Acylation of adenovirus type 12 early region 1b 18-kDa protein

Further evidence for its localisation in the cell membrane

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The 18-kDa E1b protein in Ad 12-transformed rat cells and in Ad 12-infected human cells binds lipid strongly. The lipid is not removed by boiling in the presence of SDS or by extraction with methanol/chloroform. It is, however, dissociated from the protein by treatment with methanolic KOH suggesting that attachment is through an ester linkage. The acylated 18-kDa protein is detected only in the membrane fraction. Labelling cell surface proteins on Ad 12-transformed cells with [^{125}I]iodosulphanilic acid shows that some of the Ad 12 18-kDa E1b protein is present on the outside of the cell. It is concluded that this protein is responsible for cell surface T-antigen activity.

Adenovirus 12 *Ad 12 E1 protein* *Tumor antigen* *Acylation* *Post-translational modification*

1. INTRODUCTION

For the morphological changes associated with transformation to occur the transforming proteins of tumour viruses must exert considerable influence on different organelles in the host cell. It seems likely that these changes are achieved through a series of interactions of the viral proteins with specific sites in the cell. For example, a proportion of the transforming protein of Rous sarcoma virus (RSV) (pp 60 src) [1,2] and most of middle-T antigen of polyoma virus are primarily associated with plasma membranes [3], whereas simian virus 40 (SV40) large-T is mainly present in the nucleus [4]. It has recently become apparent that a number of the viral transforming proteins which are present in the membrane of the host cell have been specially modified by the attachment of lipid at specific points in the amino acid sequence [5,6].

The region of the adenovirus 12 (Ad 12) chromosome whose expression is sufficient to induce transformation, early region 1, consists of two transcriptional units: E1a and E1b [7,8]. The former codes for a family of proteins of molecular mass of about 41 kDa, as determined by SDS-polyacrylamide gel electrophoresis (SDS–PAGE), whereas the latter codes for two proteins of molecular masses 19 and 60 kDa [9,10] (equivalent to proteins of 18 and 52 kDa, respectively, in our hands). Although under certain circumstances rat cells expressing only the E1a proteins may exhibit a limited transformed phenotype, transformation is accomplished much more efficiently if the E1b proteins are present as well [11,12]. In the light of the results which showed that lipid is bound to various transforming proteins [5,6] we have investigated whether any of the tumour antigens of Ad 12 are subject to a similar post-translational modification particularly as at least some of the 18-kDa protein has been shown to be membrane-bound [13].

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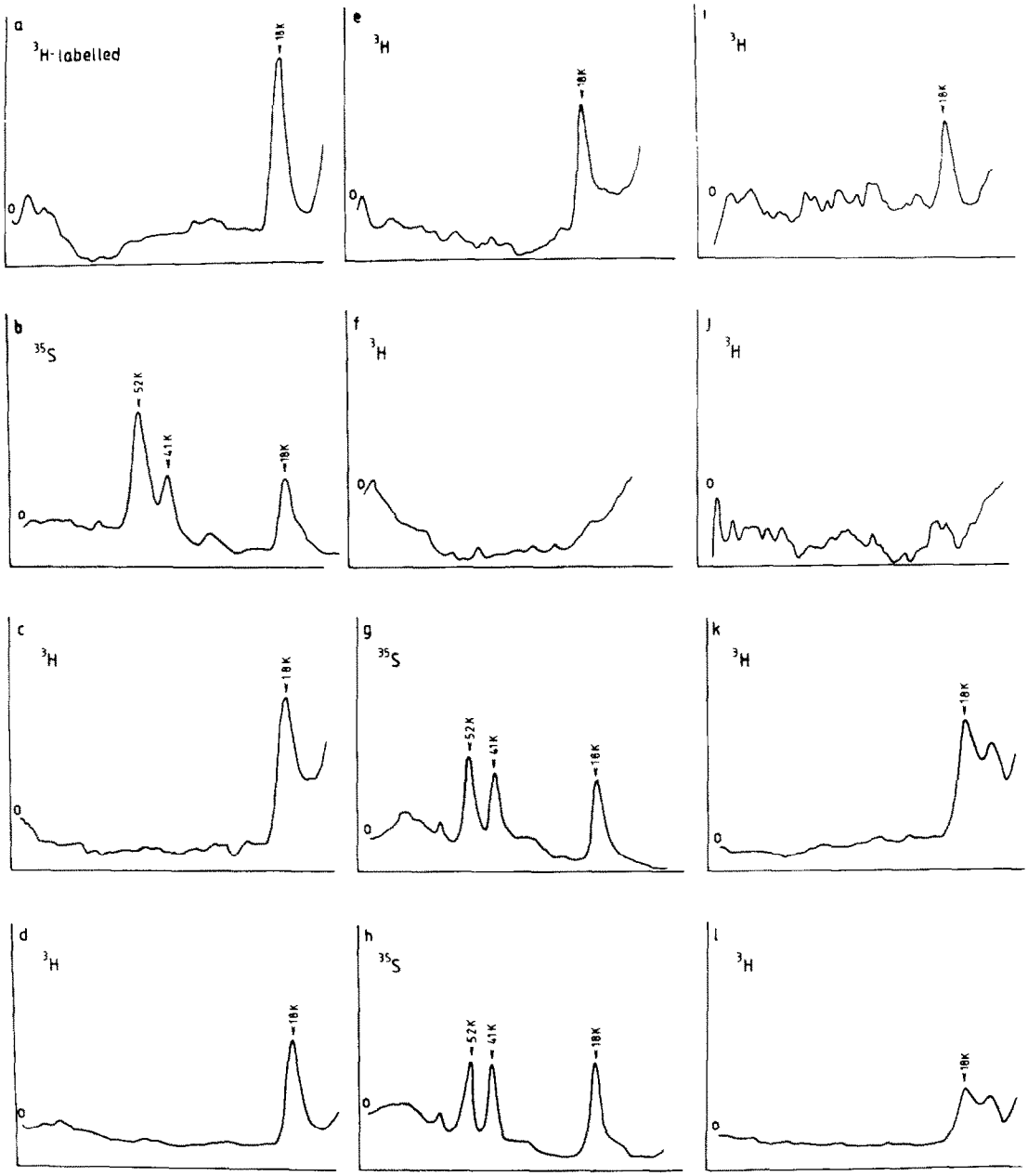
2. MATERIALS AND METHODS

A number of different cell lines were used in these experiments: Hooded Lister baby rat kidney cells (HLBRK) and Hooded Lister rat embryo brain (HLREB) cells each are transformed with the *Eco*RIC or *Hin*dIIIG fragments of Ad 12 DNA [12] (submitted), transformed rat liver cells containing the left-hand 16% of the Ad 12 genome (Ad 12/C3/100-1) [14], and human embryo kidney cells transformed with the *Eco*RIC fragment of Ad 12 DNA (Ad 12 HEK2) [15].

Transformed cells were labelled with [9,10-^{3}H(n)]palmitic acid, as described by Schmidt and Schlesinger [16] and Sefton et al. [5], for 6 h at 37°C (3 mCi/10^7 cells); [9,10-^{3}H(n)]palmitic acid



was also added to dishes of Ad 12-infected human embryo kidney cells (HEKs) 24 h post-infection and the cells harvested 30 h post-infection. Ad 12-transformed cells were also labelled with [^{35}S]methionine for 2 h in methionine-free medium. After radioactive labelling the cells were scraped off the dishes and dissolved in immunoprecipitation lysis buffer [17]. Immunoprecipitation was by the procedure of Paraskeva et al. [17] using specific Ad 12 rat tumour bearer sera (reactive only to E1a 41- and E1b 18- and 52-kDa proteins). Immunoprecipitates were dissolved in 9 M urea, 50 mM Tris–HCl (pH 7.5), 1% β-mercaptoethanol, 1% SDS (25 μl) and run on 13% polyacrylamide gels in the presence of 0.1% SDS and 0.1 M Tris, 0.1 M Bicine (pH 8.3). Radiolabelled proteins were detected by fluorography. In the case of [^{35}S]methionine-labelled proteins the films were exposed to the dried gels for 5 days, but for detection of ^{3}H-labelled proteins exposure was normal for approx. 8 weeks. Autoradiographs were scanned using an Isco gel scanner.

Membranes were prepared from Ad 12 *Hin*dIIIG HLREB cells (5 × 10 cm dishes) by homogenisation in 10% sucrose, 0.5 mM EDTA, pH 7.2 (5 ml) using a Dounce tight-fitting homogeniser (50 strokes). Nuclei were removed by centrifugation at 1000 × *g* for 5 min. The supernatant from this step was centrifuged at 100000 × *g* for 1.5 h and the precipitate taken as a crude membrane preparation.

To examine whether any of the Ad 12 E1 proteins could be labelled on the cell surfaces, rat and human cells (approx. 1 g of each) transformed with the *Eco*C fragment of Ad 12 DNA (Ad 12 *Eco*C HLBRK and Ad 12 HEK 2, respectively) were washed with saline and suspended in PBS (2.5 ml). Surface proteins were labelled with diazotised [^{125}I]iodosulphanilic acid (0.2 mCi) for 20 min at 5°C using the method of Schlager [18]. After extensive washing with PBS containing 1% BSA most of the cells were homogenised in 10% sucrose and nuclear and membrane fractions separated by differential centrifugation. A second experiment was performed using similar amounts of cells in which radiolabelling occurred after disruption and subcellular fractionation. Aliquots (50 μl) from each fraction were immunoprecipitated and the adenovirus proteins electrophoresed on polyacrylamide gels in the presence of SDS. Autoradiographs were scanned using an Isco gel scanner.

3. RESULTS AND DISCUSSION

3.1. *Labelling of Ad 12 E1b 18-kDa protein with [^{3}H]palmitic acid*

It can be seen from fig.1, scans a–d that, after

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Fig.1. Binding of [^{3}H]palmitate to Ad 12 E1b 18-kDa protein. Ad 12-transformed rat cells were grown on 10-cm Petri dishes in DME medium supplemented with 10% foetal calf serum. [9,10-^{3}H(n)]Palmitic acid (23.5 Ci/mM, New England Nuclear), which had been dried in a stream of N_2 and redissolved in DMSO (1 mCi/16 μl), was added (3.2 mCi/dish) and after 6 h the cells were washed with ice-cold saline, harvested with a rubber policeman and solubilized in immunoprecipitation lysis buffer [17]. Other dishes were also labelled with [^{35}S]methionine (800 Ci/mM, Amersham International) in methionine-free medium (200 μCi/dish); 13% polyacrylamide gels were run in the presence of 0.1% SDS, 0.1 M Tris, 0.1 M Bicine (pH 8.3). Autoradiographs were subjected to densitometric scanning and the Ad 12 tumour antigens are indicated by arrows. 0 indicates the origin. No proteins were immunoprecipitated with non-immune rat serum, and no proteins were immunoprecipitated from mock-infected HEKs. Scans a–d, immunoprecipitation of ^{3}H-labelled (a, c, d) and ^{35}S-labelled (b) cells. Scans a, b, Ad 12-transformed rat liver cells (Ad 12/C3/100-1 cells); scan c, Ad 12-infected (50 pfu/cell) HEKs; scan d, Ad 12 *Hin*dIIIG HLREB-transformed cells. Scans e–h, effect of methanolic KOH on ^{3}H- and ^{35}S-labelled Ad 12 proteins. Ad 12-transformed rat liver cells (Ad 12/C3/100-1) were labelled with [^{3}H]palmitate (e, f) or [^{35}S]methionine (g, h) and immunoprecipitated with rat tumour bearer serum. Scans f and h show the results obtained after incubation of the immunoprecipitates in 0.1 M KOH for 20 min at 23°C. The immunoprecipitates shown in scans e and g were untreated. Scans i and j, effects of trypsin on ^{3}H-labelled Ad 12 18-kDa E1b protein. Membranes prepared from Ad 12 *Hin*dIIIG HLREB cells (see text) were treated with trypsin (20 μg for1 h at 30°C) before solubilisation and immunoprecipitation. Scan i, untreated; scan j, treated with trypsin. Scans k and l, effect of tunicamycin on the incorporation of [^{3}H]palmitate into Ad 12-infected HEKs. Tunicamycin (2 μg/ml) was added to Ad 12-infected HEKs at the same time as the [^{3}H]palmitate. Scan k, untreated cells; scan l, treated with tunicamycin.

labelling with [^{3}H]palmitic acid, radioactivity was incorporated into the Ad 12 18-kDa E1b protein in both Ad 12-transformed rat cells and Ad 12-infected human embryo kidney cells. A number of different rat cell lines were used in this series of experiments and in all cases the 18 kDa E1b protein was acylated. Similar results were obtained for baby rat kidney cells and rat embryo brain cells transformed with the *Eco*C and *Hin*dIIIG fragments of Ad 12 DNA and rat liver epithelial cells (Ad 12/C3/100-1 cells). There seems little doubt that the lipid moiety was covalently bound to the Ad 12 18 kDa protein since boiling for 5 min in 1% SDS or repeated extraction of the immunoprecipitates with a mixture of chloroform and methanol (2:1, v/v, 1 ml) failed to remove any of the radiolabel although the latter procedure did reduce the radioactivity visible at the bottom of the gel which was probably due to unbound lipid, non-specifically absorbed either onto the proteins or onto the *Staphylococcus aureus* used for immunoprecipitation.

3.2. *Treatment of [^{3}H]palmitate labelled Ad 12 E1b 18-kDa protein with methanolic KOH*

Schmidt et al. [19] originally showed that labelled lipid could be removed from Sindbis virus glycoproteins by treatment with methanolic KOH. Therefore, immunoprecipitates from Ad 12-transformed cells labelled with [^{3}H]palmitate were incubated in 0.1 M KOH in methanol at 23°C for 20 min and then washed with chloroform/methanol before SDS–PAGE (fig.1, scans e–h). This resulted in the removal of ^{3}H radiolabel from the 18-kDa protein (scan f) although no difference could be seen in samples labelled with [^{35}S]methionine after KOH treatment (scans g,h). It is therefore likely that the lipid is attached to a hydroxyl residue (e.g., serine, threonine or tyrosine) on the viral protein through an ester linkage as appears to be the case for Sindbis virus glycoprotein [19] and Harvey Sarcoma virus p21 [5]. Sefton et al. [5] found, however, that the lipid bound to pp60 from RSV was not labile under these conditions and they concluded that in that case the lipid-protein linkage was possibly through an amide bond. Whether such differences in lipid binding are indicative of a different distribution of viral proteins in the membrane (e.g., whether it is located on one side only or penetrates right through) is not yet clear.

3.3. *Subcellular distribution of [^{3}H]palmitate-labelled Ad 12 E1b 18-kDa protein*

A crude membrane preparation was made from Ad 12 *Hin*dIIIG HLREB cells as described in section 2. Immunoprecipitation confirmed that appreciable amounts of the acylated Ad 12 18-kDa E1b protein were present in this fraction but not in the cytosol (not shown). If the membranes were treated with trypsin (20 μg for 1 h at 30°C in 0.1 M NH_4HCO_3, pH 7.9) before immunoprecipitation, no radiolabelled 18-kDa protein could be detected (fig.1, scan j). It is tentatively suggested therefore that part of the protein protrudes out of the membrane, either on the inside or outside, and when this is digested by the protease the radiolabelled residue(s) is (are) lost. Alternatively it is possible that a relatively large proportion of viral protein is not incorporated in the membrane and is therefore sensitive to proteolysis in which case immunoprecipitation would not necessarily result in the purification of the surviving acylated peptide due to loss of the antigenic site.

3.4. *Effect of tunicamycin on [^{3}H]palmitate labelling*

Many of the viral structural proteins which have been shown to be acylated are glycoproteins (review [20]). However, it is apparent from labelling experiments using [^{3}H]glucosamine added to Ad 12-transformed rat cells that neither the Ad 12 18-kDa E1b protein nor the 41- or 52-kDa E1 proteins are glycosylated (not shown). This result is in agreement with the findings of Persson et al. [21] who could not detect any glycosylation of the Ad 2 15-kDa membrane protein (equivalent to the Ad 12 18-kDa).

Tunicamycin is known to be a potent inhibitor of protein glycosylation, but has also been shown to inhibit the acylation of the G glycoprotein of a *ts* mutant of vesicular stomatitis virus under certain conditions [16]. We have found, however, that the addition of tunicamycin (2 μg/ml) to Ad 12-transformed rat cells or to Ad 12-infected HEKs at the same time as the addition of [^{3}H]palmitate, did not abolish binding of the lipid to the 18-kDa protein although the radioactivity was somewhat reduced in the infected cells (fig.1, scans k and l). Whether this indicates a different pathway for acylation in the VSV G protein at the non-permissive temperature and the Ad 12 18-kDa

protein is not clear at present although in both cases the lipid is probably attached to the protein through an ester linkage (i.e., it is labile in methanolic KOH). It seems probable however that in the case of VSV G protein, acylation cannot be considered separately from glycosylation and the inhibition of the latter process may affect the former.

It can be seen that when cells infected with Ad 12 were labelled with [^{3}H]palmitate a minor radioactive band of about 15 kDa could be detected (fig.1, lanes c, k and l). No comparable

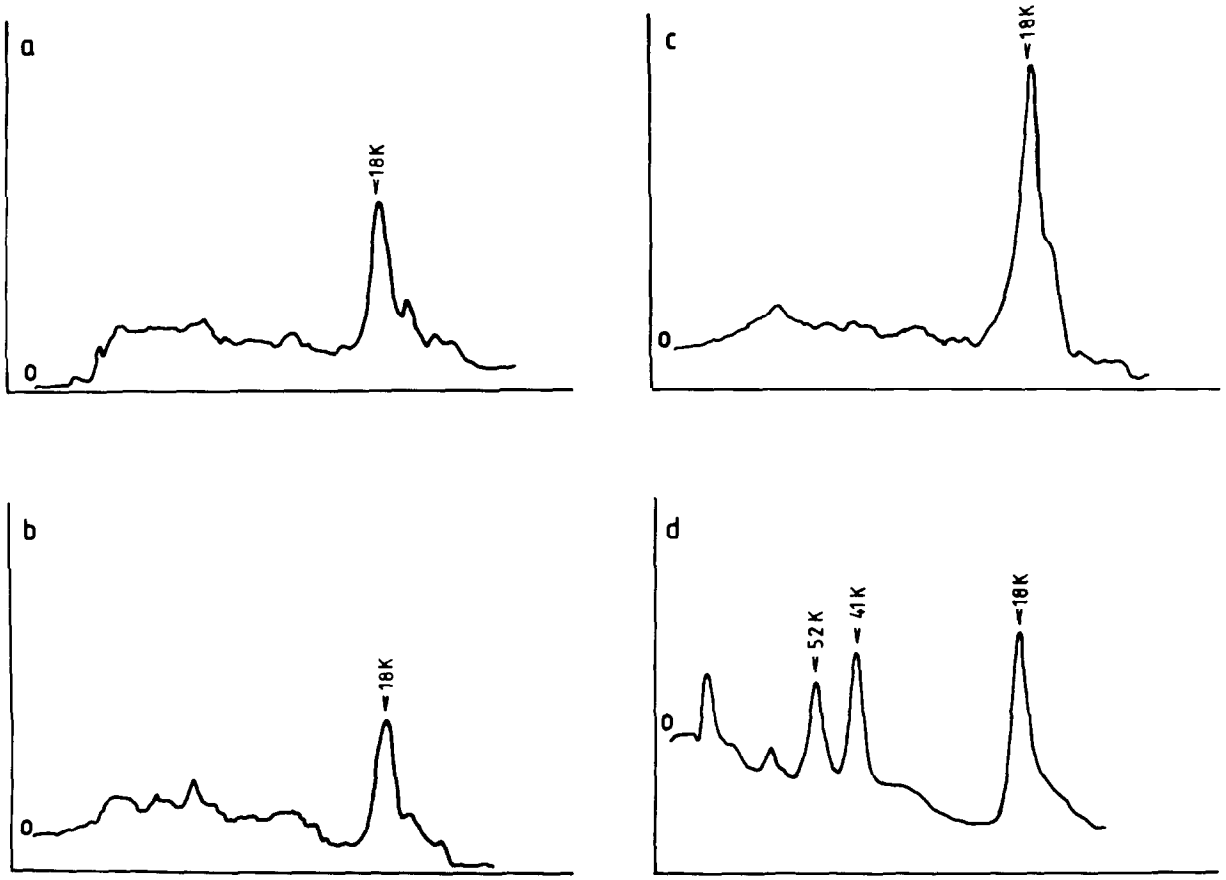

Fig.2. Surface labelling of Ad 12 HEK 2 cells with [^{125}I]iodosulphanilic acid. Ad 12 HEK2 cells (1 g) were suspended in 2.5 ml PBS and labelled with [^{125}I]iodosulphanilic acid. After washing the cells were homogenised in 5 ml of buffered 10% sucrose and fractionated into nuclear, membrane and cytosolic fractions by differential centrifugation. The nuclear and membrane fractions were resuspended in the immunoprecipitation extraction buffer (0.5 ml) used by Paraskeva et al. [17]. The cytosolic fraction was diluted 1 : 1 with the same buffer. A similar sample of cells was fractionated in the same way and then subcellular fractions were labelled with [^{125}I]iodosulphanilic acid. After labelling these nuclear and membrane fractions were washed in PBS. Cells were also disrupted and the whole cell extract labelled. The Ad 12 E1 proteins were immunoprecipitated using rat tumour bearer sera and run on 13% polyacrylamide gels. Scan a, immunoprecipitation of whole Ad 12 HEK2 cells labelled with [^{125}I]iodosulphanilic acid; scan b, immunoprecipitation of the membrane fraction from Ad 12 HEK2 cells labelled with iodosulphanilic acid before subcellular fractionation; scan c, immunoprecipitation of membrane fraction labelled with [^{125}I]iodosulphanilic acid after subcellular fractionation; scan d, immunoprecipitation of a whole cell extract labelled after disruption. No labelled proteins were immunoprecipitated from the cytosolic fraction labelled before subcellular fractionation. No proteins were immunoprecipitated with non-immune rat serum. Similar results to those presented here were obtained when the experiment was repeated with Ad 12 *EcoC* HLBRK cells. 0 indicates the origin.

protein was labelled in Ad 12-transformed cells. Since this protein is immunoprecipitated with tumour bearer serum of known specificity it must be encoded by Ad 12 E1 DNA but, at present, its relationship to the other, well characterised Ad 12 E1a (41 kDa) and E1b (18 and 52 kDa) proteins is not clear.

3.5. *Surface labelling of Ad 12 E1b 18-kDa protein*

It was found for both the Ad 12-transformed human and rat cell lines (Ad 12 HEK2 and Ad 12 *Eco* HLBRK) that the major iodinated protein, observed after surface labelling and immunoprecipitation with tumour bearer sera, was the Ad 12 18-kDa E1b protein. This was detected in the whole cell extract and the membrane fraction but not in the cytosol (fig.2). When the membrane fraction was iodinated after fractionation rather more radioactivity was incorporated into the 18-kDa protein than when whole cells were treated (cf. fig.2, scans a–c). When the whole cell homogenates and the subcellular fractions were radiolabelled after fractionation the three Ad 12 E1 proteins were iodinated (fig.2, scan d). It has been shown that most of the 52- and 41-kDa proteins are present in the cytoplasmic fraction with rather less in the nuclear fraction, and the majority of the 18-kDa protein in the membrane fraction [13].

A number of workers have now shown that the smaller E1b protein of adenovirus (15 kDa in Ad 2, 18–19 kDa in Ad 5 and 18 kDa in Ad 12) is mainly associated with a crude membrane fraction in transformed and infected cells [13,21,22]. The results presented here confirm this finding, although we have been able to detect only a single E1b component of about 18 kDa. This contrasts with the results of Rowe et al. [22] who found that after Ad 5 infection two related E1b proteins of 18.5 and 19 kDa were produced in equal amounts and both were membrane-bound. Reasons for this discrepancy are not clear although it might be indicative of group differences between Ad 12 and Ad 5. However, a number of workers [9,23] have demonstrated two E1b proteins of 17–19 kDa in Ad 12 in vitro translation studies. Here we have observed that on certain occasions after Ad 12 infection of HEKs a second minor band of about 15 kDa is labelled with [^{3}H]palmitate (fig.1, scans k, l).

In the results presented here we have demonstrated for the first time that the Ad 12 18 kDa protein has lipid covalently bound to it through an ester linkage. Although the function of acylation is not yet clear it is probable that it represents a post-translational modification required to anchor the protein in the plasma membrane. The 18-kDa protein is not totally enclosed in the cell membrane, but extends through to the outside of the cells as shown by the iodination experiments in which only surface proteins are labelled. As none of the other Ad 12 E1 proteins are detectable on the surface of transformed cells, or are even present within the membrane, it is reasonable to suppose that the 18-kDa protein is responsible for cell surface T-antigen activity in Ad 12-transformed cells. This result confirms the findings of Fohring et al. [24] who came to a similar conclusion on the basis of a study of cytolytic T cell killing of a number of different Ad 12-transformed rat cell lines. These workers found that the only Ad 12 E1 protein shared by all cell lines killed in cytolytic assays, is the 18-kDa E1b polypeptide.

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