyzed by 5% SDS-PAGE (A). At the end of the electrophoresis, one-half of the gel was incubated for 18 h in either 1 M Tris-HCl, pH 7.0 (control, lanes 1 and 2), or in 1 M hydroxylamine, pH 10.0 (NH2OH, lanes 3 and 4), followed by fluorography. B and C, quantitation of absorbance by scanning laser densitometry and of recovered 3H activity (mean ± S.D., n = 4), respectively.](http://www.jbc.org/)
ity in HS-PG, the proteoglycan bands pooled from SDS-PAGE (similar to those shown in Fig. 5A) were homogenized in OH/BH₄, incubated at 45 °C for 48 h, neutralized with acetic acid, filtered, lyophilized, and subjected to acid methanolysis at 110 °C for 62 h. Released fatty acid methyl esters were extracted into petroleum ether and the radioactivity in the aqueous and organic phases was determined by scintillation counting. Recovery of 3H activity into the hexane phase consistently exceeded 90% and, in the case of double-labeled samples, 35S activity remained in its entirety in the aqueous phase (not shown). These data are in agreement with previous results (11, 40), and indicate that little interconversion of [3H]myristic acid into amino acids occurs when colon carcinoma cells are cultured in medium supplemented with 5 mM sodium pyruvate. The hexane phase was dried under nitrogen, resuspended in methanol and analyzed by reverse-phase HPLC. The majority of the radioactivity (about 60%) co-eluted with standard methylmyristate (Fig. 6A) and with standard methylpalmitate. Variable amounts of 3H activity co-migrating with authentic myristate and palmitate (Fig. 6A) were often observed; this material, which likely derives from partial acid methanolysis, always accounted for a significant proportion of total radioactivity. Similar HPLC elution profiles were obtained from the other pooled acylated proteins (not shown) indicating that elongation of myristate to palmitate in colon carcinoma cells is not restricted to HS-PG acylation. When samples were first treated with NH₂OH, at pH 10.0, and then subjected to acid methanolysis, the peak co-eluting with methylpalmitate (Fig. 6B) totally disappeared, indicating an ester or thioester bond (1–3).

Next we wanted to determine whether the same distribution of 3H activity was present after a short labeling time. To accomplish this, we labeled cells with [3H]myristic acid for only 2 h and then purified the HS-PG as above. The results (Fig. 7) showed a HPLC profile of fatty acids nearly identical to that obtained after 18 h of continuous labeling. Collectively, these results indicate that the HS-PG of human colon carcinoma cells contains both myristate and palmitate and suggest that palmitate derives from metabolic elongation of myristate, a process that occurs within the first 2 h. Furthermore, these data support the results obtained with cycloheximide (see above) and provide an explanation for the presence of hydroxylamine-sensitive 3H activity in the HS-PG (cf. Fig. 5).

**Metabolic Incorporation of Palmitate into Heparan Sulfate Proteoglycan**—The results presented above warranted experiments to determine whether we could metabolically label the HS-PG with [3H]palmitic acid as precursor. The results (Fig. 8) showed a double-labeled peak eluting from Sepharose CL-4B at the same position as that presented in Fig. 3B. The unfractionated material revealed a large number of bands on a 12.5% SDS-PAGE (Fig. 9, lane 1) with the contaminating lipids migrating ahead of the dye front (asterisk, Fig. 9) as in the case of [3H]myristic acid-labeled samples (cf. Fig. 4). The purified HS-PG was retained in the stacking and separating gel (Fig. 9, lane 2) and, as in the case of labeling with [3H]myristic acid (cf. Fig. 4), showed no significant contaminations with either acylated proteins or free fatty acids. The SDS-PAGE profile of total cellular proteins (Fig. 9, lane 1) was quite different from that obtained with [3H]myristic acid as a precursor (Fig. 9, lane 3), thus confirming the specificity of the two metabolic precursors.

Reverse-phase HPLC analysis of the acid methanolysis extracts of acylated HS-PG recovered from SDS-PAGE revealed predominantly methylpalmitate and palmitate (Fig. 10), in addition to two minor peaks that did not co-elute with either the myristate or the methylmyristate standard. These findings are consistent with previous studies which have shown a lack of interconversion of palmitate to myristate (1–3).

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![Fig. 6. HPLC elution profiles of fatty acids covalently bound to the heparan sulfate proteoglycan of human colon carcinoma cells.](http://www.jbc.org/)

![Fig. 7. HPLC elution profiles of fatty acids covalently bound to the heparan sulfate proteoglycan of human colon carcinoma cells following a short labeling period.](http://www.jbc.org/)
Cell cultures were incubated with [3H]palmitic acid and [35S]sulfate in DMEM/BSA supplemented with 5 mM sodium pyruvate. The cell layer was then delipidated as described before, with V1 and V2 at fractions 20 and 61, respectively. The HS-PG was purified as detailed in the "Experimental Procedures." The Sepharose CL-4B column was equilibrated and run in 4 M guanidine HCl, containing 0.2% Triton X-100.

Fig. 8. Sepharose CL-4B elution profiles of [3H]palmitic acid/[35S]sulfate-labeled products of human colon carcinoma cells. Confluent cultures were incubated for 18 h with 100 pCi/ml [3H]palmitic acid and 40 pCi/ml [35S]sulfate in DMEM/BSA supplemented with 5 mM sodium pyruvate. The cell layer was then delipidated as in Table I, and the HS-PG purified as detailed in the "Experimental Procedures." The Sepharose CL-4B column was equilibrated and run in 4 M guanidine HCl, containing 0.2% Triton X-100, as described before (61), with V1 and V2 at fractions 20 and 61, respectively.

Alkaline Methanolysis of Acylated Heparan Sulfate Proteoglycan—To investigate in more detail the nature of HS-PG acylation, we purified proteoglycan samples labeled with [3H]palmitic acid ("H-Pal) or [3H]myristic acid ("H-Myr), purified as described in the text, and subjected them to alkaline methanolysis in 0.2 M KOH in methanol. The hexane phases were placed directly into scintillation vials and dried under a stream of nitrogen. Each sample contained 200 g each of the four standard fatty acids used for the HPLC analysis. At the end of the incubation, the samples were acidified with 60 mM HCl and extracted four times with petroleum ether. Methanolic KOH treatment of samples labeled with [3H]palmitic acid or [3H]myristic acid was performed for 30 min at 37°C, followed by acidification with 60 mM HCl and extraction with petroleum ether as above. The hexane phases were placed directly into scintillation vials and dried under a stream of nitrogen. Both the aqueous and dried organic phases were counted in total and the results expressed as percentage of total [3H] activity recovered in each phase.

TABLE II

| Treatment               | Aqueous/methanolic phase | Hexane phase |
|-------------------------|--------------------------|--------------|
| [3H]Palmitic acid as precursor |                          |              |
| 1 M Tris-HCl, pH 7.0    | 99.5                     | 0.5          |
| 1 M NH4OH, pH 7.0       | 95                       |              |
| 1 M NH4OH, pH 10.0      | 92                       |              |
| 0.2 M KOH in methanol   | 2                        |              |
| [3H]Myristic acid as precursor | 65                       | 35           |
| 0.2 M KOH in methanol   |                          |              |

The values represent the mean of duplicate analyses with variance < 15%.

was analyzed by HPLC and found to comprise methylpalmitate with only trace amounts of methylmyristate (not shown). These data are in agreement with those obtained using hydroxylamine at pH 10.0, a procedure that removed nearly identical amounts of [3H] activity from the SDS-PAGE (cf. Fig. 5). Conversely, when [3H]palmitate-labeled HS-PG was subjected to alkaline methanolysis, 98% of [3H] activity was recovered in the hexane phase (Table II) and it was found to co-elute (>95%) with standard methylpalmitate and palmitate by HPLC (not shown).

In order to distinguish between ester and thioester bonds, we subjected HS-PG samples labeled with [3H]palmitic acid to hydroxylamine treatment under neutral or alkaline conditions. It is known (9) that alkaline hydroxylamine treatment cleaves both ester and thioester bonds, while neutral hydroxylamine treatment cleaves preferentially thioester bonds. The results (Table II) showed that under neutral or alkaline conditions, nearly all the [3H] activity could be displaced by hy-
The metabolic elongation of myristate to palmitate in the colon carcinoma cells occurred after a 2-h labeling with [\textsuperscript{3}H]myristate to the same extent as after long-term labeling period. Our results further indicate that in those cells elongation of myristate to palmitate occurs independently of protein synthesis and suggest that palmitate is linked to the HS-PG at a later stage of maturation, such as in the Golgi complex. In future experiments, it would be interesting to test whether agents that disrupt Golgi functions, such as tunicamycin, would also affect acylation of HS-PG. We have previously shown (33) that colon carcinoma cells have a large and long-lived pool (t\textsubscript{1/2} ~ 75 min) of HS-PG precursor protein which is in part translocated to the cell surface and secreted without the addition of glycosaminoglycan side chains. An important question is whether acylation is required for further glycosylation of HS-PG since blocking fatty acylation prevents addition of sialic acid to Ia-associated invariant chain (46).

**Nature of Fatty Acyl Linkage to Heparan Sulfate Proteoglycan**—The covalent binding of fatty acids to proteins occurs via two major pathways (1–3). Bonds are usually formed between palmitate and either the hydroxyl group of serine/threonine to form oxygen ester bonds or the thiol group of cysteine to yield thioester bonds. Both linkages are sensitive to treatment with either methanolic KOH or hydroxylamine under alkaline (pH 10.0) conditions; however, only thioester bonds are sensitive to hydroxylation under neutral conditions. In contrast, hydroxylamine- and alkali-resistant amide bonds are formed primarily between myristate and amino-terminal glycine residues (1–3, 42) but can also be formed between myristate and lysine side chains (22). In the present study, the loss of [\textsuperscript{3}H]palmitate label following hydroxylation and quantitative recovery of [\textsuperscript{3}H]activity into the organic phase after acid methanolysis indicate that, under the labeling conditions used here, little or no interconversion of fatty acids into amino acids occurred. We found that myristate was the major fatty acid of HS-PG, bound to the protein core via a hydroxylamine-resistant amide bond presumably to amino terminal glycines as in other membrane-binding proteins (47). In contrast, palmitate was displaced by treatment with methanolic KOH or hydroxylamine at either pH 7.0 or 10.0, indicating a thioester bond, presumably to cysteine. It is noteworthy that colon carcinoma cells incorporate a significant amount of [\textsuperscript{35}S]cysteine into the HS-PG (34) and that this molecule is immunologically and structurally related (33) to a cysteine-rich HS-PG from a murine basement membrane (48).

The presence of both esterified and amide-linked fatty acids in the same molecule has been reported in murein-lipoprotein of *Escherichia coli* (49) and the human insulin receptor (13), although, a recent report (14) has detected only palmitate in the latter gene product. Our data represent the first example of a human proteoglycan with both types of fatty acid bound to the protein core apparently through two distinct linkages. In contrast, palmitate was displaced by treatment with methanolic KOH or hydroxylamine at either pH 7.0 or 10.0, indicating a thioester bond, presumably to cysteine. It is noteworthy that colon carcinoma cells incorporate a significant amount of [\textsuperscript{35}S]cysteine into the HS-PG (34) and that this molecule is immunologically and structurally related (33) to a cysteine-rich HS-PG from a murine basement membrane (48).

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An attempt to obtain amino acid sequence from the [\textsuperscript{3}H]myristate labeled HS PG and 16-kDa band (Fig. 4A) blotted into polyvinylidene difluoride membrane (Immobilon) gave negative results, suggesting that in both instances the amino termini were blocked. In contrast, we could sequence from the same gel the first eight amino acids of bovine serum albumin which was used as a carrier (G. R. Dodge, P. Roughley, and R. V. Iozzo, unpublished results).
mitoyl transferase with relaxed specificity for fatty acyl substrates (45).

The covalent addition of lipids to HS-PG synthesized by rat hepatocytes (50) and Schwann cells (51) has been reported. Specifically, these hydrophobic proteoglycans appear to be associated to the plasma membrane via glycosyl-phosphatidylinositol moiety that is covalently linked to the protein core and has its fatty acyl chains embedded into the lipid bilayer (41). Three lines of evidence indicate that the fatty acids in the colon carcinoma HS-PG are not linked via myo-inositol phosphate. First, no 3H-labeled fatty acids could be displaced by phosphatidylinositol-specific phospholipase C. Second, millimolar amounts of exogenous inositol hexaphosphate failed to displace any [35S]sulfate-labeled HS-PG from intact cells. Third, the myristate was bound primarily through an alkali-resistant bond in contrast to those described in glycosyl-phosphatidylinositol structure in which fatty acids are bound primarily via alkali-labile phosphodiester bonds (41).

Possible Roles of Fatty Acylation of Proteoglycan—The incorporation of two different fatty acids into the heparan sulfate proteoglycan raises the interesting possibility that each has a distinct function. For example, it is possible that myristate may be necessary for membrane targeting and anchorage. The recent discoveries of high-affinity membrane receptors for myristoylated p60^NTR protein (4, 5) and of a major class of myristate in hydrophobic interactions between guanine-binding proteins and plasma membranes (52, 53) open the possibility that cell surface proteoglycans may interact with plasma membranes in a similar fashion. It is noteworthy that only 60–65% of the cell surface HS-PG of colon carcinoma cells is accessible to trypsin (31) and, based on pulse-chase and structural studies (28–34), we proposed that part of the completed HS-PG resides in a compartment between the Golgi and the cell surface. Our present results raise the possibility that the HS-PG may be anchored internally to specific membrane receptors that recognize the fatty acyl moiety. As proposed earlier (1), the lower hydrophobicity of myristate as compared to the longer acyl chain of palmitate may permit reversible association of myristoylated HS-PG with various membrane compartments. This reversible association would make this molecule quite versatile and would allow targeting to the cytoplasmic face of the exocytotic pathway. The addition of palmitate could further stabilize the hydrophobic interactions of the transmembrane or loop structure of the protein core (29), similar to the role proposed for other transmembrane proteins (1–3). Palmitoylation can also have marked structural and functional consequences since it can prevent disulfide bond formation (18) and plays a crucial role in the coupling of p2-adrenergic receptor to the adenyl cyclase signal transduction system (54). Blocking palmitoylation by the antibiotic cerulenin (55) or by site-directed mutagenesis (50) prevents replication and budding of Sindbis virus, respectively. Therefore, it is possible that subtle changes in the degree of palmitoylation of the protein core may be a signal for secretion of a subpopulation of proteoglycans. Finally, it has been proposed (8, 57) that acylation of gastric glycoproteins may protect them from peptic digestion and that in the alimentary tract secretions the covalently bound fatty acids may serve as an anchor for the interactions of mucin with lipids which ultimately protect the underlying epithelium. This is a plausible function for the colon carcinoma cell HS-PG since this gene product is derived from an enteric epithelium and is structurally related to colonic mucins, particularly in its large number of O-linked oligosaccharides (32).

In conclusion, while fatty acylation of HS-PG can provide an additional explanation for the hydrophobic properties reported in the past for this family of macromolecules, it opens a number of fundamental questions regarding specific functions of the two fatty acids and intracellular site of acylation of the protein core. Important to our basic understanding of membrane proteoglycans is the question whether fatty acylation is required for proper membrane insertion and intracellular trafficking, and whether proteoglycan acylation is a more general modification that occurs in other cell surface proteoglycans. Our current studies are attempting to address experimentally some of these questions and will determine whether interfering with fatty acylation has any effect on biosynthesis, turnover and membrane targeting of HS-PG.

Acknowledgments—We thank Dong Nham for excellent technical assistance and Dr. J. Erek for valuable suggestions.

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*J. Biol. Chem.* 1990, 265:19980-19989.

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