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Dolichol-Like Lipids With Stimulatory Effect on DNA Synthesis: Substrates for Protein Dolichylation?

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Abstract Substantial evidence has suggested that a nonsterol product of mevalonic acid (MVA) is essential for the initiation of DNA synthesis in mammalian cells. Several possible isoprenoid candidates have been suggested, but the identity of this compound still remains unknown. In this study we have isolated and purified MVA products from SV40-transformed human fibroblasts and identified fractions with a growth-stimulatory effect. The cells were labelled with [^{14}C]MVA in the presence of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. After lipid extraction, the [^{14}C]MVA-labelled lipids were subjected to high performance liquid chromatography and size-exclusion chromatography, and the effect of the fractionated eluate on the DNA synthesis of arrested MVA-depleted target cells was tested. Thereby we found a fraction of [^{14}C]MVA-labelled lipids with a substantial stimulatory effect on DNA synthesis. The chromatographic behavior suggested that the growth-stimulating fractions contained dolichol-20. This was confirmed by mass spectrometric analysis. Similar results were obtained when lipids from hepatocellular carcinoma cells and a sample from breast tumor were isolated and analyzed by the same procedure. The mechanisms by which these compounds induce DNA synthesis are unknown. Recent data obtained in our laboratory have provided evidence that dolichyl groups are covalently linked to tumor cell proteins, which implicates a new biological function for long-chain polyisoprenoid alcohols (Hjertman et al. [1997] FEBS Lett 416:235–238). In this study we demonstrate that tumor cells containing dolichol-like growth-stimulatory lipids also contained dolichylated proteins. This raises the question whether the growth-stimulatory dolichol-like lipids serve as substrates for the dolichylation reaction. J. Cell. Biochem. 71:502–514, 1998.

Key words: HMG-CoA; MVA; HPLC; dolichol-like lipids; DNA synthesis

The synthesis of MVA is the major rate-limiting step in the biosynthesis of sterols and nonsterol isoprenoid molecules, such as dolichol, ubiquinone, and isopentenyladenine. MVA or products of the MVA metabolism are essential for cell proliferation [Brown and Goldstein, 1980]. Several studies have suggested that a nonsterol isoprenoid is required to restore cell growth in MVA-depleted cells and that initiation of DNA synthesis is the process by which MVA regulates cell proliferation [Brown and Goldstein, 1980, 1990; Habenicht et al., 1980; Sinensky and Logel, 1985]. Moreover, studies from several laboratories have demonstrated that increased production of MVA and deranged feedback control of HMG-CoA reductase is a characteristic feature of malignant cells [Siperstein, 1984].

During the last 10 years much attention has been focused on prenylation of cellular proteins [Schmidt et al., 1984; Glomset et al., 1990]. In particular, farnesylation of the proto-oncogene product p21^{ras} has attracted great interest [Schafer et al., 1989; Casey et al., 1989]. In some cell systems, growth arrest induced by HMG-CoA reductase inhibition is correlated with an inactivation of the ras protein due to absence of farnesylation [Jakobisiak et al., 1991],

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; MVA, mevalonic acid.

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whereas in other cell systems cell growth appears to be independent of Ras farnesylation [DeClue et al., 1991]. Thus, it is still unclear which isoprenoid molecules are involved in the regulation of cell growth. Recently, we provided evidence for modification of cellular proteins with dolichyl groups [Hjertman et al., 1997]. This may implicate a new biological function of isoprenoid lipids and raises the possibility of a mechanism involved in MVA-regulated cell growth.

Previously, we have demonstrated that HPLC fractions containing [3H]-labelled lipids, which chromatographically correspond to long-chain dolichols, can stimulate DNA synthesis [Wejde et al., 1993]. These lipids were isolated from human breast cancer cells [Wejde et al., 1993]. In the present study, we have extended this work and have purified and partially characterized growth-stimulatory dolichol-like lipids from various types of malignant cells (including virus-transformed fibroblasts and hepatocellular carcinoma cells). We also tried to investigate the possible link between these dolichol-like lipids and the dolichylation reaction.

MATERIALS AND METHODS

Chemicals

Mevinolin, obtained from Merck, Sharp, and Dohme (West Point, PA), was converted to its sodium salt before experimental use. RS[2–14C]mevalonolactone (MVA), [2–14C]HMG-CoA, L-[35S]cysteine, and *methyl*-[3H]thymidine were from Amersham (Buckinghamshire, UK), RS-[2–3H]MVA was from New England Nuclear, DuPont (Sweden). Farnesyl-cysteine, geranylgeranyl-cysteine, and [1–3H]dolichol (dolichol-16-dolichol-22) (1–2 Ci/mmol) were from American Radiolabelled Chemicals Inc., through Larodan (Malmö, Sweden). HPLC-grade solvents were purchased from Merck (through Kebo Inc., Stockholm, Sweden). All other chemicals, if not otherwise specified, were from Sigma Co. (through Kemila, Stockholm, Sweden).

Cells and Media

Simian virus-40 transformed human fibroblasts (line 90VAVI) were kindly given to us by Dr. Gretchen Stein, University of Colorado (Boulder, CO). The human hepatocellular cancer cell line HepG2 was from American Type Culture Collection (Rockville, MD). Both cell lines were cultured in Minimal essential medium with Eagle's salts supplemented with non-essential amino acids and 10% (v/v) fetal calf serum. The 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. For experimental purposes, the cells were cultured in 35, 50, or 140 mm dishes. The cells were seeded at a density of 3,000–5,000 cells/cm^2. The experiments started when the cells had reached subconfluence.

Determination of DNA Synthesis

The stimulatory effects of added compounds on DNA synthesis were evaluated by measurement of the incorporation of [3H]thymidine. Arrested target cells were cultured in duplicate 35 mm dishes containing 2 ml medium/dish and [3H]thymidine (5 Ci/mmol) was added for 1 h (1 µCi/ml) before termination of the experiments. The cells were then rinsed with phosphate-buffered saline (PBS), fixed in 10% (w/v) ice-cold trichloroacetic acid, rinsed with PBS, and finally dissolved in 1 ml of 0.1 M NaOH (20°C). Parallel samples from the duplicate dishes were taken for scintillation counting and for spectrophotometric determination of protein content. The level of DNA synthesis was expressed as disintegrations per minute (DPM)/mg cell protein.

Lipid Isolation

Exponentially growing subconfluent 90VAVI cells or HepG2 cells, cultured in 15 cm dishes, were shifted to serum-free medium containing 1.25 µM 25-hydroxycholesterol, 1 µM mevinolin, 0.33 µCi [^{14}C]MVA (50.1 mCi/mmol)/ml, or 2 µCi [3H]MVA (1.26 Ci/mmol)/ml and 0.77 mM unlabelled MVA. The incubations were stopped after 24 h. The medium was then removed and the monolayer cultures carefully rinsed twice with PBS (20°C), after which the cells were scraped by a plastic cell scraper and collected in 2 ml of PBS. Dolichol-23 (10 µg per sample) (Larodan Fine Chemicals Inc.) was added to the cell material as an internal standard. After vigorous vortexing, a small sample was taken from the homogenous cell suspension for determination of protein content. The lipids were extracted from the cell material using hexane/isopropanol, 3:2 (v/v). The organic solvents were evaporated under a stream of nitrogen, whereupon the lipid material was redissolved in methanol/isopropanol, 1:3 (v/v). The samples were filtered through a 0.45 µm Millex filter

(Waters, Rochester, MN) prior to chromatography.

Material from a human breast tumor (ductal carcinoma) was also analyzed. Tumor tissue samples, 7.5 g each, were homogenized in 20 ml of 80% aqueous isopropanol, whereupon lipid extraction was performed as described above.

Isolation and Delipidation of Prenylated Proteins

Cells cultured in 15 cm dishes were shifted to fresh serum-free medium containing [3H]MVA and/or [35S]cysteine (1,297 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 with acetone, chloroform/methanol, 2:1 (v/v), and absolute ethanol as described [Schmidt et al., 1984; Maltese and Erdman, 1989; Sagami et al., 1995]. In order to remove any residual dolichyl pyrophosphoryl oligosaccharides, we also washed the precipitate five times with chloroform/methanol/water, 10:10:3 (by vol) [Bhat and Waechter, 1988].

Sulfonium Salt Cleavage With Methyl Iodide

The procedure described by Maltese and Erdman [1989] was followed.

Proteolytic Cleavage of Radiolabelled Proteins

Proteolytic digestion was performed using protease type XIV from *Streptomyces griseus (Pronase E)* [Crick et al., 1994]. It was confirmed by SDS-PAGE that this treatment resulted in total degradation of the cellular proteins. The hydrophobic products were extracted with butanol for HPLC analysis.

High Performance Liquid Chromatography (HPLC)

The extracted lipid material was purified by three different HPLC systems—reversed-phase system I, normal-phase HPLC and reversed-phase system II—as described elsewhere [Griffiths et al., 1996]. In brief, the reversed-phase system I, consisted of a Resolve C18 Radial Pak column (100 × 8 mm, 5 µm) (Waters) and an isocratic mobile phase composed of methanol/isopropanol, 1:3 (v/v). The flow rate was 1 ml/min, and 2 ml fractions were collected. Samples of 100 µl and 400 µl were taken for measurements of radioactivity and growth-stimulatory effects, respectively. The remainder of the fractionated material was utilized for further purification by normal-phase HPLC, which was performed on a Resolve CN Radial Pak column (100 × 8 mm, 10 µm) (Waters) using 0.25% (v/v) isopropanol in hexane as mobile phase. The flow rate was 1 ml/min, and 2 ml fractions were collected. Samples (500 µl) were taken for assay of radioactivity and for growth-stimulatory experiments. The final purification was performed by the reversed-phase HPLC system II, which comprised an analytical Resolve steel C18 column (150 × 3.9 mm, 5 µm) (Waters). Methanol/isopropanol, 1:1 (v/v), was used as mobile phase, and the flow rate was 1 ml/min. Fractions of 0.5 ml were collected, and aliquots of the fractionated eluate were used for assay of radioactivity, growth stimulatory experiments, and mass spectrometry.

Size-Exclusion Chromatography

Sephadex LH-60 (Pharmacia LKB, Uppsala, Sweden) was sieved, and the 200–325 mesh fraction was used for preparation of columns in 1.5 mm i.d. Teflon tubing [Sjövall et al., 1968]. The gel was slurried in methanol/isopropanol/heptane, 1:1:2 (by vol), and allowed to settle from a glass reservoir attached via a steel capillary to the Teflon tubing. The column end consisted of a piece of stainless steel tubing covered with Teflon gauze. A slight pressure of nitrogen was applied to the glass reservoir to give a suitable flow rate. When the height of the gel bed was 1–1.5 m, the slurry was removed from the reservoir. The Teflon tubing was cut at the top, and an injection port was attached to the reservoir filled with solvent. The nitrogen pressure (about 50 kPa) was adjusted to give a constant flow of 350–450 µl/h. The column end was attached to a variable UV detector with an 8 µl cell (Uvidec-100–2; Jasco, Tokyo, Japan). The column was calibrated with commercial dolichols of different chain lengths and coenzyme Q_{10}. Dolichol-20 and dolichol-11 had uncorrected retention volumes corresponding to about 40% and 50%, respectively, of the total column volume. Samples were injected in 10 µl of the solvent.

Mass Spectrometry

Preliminary studies were carried out by recording electron impact (EI) mass spectra on either a VG 7070 (VG Analytical, Manchester, UK) or a JEOL JMS AX5050W (JEOL Ltd,

Tokyo, Japan) mass spectrometer. Sample introduction was either via a heated direct inlet probe or a gas chromatography (GC) column which terminated in the ion source. Samples for GC/MS were analyzed as trimethylsilyl ethers, while samples introduced on the probe were either underivatized or tert-butyldimetylsilyl (TBDMS) ethers [Griffiths et al., 1996].

Detailed mass spectrometric studies were carried out on a VG AutoSpec Q mass spectrometer (VG Analytical, now Micromass, Manchester, UK). Samples were ionized by either electron impact (EI) or fast atom bombardment (FAB) [Griffiths et al., 1996]. Positive ion EI mass spectra were recorded either from underivatized samples or from TBDMS ethers. Samples were introduced into the EI source on the direct inlet probe which was heated 200°C to 300°C in 10° intervals at a rate of 10°/10 scans for the underivatized samples or from 200°C to 550°C in 50° intervals for the TBDMS ethers. Source conditions were 200 µA trap current, 70 eV electron impact energy, and a temperature of 200°C. The accelerating potential was 8 kV, and the mass ranges scanned were 100–2,000 Da and 1,000–2,000 Da. Scan rate was 2 sec per decade.

Chromatographic fractions containing the unknown growth-stimulating compound(s) were sulphated and analyzed by fast atom bombardment mass spectrometry (FABMS) as described previously [Griffiths et al., 1996]. In brief, dolichol and dolichol-like lipids were dissolved in dichloromethane containing 0.05 M N,N-dicyclohexylcarbodiimide The solution was held on ice, and 50 ml of ice-cold 0.015 M sulphuric acid in dry, restilled dimethylformamide was added. The mixture was diluted with 60% aqueous ethanol and then applied to a Lipidex-DEAP column (Packard, Meriden, CT). Dolichol sulphates were eluted with 0.3 M ammonium acetate, whereupon they were passed through Sep-Pak t-C18 column (Waters). The dolichols were eluted with absolute ethanol. The eluate was dried and then subjected to mass spectrometry.

Addition of Lipids to Cells

After evaporation of the solvents, the lipid material from the indicated HPLC fractions was resuspended in 50 µl ethanol (99.5% by vol), and aliquots were added to the target cells (0.5–2.0 µl per 2 ml dish). Equivalent volumes of ethanol were added to the controls.

Determination of HMG-CoA Reductase Activity

Cells cultured in 50 mm dishes were, after the indicated experimental conditions, rinsed twice in prewarmed (37°C) PBS (without divalent cations) and harvested for determination of cellular activity of HMG-CoA reductase, essentially as described elsewhere [Cavenee et al., 1981].

Determination of Protein Concentration

Protein concentration of cell lysates was determined by a dye-binding assay [Bradford et al., 1976] with a reagent purchased from Bio-Rad (Stockholm, Sweden). Bovine serum albumin (fatty acid–free) was used as a standard.

RESULTS

An MVA-Derived Compound Is Limiting for Cell Growth

The SV40-transformed human fibroblast cell line 90VAVI has the capacity to proliferate at an undiminished rate in serum- or growth factor–free medium for at least 72 h [Larsson et al., 1989]. However, upon inhibition of MVA synthesis by exposing the cells to inhibitors of HMG-CoA reductase, the proliferation was drastically decreased, and the cells were growth-arrested after 20–30 h (data not shown). DNA synthesis was rapidly restored and had reached 75–80% of its maximum level 8 h after addition of MVA (final concentration 0.77 mM) to the arrested cells (data not shown). The combined treatment with low doses of 25-hydroxycholesterol (2.5 µM) and mevinolin (1 µM) was found to be the best inhibitory regime since it resulted in an efficient decrease of HMG-CoA reductase activity, while the cytotoxic effects were small. The combination of 25-hydroxycholesterol and mevinolin has also been used by others [Kabakoff et al., 1990]. Its efficiency is explained by the different mechanisms of action of the two compounds. The expression of the HMG-CoA reductase gene and the biosynthesis of the enzyme are repressed by 25-hydroxycholesterol [Luskey et al., 1983], while the enzyme is directly inhibited by mevinolin [Alberts et al., 1980].

Isolation and Purification of Growth-Stimulatory Lipids Derived From MVA

Our approach was to extract isoprenoid lipids without focus on any specific type of compounds. Harsh conditions like heating and alka-

line hydrolysis were avoided in order to minimize possible inactivation. The lipids were extracted from large-scale cultures of 90VAVI cells (approximately 2×10^7 cells/experiment) using hexane/isopropanol. In order to detect the endogenous isoprenoid compounds, the 90VAVI cultures had been labelled with [^{14}C]MVA. The endogenous synthesis of MVA was blocked by inhibition of HMG-CoA reductase during the labeling period (24 h). Thus, the source of MVA for isoprenoid synthesis would solely be exogenous (final concentration of exogenous MVA was 0.77 mM), and the produced potentially growth-stimulatory isoprenoids would be labelled with [^{14}C]MVA. The labelled material was first separated on reversed-phase HPLC system I, as described elsewhere [Wejde et al., 1993]. The retention times of several MVA-derived products in this system are shown in Table I. The elution of radioactivity, reflecting [14C]MVA incorporated into isoprenoids, was related to the effects of material in the fractions on the DNA synthesis in arrested target cells. The elution of [^{14}C]MVA-labelled material in HPLC fractions 11–13 (retention time 20–26 min), corresponding to long-chain dolichols (especially dolichol-20), coincided with elution of material inducing a substantial increase in DNA synthesis (data not shown). In contrast, lipids in other fractions did not elicit any significant stimulatory effect.

The possibility that the lipids in fraction 11–13 exerted their effect by eliminating the inhibitory effect of mevinolin and 25-hydroxycholesterol on HMG-CoA reductase activity in the target cells was excluded by the finding that the lipids had no effect on HMG-CoA reductase activity in arrested 90VAVI (data not shown).

TABLE I. Retention Times of Some Isoprenoid Compounds in Two of the Preparative HPLC Systems Used[a]

Compound	Retention time (min)	
	RP HPLC	NP HPLC
MVA	4.0	ND
Farnesol	4.0	16.4
Geranylgeraniol	ND	14.8
Isopententenyladenine	5.0	ND
Cholesterol	5.3	22.0
Lanosterol	5.4	10.3
Ubiquinone (coenzyme Q_{10})	8.3	7.3
Dolichyl acetate	ND	4.2
Dolichol-11	6.4	12.2
Dolichol-17	14.2	11.2
Dolichol-19	18.7	11.0
Dolichol-20	21.6	10.9
Dolichol-23	33.0	ND

[a]For details, see Materials and Methods. ND, not determined; NP, normal-phase; RP, reversed-phase.

The magnitude of the stimulatory effect of the lipids varied between different preparations. In some preparations, the stimulation exceeded the negative control only by a factor of 2, whereas material from the most active preparations gave a twentyfold stimulation. Only material inducing more than a fourfold growth-stimulatory effect after the first HPLC step was subjected to further purification. Approximately one-third of all isolated materials (40/118) fulfilled this criterion.

For further purification, the material from fractions 11–13 in the first reversed-phase HPLC was subjected to normal-phase HPLC. Table 1 shows the retention times of different reference compounds in this system. Due to the principally different separation mechanisms, the combination of these two HPLC systems gives a considerable purification of the growth-stimulatory compounds. As shown in Figure 1, the major portion of the [^{14}C]-labelled lipids eluted rapidly in the normal-phase system (fractions 2 and 3). This may represent sterol esters which are eluted late like dolichols in a reversed-phase system but early in a normal-phase system. The remainder (5%) of radioactivity appeared mainly in fraction 6 (Fig. 1B). As is shown in Figure 1A, there was a small but distinct peak of UV absorption in fraction 6. The fractionated eluate was then added to the target cells. In Figure 1C, it is clearly demonstrated that only material from fraction 6 (retention time 10–12 min), corresponding to long-chain dolichols, induced stimulation of DNA synthesis in the target cells.

Characterization of the Growth-Stimulatory Effect

In Figure 2A, the reproducibility of the growth-stimulatory effects of the lipids in normal-phase HPLC fraction 6 is confirmed. The stimulatory effects obtained with material from ten different preparations are shown.

As shown in Figure 2B, the active lipids were not produced in cells which had been depleted of MVA prior to the isolation of lipids. In this experiment, large-scale cultures of 90VAVI were first exposed to MVA depletion over a period of 30 h. Thereafter, the cells remained either de-

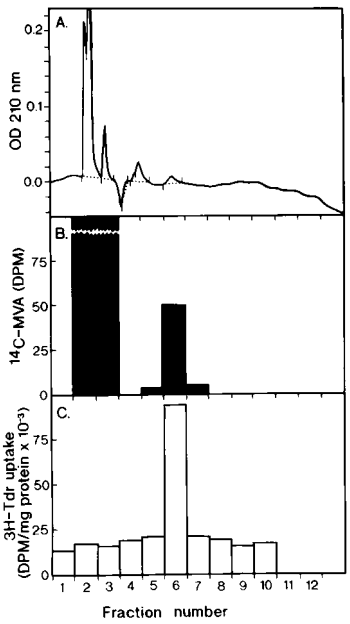

Fig. 1. HPLC purification of growth-stimulating lipids. UV absorption (**A**), [^{14}C]MVA incorporation (**B**), and effects on DNA synthesis (**C**) after normal-phase HPLC are shown. The material in reversed-phase HPLC fractions 11–13 was pooled and subjected to normal-phase HPLC. Aliquots of fractions from the latter were taken for assay of radioactivity and growth stimulation. For the latter, lipid material corresponding to 180,000 cells was added to the target cells (90VAVI blocked by 25-hydroxycholesterol and mevinolin for 30 h), and DNA synthesis was assayed after 24 h.

pleted for an additional 24 h period or were replenished with MVA (0.77 mM) for 24 h. Lipids were then isolated from the cells and subjected to reversed-phase and normal-phase HPLC as described above. As shown, the material obtained from the depleted cells exhibited no peak of UV absorption corresponding to dolichols, and this fraction did not induce any stimulatory effects on DNA synthesis of the target cells. In contrast, the purified lipid material from MVA-stimulated cells exhibited a distinct peak of UV absorption corresponding to dolichols and induced DNA synthesis in the target cells. Taken together with the incorporation of [^{14}C]MVA in this material (Fig. 1B), these data provide evidence that the growth-stimulatory compound(s) is derived from MVA.

Purification of Growth-Stimulatory Lipids by Size-Exclusion Chromatography

In order to estimate the approximate molecular mass of the growth-stimulatory compound(s), we subjected material in the appropriate HPLC fraction from experiments with [14C]MVA to size-exclusion chromatography on a column of Sephadex LH-60. Different long-chain polyprenols and coenzyme Q_{10} were used as references. As can be seen in Figure 3A, there was a substantial peak of [^{14}C] eluting in the range of reference compounds with a molecular weight of 1,200–1,400 Da (Fig. 3B). The material in this fraction was confirmed to be biologically active (data not shown).

Functional-Group Analysis

In order to investigate whether the growth-stimulatory lipids contained epoxy, carbonyl, or hydroxyl groups, we treated the samples with sodium borohydride in ethanol or with TBDMS chloride [Griffiths et al., 1996]. The retention times of the radiolabelled growth-stimulatory lipids were determined on the normal-phase HPLC system before and after each reaction. The retention time was not changed by exposure to sodium borohydride (data not shown), indicating absence of carbonyl or epoxy groups which would have been converted to more polar products. In contrast, reaction with TBDMS chloride resulted in the formation of a less polar derivative with drastically shorter retention time (data not shown). This is compatible with the presence of a hydroxyl group. The behavior of the parent compound in the normal-phase HPLC system excludes the presence of a carboxyl group. Furthermore, the mobility in this system argues against a dihydroxy structure (data not shown). The introduction of only one TBDMS group was also indicated by the small change of the molecular weight as estimated by size-exclusion chromatography of the derivative (data not shown). Thus, the results strongly suggest that the growth-stimulatory lipids contain one hydroxy group and a large nonpolar hydrocarbon skeleton.

Mass Spectrometry of the Growth-Stimulatory Compounds

Fast atom bombardment mass spectrometry (FABMS). The material in the chromatographic fraction containing the growth-stimulatory compound(s) obtained after preparative reversed-phase HPLC I and II and normal-phase HPLC was sulphated and analyzed by FABMS. The resultant spectrum was analyzed in the mass range 1,200–2,000 Da on the basis of the mobility in the size-exclusion chromatography. The most intense peak, as shown in Figure 4, was at 1,461 Da (relative abundance =

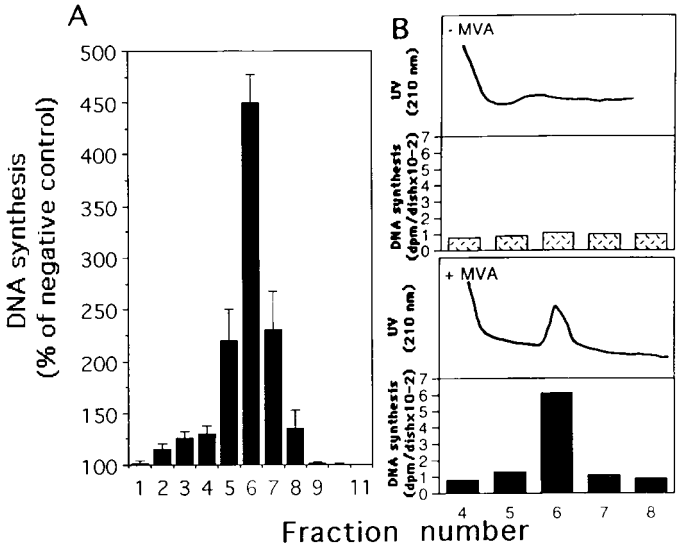

Fig. 2. Reproducibility and generality of the growth-stimulatory effects. Requirement for MVA. **A:** The stimulatory effects of fractionated lipids, purified by normal-phase HPLC, on DNA synthesis in 90VAVI blocked by HMG-CoA reductase inhibitors. The mean values, indicated as percentages of the negative control, from ten independent experiments are shown. SEM values are also indicated. **B:** The biosynthesis of growth-stimulatory lipids is dependent on the access of MVA. Cultures of 90VAVI blocked by a 30 h treatment with mevinolin and 25-hydroxycholesterol in the absence of serum either remained blocked for an additional 24 h (- MVA) or were supplemented with 0.77 mM MVA for 24 h (+ MVA). The lipid material was then purified by reversed-phase and normal-phase HPLC, as described in Fig. 1. The UV absorbance and the stimulatory effect on DNA synthesis for each condition (absence or presence of MVA during the incubation period) are shown.

100%). This corresponds to the most abundant ion in the isotopic envelope of the anion of dolichol-20 sulphate. This envelope of anions was analyzed by FAB/CID tandem mass spectrometry [Griffiths et al., 1996]. The resultant daughter ion spectrum confirmed that the compound analyzed was dolichol-20 sulphate.

Electron impact ionization mass spectrometry (EIMS). The growth-stimulatory fraction obtained after HPLC as described above was also analyzed by EIMS. Positive ion mass spectra were recorded from the material both as TBDMS ether derivative and in underivatized form.

The derivatized polyprenol standards gave the expected pattern of ions for TBDMS derivatives of organic alcohols (i.e., peaks at M^+, $[M-57]^+$, and $[M-132]^+$) (data not shown). The dolichols, however, gave predominantly the radical molecular ion of the intact TBDMS ether, as shown for dolichol-20 TBDMS ether (mass range 1,050–2,000) in Figure 5A.

The fraction containing the growth-stimulatory activity was derivatized with TBDMS chloride. The material evaporated from the probe in the range 250–320°C. The most prominent ion cluster in the mass range 1,000–2,000 Da ap

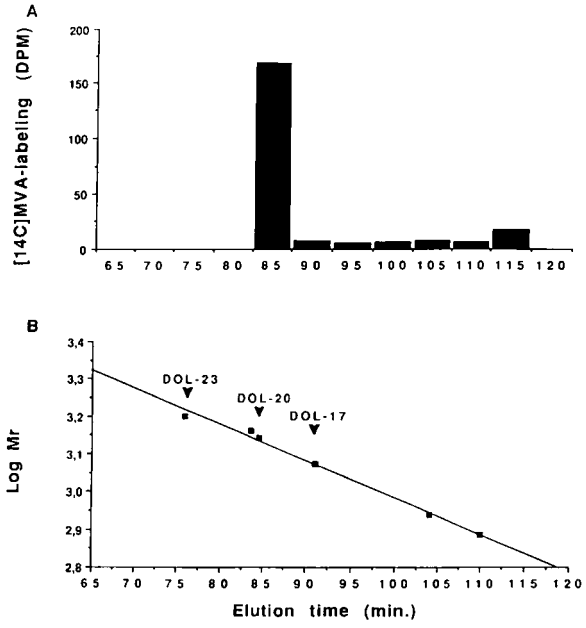

Fig. 3. Estimation of molecular weight of the growth-stimulatory compound. [3H]-labelled growth-stimulatory lipids, which were purified by HPLC, were separated on Sephadex LH-60. The radioactivity in the fractions was detected (**A**). Peak elution positions of molecular mass standards (i.e., dolichol-11 (M_r 769.3), coenzyme Q_{10} (M_r 863.4), dolichol-17 (M_r 1178), dolichol-19 (M_r 1314.3), dolichol-20 (M_r 1382.4), dolichol-21 (M_r 1450.5), and dolichol-23 [M_r 1586.7]) are shown (**B**).

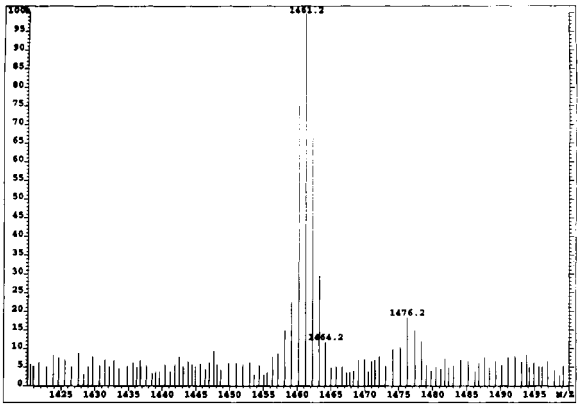

Fig. 4. Negative-ion FABMS after sulphation of the HPLC-purified fraction containing the growth-stimulatory lipids. The mass range 1,420–1,500 Da, where the cluster of anions of the dolichol-20 sulphate is located, is shown.

peared at *m/z* 1,495.36–1,498.38, corresponding to the molecular ions of dolichol-20 TBDMS ether (Fig. 5C). Mass spectra of material in the HPLC fractions collected immediately before and after the biologically active fraction are shown in Figure 5B,D, respectively.

Material from a human breast cancer was prepared in essentially the same way as the material from cell cultures, except for an initial homogenization step. This material also had the capacity to induce DNA synthesis in MVA-depleted cells. After TBDMS derivatization, the most prominent ion in the mass range 1,050–2,000 Da corresponded to dolichol-20 TBDMS (Fig. 5E). Figure 7F illustrates the growth-stimulatory effects of the fractions analyzed above.

Nonderivatized growth-stimulatory compound(s) was also analyzed by EIMS. The material evaporated from the probe at approximately 240–280°C. Mass spectra were recorded in the mass range between 1,000 and 2,000 Da and showed the presence of dolichol-20 and to some extent dolichol-19 (data not shown).

Covalent Binding of Dolichol-Like Lipids to Tumor Cell Proteins

Against the background that dolichylation of proteins has been demonstrated [Hjertman et al., 1997], we found it interesting to investigate whether 90VAVI cells were capable of synthesizing dolichol-modified proteins. Such proteins might hypothetically explain some of the growth-stimulating properties of dolichol-like compounds.

First, we incubated 90VAVI cells with a mixture of [3H]-labelled dolichol-17, -19, and -20 for 48 h. After delipidation, the [3H]-labelled proteins were analyzed by SDS-PAGE. Similar to the case with colonic carcinoma cells [Hjertman et al., 1997], bands of low-molecular weight proteins labelled with [3H] were seen (data not shown).

Sulfonium salt cleavage was used to release [3H]-labelled lipids from delipidated proteins isolated from 90VAVI cells grown in the presence of [3H]MVA. The lipids were purified by HPLC, and fractions corresponding to dolichols were found to be [3H]-labelled. However, the amounts obtained were too small for detection by EIMS.

90VAVI cells were also cultured in the presence of [3H]MVA and [35S]cysteine. The labelled proteins were isolated and delipidated under conditions also removing dolichyl phosphoryl oligosaccharides [Hjertman et al., 1997]. They were then subjected to extensive proteolysis. The products were extracted with n-butanol and separated by reversed-phase HPLC using a C_4 column. The [3H]-labelled material was separated into two radioactive peaks (Fig. 6). The most hydrophobic of these (appearing after 45–60 min) coeluted with the most hydrophobic [35S]-labelled peak (Fig. 6). Farnesyl-cysteine and geranylgeranyl-cysteine standards coeluted with the earlier [3H]- and [35S]-labelled peaks. It therefore seems likely that these peaks represent short-chain prenyl-cysteines (i.e., geranylgeranyl-cysteine and farnesyl-cysteine). A dolichyl phosphate standard was found to elute somewhat earlier than the hydrophobic [3H]/[35S]-labelled peak, whereas the dolichol standard eluted later (Fig. 6). These data suggest that the hydrophobic radioactive peak represents dolichyl-cysteine.

Since dolichols could not be detected by mass spectrometry of fractions obtained after sulphonium salt cleavage of proteins from 90VAVI cells, we repeated the experiment using HepG2 cells. These were found to produce much more dolichols from [3H]MVA than the 90VAVI cells (data not shown). It was confirmed that dolichol-like lipids isolated from HepG2 cells stimulated DNA synthesis in MVA-depleted target cells (data not shown).

EIMS of underivatized dolichol-like lipids released from HepG2 cell proteins by sulfonium salt cleavage demonstrated the presence of peaks with *m/z* 1,177 and 1,379. These peaks

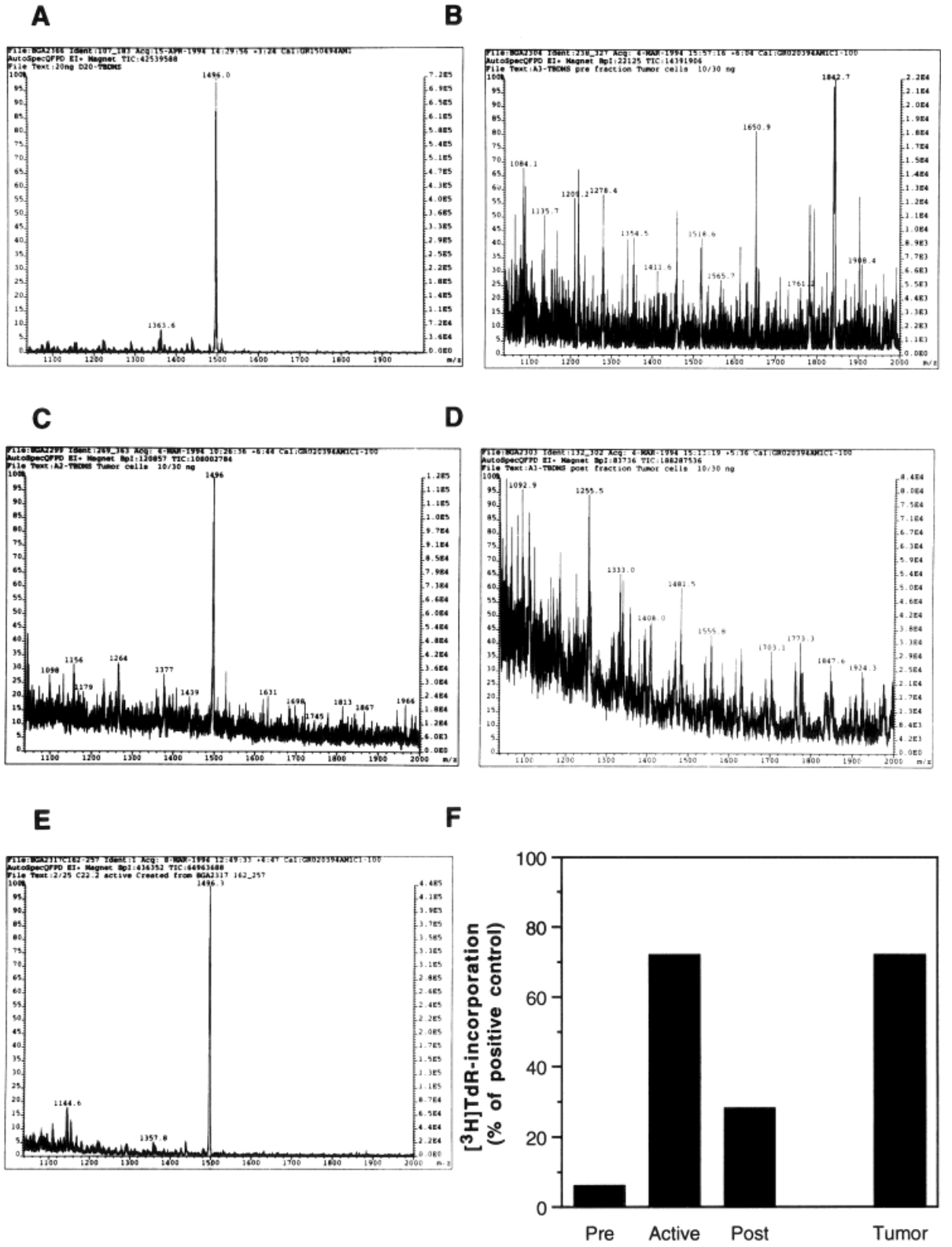

Fig. 5. Positive-ion EIMS of the TBDMS ethers from the HPLC fraction containing the active compounds. Mass ranges between 1,050 and 2,000 Da are shown. **A:** Dolichol-20-TBDMS standard. **B:** Fraction preceding the active fraction (fraction 1). **C:** The active fraction (fraction 2). **D:** Fraction following the active fraction (fraction 3). **E:** Active material obtained from a total of 15 g of a human breast tumor. **F:** Growth-stimulatory effects of the lipid material analyzed in B–E. After labelling with [3H]thymidine, the level of DNA synthesis was determined. The effects are expressed as percent of positive control (MVA). The indicated values represent means from duplicate dishes.

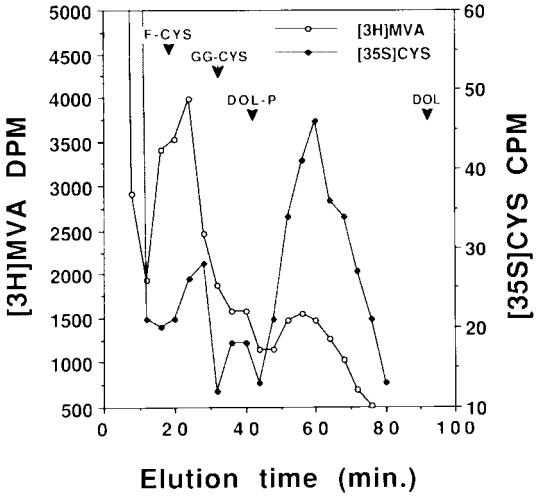

Fig. 6. Reversed-phase HPLC of [3H]MVA- and [35S]cysteine-labelled protein digest. 90VAVI cells were incubated in medium containing [3H]MVA (50 µCi/ml) or [35S]cysteine (5 µCi/ml) for 48 h. After delipidation, removal of dolichyl pyrophosphoryl oligosaccharides, and extensive proteolysis, the butanol-extractable labelled compounds were separated on a C_4 reversed-phase HPLC system. The elution of external standards for farnesyl-cysteine (F-cys), geranylgeranyl-cysteine (GG-cys), dolichyl phosphate (DOL-P), and dolichol (DOL) is indicated.

correspond to dolichol-17 and to dolichol-20 lacking two hydrogens (i.e., polyprenol-20) (Fig. 7A). A mass spectrum of authentic dolichol-17 is shown for comparison (Fig. 7B). Fractions adjacent to those containing the dolichol-like lipids did not give rise to any peaks in the investigated mass ranges (data not shown). Furthermore, analysis of a sample treated by alkaline hydrolysis without methyl iodide did not give rise to any peaks (data not shown).

DISCUSSION

Previous results from our laboratory have suggested that dolichol may be of regulatory importance for cell proliferation [Larsson, 1987; Larsson et al., 1989; Larsson and Wejde, 1992]. In one study we showed that DNA synthesis was not decreased in virus-transformed human fibroblasts (90VAVI) but was effectively blocked in normal human diploid fibroblasts when the HMG-CoA reductase activity in both cell lines was inhibited to a similar degree by mevinolin [Larsson et al., 1989]. As also distinguished from the normal fibroblasts, in virus-transformed cells the incorporation of [3H]acetate into dolichols was unaffected by mevinolin [Larsson et al., 1989]. However, further inhibition by adding 25-hydroxycholesterol to the cells resulted in both a decreased dolichol production and an inhibited proliferation of 90VAVI cells [Larsson et al., 1989]. The decrease in DNA synthesis could partially be reversed by the addition of dolichol-20 [Larsson et al., 1989]. Uptake of dolichol in cultured cells is well-documented [Carson and Lennarz, 1979; Chojnacki et al., 1980; Rip et al., 1985; Dricu et al., 1997]. In another study we showed that dolichol could delay cell cycle arrest in human diploid fibroblasts when the HMG-CoA reductase activity was decreased [Larsson and Wejde, 1992]. We have also demonstrated growth-stimulatory lipids with chromatographic mobilities corresponding to long-chain dolichols in human breast cancer cells [Wejde et al., 1993].

The only established biological role of dolichols is their function as carriers (in phosporylated form) of oligosaccharide groups in the biosynthesis of glycoproteins in the endoplasmic reticulum [Hart, 1992]. N-linked glycosylation has been shown to be essential for normal embryogenesis [Carson and Lennarz, 1981] and cell growth [Kabakoff et al., 1990]. In a recent study of melanoma cells, we demonstrated that the translocation of the insulin-like growth factor-1 receptor (IGF-1R), which is a glycoprotein, to the cell surface requires MVA [Carlberg et al., 1996]. A reduced MVA synthesis, produced by the HMG-CoA reductase inhibitor lovastatin (mevinolin), drastically reduced the plasma membrane expression of IGF-1R [Carlberg et al., 1996]. This effect was correlated to a decrease in dolichyl phosphate synthesis and N-linked glycosylation of intracellular IGF-1R [Carlberg et al., 1996]. In a more recent study, we showed that addition of exogenous dolichyl phosphate increased the cell surface IGF-1R expression in lovastatin-treated melanoma cells [Dricu et al., 1997]. Taken together, these data suggest that the translocation of IGF-1R from endoplasmic reticulum to the plasma membrane is regulated by dolichyl phosphate–dependent glycosylation of the receptor proteins. In melanoma cells, this event seems to be necessary for cell growth [Carlberg et al., 1996]. However, unpublished results in our laboratory have shown that IGF-1R expression is not required for growth of 90VAVI cells. Therefore, MVA-dependent growth of these cells seems to be mediated through other mechanisms.

The present study has demonstrated the formation of growth-stimulatory compound(s) from [3H]MVA in 90VAVI and HepG2 cells. These

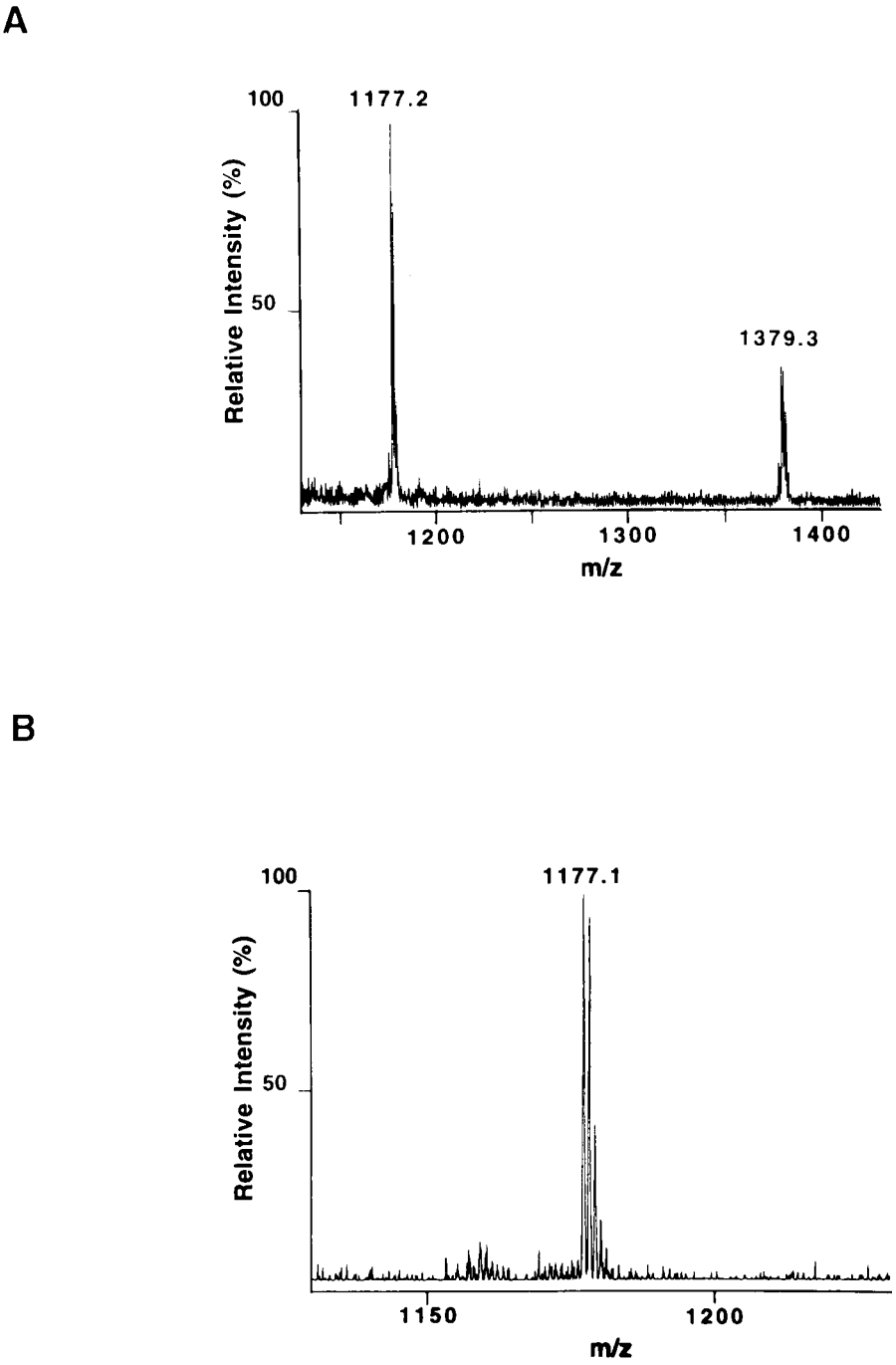

Fig. 7. **A:** Positive-ion EIMS of underivatized material derived from HepG2 cells after sulfonium salt cleavage followed by HPLC. **B:** Positive-ion EIMS of underivatized standard dolichol-17.

compounds have the chromatographic characteristics of dolichols or polyprenols, and corresponding growth-stimulatory compound(s) was obtained from human breast cancer tissue. When HPLC fractions containing this biological activity were analyzed by mass spectrometry, the common finding was the presence of dolichol-20 and in some cases dolichol-19. No other compounds were consistently seen above the detection level (about 4–8 pmol in an HPLC fraction [Griffiths et al., 1996]). It is not known with certainty if dolichol-20 (or dolichol-19) is the growth-stimulatory compound. Thus, mass spectrometry may not distinguish between dif-

ferent isomeric forms, and it is still possible that compound(s) present below the limit of detection is responsible for the biological activity. However, if this is the case, such compound(s) should be closely related to dolichols or polyprenols. Thus, even if the magnitude of the growth-stimulatory effect varied considerably between different preparations, we can conclude that the compounds inducing DNA synthesis belong to the dolichol group of lipids. The reason for the variation in biological activity is not clear but may be due to different yields of active compounds between different preparations.

Recently we provided evidence for thioether linkage of dolichyl groups to cysteine residues in tumor cell proteins [Hjertman et al., 1997]. Our evidence was based on three major findings. First, despite extensive delipidation procedures, radioactive dolichols were found to be associated with cellular proteins. Gel electrophoresis showed dolichol-labelled proteins with molecular sizes of, for example, 5, 10, 27, and 75 kDa [Hjertman et al., 1997]. Second, extensive proteolysis of prenylated proteins revealed hydrophobic products containing both dolichol and cysteine. These products were much more hydrophobic than geranylgeranyl-cysteine and farnesyl-cysteine [Hjertman et al., 1997]. Third, treatment of cellular proteins with methyl iodide and alkali released [3H]-labelled lipids, which chromatographically behaved like dolichols, and mass spectrometry disclosed a compound corresponding to dolichol [Hjertman et al., 1997].

In this study we investigated the possibility that 90VAVI cells contain dolichylated proteins. We could show that proteins from this cell line were labelled with [3H]dolichol. We did not succeed in releasing dolichyl groups from the 90VAVI cell proteins in sufficient amounts to be able to detect them with mass spectrometry. However, after extensive proteolysis of [3H]- and [35S]-labelled proteins from 90VAVI cells grown in the presence of [3H]MVA and [35S]cysteine, a hydrophobic product chromatographically corresponding to dolichyl-cysteine was found. In another malignant cell line, HepG2, we could release dolichol-like compounds (corresponding in molecular mass to dolichol-17 and polyprenol-20) from the cell proteins by sulphonium salt cleavage and also detect them by mass spectrometry. It was confirmed that this cell line, like 90VAVI, produced growth-stimulatory dolichol-like lipids. Compared to 90VAVI cells, the HepG2 cell line synthesized much more dolichols. Therefore, it is possible that the level of protein dolichylation may be related to the rate of dolichol synthesis in the tumor cells.

In conclusion, the data presented here suggest that long-chain dolichols or closely related compounds play an essential role in growth regulation. Our data suggest the presence of dolichylated proteins in virus-transformed fibroblasts and hepatocellular carcinoma cells, which in turn raises the possibility that such modified proteins may be involved in growth control of these cells. An interesting question for future research is whether the growth-stimulatory dolichol-like lipids serve as lipid substrates for the dolichylation reaction.

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