Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 209-214

PRENYLATED PROTEINS FROM KIDNEY*

E. Bruenger and H. C. Rilling‡

Department of Biochemistry, School of Medicine
University of Utah, Salt Lake City, UT 84108

Received July 14, 1986

SUMMARY: When [5-^{3}H]mevalonate is injected into mice, it is incorporated into macromolecules in the kidney. The incorporated material is stable to treatment with RNase or DNase but not protease, indicating that the radioactivity is associated with protein. Electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels indicates a molecular weight of about 25,000. The incorporated radioactivity can be released from the polypeptide and extracted into organic solvents after hydrolysis with acid or base or by treatment with protease. The conditions required for hydrolysis strongly suggest that the linkage between the protein and the mevalonate-derived material is an allylic ether. The chromatographic mobility of the incorporated material in several systems is similar to that of dolichol C_{95}.

Proteins are frequently derivatized by one of a number of ligands. One class of these ligands consists of lipids covalently linked to the side chain of an amino acid (1,2,3). These lipids have usually been fatty acids or glycophospholipids (4). More recently, a new class of protein-lipids has been demonstrated. Schmidt et al. (5) found proteins that were labeled by exogenous mevalonate or one of its metabolites. The labeled peptides obtained by proteolysis of these proteins were nonpolar, indicating that a nonpolar, i.e., polyisoprenoid, derivative of mevalonate was covalently linked to protein. Neither the nature of the linkage nor the structure of the polyisoprenoid molecule(s) was established.

We have investigated labeling of proteins by mevalonate in the whole animal and have found that some proteins in kidney are covalently linked to

*This research was supported by Grant AM 13140 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

‡To whom all correspondence should be addressed: University of Utah, Department of Biochemistry, 410 Chipeta Way, Salt Lake City, UT 84108.

The abbreviation used is: SDS, sodium dodecyl sulfate.

0006-291X/86 $1.50

high molecular weight polyprenols. Evidence for the kind of linkage and the prenol involved are presented.

MATERIALS AND METHODS

Fasted mice (17 h) were injected intraperitoneally with 50 μCi RS-[5-^{3}H]-mevalonolactone[1]. After 2 h, the mice were sacrificed and the kidneys removed. The kidneys were homogenized in water with a Polytron homogenizer and macromolecules were precipitated with 10 volumes of cold acetone. The resulting precipitate was sequentially extracted three times each with 10 mL acetone, three times with 5 mL $CHCl_3$:CH_3OH (2:1 v/v), and finally twice with 5 mL absolute ethanol. The residual pellet was suspended in 2% SDS , 10 mM Tris buffer pH 7, and incubated at 37^0 overnight. The mixture was clarified by centrifugation at 12,000 x g for 5 min. The radioactivity, 90% of which was in the supernatant, was chromatographed on a 1.5 x 30 cm column of Sephadex G-50 in 10 mM Tris buffer pH 7 containing 1% SDS.

Proteins that had been solubilized in SDS were hydrolyzed with 6 N HCl by heating in a boiling water bath for 10 min. An equal volume of saturated NaCl added, and nonpolar organic materials were extracted into toluene which was concentrated under a nitrogen stream.

RESULTS AND DISCUSSION

To determine if protein could be labeled *in vivo* by mevalonic acid, fasted mice (CF-1) were injected with [5-^{3}H]mevalonate and the kidneys, liver and a segment of the intestine removed for analysis. The samples were homogenized and then extracted exhaustively with organic solvents to remove noncovalently bound lipids. The incorporation of radioactivity found in residual material from intestine, liver, and kidney was 30, 270±100, and 1030±200 cpm mg^{-1} wet tissue, respectively. To ascertain that protein was being labeled by mevalonate rather than an impurity in the radioisotopic sample, mevalonate was diluted several thousand fold with nonradioisotopic

---

[1]This isotopic derivative of mevalonate was selected because the label would be lost as a proton if mevalonate were metabolized via the shunt pathway (6).

mevalonate on injection. Isotope incorporation into macromolecular bound material was reduced about 10-fold, strongly indicating the isoprenoid nature of the labeling. Full dilution is not anticipated since the injected mevalonate always is diluted by endogenous mevalonate.

When the residue remaining after extraction of the kidneys with organic solvents was incubated overnight in 2% aqueous SDS, about 90% of the radioactivity was solubilized. When this extract was chromatographed on Sephadex G-50 in buffer containing 1% SDS, the radioactivity eluted in the excluded volume. Radioactive material thus obtained was subjected to SDS polyacrylamide gel electrophoresis in 12.5% gels (7). The majority of the radioactive material migrated as though it had a molecular weight of about 25,000 (Fig. 1). Gel exclusion chromatography, in conjunction with electrophoresis, indicates that the radioactivity from mevalonate was covalently associated with protein. The following experiments provide additional evidence for covalent binding. The SDS solubilized protein was dialyzed 18 h against 10 mM Tris buffer pH 7.0. Aliquots then were treated with protease, deoxyribonuclease 1, or ribonuclease-A following the procedure of Schmidt et al. (5) and then subjected to SDS polyacrylamide gel electrophoresis. The samples that had been treated with the nucleases showed no change in the pattern of radioactivity. However, if the extract had been treated with protease, all of the radioactivity now migrated between the tracking dye and the 14 Kd molecular weight marker, indicating a large reduction in molecular weight.

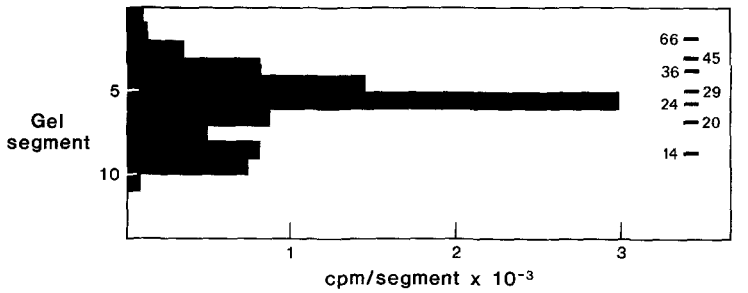

Figure 1. Electrophoresis of the protein-bound radioactivity in SDS polyacrylamide gels. After electrophoresis, the lanes containing the labeled proteins were cut into 1-cm segments for determination of radioactivity. The bands on the right indicate the migration of molecular weight standards (Sigma Dalton Mark VII L).

In another experiment, labeled kidneys were homogenized and the resulting extract subjected to differential centrifugation. The bound radioactive material was distributed throughout the cellular fractions (data not shown). Almost 90% of the radioactivity was recovered in the cell debris, and particulate fractions with the low-speed pellet (undisrupted cells and nuclei) and 25,000 x g pellet (mitochondria and Golgi) contained the majority of radioactivity. This experiment shows that the proteins bearing the label from mevalonate are mostly particle bound.

Preliminary experiments had revealed that the radioactive material attached to protein was rendered toluene soluble by strong acid hydrolysis. The following experiments more closely defined the nature of the bond being cleaved. An SDS extract of solvent-extracted protein was subjected to several different conditions of hydrolysis and then extracted with toluene. The amount of radioactivity extracted into toluene is reported in Table I. The predominant ligand between protein and the lipid is clearly labile to both acid and base, with greater susceptability being shown towards acid. Since the material of concern is most likely the product of the condensation of an

TABLE I

Hydrolysis of radioactive protein

Solution	Conditions of hydrolysis	% radio-activity extracted into toluene
Buffer, pH 7	-	3
Buffer, pH 7	95°, 15 min	10
.5 N HCl	30°, 15 min	3
.5 N HCl	95°, 15 min	37
6 N HCl	95°, 15 min	44
6 N $HClO_4$	95°, 10 min	75
.5 N NaOH	30°, 15 min	8
.5 N NaOH	95°, 15 min	21
5 N NaOH	95°, 15 min	26

allylic pyrophosphate with the side chain of an amino acid or a sugar and since treatment with $NaIO_4$ did not change the electrophoretic mobility of the labeled molecule, the most probable products would be the allylic moiety linked by either a nitrogen, sulfur, or oxygen atom to protein. Saturation of the allylic double bond to give the corresponding alkyl ether is unlikely because the product would be extremely stable. Since allylic ethers are considerably more acid labile than the corresponding nitrogen- or sulfur-containing bond, the most likely bond is an allylic ether to the hydroxyl of serine, threonine, or tyrosine, in the protein.

The toluene-soluble material obtained by acid hydrolysis was chromatographed on a Bio-Bead S-X4 gel exclusion column with toluene as a solvent. The radioactivity migrated as a trailing peak with a maximum at the dolichol marker (Fig. 2). The toluene-soluble material also was chromatographed on silica gel 60 plates with 15% ethylacetate in toluene as solvent. Two zones of radioactivity were observed; about 25% of the radioactivity traveled slightly behind the solvent front, while the remaining

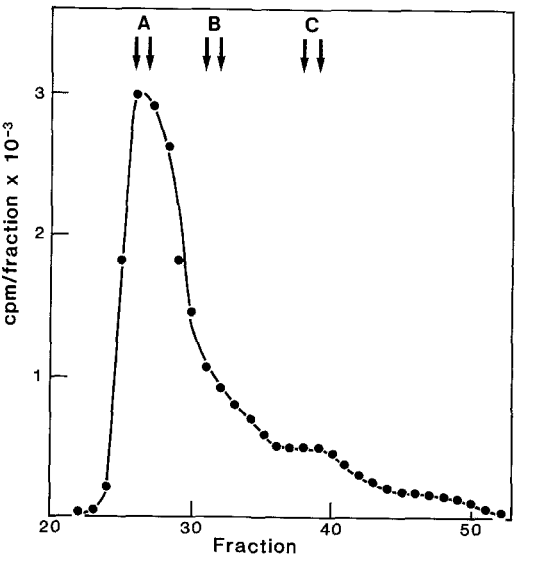

Figure 2. Chromatography on Biobeads S-X4 of the toluene-soluble material. Toluene was the solvent and 1-ml fractions were collected. The elution position of the markers are indicated by arrows. The markers are A, dolichol C_{95}; B, solanesol; and C, ergosterol.

75% had an Rf of .55. Dolichol and solanesol had Rf values of .7 and .47, respectively in this system. Reversed phase thin layer chromatography with 10% water in acetone was also utilized. The alcohols produced by hydrolysis migrated as a spread of radioactivity between the dolichol and solanesol markers. Thus the prenol is most likely an unsaturated dolichol or dolichol like material linked via an allylic ether to a hydroxyl group in the protein.

The results presented in this paper indicate that polyprenols can be covalently linked to protein. This observation corroborates the observation of Schmidt et al. (5) and a) extends it to a specific tissue location, b) indicates that the prenol is high molecular weight; i.e., similar to dolichol and suggests that an allylic ether link is involved in bonding to protein.

Acknowledgments--The authors are grateful to Professor W. W. Epstein, Department of Chemistry, University of Utah, for most helpful discussions.

REFERENCES

1. Schlesinger, M. J. (1981) Ann. Rev. Biochem. 50, 193-206.
2. Magee, A. I., and Schlesinger, M. J. (1982) Biochim. Biophys. Acta 694, 279-289
3. Olson, E. N., Towler, D. A., and Glaser, L. (1985) J. Biol. Chem. 260, 3784-3790
4. Ferguson, M. A. J., Haldar, K., and Cross, G. A. M. (1985) J. Biol. Chem. 260, 4963-4968
5. Schmidt, R. A., Schneider, C. J., and Glomset, J. A. (1984) J. Biol. Chem. 259, 10175-10180
6. Landau, R. G., and Brunengraber, H. (1985) Methods Enz. 110, 100-116.
7. Laemmli, U.K. (1970) Nature 227, 680-685.