Biosynthesis and glycosylation of the carcinoembryonic antigen

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Human gastric adenocarcinoma MKN-45 cells were found to synthesize actively carcinoembryonic antigen (CEA). The biosynthesis and carbohydrate processing of CEA were studied in these cells by means of metabolic labelling followed by immunoabsorption with a specific polyclonal-antibody preparation and gel electrophoresis. Pulse-chase studies with [^{14}C]leucine and [^3H]mannose (shortest pulse 3 min) showed that *N*-linked oligosaccharide side chains are added to the protein co-translationally, producing a high-mannose immature CEA; the average molecular mass of this form is 145 kDa. The protein is later translocated to the Golgi apparatus and here undergoes additional processing; these modifications are visible in our system as a broadening of the CEA band and require about 4 h. The upper limit of mature CEA band reaches 200 kDa, but radioactivity is maximally incorporated at 168 kDa. The extent of co-translational glycosylation was measured by treating the cells with tunicamycin; in the presence of this inhibitor, a 74 kDa aglyco-CEA was produced and was still recognized by the antibody. Monensin, an ionophore which interferes with glycoprotein maturation and terminal sugar addition, blocked broadening of the CEA band, producing a sharp 141 kDa peak. In conclusion, CEA appears to be synthesized as a 145 kDa high-mannose immature form, the protein core accounting for about half of its molecular mass. Full maturation results in a broad band at 168 kDa.

INTRODUCTION

Carcinoembryonic antigen (CEA) is an oncofetal glycoprotein first identified by Gold & Freedman (1965a) as a tumour-associated antigen present in the cellular membrane of colonic adenocarcinomas. CEA was also found to be normally expressed in the fetal digestive tissue between 2 and 6 months of gestation (Gold & Freedman, 1965b) and is now known to appear in several types of human adenocarcinomas from endodermally derived epithelia (Marchand *et al.*, 1975; Shonsha & Lyssiotis, 1978; Waalkes *et al.*, 1980), with a most significant increase in tumours of gastrointestinal tract (Moore *et al.*, 1971). CEA was chemically characterized after HClO_4 extraction of liver metastases from primary colon carcinoma (Krupey *et al.*, 1968; Chandrasekaran *et al.*, 1983); it has an M_r of approx. 180 000 and appears as a diffuse band on SDS/PAGE. It is formed by a single polypeptide with 28 possible *N*-linked oligosaccharide chains, as indicated by the putative amino acid structure derived from a cDNA sequence (Oikawa *et al.*, 1987) and confirmed by structural studies (Yamashita *et al.*, 1987). These carbohydrates amount to 50–60% of the molecule (Chandrasekaran *et al.*, 1983; Laurence *et al.*, 1975; Terry *et al.*, 1972) and exhibit a certain extent of heterogeneity in different preparations, particularly regarding the number of fucose and sialic acid residues. Isoelectric focusing of purified CEA shows multiple species with acidic pI values (Coligan *et al.*, 1973), suggesting differences in sialylation, and CEA heterogeneity has also been described with regard to lectin affinity (Rogers *et al.*, 1974).

The presence of a limited number (five to nine;

Shively, 1984; Haggarty *et al.*, 1986) of CEA-associated antigenic determinants has been established by extensive studies carried out using specific monoclonal antibodies. These epitopes appear to be formed by the protein chain and are under intensive investigation because of CEA immunological cross-reactivity with a number of related antigens (Grunert *et al.*, 1985; Neumaier *et al.*, 1985). The purpose of the present study was to characterize some of the steps of biosynthesis and processing of the oncofetal protein CEA, using pulse-chase labelling studies and specific inhibitors of glycosylation and glycoprotein transport. The gastric adenocarcinoma MKN-45 cells were employed as active producers of CEA, and a specific immunoabsorbent was prepared for the identification of the antigen.

MATERIALS AND METHODS

Materials

L-[3,4,5- ^3H (n)]Leucine (150 Ci/mmol), D-[2- ^3H (n)]mannose (25 Ci/mmol), L-[U- ^{14}C]leucine (300 Ci/mmol) and L-[U- ^{14}C]valine (250 mCi/mmol) were from New England Nuclear (NEN). Tunicamycin and monensin (sodium salt) were purchased from Sigma. Proteinase inhibitors were obtained as follows: aprotinin and phenylmethanesulphonyl fluoride were from Boehringer; leupeptin was from Fluka. Media and fetal-calf serum for the cell culture were purchased respectively from Flow Laboratories and Biochrom KG (Berlin, Germany).

Cell culture

Human gastric adenocarcinoma MKN-45 cells (a gift

Abbreviations used: CEA, carcinoembryonic antigen; PAGE, polyacrylamide-gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline (0.008 M-phosphate/0.13 M-NaCl, pH 7.4); e.l.i.s.a., enzyme-linked immunoabsorbent assay.

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from Dr. T. Motoyama and Dr. K. Aizawa, Niigata University, Niigata, Japan) were grown on 75 cm² Petri dishes in the presence of DMEM (5 ml/dish) containing 10% (v/v) fetal-calf serum and 2 mM-L-glutamine. Approx. 15×10^4 cells were seeded in each plate and were utilized for the metabolic labelling experiments when the cells reached 80% confluency.

Preliminary studies were carried out to test the effect of different concentrations of tunicamycin on protein synthesis and mannose incorporation. We observed that tunicamycin, at a concentration of 2 µg/ml, inhibited total cellular protein synthesis by 10–15%, but prevented the incorporation of mannose into glycoproteins by 80–90%. Tunicamycin stock solution (1 mg/ml) was freshly prepared in 10 mM-NaOH. The protein contents of the samples were determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard, and measurement of radioactivity performed after neutralization of the material; the results were expressed as c.p.m./mg of protein.

Control experiments on the effect of monensin were performed at 1 µM concentration of the drug; a 1 mM stock solution in DMSO was freshly prepared each time, and incubations were carried out for 14–48 h at 37 °C. Viability was tested by Trypan Blue exclusion.

Metabolic labelling of MKN-45-cell CEA and cell solubilization

Cell monolayers were rinsed three times with 5 ml of PBS at 37 °C and then covered with 5 ml of serum-free DMEM containing 2 mM-L-glutamine. A preincubation was carried out for 3 h at 37 °C, followed by two quick rinses with PBS; cells were then pulsed in 5 ml of serum-free, leucine-(or valine-)free DMEM supplemented with 2 mM-L-glutamine, 40 µM-leucine (or 20 µM-valine) and 50–200 µCi of either [³H]leucine (or [¹⁴C]-valine) or 50–200 µCi of [³H]mannose, plus an equivalent amount of [¹⁴C]leucine, per dish. Labelling was performed at 37 °C for different periods and terminated by three washes with cold PBS and solubilization of the cells, or followed by a period of 'chase'. For studies involving a chase, radioactivity incorporation by the cell monolayers was stopped by replacing the labelling medium with 5 ml of serum-free complete DMEM with 2 mM-L-glutamine/dish after three rinses with this same buffer. The chase was allowed to proceed for the scheduled time, then the cells were finally washed with cold PBS and scraped off into 1 ml of extraction buffer [50 mM-Hepes (pH 7.4)/1% (v/v) Triton X-100/leupeptin (20 µg/ml)/aprotinin (10 µg/ml)/0.1 mM-phenylmethanesulphonyl fluoride].

When included in the experimental protocol, tunicamycin (2 µg/ml) or monensin (1 µM) was added in the medium for a 3 h preincubation period, and this concentration of inhibitor was maintained throughout pulse and chase periods. In these experiments the absence of serum did not influence our results.

Preparation of an immunoaffinity resin using anti-CEA Ig

Anti-CEA immunoresponse was raised in 3 kg albino rabbits and a goat by subcutaneous injections of purified CEA. The first injection was carried out with the antigen emulsified with an equal volume of Freund's complete

adjuvant and all the others with Freund's incomplete adjuvant. At the end of the immunization period the Ig fraction was partially purified (Garvey *et al.*, 1977) and redissolved in about one half of the initial serum volume to give a protein concentration of 25–50 mg/ml. Control Ig was isolated from preimmune animals by the same procedure.

The resin for immunoabsorption was prepared by covalent linkage of the Ig fraction with CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Coupling efficiency, as shown by protein determination, was around 90%, resulting in a final ratio of 4 mg of Ig protein/ml of packed resin; the preparations were stored in PBS/0.05% NaN₃ at 4 °C.

Immunoabsorption of labelled CEA

Cell extracts were clarified by centrifugation at 40000 g for 30 min, and the supernatants were used for affinity adsorption on anti-CEA Ig-Sepharose. Usually each sample was derived from one dish of cells. The clear extracts were first incubated with control Ig-Sepharose (100 µl of packed resin/sample) for 60 min at 22 °C (end-over-end mixing). This resin and anti-CEA Ig-Sepharose had been washed before use in 50 mM-Hepes (pH 7.4)/0.15 M-NaCl/0.1 mM-phenylmethanesulphonyl fluoride. At the end of this first incubation the samples were separated by centrifugation from the resin and transferred to new tubes, each containing 100 µl of packed anti-CEA Ig-Sepharose. The tubes were placed on an end-over-end mixer for 16 h at 4 °C; extensive washing of the resin pellets was then accomplished by using 3 × 10 ml of cold 50 mM-Hepes (pH 7.4)/0.2% (v/v) Triton X-100/1 M-NaCl, and 3 × 10 ml of cold 50 mM-Hepes, pH 7.4. Finally the resin pellets were added directly to modified Laemmli (1970) sample buffer for electrophoresis (100 µl/sample): 63 mM-Tris (pH 6.8)/50 mM-dithiothreitol/5% (w/v) SDS/0.2% Bromophenol Blue/10% (w/v) glycerol, and boiled for 10 min before being loaded on to the gel. Usually each sample was divided into two halves, which were run on parallel gels (one for fluorography and one to be sliced and counted for radioactivity).

Gel electrophoresis and fluorography

The samples were applied on top of 5–16% or 5–10%-(v/v)-polyacrylamide gradient slab gels [75:2 (w/w) acrylamide/bisacrylamide ratio]. The high-*M_r* calibration kit of Bio-Rad and a purified preparation of neurofilaments (a gift from Dr. Massimo Bugnoli of this Research Center) was used as standards. The electrophoretic separation was carried out in the presence of 0.1% (w/v) SDS as described by Laemmli (1970), and the portion containing the standard cut from the gel and stained with Coomassie Blue. When used for fluorography, the gels were treated with En³Hance (NEN) according to the method accompanying the product, sealed in plastic foil without drying, and exposed to Curix RPI X-ray film (Agfa) with Curix MR 800 (Agfa) enhancing screens at –40 °C. In other experiments, radioactivity was measured by cutting each gel lane into 2 mm slices. Extraction of the labelled proteins took place in 10 ml of 3% (v/v) Protosol in Econofluor (both NEN products) at 37 °C for 16 h. Samples were counted for radioactivity, after the addition of an extra 10 ml of

Fig. 1. Specificity of the immunoabsorbent

Cells were pulsed with [^{14}C]valine for 14 h; the profile of labelled protein distribution on a 5–10% gradient gel is shown after extraction and immunoabsorption to anti-CEA Ig–Sepharose. The positions of molecular mass (in kDa) markers are indicated.

Econofluor, on an LS 7500 Beckman liquid-scintillation counter. When double labelling had been performed, the values for ^3H were corrected for a 9% interference of ^{14}C in that channel.

RESULTS

Identification of a CEA-producing cell line and characterization of the antibody

Human gastric adenocarcinoma MKN-45 cells were chosen as the experimental model for this study because of the particularly high expression of CEA in these cells when compared with a number of other tumour-cell lines screened in our laboratory. The antigen was identified by indirect immunofluorescence assay and appeared to be evenly distributed in association with the plasma-membrane outer structure. Permeabilization of this membrane before immunoreaction also allows the staining of an abundant pool of cytoplasmic CEA (C. Mottola-Hartshorn & P. Lorenzoni, unpublished work).

The immunoabsorbent system used in our studies was able to capture approx. 200 μg of CEA equivalent when compared with the Abbott CEA-EIA monoclonal test. By using this test we found that the MKN-45 cells produced 7 μg of CEA/mg of total cellular protein. As indicated in the Materials and methods section, we measured the biosynthesis of CEA in 100 mm-diameter plates, and, since each plate contained on average less than 5 mg of total cell protein, we conclude that all the CEA present would have been adsorbed.

The specificity of the rabbit solid-phase immuno-

Fig. 2. Identification of CEA precursor

Incorporation of [^{14}C]leucine in the cells was allowed to proceed for 3 min (O) or 3 min, followed by 7 min of chase (●); results were analysed on a 5–16%-polyacrylamide gradient gel. Molecular masses (in kDa) calculated by the linear-regression method are indicated.

adsorbent system is illustrated by the profile of radioactivity distribution in a polyacrylamide gradient gel (Fig. 1). A single main peak was found, migrating at the expected molecular mass for CEA; no similar radioactivity peak was adsorbed when pre-immune serum was used.

The partially purified rabbit antiserum in an e.i.s.a. was capable of recognizing the CEA-like material present in meconium and only weakly, or not at all, the CEA-like material present in human bile, lung and spleen extracts. More recently, using a highly specific anti-CEA monoclonal antibody, we have found that the cell line used in these studies does not synthesize appreciable amounts of any of the CEA-like material (results not shown).

Biosynthesis labelling of CEA and its precursor in MKN-45 cells

Synthesis *de novo* of CEA was determined by metabolic incorporation of [^{14}C]leucine and [^3H]mannose.

The results presented in Fig. 2 were obtained by labelling the cells for 3 min; this short pulse time allows a very limited incorporation of radioactivity into the protein, but a single small peak, migrating in the upper half of the gel, is specifically recognized by anti-CEA Ig–Sepharose and becomes clearly visible after 7 min of chase. The average M_r of this early CEA form (that we will call 'immature') was calculated to be $145\,000 \pm 2\,500$ (mean $\pm$ S.E.M.) for seven experiments including 29 determinations. When the labelling was carried out for 10 min or less, [^3H]mannose could not be recovered above background levels in association with the immature CEA

Fig. 3. Time course of CEA processing as it appears on a fluorogram

Double labelling of the cells was performed with [^3H]-mannose and [^{14}C]leucine, and proteins were separated by SDS/PAGE on a 5–16 % polyacrylamide gradient gel. (a) 60 min pulse; (b) 30 min pulse. Chase was carried out for the times indicated in the Figure.

band, possibly because of the low (in respect of the glycoprotein composition) specific radioactivity of the [^3H]monosaccharide employed.

The 145 kDa form of CEA appeared to be stable for several hours. Labelling for either 30 min, followed by 30 min of chase, or 60 min, followed by 60 min of chase, still produces the same single band on fluorogram (Figure 3, lanes 1 and 2 in *b* and *a* respectively). Radioactivity incorporation during the previous experiment was quantified by gel slicing and counting of ^3H and ^{14}C radioactivity on separate channels. The profiles show that mannose is now clearly present in the precursor peak, starting from 30 min of pulse. Maturation of the CEA molecule resulted in a broadening of the 145 kDa band, with a shift of its upper limit toward a higher molecular mass; this effect could first be discerned after 1 h of labelling and 3 h of chase [Fig. 3, lane 3 in *a*; also visible for a longer chase time (lane 3 in *b*)]. The mature form of CEA, average M_r 168 000 $\pm$ 3500 (mean $\pm$ S.E.M. for nine experiments including 35 determinations), could be detected after either a 14 h chase (results not shown) or a 14 h labelling period (see Fig. 1). Prolonging the chase or the labelling period did not modify these results. Furthermore, the small amount of CEA which was released by the cells in the culture medium appeared to have the same molecular mass as that associated with the cells (C. Mottola-Hartshorn & P. Lorenzoni, unpublished work).

Synthesis of aglyco-CEA in the presence of tunicamycin

In order to gain further insight into the biosynthetic pathway of CEA, we investigated the effect of tunicamycin, which is known to block the addition of *N*-linked oligosaccharide chains to polypeptides undergoing translation, leading to the synthesis, when *O*-linked carbohydrates are absent, of the totally deglycosylated protein core of glycoproteins (Elbein, 1981).

Fig. 4. Synthesis of aglyco-CEA in the presence of tunicamycin

After a 3 h preincubation, cell protein was labelled with [^3H]leucine for 60 min in the presence (●) or in the absence (○) of tunicamycin (2 $\mu\text{g}/\text{ml}$), as explained in detail in the Materials and methods section. Molecular masses (in kDa), calculated with the linear-regression method, are shown. Electrophoresis was performed on a 5–16 % polyacrylamide gradient gel.

When cells were preincubated with the inhibitor for 3 h and then pulsed with [^3H]leucine for 1 h, always in the presence of tunicamycin, a peak of 74 $\pm$ 3 kDa (average $\pm$ S.E.M. for three experiments) was specifically immunoadsorbed to the resin (Fig. 4 shows the results of a typical experiment). This peak is absent in the control (in this case represented by immature CEA, owing to the rather short pulse time chosen to avoid toxic side effects of tunicamycin) and, as seen in a parallel experiment, does not incorporate mannose (results not shown). From these observations, and knowing that CEA is probably devoid of *O*-linked carbohydrates (Chandrasekaran *et al.*, 1983; Yamashita *et al.*, 1987), we assume that the 74 kDa form represents aglyco-CEA.

Effect of monensin on CEA processing

Monensin is a lipophilic molecule that binds Na^+ , K^+ and H^+ ; it can therefore redistribute charges across biological membranes, perturbing the intracellular vesicular traffic. One of the effects of monensin is to produce swelling of Golgi cisternae, interfering with the transport of immature proteins from endoplasmic reticulum to the

Fig. 5. Accumulation of partially processed forms of CEA in the presence of monensin

Fluorograms of the monensin-induced 141 kDa form are shown. After a preincubation of 3 h, as described in the Materials and methods section, cells were labelled for either 1 h ($[^3\text{H}]$ leucine) or 14 h ($[^{14}\text{C}]$ leucine and $[^3\text{H}]$ -mannose), with or without $1\text{ }\mu\text{M}$ -monensin, as indicated in the Figure.

Golgi complex (Tartakoff, 1983). Glycoproteins synthesized in the presence of this ionophore are only partially processed and lack terminal sugars.

Monensin was employed in our study at a concentration of $1\text{ }\mu\text{M}$; this dosage of the drug produces only reversible effects (Tartakoff, 1983), and appropriate controls showed that it did not diminish cell viability. The MKN-45 cells were labelled for 1 and 14 h with $[^{14}\text{C}]$ leucine in the presence or absence of the drug. The results are presented in Fig. 5. After the short labelling period, no apparent difference in the average molecular mass of CEA was evident between that synthesized by the control cells and that synthesized by monensin-treated cells. However, after 14 h of labelling, the CEA of the control cells had progressed through the expected maturation steps, whereas that synthesized by the ionophore-treated cells failed to go through the final processing. The same results were obtained when the cells were labelled with $[^3\text{H}]$ mannose (C. Mottola-Hartshorn & P. Lorenzini, unpublished work).

DISCUSSION

The present results permit the elucidation of some steps in the biosynthetic pathway of CEA, an important tumour marker. This antigen, characterized in human carcinoma, has also been demonstrated to be present in some cultured tumour-cell lines (Alderman *et al.*, 1985; Beauchemin *et al.*, 1987; Philben *et al.*, 1986; Tsao & Kim, 1978); we describe here a study on MKN-45, a CEA-producer-cell type from human gastric adenocarcinoma.

In order to monitor the metabolic synthesis of CEA in MKN-45 cells we have developed an immunoadsorption assay that meets the necessary requirements of sensitivity and specificity towards the radioactively labelled glycoprotein. The characteristic diffuse and poorly defined broad band produced by the antigen on electrophoretic gels (Tsao & Kim, 1978; Grunert *et al.*, 1985; Neumaier *et al.*, 1985; Miura & Kim, 1986) has presented problems in the identification of changes occurring in its molecular structure. For this reason, we have adopted the parallel use of autoradiography (to reveal radioactivity visually) and gel slicing (for precise radioactivity quantification) and the results were confirmed, when possible, by immunoblotting.

Our experiments provide evidence for the presence of

an early form of CEA, termed 'immature', which is recovered immediately after translation. In fact, a single peak of about 145 kDa is the first species recognized by our immunoadsorbent after radioactivity incorporation into the cell proteins had commenced. Both leucine and mannose are detected in the precursor molecule, confirming the hypothesis of a co-translational addition to the nascent protein of high-mannose *N*-linked oligosaccharide chains (Hubbard & Ivatt, 1981). The molecular mass of the immature CEA appears considerably high when compared with the 74 kDa size of aglyco-CEA, as it is synthesized in the presence of tunicamycin. This result is also consistent with preliminary studies, concomitant with ours, communicated by another group (Todderud *et al.*, 1986) and with a work reporting the presence of a 70 kDa band as the smallest species derived from tunicamycin treatment of CEA during synthesis (Miura & Kim, 1986).

Understanding of the biochemical events involved in the formation of CEA may be very useful in order to clarify problems connected with the heterogeneity of this molecule, its homology with a number of related antigens and, most importantly, its relevance in perturbing the surface properties of transformed cells (Oikawa *et al.*, 1987). The observations described here should be helpful in the development of future studies involving the localization of the intracellular itinerary of CEA and its response to different inhibitors.

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