# Evidence for protein dolichylation

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**Abstract Labeling of human colon carcinoma cells with [$^3$H]dol, followed by extensive delipidation and removal of dol-P oligosaccharides, showed that dol are bound to cellular proteins with sizes of 5, 10, 27, 75 and >140 kDa. HPLC purification of proteolytic products of [$^3$H]dol- and [$^{35}$S]cys-labeled proteins revealed a hydrophobic peak containing both dol and cysteine. The dol/cys-labeled products were clearly separated from GG-cys, and exhibited a hydrophobicity between that of dol-P and dol. In another set of experiments delipidated proteins were treated with methyl iodide, which cleaves thioether bonds. After HPLC purification of released dol-like lipids, these were subjected to mass spectrometry. This demonstrated molecular ions with the same mass as that of dol. Taken together our data provide evidence for the existence of proteins covalently modified with dol.**



*Key words:* Prenylation; Protein modification; Dolichol

## 1. Introduction

Thirteen years ago Glomset and coworkers reported that compounds derived from MVA are covalently linked to proteins in mouse 3T3 cells [1]. It was later shown that farnesyl (C15; three isoprene units) or GG (C20; four isoprene units) groups were coupled to the carboxy terminal of certain proteins through a thioether bond [2–4].

Theoretically, protein prenylation might include modifications with isoprene moieties other than farnesyl or GG groups, e.g. long-chain compounds such as polyprenols or dol (C85–110; 16–22 isoprene units). Although some studies have indicated that dol may be bound to proteins [5–7], the existence of protein dolichylation still remains to be established. The present paper provides evidence that dol is covalently bound to human cell proteins.

## 2. Materials and methods

### 2.1. Chemicals

GG-cys and [1-$^3$H]dol were from American Radiolabeled Chemicals Inc. *R,S*-[5-$^3$H]MVA was purchased from DuPont New England Nuclear and L-[$^{35}$S]cys from Amersham. All other chemicals, if not otherwise specified, were from Sigma.

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*Abbreviations:* CM, chloroform:methanol; CMW, chloroform:methanol:water; cys, cysteine; dol, dolichol; dol-P, dolichyl phosphate; GG, geranylgeranyl; HPLC, high performance liquid chromatography; MVA, mevalonic acid; PAGE, polyacrylamide gel electrophoresis

### 2.2. Cell culture

The human colon carcinoma cell line WiDr, the human melanoma cell line SK-MEL-2 and the human hepatoblastoma cell line HepG2 were purchased from American Type Cell Collection, Rockville, MD, USA. All cell lines were grown in monolayers in tissue culture flasks maintained in a 95% air/5% $CO_2$ atmosphere at 37°C in a humidified incubator. The cells were cultured in minimum essential medium supplemented with 10% fetal calf serum.

### 2.3. Isolation of prenylated proteins

Cells cultured in 15-cm dishes were shifted to fresh serum-free medium containing 50 μCi [$^3$H]MVA/ml (50.1 Ci/mmol) or 6.0 μCi [$^3$H]dol (dol-16–dol-22)/ml (1–2 Ci/mmol) or 5.0 μCi [$^{35}$S]cys/ml (1297 Ci/mmol). Proteins were isolated in the presence of protease inhibitors (1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin, 0.7 μg/ml pepstatin and 2 mM phenylmethylsulfonyl fluoride). Delipidation was performed by washing the protein precipitate three times with acetone, CM (2:1) and absolute ethanol, as described elsewhere [1,8,9]. In order to remove any residual dol-P oligosaccharides the precipitate was also washed five times with CMW (10:10:3) [10,11].

### 2.4. Sulfonium salt cleavage with methyl iodide

The procedure described by Maltese and Erdman [9] was followed.

### 2.5. Proteolytic cleavage of radiolabeled proteins

Proteolytic digestion was performed using protease type XIV from *Streptomyces griseus* (Pronase E) [12]. It was confirmed by SDS-PAGE that this treatment resulted in total degradation of the cellular proteins. The hydrophobic products were extracted by butanol for HPLC analysis.

### 2.6. HPLC

Methyl iodide-released dol-like lipids were purified by reversed-phase and normal-phase HPLC for mass spectrometry as described elsewhere [13]. The flow rate in both systems was 1 ml/min and 2-ml fractions were collected. Labeled proteolytic butanol-extractable products were purified on a reversed-phase HPLC system consisting of a Hypersil WP butyl ($C_4$) column (150×4.6 mm) (Waters). Solvent A was methanol/water (1/1) containing 10 mM ammonium acetate, and solvent B was 100% isopropanol containing 10 mM ammonium acetate. A linear gradient starting with 95% solvent A and 5% solvent B reaching 50% solvent B after 60 min was used. The flow rate was 0.8 ml/min. In one experiment rechromatography was done on a reversed-phase HPLC system consisting of a Radial Pak Resolve (5 μm) $C_{18}$ cartridge (Waters). Solvent A was methanol/water (95/5) containing 10 mM ammonium acetate, and solvent B was 100% isopropanol containing 10 mM ammonium acetate. After a 5-min isocratic period in 95% solvent A and 5% solvent B, a linear gradient was started. After 45 min a solvent B concentration of 95% was reached. The flow rate was 1.0 ml/min and 2-ml fractions were collected for radioactive assay.

### 2.7. Gel electrophoresis

Labeled proteins were fractionated by SDS-PAGE [14] using 10% or 15% polyacrylamide gels. The lanes were excised and sliced into 2-mm segments which were solubilised in 0.5 ml Soluene-350 (Packard). 8 ml Hionic-Fluor (Packard) was added and the radioactivity determined by scintillation counting.

### 2.8. Mass spectrometry

Mass spectra were recorded on a VG AutoSpec (VG Analytical, Manchester, UK) mass spectrometer. Standard dol (dol-17 and dol-


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19) and biological samples at different sample levels (1 ng–10 μg) were introduced individually into the spectrometer electron impact (EI) source via the direct inlet probe which was heated stepwise from 200°C to 300°C in 10°C intervals at a rate of 10°C/10 scans as described elsewhere [13].

## 3. Results

[$^3$H]Dol-labeled proteins from WiDr cells were harvested for analysis. It has been shown extensively elsewhere that dol are taken up by cells when added to the culture medium [15]. Approximately 10% of the added [$^3$H]dol was found in the cell extract. The [$^3$H]dol-labeled proteins were delipidated extensively, including extraction of dol-P oligosaccharides using CMW. Delipidated proteins from control cells demonstrated a substantial incorporation of [$^3$H]dol (173 000 DPM/mg protein). The same level of [$^3$H]dol incorporation was seen in cells treated with tunicamycin (TM) during the labeling period. Since TM is a specific inhibitor of *N*-linked glycosylation through blocking of the production of *N*-acetylglucosamine-dol-P [16], this result confirms that the delipidation procedures used efficiently removed dol-P oligosaccharides from the proteins. It was also found that treatment with cycloheximide, an inhibitor of de novo protein synthesis, did not decrease the [$^3$H]dol labeling of the proteins. This result suggests that the binding of dol to the proteins comprises a post-translational event. The delipidated [$^3$H]dol-labeled proteins were fractionated by SDS-PAGE (15% or 10% polyacrylamide). This gave rise to four peaks with regard to the molecular size: 75 kDa, 27 kDa, 10 kDa and 5 kDa, and also an uncharacterised peak above 140 kDa (Fig. 1).

WiDr cells were next labeled with either [$^3$H]MVA, [$^3$H]dol or [$^{35}$S]cys. After delipidation and removal of dol-P oligosaccharides the labeled proteins were subjected to extensive proteolysis. Fifty to 85% of the [$^3$H]dol labeling of the protein digest was extractable with butanol. The corresponding value for [$^{35}$S]cys was only 2%. It was confirmed by SDS-PAGE that the aforementioned [$^3$H]dol-labeled peaks (5, 10, 27 and 75 kDa) were deleted by the proteolytic treatment (data not shown).

The butanol-extractable [$^{35}$S]cys-, [$^3$H]MVA- and [$^3$H]dol-labeled proteolytic products were each subjected to reversed-phase HPLC using a $C_4$ column. The [$^3$H]MVA-labeled material separated into three radioactive peaks (Fig. 2A). The most hydrophobic of these (appearing after 45–60 min) co-eluted with the most hydrophobic [$^{35}$S]cys-labeled peak (Fig. 2B). These compounds required 47% isopropanol to be eluted. The two less hydrophobic [$^3$H]MVA-labeled peaks (the first and second peaks) coeluted with a broad [$^{35}$S]cys-labeled peak. A GG-cys standard coeluted (at 29% isopropanol) with the second [$^3$H]MVA-labeled peak and the late aspect of the first [$^{35}$S]cys-labeled peak. It seems likely that the second [$^3$H]MVA-labeled peak represented GG-cys, and presumably also farnesyl-cysteine, which would behave similarly to GG-cys on this HPLC system. The chromatographic behaviour of the [$^3$H]dol-labeled butanol-soluble protein digest is shown in Fig. 2C. As demonstrated, a hydrophobic peak co-eluting with the late [$^3$H]MVA- and [$^{35}$S]cys-labeled peaks (cf. Fig. 2A,B) appeared. The shapes of the [$^3$H]dol-labeled and the [$^{35}$S]cys-labeled peaks differed somewhat (Fig. 2C). The explanation for this might be differences between the distribution of dol homologues bound to cysteine residues and the distribution of [$^3$H]-labeled dol homologues. A dol-P standard was found to elute somewhat earlier (at 36% isopropanol) than the [$^3$H]dol/[$^{35}$S]cys-labeled peak, whereas the dol standard eluted later (at 65% isopropanol) (Fig. 2C). An aliquot from fractions corresponding to the hydrophobic peak of [$^3$H]MVA-labeled and [$^{35}$S]cys-labeled proteolytic products (see Fig. 2A,B) was rechromatographed on a reversed-phase $C_{18}$ column. As shown in Fig. 3, the [$^3$H]MVA- and [$^{35}$S]cys-labeled compounds behaved similarly, and eluted later (at approximately 40% isopropanol) than dol-P (at 5% isopropanol) but earlier than the neutral dol (at 95% isopropanol) on this HPLC system.

In another set of experiments large amounts of [$^3$H]MVA-labeled proteins isolated from WiDr, SK-MEL-2 and HepG2 were, after delipidation and removal of dol-P oligosaccharides, treated with methyl iodide. The released [$^3$H]MVA-labeled material was first purified by reversed-phase HPLC. Labeled material coeluting with dol standards were rechromatographed on a normal-phase HPLC system, as described elsewhere [13]. Fractions corresponding to dol were taken for mass spectrometry. Control material was obtained from proteins that had undergone the same hydrolytic treatment but with the omission of methyl iodide.

The molecular ion regions of the EI mass spectra of reference dol-19 and dol-17 were first analysed. Dol-19 vapourises at approximately 240°C and has a monoisotopic mass $[M]_+$ of 1313.2 Da (data not shown). Dol-17 vapourises from the probe at around 230°C and has a monoisotopic mass $[M]_+$

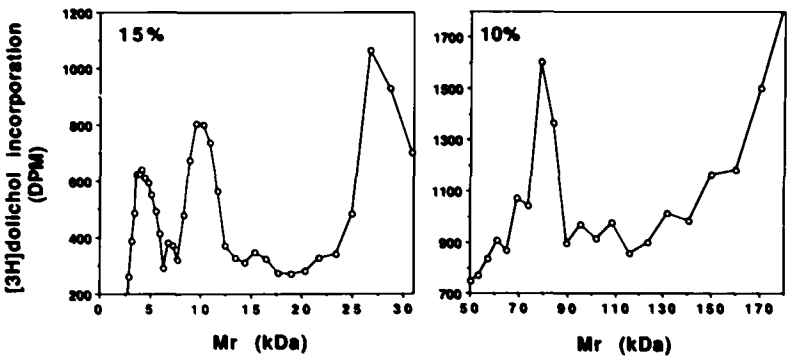


Fig. 1. Electrophoretic pattern of [$^3$H]dol-labeled proteins. WiDr cells were incubated in medium containing [$^3$H]dol (6 μCi/ml) for 48 h. After delipidation procedures two parallel protein samples (200 μg each) were fractionated by SDS-PAGE (10% or 15% polyacrylamide). The protein-containing lanes were sliced and assayed for radioactivity. The molecular weights are based on standard proteins. The experiment was repeated with similar results.

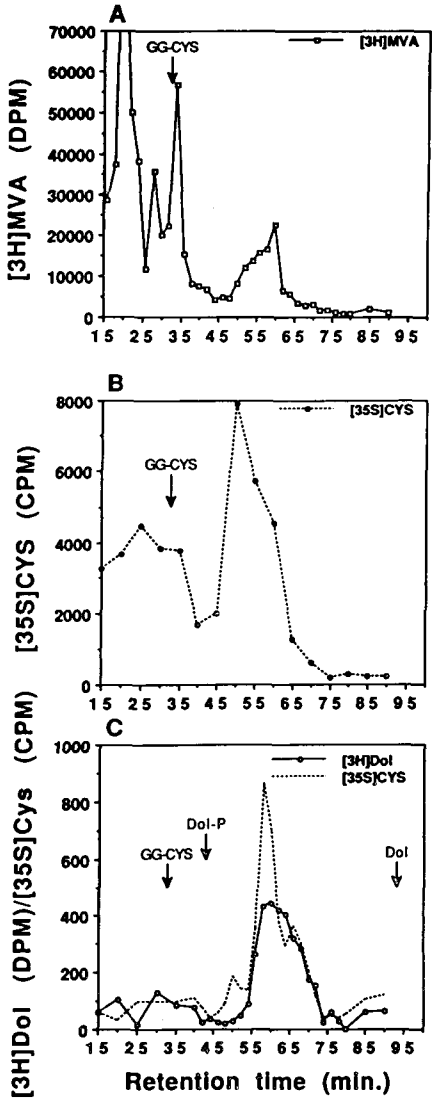


Fig. 2. $C_4$ reversed-phase HPLC of [$^3$H]dol-, [$^3$H]MVA- and [$^{35}$S]cys-labeled protein digest. WiDr cells were incubated in medium containing [$^3$H]MVA (50 μCi/ml) (A), [$^{35}$S]cys (5 μCi/ml) (B) or [$^3$H]dol (6 μCi/ml) (C) for 48 h. After delipidation, removal of dol-P oligosaccharides and extensive proteolysis, the butanol-extractable labeled compounds were run on a $C_4$ reversed-phase HPLC system. In C the appearance of rechromatographed [$^{35}$S]cys-labeled products is compared with [$^3$H]dol-labeled proteolytic products. The elution of external standards for GG-cys, dol-P and dol is indicated. The experiment was repeated several times with similar results.

of 1177.1 Da (data not shown). The isotope patterns were almost identical to those calculated from known isotope abundances for $C_{95}H_{156}O$ (dol-19) and $C_{85}H_{140}O$ (dol-17). Fig. 4A,B shows the molecular ion regions of the dol-like lipids removed by methyl iodide of delipidated proteins from WiDr and SK-MEL-2, respectively (see above). The material from WiDr (Fig. 4A), which vapourised in the range 200–240°C, gave a peak at *m/z* 1177. Closer inspection of these mass regions showed a molecular mass and an isotope pattern similar to that calculated for dol-17 ($C_{85}H_{140}O$). The dol-like material from SK-MEL-2 vapourised in the range 230–270°C and gave a molecular ion envelope corresponding to $C_{86}H_{142}O$ with a monoisotopic mass peak at *m/z* 1191 (Fig. 4B). It is possible that this is a methylation product of dolichol-17, resulting from the methyl iodide treatment of the

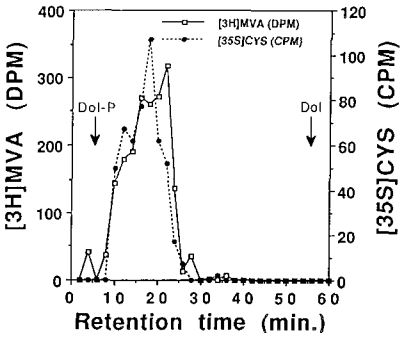


Fig. 3. $C_{18}$ reversed-phase HPLC of [$^3$H]MVA/[$^{35}$S]cys-labeled proteolytic products. Fractions corresponding to the late [$^3$H]MVA and [$^{35}$S]cys peaks shown in Fig. 4A,B were rechromatographed on a HPLC system with a $C_{18}$ reversed-phase column. The elution of external dol-P and dol standards is indicated. The experiment was repeated with similar results.

original sample. A smaller dol-17 envelope was also present (Fig. 4B). In the HepG2 cell material, which vapourised in the range 200–240°C, there was also a peak at *m/z* 1177 (data not shown). In all three cases fractions adjacent to those containing the dol-like lipids did not give rise to any peaks in the investigated mass ranges. Furthermore, analysis of a sample treated by alkaline hydrolysis without methyl iodide did not give rise to any peaks (data not shown).

## 4. Discussion

An intriguing question is whether protein prenylation also includes modification with isoprenes other than farnesyl or

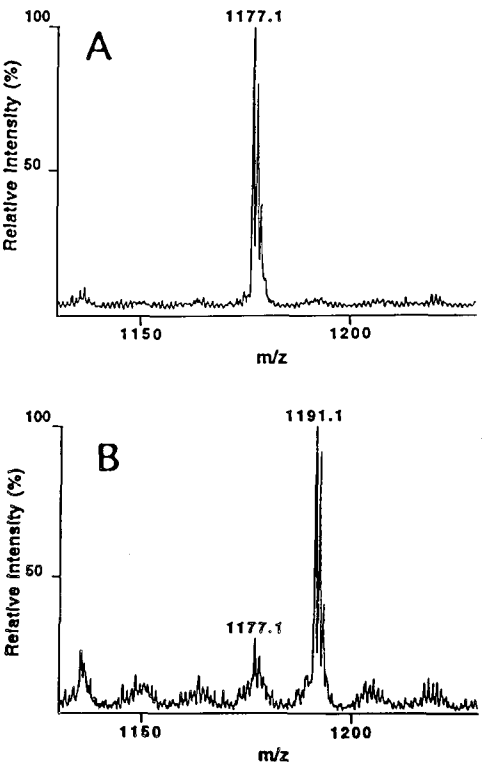


Fig. 4. Mass spectrometry. [$^3$H]MVA-labeled lipids removed from WiDr proteins by methyl iodide were purified by HPLC. Fractions corresponding to dol were subjected to EI mass spectrometry. A: Material from WiDr. B: Material from SK-MEL-2.

GG groups. Evidence for protein modification of a cysteine residue in *Halobacteria halobium* with a diphytanylglyceryl group through a thioether linkage has recently been demonstrated by Sagami et al. [9]. Furthermore, Shipton et al. [17] showed that methyl iodide hydrolysis of [$^3$H]MVA-labeled proteins in spinach releases phytol and some other unidentified isoprenoid compounds.

In this study we provide evidence for thioether linkage of dol groups to cysteine residues in human cell proteins. Our evidence is based on the following three main findings. Firstly, [$^3$H]dol was incorporated into cellular proteins, and was not released by procedures for extensive delipidation and removal of dol-P oligosaccharides. Secondly, extensive proteolysis of proteins labeled with [$^3$H]dol, [$^3$H]MVA and [$^{35}$S]cys resulted in hydrophobic products containing both [$^3$H]dolichol/[$^3$H]MVA and [$^{35}$S]cysteine. The hydrophobicity of these products was between that of dol-P and dol. Thirdly, mass spectrometry of lipids released by methyl iodide disclosed a compound corresponding to dol.

In the mass spectrometry of the dol-like lipids released by methyl iodide, only one homologue (dol-17) could be detected. This does not, however, exclude the possibility that other dol homologues are linked to proteins. The reason why only dol-17 was detected could be that this homologue may constitute the predominant substrate in the dolichylation reaction, and therefore be the only homologue exceeding the mass spectrometric detection limit. Support for this idea is provided by our finding that the peak of [$^{35}$S]cys-labeled proteolytic products was somewhat skewed towards more polar compounds indicating that shorter dol homologues are preferentially linked to cysteine residues. Another possibility is that dol-17 may be more stable than the longer homologues upon treatment with methyl iodide. It has been reported that sulfonium salt cleavage gives rise to hydrophobic rearrangement products derived from farnesol or GG [8]. Similar rearrangements most likely also occur in dol, and perhaps longer homologues are more sensitive to such reactions.

An important question which can be raised is whether dol, dol-P, or dol-PP constitutes the lipid substrate in this reaction. Generally, establishment of a thioether linkage necessitates the presence of a pyrophosphate group, because of the requirement for energy in this type of reaction. It therefore seems probable that phosphorylated derivatives, rather than dol in itself, comprise the substrates in the dolichylation reaction. Because we labeled the cells with [$^3$H]dol, it is likely that phosphorylation takes place before linkage to the proteins. That exogenous dol can undergo phosphorylation has been reported in other studies [15,18].

Similarly to the covalent linkages of GG and farnesyl groups to proteins, the dolichylation reaction seems to occur post-translationally because the reaction was not blocked by inhibition of de novo protein synthesis.

In this study evidence for protein dolichylation is demonstrated in three human tumour cell lines. Unpublished results in our laboratory have, however, shown no evidence for protein dolichylation in normal fibroblasts. This preliminary result, taken together with our present data, is interesting since it raises the question of whether protein dolichylation is a reaction involved in tumour transformation or progression.

Studies to disclose the nature and intracellular distribution of dol-modified proteins and the chemical properties of the dolichylation reaction are in progress in our laboratory.

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