Heterogeneous N-Acylation Is a Tissue- and Species-specific Posttranslational Modification*

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Heterogeneous N-terminal glycine acylation recently has been reported for two proteins involved in visual signal transduction. Similar N-acylations have typically involved only myristate; however, none of the previously examined proteins were isolated from retinas. To determine whether heterogeneous N-acylation is tissue-specific or protein sequence-specific, the N-terminal modifications of the catalytic subunit of cAMP-dependent protein kinase, partially purified from bovine retinas, heart, and brain tissues, were characterized. Using tandem mass spectrometry and liquid chromatography coupled directly to an electrospray mass spectrometer, we found only myristate at the N termini of catalytic subunits from brain and heart tissue, whereas the N termini of the retina-derived subunits were heterogeneously acylated in a manner similar to recoverin and transducin. Thus it appears that the nature of N-terminal glycine acylation is determined by the cell or tissue type in which it is located, and not by the sequence of the modified protein. We also examined the N-acylation of recoverin purified from human retinas, as well as transducin purified from frog retinas, to determine if heterogeneous acylation of retinal proteins is a uniquely bovine phenomenon. Interestingly, human recoverin was modified by the same family of fatty acids found on the bovine retinal proteins, while frog transducin was modified homogeneously not with myristate, but with a doubly unsaturated (C14:2) fatty acyl group.

N-terminal protein acylation by myristic acid, a fully saturated unbranched 14-carbon fatty acid (C14:0), was first observed on the catalytic subunit of cAMP-dependent protein kinase (1). Since this first report, a variety of myristoylated proteins have been identified (reviewed in Refs. 2–4). There seems to be no single function associated with this posttranslational modification, nor does myristoylation by itself appear to direct proteins to particular cellular locations. Myristoylated and, in some cases myristoylation may mediate protein-protein interactions, and in the case of the catalytic subunit of cAMP-dependent protein kinase, it appears to act as the core of a folding domain (5). It has also been suggested that the weak hydrophobicity of N-myristoylation, compared to longer chain fatty acids, allows for reversible modulation of membrane interactions by associated proteins (6).

There have been three reports of the heterogeneous acylation of N-terminal glycine of two proteins: recoverin (7) and transducin α-subunit (T.) (8, 9). Although both proteins have an N-terminal glycine, which is required for myristoylation, three other fatty acids were found in addition to the usual myristate (C14:0): lauroyl (C12:0), (cis-Δ⁵)-tetradecaenoyl (C14:1), and (cis,cis-Δ⁵,Δ⁷)-tetradecadienoyl (C14:2). In all of these cases, the heterogeneously acylated proteins were purified from bovine retinas and are involved in visual signal transduction. To determine whether heterogeneous N-acylation is tissue-specific or, alternatively, protein-specific, the N-terminal acyl groups of the catalytic subunit of cAMP-dependent protein kinase (C-subunit) from bovine heart, brain, and retina were characterized. In addition, variability in N-acylation between species was examined by purification and analysis of frog Tα and human recoverin in order to verify that this posttranslational modification was not a peculiarity of Bos taurus.

These proteins were digested by trypsin and analyzed by reverse phase high performance liquid chromatography coupled directly to a mass spectrometer via an electrospray interface (LC/MS) (10–12). To exclude the possibility that observed peptides were derived from contaminating proteins, peptide sequences and N-terminal modifying groups were verified by tandem mass spectrometry (MS/MS) of collected fractions (13, 14) or liquid chromatography coupled directly to a tandem mass spectrometer (LC/MS/MS) (12).

MATERIALS AND METHODS

Unless otherwise stated, all procedures were performed at 4 °C using ice-cold solutions.

Partial Purification of C-subunit—Activity was monitored using a CAMP-dependent kinase assay described by Beavo et al. (15). The C-subunit was partially purified from a retinal extract in ROS buffer (20 mM MOPS, pH 7.2, 2 mM MgCl2, 60 mM KCl, 30 mM NaCl, and 1 mM dithiothreitol) containing 2.0 g of total protein (using the procedure of Beavo et al.; Ref. 15). For the heart and brain proteins, two volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5 µg/ml pepstatin) was mixed with one volume of ground tissue in a Waring blender at high speed for 1 min. The mixture was centrifuged at 7000 x g for 30 min, and the supernatant was passed through glass wool and a Whatman No. 2 filter paper. The filtrate was centrifuged again for 1.5 h at 100,000 x g. The supernatant (3.6 ml) was diluted to 10 ml and loaded onto a Mono Q (Pharmacia Biotech Inc.) column and eluted with a 20-ml linear gradient from 0 to 500 mM NaCl. The fractions containing

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†† The abbreviations used are: Tα, transducin α-subunit; C-subunit, catalytic subunit of cAMP-dependent protein kinase; ROS, rod outer segments; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid.
the holoenzyme were pooled, diluted 5-fold, and the regulatory and catalytic subunits dissociated by the addition of 10 μM cAMP. This material was loaded on a Mono Q column and eluted with the same gradient as described above. Fractions containing the catalytic subunit (elutes earlier than the holoenzyme) were acetone precipitated, dissolved in 100 mM Tris-HCl, pH 8, 2 x urea, 1 mM CaCl2, and digested with 2% trypsin (w/w) for 2–3 h at 37°C.

**Purification of Frog Tp** —Tp was purified from frog rod outer segments (ROS) kindly donated by Vadim Arzhavsky. Bleached ROS membranes were washed extensively in ROS buffer (5 mM Tris, pH 7.5, 0.5 mM MgCl2, and 1 mM diithiothreitol), and Tp was isolated by washing the membranes four times in ROS buffer containing 400 μM GTP. The ω-subunit was purified by Blue Sepharose chromatography as described by Yamazaki et al. (16). Active fractions were concentrated in a Centricon 10 (Amicon) prior to digestion with trypsin for 1 h at 4°C using 2.5% trypsin (w/w).

**Purification of Human Recoverin** —Human recoverin was purified from 32 human eyes obtained from the Lions Eye Bank using the method of Polans et al. (17). Briefly, human retinas dissected from previously frozen eyes were homogenized with a glass/glass tissue homogenizer in 20 ml of 50 mM Hepes buffer, pH 7.5, containing 1 mM EDTA, 100 mM NaCl, and 1 mM benzamidine. The soluble proteins were separated from membranes by centrifugation at 100,000 x g for 30 min.

This extraction procedure was repeated twice. The combined retinal extracts were mixed with CaCl2 at a final concentration of 2 mM and applied to a column of phenyl-Sepharose (1.0 x 5 cm) that had been equilibrated with (50 mM Hepes buffer, pH 7.5, containing 100 mM NaCl and 2 mM CaCl2). The column was washed extensively with equilibration buffer until the absorbance at 280 nm lowered below 0.005 absorbance unit. Bound proteins were eluted with 50 mM Hepes buffer, pH 7.5, containing 100 mM NaCl and 10 mM EDTA at a flow rate of 10 ml/h, and 1-ml fractions were collected. Fractions 10-40 contained pure human recoverin, as determined by SDS-polyacrylamide gel electrophoresis. These fractions were combined and concentrated with a Centricon 10 (Amicon) before tryptic digestion.

**Mass Spectrometry** —Trypsin-digested protein (100–200 pmol) in 20 μl of 100 mM Tris buffer, pH 8, was digested with 1 μl of trypsin in a 2 x 100-mm Applied Biosystems Aquapore C18 column, equilibrated with a linear gradient of 0–95% acetonitrile (with 0.05% trifluoroacetic acid) in 30 min. A post-column splitting Tp was used to divert 10–20% of the column effluent to a triple quadrupole tandem mass spectrometer (PE/Sciex API-III, Thornhill, Ontario, Canada); the remaining 80–90% was directed to a UV detector monitoring 214 nm, and fractions were collected by hand. The mass spectrometer was operated at unit resolution; typically scanning from 100 to 1800 at a rate of 3–5 s/scan (dwell time of 0.4–5.5 ms and a step size of 0.25 Da). Selected ion plots depict the chromato-erographic elution profiles of components that produce ions of specified mass to charge ratios. These plots were produced using software provided by the manufacturer. MS/MS spectra were acquired using the original design of collision cell (redesigned after this experiment) with a resolution in the collision cell design.

Human recoverin tryptic peptides (approximately 10–20 pmol) were analyzed by LC/MS using a 500-μm packed capillary (WPOREX C18) operating at a flow of 15 pl/min. The Applied Biosystems model 140A pump was used to generate acetonitrile/trifluoroacetic acid gradients without a pre-column split. For this column all of the effluent was directed to the mass spectrometer. LC/MS/MS was employed for the analysis of recoverin peptides utilizing a redesigned high pressure collision cell (PE/Sciex). The gas thickness measured outside of this collision cell was optimized at 2.5 x 1014 atoms of argon/cm2; the actual pressure inside the cell is unknown. Scans were acquired at a rate of 1–2 s/scan; since the C14:2, C14:1, and C14:0 acyl species are separated chromatographically, the MS/MS spectra were acquired from a single injection of 10–20 pmol of tryptic peptides.

**RESULTS**

From a total of 50 mg of crude heart protein, the C-subunit was purified 200–300-fold using a single Mono Q column (see "Materials and Methods"). This material was subjected to tryptic proteolysis and analyzed by LC/MS. Fig. 1 shows the selected ion plots for mass to charge ratios corresponding to the singly protonated N-terminal tryptic peptide of the α-isozyme (18) of the C-subunit (Gly-Asn-Ala-Ala-Ala-Ala-Lys) with N-terminal C14:0, C14:1, C14:2, and C12:0 acyl groups. Only the myristoylated N-terminal tryptic peptide was detected. Its retention time was 24 min; its structure was verified by MS/MS (data not shown). Identical results with respect to N-terminal acylation were obtained from the C-subunit derived from bovine brain tissue. In addition to the α-isozyme of the C-subunit, brain tissue contained a smaller proportion of the β-isozyme, which was also exclusively myristoylated. The N-terminal tryptic peptide of the β-isozyme has the sequence Gly-Asn-Ala-Thr-Ala-Lys and coelutes with that of the α-isozyme.

Although the C-subunit preparation from bovine retinas was contaminated by other proteins, a combination of LC/MS and MS/MS provided unambiguous results. Fig. 2 depicts the selected ion plots of mass to charge ratios 812, 810, 808, and 784, which correspond to the protonated N-terminal tryptic peptides of the C-subunit α-isozyme N-acylated with C14:0, C14:1, C14:2, and C12:0 acyl groups. Due to the complexity of the mixture, a number of peaks appear in the selected ion plots that are due to unrelated peptides. Peaks c and d in Fig. 2 exhibit the characteristic co-elution times of C12:0 and C14:2 acylated peptides; peak b corresponds to the C14:1 acylpeptide, and peak a to the myristoylated peptide. These peptides were analyzed by MS/MS (Fig. 3) and positively identified as acylated forms of the N-terminal peptide. An identical acylation pattern was observed for the β-isozyme, but at a reduced signal compared to the α-isozyme.

To determine if heterogeneous N-acylation of retinal proteins occurred in species other than B. taurus, Tp from frog ROS and recoverin purified from thirty human eyes were examined by LC/MS and MS/MS. The amino acid sequence for frog Tp is unknown; however, based on interspecies comparisons of known G protein sequences, the frog Tp sequence was expected to be very similar to that of cows. In fact, it was found that the N-terminal tryptic peptides are of identical sequence. Fig. 4 shows the selected ion plots for the mass to charge ratios corresponding to the protonated N-terminal tryptic peptide with
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Acyl-Gly-Asn-Ala-Ala-Ala-Ala-Lys

Acyl-Gly-Ala-Gly-Ala-Ser-Ala-Glu-Glu-Lys

FIG. 2. Selected ion plots for mass to charge ratios corresponding to the protonated N-terminal tryptic bovine retinal C-subunit peptide with C14:0, C14:1, C14:2, and C12:0 N-acyl groups. The high pressure liquid chromatography fractions corresponding to the peaks labeled a-d were analyzed by tandem mass spectrometry (Fig. 3) to confirm their structures.

Acyl-Gly-Ala-Ala-Ala-Ala-Lys

FIG. 3. Low energy CID tandem mass spectra of the acyl-forms of the N-terminal tryptic fragment of bovine retinal C-subunit. The ions labeled b1 to b5 (ion nomenclature described in Ref. 26) are product ion fragments that contain the N-terminal acyl group and therefore exhibit a corresponding shift in mass. Most of the remaining unlabeled fragments correspond to y-type ions, immonium ions, or internal fragment ions.

FIG. 4. Selected ion plots for mass to charge ratios corresponding to the protonated N-terminal tryptic frog Tα peptide with C14:0, C14:1, C14:2, and C12:0 N-acyl groups. A signal was observed only for the mass corresponding to the C14:2-acylated peptide eluting at approximately 15 min; the peptide structure was confirmed by tandem mass spectrometry. The C14:2-acylated peptide with two 13C isotopes gives rise to the signal in the C14:1 selected ion plot.

the various acyl groups. A peak was observed only for the C14:2 acylated peptide; none of the other acyl groups were observed. A small peak of mass to charge ratio corresponding to C14:1 acylated peptide is seen, but it has an identical elution time to that of the C14:2 peptide. Since C14:1 acylated peptides are known to elute slightly later than the corresponding C14:2 peptide, this small peak in the selected ion plot appears to represent the population of C14:2 acylated peptide containing two carbon-13 isotopes. The structure of the C14:2 peptide was verified by MS/MS (data not shown); the tandem mass spectra of the bovine and frog C14:2 acylated peptides were identical.

The sequence of the N-terminal tryptic peptide from human recoverin is identical in sequence to the bovine protein. Fig. 5 depicts the selected ion plots for the acylated forms of the recoverin N-terminal peptide. All of the acylated forms were observed, and with the exception of the C12:0 peptide, their structures were verified by LC/MS/MS (Fig. 6). Humans exhibit heterogeneous N-acylation of retinal proteins.

DISCUSSION

Upon the discovery of heterogeneous acylation of bovine recoverin and Tαα, a reexamination of the first discovered example of N-myristoylation (bovine heart C-subunit) seemed warranted. Fig. 1 shows that bovine C-subunit is exclusively myristoylated. Identical data were obtained from bovine brain tissue, where only N-myristoylation of the C-subunit was observed. Thus, heterogeneous acylation is not a feature of nerve cells in general. These results are in contrast to the mixture of fatty acyl groups found at the N terminus of the C-subunit isolated from retinal tissue (Figs. 2 and 3), which compared to recoverin and Tαα is enriched in the C14:0 acyl group (Table I). Whereas the C-subunit is probably produced in all retinal cells, only photoreceptors are producing recoverin and Tαα. If heterogeneous acylation is specific to photoreceptor cells, then the photoreceptor C-subunit would be diluted by N-terminally myristoylated protein derived from other cells. Although these studies do not represent an exhaustive screening of tissues, it is apparent that the tissue location, not the protein sequence, determines if N-acylation is heterogeneous.

The observation of a protein that is N-myristoylated in most tissues, but heterogeneously acylated in retinas, suggests the origin of this unusual posttranslational modification. It has been found that the CoA ester of the same C14:1 isomer identified at the N terminus of Tα and recoverin (cis,Δ2) is superior to C14:0 as a substrate for both the human and yeast N-myristoyltransferases (20, 21). In the absence of any evidence
U\izing shift in mass, whereas the C-terminal ions labeled tryptic fragment identical mass. Most of the remaining unlabeled fragments correspond contain the N-terminal acyl group and therefore exhibit a correspond-

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3 T. A. Neubert, unpublished observations.
This appears to involve mechanisms distinct from myristoylation, because this protein is acylated at an N-terminal alanine, rather than glycine as exclusively reported for myristoylation.

In summary, heterogeneous N-acylation appears to arise through a tissue-specific mechanism, perhaps involving differences in acyl-CoA pools, and the degree of N-acyl heterogeneity varies among species.

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