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Characterisation of protein isoprenylation in procyclic form *Trypanosoma brucei*

Helen Field[a], Ian Blench[b], Simon Croft[c], Mark C. Field[a,*]

[a]*Laboratory of Cell Biology, Department of Biochemistry, Imperial College of Science, Technology and Medicine London SW7 2AY, UK*

[b]*Department of Biochemistry, Imperial College of Science, Technology and Medicine London SW7 2AY, UK*

[c]*London School of Hygiene and Tropical Medicine, Gower Street London, UK*

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Abstract

Protein modification by isoprenylation is essential in mammals and other eukaryotes, but has not been demonstrated in the parasitic protozoa of the order kinetoplastida. A key regulatory enzyme of the mevalonate pathway, hydroxymethylglutaryl-coenzyme A reductase (HMG-R), and end products of the path, including dolichols, are present in *Trypanosoma brucei*. By metabolical labelling of procyclic form trypanosomes in the presence of compactin, an efficient inhibitor of HMG-R, followed by one-dimensional gel electrophoresis, we demonstrate that protein isoprenylation indeed takes place in this organism and at least 14 polypeptides bear the modification. Further characterization of labelled isoprenyl groups by methyl iodide cleavage and high pressure liquid chromatography identified both the farnesyl and geranylgeranyl moieties found covalently attached to proteins in other eukaryotes. The latter moiety was more abundant, as found in mammalian systems. Prolonged incubation with compactin grossly affected cell morphology and altered a number of subcellular structures as seen by electron microscopy. High concentrations of compactin were toxic, whilst lower concentrations were cytostatic. The primary morphological lesion is distinct from that of synvinolin, another inhibitor of HMG-R. The morphological changes correlated with a complete inhibition of HMG-R activity by compactin. Surprisingly there was a complete lack of HMG-R activity in procyclic cells grown for 1 or several days in 100 μM compactin, suggesting that degradation of the enzyme had occurred and compensatory upregulation mechanisms could not be successfully exploited by the parasite to overcome HMG-R inhibition. Subsequent alterations to the overall cell shape are seen after 3 days of compactin exposure. Overall these data indicate that *T. brucei* has an essential protein

Abbreviations: BSF, bloodstream form; EM, electron microscopy; ER, endoplasmic reticulum; FCS, foetal calf serum; FT, farnesyltransferase; GT, geranylgeranyltransferase; HMG-R, HMG (hydroxymethylglutaryl) CoA reductase; MVA, mevalonic acid; MVL, mevalonolactone; -PP, pyrophosphate; RP, reverse-phase.

* Corresponding author. Tel: +44 171 5945277; fax: +44 171 5945207; e-mail: m.field@ic.ac.uk

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isoprenylation pathway that is conserved with the higher eukaryotes. Additionally, products of the MVA pathway are implicated in maintenance of cell architecture.

Keywords: *Trypanosoma*; Mevalonate; G proteins; Prenylation; Ultrastructure

1. Introduction

The mevalonate biosynthesis pathway is essential in all eukaryotic organisms so far studied [1]. The pathway progresses from acetoacetyl coenzyme A through mevalonate (MVA), activated isoprene, farnesyl pyrophosphate (-PP) (C_{15}) to geranylgeranyl-PP (C_{20}) or to squalene. Squalene is converted to sterols, with the major mammalian product being cholesterol, from which all other steroids are synthesised. Plants, fungi and some protozoan parasites convert squalene to ergosterol using a distinct pathway [2] whilst other lower order organisms make both cholesterol and ergosterol, possibly due to lower specificity of certain enzymes [3]. Geranylgeranyl-PP is converted to ubiquinone and dolichols, the latter essential in glycoprotein elaboration and other reactions (Fig. 1). Farnesyl-PP and geranylgeranyl-PP are both conjugated isoprene groups used in covalent modification of proteins [4].

The most important enzyme regulator of the mevalonate pathway is the enzyme synthesising MVA, hydroxymethylglutaryl-coenzyme A reductase (HMG-R, EC 1.1.1.34). It is inhibited by compactin (mevastatin), synvinolin (simvastatin) and mevinolin (lovastatin). HMG-R is under very stringent regulation to control sterol biosynthesis in the presence and absence of exogenous cholesterol whilst maintaining a minimal flow of MVA products to the isoprenyl pathway [5]. It is upregulated when MVA and its immediate products are depleted by, e.g. lovastatin addition, and it is downregulated by steroid and non-steroid metabolites of HMG-R, and by a farnesylated protein [6,7]. Inhibition is overcome by providing exogenous MVA.

The consequences of blocking the MVA pathway include cessation of DNA synthesis causing cell cycle arrest in the G1 and G2/M phases, inhibition of endogenous sterol synthesis and protein isoprenylation and defects in intracellular transport. The turnover of isoprenylated proteins correlates with decreased DNA synthesis under conditions of isoprene pathway block [8]. Cell growth, movement of proteins and membranes in trafficking and cytoskeletal processes are controlled in part by isoprenylated ras-like small GTP-binding proteins which are isoprenylated with farnesyl or geranylgeranyl. This modification is prerequisite for function and allows membrane attachment by embedding of the isoprenyl group in the lipid bilayer of the appropriate organelle [9]. The ability to modulate G protein function by preventing attachment of the isoprene is a highly effective target for chemotherapeutics in the treatment of cancers [10].

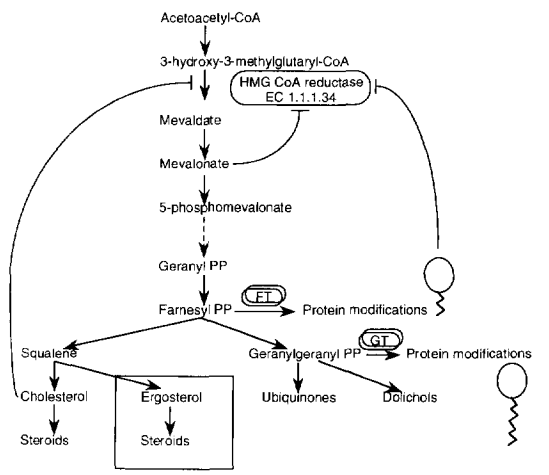

Fig. 1. The mevalonate pathway of eukaryotes. The pathway shown is that of mammals ([8,30]); divergent features of the fungal pathway are included as boxed. The procyclic form of *T. brucei* are capable of synthesis of ergosterol as well as other steroids, whilst the bloodstream form pathway more closely resembles the mammalian scheme. Blocked arrows indicate negative feedback regulatory mechanisms of metabolites upon HMG-R. Isoprenes and the proteins to which they are attached are indicated by zigzag icons and ovals respectively. Broken arrows indicate multiple steps. FT, farnesyltransferase; GT, geranylgeranyltransferase.

In *Trypanosoma brucei*, cholesterol, ubiquinone and dolichol biosynthesis from [^{3}H]-mevalonolactone (MVL) was demonstrated by HPLC and mass spectrometry in freshly isolated bloodstream form (BSF) parasites [11]. Short chain dolichols with 11–12 isoprene units were found, compared to 15–23 unit dolichols found in mammals. Shorter dolichols are also present in *T. cruzi* and *Crithidia fasciculata* [12,13].

Peptide or protein substrates are isoprenylated by the action of either farnesyltransferase (FT) or geranylgeranyltransferase (GT) [8] which add farnesyl or geranylgeranyl respectively to cysteine residues in a C-terminal motif, a CAAX box. The precise signal specifies whether a given protein is a substrate for FT or GT, with GT requiring additional signals distal to the C-terminus. The small GTP-ase class of protein is present in *T. brucei* [14–17] and nucleic acids encoding rabs isolated from *T. brucei* encode sequences appropriate for geranylgeranylation ([17]; HF and MCF manuscript in preparation), although the nature of their isoprene units has not yet been determined. Isoprenylated proteins have been detected recently in the protozoan *Giardia lamblia* [18].

HMG-R activity is ~5-fold greater in procyclic form trypanosomes compared to BSF [19] and therefore the isoprenylation pathway may be more easily examined in procyclics. In other parasites, e.g. *Schistosoma mansoni*, lovastatin has antiparasite activity [18,20]. FT is under active consideration as a target for chemotherapy of rapidly growing tumour cells [10], and we reasoned that this pathway might also provide a novel target for chemotherapy in treatment of trypanosomiasis. This paper describes the characterisation of protein-linked isoprene groups in procyclic *T. brucei* together with the morphological effects of inhibition of HMG-R by compactin.

2. Materials and methods

2.1. Materials

Geranitol, geranylgeranitol (mixed isomers) and trypsin (tissue culture grade) were from Sigma, farnesol and methyl iodide from Aldrich. All-*trans* geranylgeranitol was the kind gift of Dr. A. Magee, NIMR, Mill Hill, London. Acetonitrile and phosphoric acid (HPLC grade) were from BDH. SDS-PAGE molecular mass markers were from Amersham International. All other reagents were of analytical reagent grade.

2.1.1. Cells and parasites

Procyclic *T. brucei* strain 427 procyclics were cultured in SDM-79 medium (Sigma), containing 10% heat inactivated foetal calf serum (FCS, Gibco/BRL) and 7.5 $\mu g \cdot ml^{-1}$ haemin at 27°C [21]. BSF strain 427 were cultured in HMI-18 medium as described [22]. Hep2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL), 10% FCS at 37°C in a 5% CO_2 atmosphere.

2.2. Compactin treatment and metabolic labelling

Compactin, sodium salt (Sigma), was made up at 490 μM as described [23]. The stock solution was frozen at −20°C in 1 ml aliquots. Before use an aliquot was thawed and incubated at 50°C for 2 h to ensure complete solubilisation of the compound. Compactin was added to small volume cultures (2 ml) of freshly diluted rapidly growing log phase procyclic or BSF parasites and the cells cultured for up to 5 days. The cultures were inspected by light microscopy and the cell number determined periodically.

Cells (trypanosomes and Hep2) were labelled with RS-[5-^{3}H]-mevalonolactone (MVL; NEN-Dupont), specific activity 0.37–1.11 TBq (10–30 Ci) $mmol^{-1}$, as follows: the ethanol carrier was evaporated with a stream of nitrogen and the residue was dissolved in 1 ml of minimal essential medium (MEM) containing 10% FCS. Procyclics pretreated for 24 h at 1×10^7 cells $\cdot ml^{-1}$ in SDM-79 containing 100 μM compactin were harvested, pelleted in a bench top centrifuge and resuspended in 7 ml of MEM/FCS and 1.7 ml of 490 μM compactin was added (final concentration 89 μM). Finally 700 μCi [5-^{3}H]-MVL was added (75 $\mu Ci \cdot ml^{-1}$) and the cells cultured for 24 h. At the end of this period the cells ($\sim 3 \times 10^8$) were

washed in PBS, counted and lysed by the addition of 1 ml of ice-cold acetone and stored at −85°C. For Hep2 cells, two dishes of 80% confluent cells ($\sim 7 \times 10^6$ cells total) were pretreated with compactin at 30 μM for 24 h. Media in the dishes were then replaced with 5 ml fresh MEM/FCS containing 89 μM compactin and [5-^{3}H]-MVL at 75 $\mu Ci \cdot dish^{-1}$. Cells were labelled for 24 h at 37°C. Cells were then detached by trypsin treatment, washed in PBS, lysed with 1 ml ice-cold acetone and stored at −85°C.

2.3. Analysis of [^{3}H]-MVL-labelled moieties by HPLC

Acetone precipitated [^{3}H]-labelled cells were prepared for HPLC analysis as follows: pellets (above) were washed 3 times with ice-cold (−20°C) acetone [23], with sonication for 15 min between each addition. An aliquot was withdrawn at this stage for SDS-PAGE analysis. The final pellet was briefly dried in a Speedvac (Savant) to remove residual acetone, and dissolved in 1 ml of 100 mM Tris-HCl/5% acetonitrile by bath sonication and heating to 95°C for 10 min. The sample was then treated with trypsin and methyl iodide essentially as described in [4], with minor modifications. Solvents were evaporated with nitrogen from the final extract, which was resuspended in 50% acetonitrile/25 mM phosphoric acid (v/v) (buffer A) and analysed by reverse phase (RP)-HPLC. Internal standards were added prior to chromatography. In a pilot experiment it was found that ~85% of incorporated tritium was released by the procedure. Analysis was performed on an ODS silica column (0.3 × 25 cm) column (Phenomenix) at 40°C, flow rate 0.25 $ml \cdot min^{-1}$. A linear gradient from 100% buffer A to 100% buffer B (acetonitrile containing 25 mM phosphoric acid) was applied over 40 min followed by a plateau at 100% B for 20 min. Fractions were collected every 60 s. Elution of the internal standards was monitored at 205 nm. Radioactive material was detected by scintillation counting.

2.4. Gel electrophoresis and autoradiography

Proteins were analysed by reducing SDS-PAGE in 15% discontinuous gels. After electrophoresis gels were impregnated with En^{3}Hance (DuPont) according to the manufacturer's instructions, dried and exposed to X-ray film at −85°C.

2.5. Electron microscopy

Procyclic cells were treated with differing concentrations of compactin for 48 h prior to fixing with 3% glutaraldehyde and processing for electron microscopy. Procyclic cultures were washed in serum-free medium, and fixed in 3% glutaraldehyde in serum free SDM-79 for 1 h at room temperature. After 3 washes in 75 mM sodium cacodylate/0.2 M sucrose buffer, cells were postfixed in 1% OsO_4 for 2 h at 4°C. Following dehydration in a methanol series, including 2% uranyl acetate stain at the 30% stage, cells were embedded in TAAB resin. Sections were stained with Reynold's lead citrate and examined on a JEOL 1200EX MkII at 80 kV.

2.6. Hydroxymethylglutaryl-coenzyme A reductase assays

HMG-R activity was measured in digitonin-permeabilised procyclic *T. brucei* essentially as described [19,24], with minor modifications. All reagents in the assay buffer were from Sigma except 3-hydroxy-3-methyl-[3-^{14}C]glutaryl-coenzyme A which was from NEN Dupont. Imidazole, EDTA, EGTA and KF were made up and stored as a 10X stock, all other solutions made up freshly on the day of assay. The samples were chromatographed on Si60 TLC plates (Merck) in acetone/toluene 1:1 (v/v) solvent system in a saturated atmosphere. MVL was visualised with iodine vapour, and radioactivity located by autoradiography at −85°C. Regions corresponding to MVL were scraped into glass scintillation vials and counted on a Beckman LS5801 scintillation counter. Mock assays were

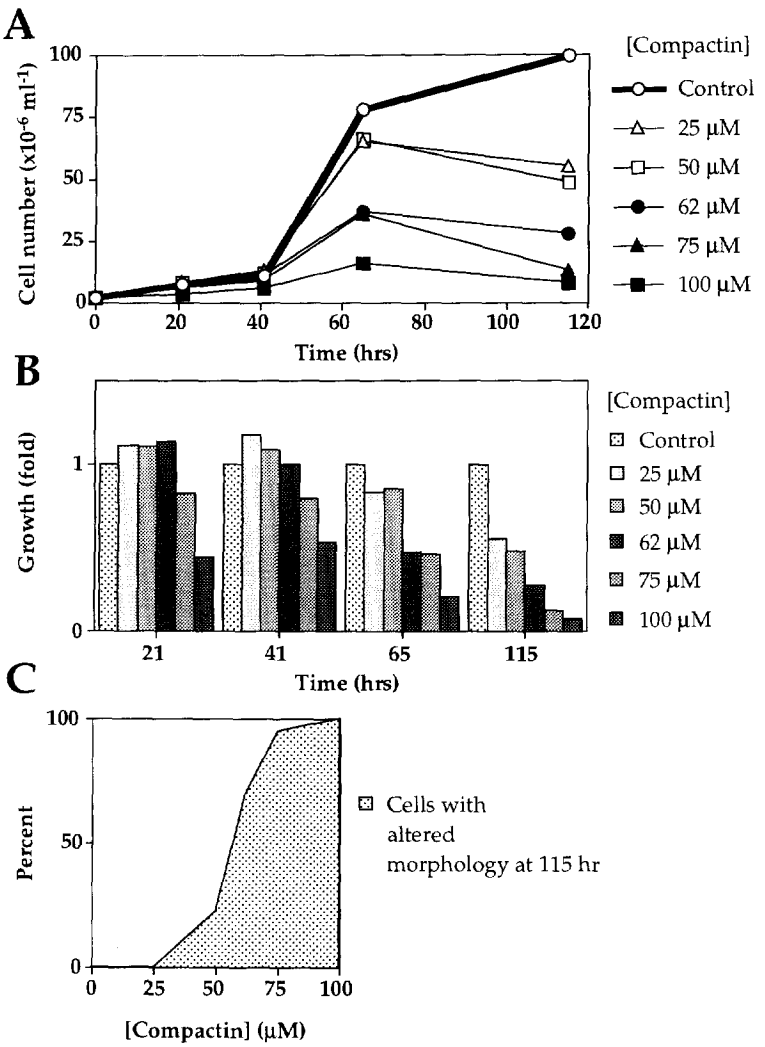

Fig. 2. Cytostasis and altered morphology of procyclic *T. brucei* grown in the presence of compactin. Panel A; Growth curves for procyclic *T. brucei* at various concentrations of compactin. Log phase cultures were subcultured and supplemented with the indicated concentrations of compactin. At intervals, cell density was determined by hematocytometer counting. After about 70 h cultures began to enter stationary phase. At higher concentrations (75–100 μM) lysis becomes apparent at this stage. From these data the IC_{50} for compactin is 60.1 μM for procyclic cells. Panel B; Growth rate of procyclic cells at different times and concentrations of compactin. Higher concentrations of compactin are seen to affect growth within 21 h, but at lower concentrations more time is required for this to become significant. Panel C; Prevalence of procyclic cells with comma-type morphology. Cellular morphology was scored microscopically from inspection of at least 400 cells for each concentration. After prolonged treatment with compactin at 100 μM essentially all cells show the abnormal comma morphology. The data presented are from a representative experiment; the analysis has been repeated twice with essentially identical results.

performed by omitting cells from the assay, but otherwise treating the reagents as normal. Activity is expressed in pmol·mg cell protein^{-1}·min^{-1}, assuming 1 mg as equivalent to 10^8 cells [19]. Cells that were pretreated with compactin in culture were assayed as above. Compactin was heated for at least 2 h at 50° C before addition to the assay (immediately prior to adding cell suspension).

3. Results

3.1. Differential sensitivity to compactin in procyclic and BSF T. brucei strain 427

We chose cell growth as a parameter to determine the compactin concentration required for effective inhibition of isoprene biosynthesis in *T. brucei*. Compactin was titered up to 200 μM for procyclic cells, and cell density determined at various times. As shown in Fig. 2A, the cytostatic concentration of compactin was 100 μM for procyclics. At greater concentrations significant cytolysis was observed (data not shown). We found that cytostasis became more apparent with prolonged exposure (Fig. 2B), consistent with an endogenous pool of material being utilised and eventually depleted. For example, in the presence of 25 μM compactin significant inhibition of cell proliferation was observed only after 115 h treatment.

After about 3 days, the morphology of procyclics exposed to high levels of compactin (≥ 75 μM) became dramatically altered. Cells became spherical with small active flagella, resembling motile commas when viewed by phase contrast microscopy. The percentage of cells exhibiting this morphology was proportional to the amount of drug initially added to the medium (Fig. 2C). Remaining procyclic cells appeared unaffected, suggesting that cells rapidly progressed through a transition period from normal morphology to comma form.

The activity of HMG-R is approximately five-fold lower in BSF trypanosomes compared with procyclics, and additionally steroid biosynthesis is differentially regulated in the two life forms. We therefore considered that BSF may exhibit greater sensitivity to compactin than procyclics. This was found to be the case; cultured BSF *T. brucei* 427 proved an order of magnitude more sensitive to the drug than procyclics, with cytostasis observed at 10 μM (Fig. 3). Morphological changes noted for procyclics were not observed, although at low concentrations (5 μM) cells appeared to be smaller than in control cultures presumably reflecting a defect in cell cycle control.

Additionally we observed some inhibition ($\sim$25%) of ^{35}S-methionine incorporation into total cell protein when procyclic cells were cultured in cytostatic concentrations of compactin (data not shown). This finding is clearly consistent with the growth arrest, but confirms that polypeptide synthesis remains ongoing. This explains the ability of the cells to incorporated ^{3}H-MVL into protein (below).

3.2. [^{3}H]-mevalonolactone is incorporated into a spectrum of polypeptides in procyclic T. brucei

We next examined the spectrum of polypeptides in procyclic *T. brucei* which were isoprenylated. Procyclic and Hep2 cells were labelled with [^{3}H]-MVL in the presence of compactin and an aliquot of each analysed by SDS-PAGE. Note that the Hep2 cells were more lightly labelled than procyclics due to different labelling conditions (Fig. 4 legend and Section 2) and were included as a positive control for the procedures. Incorporation of [^{3}H]-MVL into trypanosome proteins was severely impaired in the absence of compactin, but not abolished (data not shown), suggesting that the endogenous MVA pool is smaller in trypanosomes than in mammals which cannot be labelled without HMG-R inhibition. In addition, the same try-

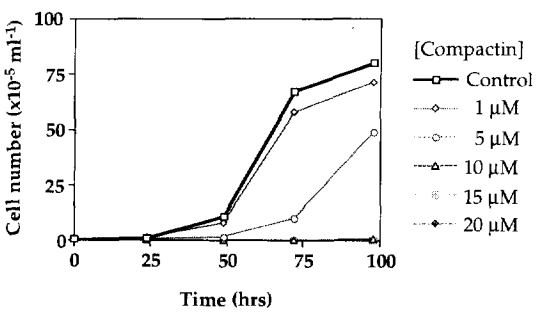

Fig. 3. Compactin sensitivity of BSF trypanosomes. Growth of BSF *T. brucei* in various concentrations of compactin was studied as described in Fig. 2. During the first 24 h the cells typically enter a lag phase before initiating log phase growth. At inhibitory concentrations of compactin the lag phase is prolonged. After reaching a limiting concentration of $\sim 1 \times 10^{7}$ ml^{-1} the cells die in the absence of compactin. Cell numbers were determined microscopically using a hematocytometer. The data are from a representative experiment of two separate studies. From these data the IC_{50} for compactin is 7.8 μM on bloodstream form cells; 15 μM compactin is required to achieve complete inhibition of growth.

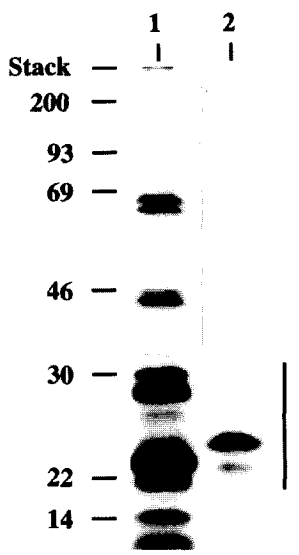

Fig. 4. Several *T. brucei* proteins are labelled with [^{3}H]-mevalonolactone. Procyclic *T. brucei* (lane 1) and Hep2 cells (lane 2) were labelled with [^{3}H]-MVL for 24 h and then analysed by reducing SDS-PAGE. Approximately 1×10^6 cells were loaded in lane 1 and 2×10^5 cells in lane 2. Note that the concentration of [^{3}H]-MVL used for the mammalian cells was ~five-fold less than for the trypanosomes. The migration positions of molecular mass markers are indicated in kDa (left) and of putative small G proteins by the bar (right). Both tracks were autoradiographed at −85°C for 3 weeks.

panosome bands were labelled in the absence of compactin, indicating that the drug did not alter which protein species became isoprenylated.

Both cell types yielded a number of putative isoprene-labelled polypeptides. In mammals, three size classes of proteins bearing isoprene moieties can be defined; ~69 kDa corresponding to the polyisoprenylated nuclear lamins [8], an unassigned 46 kDa protein, and ~20–30 kDa ras superfamily members (which include rabs), and low molecular mass subunits of trimeric GTPases [8]. This 20–30 kDa group is a complex cluster of a large number of polypeptides having very similar molecular mass, and in mammals may correspond to over thirty distinct species. In our analysis of Hep2 cells (Fig. 4, lane 2) the ~46 kDa band and G protein complex can be seen (but see [25] for a stronger profile). In the trypanosome sample (Fig. 4, lane 1) again three overall groups of polypeptides can be distinguished, a ~67 kDa doublet, a ~45 kDa doublet and a complex between 20–30 kDa. Additional low molecular mass species are also present migrating at or below 14 kDa. At least 14 separate polypeptides can be resolved. It is tempting to speculate that the 67 and 46 kDa bands are homologues of the mammalian isoprenylated species, and more specifically that the 67 kDa doublet represents *T. brucei* lamins. However, additional data are required before this assertion can be made with confidence.

Close examination of the putative small GTPase region at 20–30 kDa (Fig. 4) indicates at least eight distinct species were labelled in *T. brucei*. A highly labelled species was detected at 23 kDa; high incorporation may be a consequence of a high abundance of the polypeptide, several co-migrating polypeptides or the addition of more than one isoprene, as in rab3A and some other mammalian G proteins. The most heavily labelled species in the mammalian extract migrates at 25 kDa. Most recently, molecular cloning of nucleic acids encoding rab proteins has been reported in *T. brucei* and a candidate trimeric G protein subunit identified [15–17]. The rab sequences have canonical geranylgeranylation signals ([17], HF and MCF, in preparation). It is therefore most likely that the 20–30 kDa trypanosomal labelled bands include this class of protein. Indeed, we have recently found that two rab protein homologues from *T. brucei*, Trab1 and Trab7, migrate at 27 kDa and 29 kDa respectively on SDS-PAGE (HF and MCF, in preparation). In *G. lamblia* the same groups of high and low molecular mass bands were observed [18], suggesting that isoprenylation is a conserved feature amongst the eukaryotes including the general classes of protein receiving the modification.

3.3. Identification of protein-linked [^{3}H]-MVL-labelled species shows the same isoprene groups are conjugated to proteins in trypanosomes and mammals

[^{3}H]-MVA-labelled moieties attached to both the procyclic *T. brucei* and Hep2 proteins were released by methyl iodide treatment and then co-chromatographed with isoprene standards by RP-HPLC. The radiochromatogram for procyclic *T. brucei* contained two major peaks eluting at 32 and

50 min (Fig. 5, upper panel). The first peak eluted with the co-injected farnesol standard. A second peak just resolved from this farnesol peak at 36 min is a rearranged farnesol species, i.e. nerolidol, which elutes from an ODS column just after farnesol [20,26]. The peak at 50 min eluted slightly after the co-injected geranylgeranitol standard, and was just resolved from a small peak at the C_{20} position (47 min); analogously to farnesol the 50 min peak represented a rearrangement product of geranylgeranyl [23]. These assignments were confirmed by analysis of the Hep2 cell-derived material where it is known that farnesol and geranylgeranitol are the protein-linked isoprene species. The detection of rearrangement products is most likely to be the result of increased resolution under the chromatography conditions used in this study.

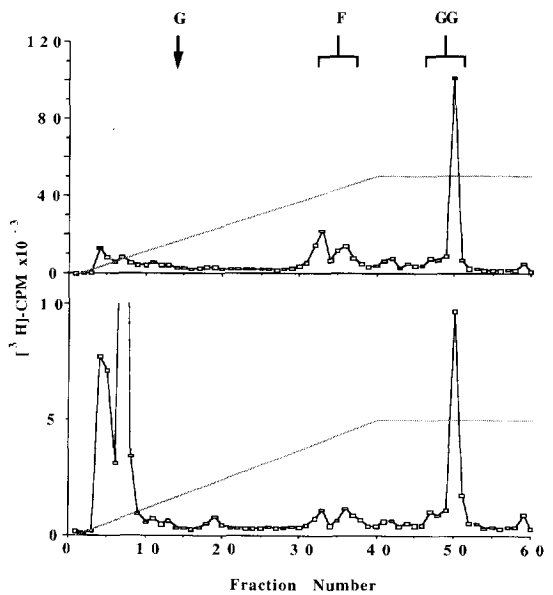

Fig. 5. *T. brucei* proteins are modified with farnesyl and geranylgeranyl groups. [^{3}H]-Labelled cells were processed for HPLC analysis by trypsin digestion and methyl iodide cleavage. Upper panel; radiochromatogram of material derived from procyclic *T. brucei*. Lower panel; radiochromatogram of material derived from Hep2 cells. G, F and GG indicate the elution positions of authentic standards (geranitol, farnesol and geranylgeranitol respectively; detected by absorbance at 205 nm) and the associated peaks of rearrangement products. The dotted line represents the acetonitrile gradient, from 50 to 100%. Substantial breakthrough in the mammalian sample was observed, but this profile is a control to confirm the elution positions of the farnesol and geranylgeranitol from an authentic sample and therefore is of no relevance.

Table 1
Comparison of levels of farnesyl and geranylgeranyl groups from human and procyclic *T. brucei* cells

	Isoprene (% incidence)	
	Farnesyl (C_{15})	Geranylgeranyl (C_{20})
T. brucei brucei procyclic 427	36.6	63.3
H. sapiens Hep2	21.6	78.4

Quantitation of RP-HPLC analysis of the released prenyl alcohols in Fig. 5 was obtained from scintillation counting for the relevant peaks and expressed as percent incidence of total isoprene recovered in farnesyl and geranylgeranyl products.

Therefore, trypanosomes modify proteins using the same isoprene groups as higher eukaryotes and other protozoa [18], both qualitatively and quantitatively. Farnesyl and geranylgeranyl are the major species of isoprene used to modify trypanosome proteins. Essentially identical results were obtained from procyclic *T. brucei* in two other separate analyses (MCF, unpublished data). Quantitation of the data from these separations is shown in Table 1. The farnesol accounted for ~20% of the total isoprene recovered from the Hep2 cells, whilst in procyclic *T. brucei* this figure is ~35%. This is most likely to reflect species-specific differences in the abundance of the underlying prenylated proteins, and therefore is not likely to be significant.

3.4. Morphological changes on compactin treatment correlate with HMG-R inhibition

After 3 days in cytostatic concentrations of compactin, procyclic trypanosomes become rounded yet motile (comma morphology, see above). We tested the effect of these concentrations of compactin on HMG-R activity in situ in digitonin-permeabilised procyclic trypanosomes to ascertain if HMG-R inhibition was correlated with the alterations to morphology. As expected, HMG-R from normal trypanosomes is completely inhibited in the presence of compactin in vitro (Table 2), and effective inhibition was obtained at 10 μM, the lowest concentration tested, confirming that endogenously added compactin at 100 μM would be

expected to efficiently shut down HMG-R activity. In many other systems inhibition of HMG-R results in upregulation of the enzyme; therefore we tested residual enzyme activity in trypanosomes which had been exposed to 100 μM compactin for 1 and 3 days. Unexpectedly there was no activity in the cells after 1 or 3 days treatment. Thus, enzyme activity was essentially abolished in long-term compactin-treated cells. Therefore the morphological alterations are most likely a consequence of inhibition and loss of HMG-R and a corresponding lack of synthesis of the downstream products. Clearly, the loss of HMG-R activity from the treated cells indicates that the parasites are unable to overcome the compactin block by an upregulation of expression.

3.5. Ultrastructure of compactin-treated cells: a primary lesion in the digestive area of trypanosomes

The observation of abnormal morphology following prolonged incubation of procyclic *T. brucei* with compactin prompted further investigation of the effects of this compound and was studied by electron microscopy (EM). In order to find the earliest effects of HMG-R inhibition by compactin we examined a sample of trypanosomes containing normal and altered cells at concentrations subcytostatic and cytostatic and at time points before comma morphology was observed, i.e. at 50 and 100 μM compactin and 2 days. Examination of many cells allowed an assignment of the primary ultrastructural lesions. Changes were observed in the digestive-vacuolar network between the flagellar pocket and the nucleus (Fig. 6). In untreated controls, discrete digestive vacuoles, connecting membranes and multivesicular bodies were observed close to the Golgi apparatus (Fig. 6A). After 2 days at 50 μM compactin, about 50% of the cells appeared normal by EM, making these ideal conditions for determining the primary lesion affected by disrupting the isoprene biosynthetic pathway: damage to digestive vacuoles appeared with limited cytoplasmic vacuolation (Fig. 6B). In cells with more extensive damage, at 50 μM and 100 μM, developing vacuoles were frequently contiguous (Fig. 6C–D). Extensive vacuolation was associated with increasing numbers of multivesicular bodies and a loss of digestive vacuoles. At these concentrations other cell structures, including the Golgi apparatus, appeared normal (Fig. 6D). When these vesicles first appeared, lumenal contents were present, but at higher concentrations of compactin the same lesions often appeared empty. We believe this represents localised bursting of this system and surmise that the primary lesion resulting from HMG-R inhibition by compactin is this vacuolisation in the digestive region of the trypanosome between the Golgi, the nucleus and the flagellar pocket. Vacuoles were in some cases observed at or within the Golgi apparatuses at 100 μM (not shown). Pyrophosphate vesicles had an altered appearance which is also associated with other inhibitors and probably an effect associated with cell death (S.C., unpublished). We observed no changes in the mitochondrion, the nuclear structure, nor in the cytoskeleton.

Table 2
Procyclic hydroxymethylglutaryl-coenzyme A reductase is highly sensitive to compactin

Condition	Activity (pmol·mg protein^{-1}·min^{-1})	Number of determinations
Control	29.8 (4.2)	4
10 μM compactin	4.8 (0.2)	2
20 μM compactin	5.0 (0.0)	2
50 μM compactin	4.4 (0.2)	4
100 μM compactin	4.8 (0.8)	2
24 h adapted	4.4 (0.4)	2
72 h adapted	5.0 (0.4)	2
No cells added	4.8 (0.2)	3

Activity was measured in digitonin-permeabilised procyclic cells in the presence or absence of compactin [19]. Adapted cells were grown in 100 μM compactin for the indicated period of time, washed and assayed in the absence of the inhibitor. The negative control assay was with no cells added. Activity is expressed in pmol·mg cell protein^{-1}·min^{-1} and the standard deviation/error of the mean shown in parenthesis.

4. Discussion

In this report we have established that protein isoprenylation is a feature of post-translational

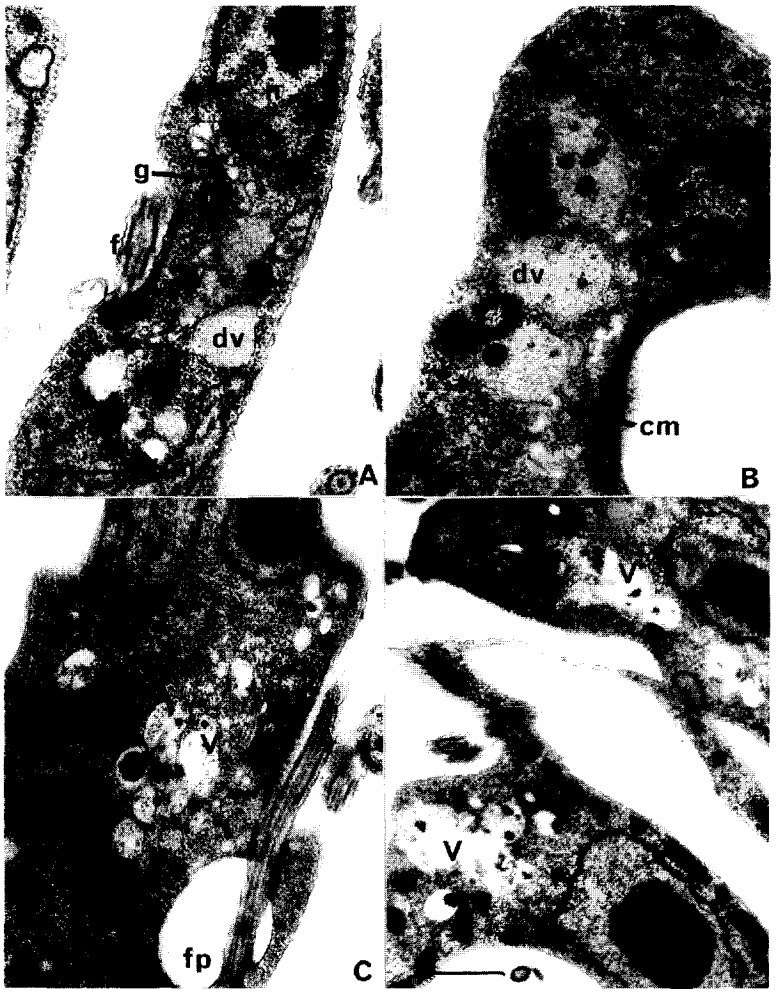

Fig. 6. Ultrastructural effects of compactin on procyclic *T. brucei*. Gallery of transmission EM images of cells treated with 50 μM or 100 μM compactin. A; Untreated *T. brucei* procyclics showing the Golgi apparatus (g), digestive vacuoles (dv) and membrane systems between the nucleus (n) and the flagellar pocket; the flagellum (f) and mitochondrion (m) are also shown (scalebar: 0.5 μm). B; Procyclics exposed to 50 μM compactin: debris is present in digestive vacuoles (dv) and there is swelling of connected collecting membranes (cm); an autophagic vacuole (a) is present (scalebar: 0.5 μm). C; Procyclics exposed to 50 μM compactin: vacuolation (v) is present in the region between the flagellar pocket (fp) and the nucleus (n), close to lipid bodies (l); multivesicular bodies (mvb) are present (scalebar: 1 mm). D; Procyclics exposed to 100 μM compactin: extensive vacuolation (v) is seen, with multivesicular bodies replacing digestive vacuoles; nuclei, mitochondria, ribosomes and other cytoplasmic structures appear normal (scalebar: 1 μm).

protein modification in the protozoan *T. brucei*. Secondly, we have demonstrated that the HMG-R inhibitor, compactin, is active against the *T. brucei* enzyme, and that prolonged exposure to the drug in vivo leads to loss of HMG-R activity, and thirdly, shown that compactin treatment results in an altered morphology and a cessation of growth in procyclic cultures. Growth inhibition was also observed for BSF cultures.

Metabolic labelling of procyclic trypanosomes with [^{3}H]-MVL allowed the detection following

SDS-PAGE, of a spectrum of polypeptides incorporating the radiolabel. That this reflected the presence of isoprenes was confirmed following release of the radiolabel and analysis by RP-HPLC. Both farnesyl and geranylgeranyl moieties were conclusively identified in a similar proportion to a mammalian cell sample. *T. brucei* isoprenylated species yielded an SDS-PAGE profile consisting of at least 14 bands. Labelled bands at ~46 kDa and ~67 kDa are present in all cell types; the latter has been identified as a lamin in mammals. Trypanosome labelling differs from mammalian and *G. lamblia* in the region of the gel ascribed to GTPases (20–30 kDa) and contains at least 8 bands [18,25]; the most prominent in *T. brucei* (23 kDa) migrates faster than the major mammalian band (25 kDa). These differences are unlikely to have major biological significance. Antisera directed against two *T. brucei* rab homologues (containing C-terminal isoprenylation signals) recognise native trypanosome proteins migrating on SDS-PAGE at 27 kDa and 29 kDa (HF and MCF, in preparation) consistent with the 20–30 kDa cluster containing small G proteins. Further study will allow identification of other trypanosome proteins in this region.

Farnesyl and geranylgeranyl are the isoprenoids linked to protein in *T. brucei*, with farnesol as the minor product, a typical eukaryotic system for protein isoprenylation indicative of the conserved and essential nature of this modification. The conservation of this arm of the MVA pathway is in marked contrast to the truncated dolichol species found in BSF trypanosomes [11–13] and other trypanosomatids. The significance of this latter peculiarity is not known, but it is possible that the functional constraints on dolichol species, which are synthesised in a range of sizes in most eukaryotes [1] are less stringent than for the protein-linked isoprenes.

To label the trypanosomes, conditions for blocking the MVA biosynthetic pathway were established for the HMG-R inhibitor compactin. Cytostasis of procyclic trypanosomes required approximately three-fold greater concentrations of compactin than did mammalian cells; this most probably reflects evolutionary distances between the respective HMG-Rs or differences in uptake of the drug by the two cells. Conversely, the cytostatic concentration of compactin for BSF *T. brucei* of the same strain (427) was ten-fold lower than for procyclics, and in this case can probably be attributed to five-fold lower activity of HMG-R in BSF compared to procyclic cells [19].

We observed that morphological changes began to appear in procyclic trypanosomes exposed to cytostatic concentrations of compactin for prolonged periods. The acquisition of a motile spheroid-with-tail cell shape (comma) correlated with compactin concentration and was a function of time of exposure, i.e. abnormal cells were present with increased frequency at higher concentrations or at later times during the experiment. This suggests that a critical point is reached, possibly depletion of an important metabolite or protein, beyond which the ability to retain normal morphology is lost. As procyclic cells will label with [^{3}H]-MVA in the absence of compactin, albeit with reduced efficiency, the MVA pool is most probably small in this organism. MVA would be rapidly depleted in the presence of compactin, thereby lowering the concentration of downstream MVA pathway products, so that a slow onset of abnormal morphology is likely to correlate with turnover of an important structural component, i.e. a protein or lipid species. This is consistent with the observation that growth inhibition caused by mevinolin, another HMG-R inhibitor, is not relieved by transformation of NIH-3T3 cells with a myristoylated ras, suggesting that inhibition of ras function cannot be solely responsible for effects on cell growth [8].

To correlate morphological alterations with compactin activity as an inhibitor of HMG-R we demonstrated that compactin abolished HMG-R activity in digitonin-permeabilised procyclic cells. In vitro, the concentration providing complete block of HMG-R activity was far lower than that required for cytostasis or morphology changes. Indeed, at 10 μM, the lowest concentration tested, there is essentially no effect on growth of procyclics, whilst HMG-R activity was abolished in the permeabilised cell assay. One clear possibility

for this discrepancy is that the true intracellular concentration may differ substantially from the concentration in the media due to uptake difficulties or active transport out of the cell. This may also partly explain the differences between the procyclic and BSF sensitivities in vivo, i.e. the ability of the two life stages to modulate $[compactin]_{in}$ are possibly not identical. At the compactin concentrations used to produce cytostasis, and consequent structural alterations, HMG-R activity would be fully inhibited.

Most interestingly, HMG-R activity in trypanosomes grown for 1 or 3 days in the presence of 100 μM compactin was undetectable. HMG-R is a multi-spanning transmembrane ER-resident protein with catalytic activity in the cytosolic C-terminus [5]. Upregulation of HMG-R by drug or genetic manipulations in mammalian cells results in elaborated ER membranes, and crystalloid ER when over expressed [27]. In *S. pombe* perinuclear karmellae are formed, and down-regulation of the HMG-R resulted in degradation of these membranes [6,7]. It is possible that HMG-R cannot be induced in *T. brucei* by depletion of MVA pathway products as in mammals and yeasts, but only down-regulation or irreversible inhibition can explain the loss of the basal activity. A likely explanation is that before achievement of cytostasis, the cells are stressed and upregulate several degradative mechanisms, including ER turnover, a common response to metabolic challenge in eukaryotes. Therefore, inhibition of MVA biosynthesis would result in depletion of downstream products and a stress response. The effect of chronic compactin exposure on the synthesis of ER or secreted proteins has not been analysed, but early appearance of interconnected digestive vacuoles (see below) is consistent with the induction of an autophagic-type ER degradation.

Investigation of morphological changes induced by compactin at the EM level revealed ultrastructural alterations after 2 days in 50–100 μM compactin. Early on in treatment, a region that may correspond to ER in the digestive area of the cell between the flagellar pocket, the Golgi apparatus and the nucleus, develops large vacuoles which appear to burst as the effects become more severe. There were no observable effects on the other major organelles, i.e. mitochondria, nucleus, Golgi or the cytoskeleton. It is possible that these changes represent some of the earliest events associated with loss of HMG-R activity. The gross morphological alterations visible by light microscopy occur later than the EM-observable changes. Thus, the digestive area vacuolisation later gives way to rounding of the procyclic cells but these two events may not be directly connected. For example, rho, a member of the ras superfamily, is involved in regulation of the cytoskeleton. Prevention of isoprenylation of rho causes major distortions of cellular surfaces [28]. Therefore, the gross alterations to cell shape may be the result of turnover of isoprenylated proteins important in maintaining overall cell architecture which cannot be replenished due to the loss of HMG-R activity, whilst the earlier effects on digestive vacuoles may be a more direct result of the inhibition of HMG-R by compactin by induction of autophagic mechanisms. Synvinolin, also an inhibitor of trypanosome HMG-R, causes small vesicles to accumulate in both the cytoplasm and the lumen of the flagellar pocket, and also results in an expanded mitochondrion and engorged ER [29]. The pattern of structural alterations are clearly distinct from those obtained with compactin suggesting subtle differences in the actions of these two compound. Further work is required to clarify the mechanism by which HMG-R activity is lost from compactin-treated procyclics and the morphological changes that follow.

The potential certainly exists for exploitation of the MVA pathway and possibly protein isoprenylation as a route to chemotherapy against this organism and other kinetoplastida. However, this and other studies [11,19,29] also indicate that a clearer understanding of the fundamental biochemistry in *T. brucei* is an important prerequisite for the success of any such strategy.

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