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MESSENGER RNA FOR A PHORBOL-ESTER INDUCED 48,000 DALTON PROTEIN FROM HUMAN MELANOMA CELLS

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Messenger RNA of the phorbol ester-induced 48kDa protein from human melanoma cells (Bowes) was isolated, characterized and used to study the protein processing. The 48kDa mRNA is induced simultaneously with that of tissue-type plasminogen activator. This induction is prominent as shown by sedimentation profiles on linear sucrose gradients. The mRNA can be isolated by classical phenol extractions, has a poly(A)-tail and sediments with a coefficient of 20 S.

Translation in reticulocyte lysates yields a 48kDa protein whether the translation is modified with canine pancreas microsomal membranes or not. Analysis of 48kDa mRNA translation products by sodium dodecyl sulphate/polyacrylamide gel electrophoresis showed that the phorbol ester-induced 48kDa is a monomeric one-chain polypeptide. Glycosylation could not be detected, nor signal peptide cleaving, suggesting that it is a non-secreted intracellular protein.

It is widely known that phorbol esters have various effects on gene expression and/or regulation (1-3). For reviews see references (4) and (5).

Although 12-O-tetradecanoyl-phorbol 13-acetate interacts with a receptor molecule (6) and activates cellular phosphorylation processes (7), thereby generating second message molecules such as inositol polyphosphate (8), the precise mechanism of action remains to be elucidated. We have focussed on the regulation of the genes for tissue-type plasminogen activator and an unidentified 48,000 dalton protein in a human melanoma cell line (Bowes) as a model system to unravel the molecular mechanism of action of phorbol ester (9). Labelling of melanoma cells with L-(^{35}S)methionine showed that it could be visualized as a cytosolic low abundancy class protein (10).

In the present study we have purified the mRNA for this 48kDa molecule by affinity chromatography and sucrose gradient ultracentrifugation. It was characterized by *in vitro* translation experiments. The mRNA translation product from reticulocyte lysates is a phorbol ester-induced monomeric one-chain polypeptide. The isolation of the mRNA is the first step in the molecular cloning of this phorbol ester-induced intracellular protein.

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MATERIALS AND METHODS

Cell culture and RNA extraction

The human melanoma cell line (Bowes), obtained from Dr. D.B. Rifkin (Rockefeller University, New York, USA), was cultured as described before (11). Application of phorbol ester was at different doses and time intervals as indicated. For optimal induction of plasminogen activator we used a dose of 100 ng/ml of phorbol ester for 4-6 h.

The extraction of RNA was as described (12). Total RNA was precipitated by addition of NaCl to 0.2 M and two volumes of absolute ethanol overnight at -20°C. It was subsequently fractionated by affinity chromatography on oligo(dT)-cellulose (13) prior to the translation experiments. RNA was measured spectrofotometrically (A_{260}/A_{280}). Sucrose gradient ultracentrifugation was as described (11,14).

Translation of mRNA in reticulocyte lysates and Xenopus oocytes and assay of tissue-type plasminogen activator

RNA was dissolved in water and then translated in the nuclease treated rabbit reticulocyte lysate system or injected and translated in Xenopus oocytes as described (9). Translation in the wheat germ system was as described (15). After translation, the reticulocyte lysate translation products were separated on polyacrylamide slab gels. The oocyte supernatants and the melanoma culture fluids were tested for tissue-type plasminogen activator activity by the fibrin plate method (16). In two experiments radiolabeled proteins from translated mRNA were immunoprecipitated with an antibody against tissue-type plasminogen activator (11). The immunoprecipitation was done using Pansorbin (Calbiochem Behring Corp., La Jolla, CA, USA). When the reticulocyte lysate translations were processed 10 % v/v of canine microsomal membranes (a kind gift of Dr. J. Content, Institut Pasteur du Brabant, Brussels, Belgium) were added to the lysate. The activity of the microsomal membranes was tested by monitoring tissue-type plasminogen activator yields and molecular mass (G. Opdenakker et al., in preparation). In accordance with previous results, only the microsomal membrane aided translations revealed biologically active plasminogen activator (0.150 and 0.70 urokinase-like units on fibrin plate assays for mRNA from untreated and phorbol ester-treated cells, respectively), and increases in molecular mass.

Sodium dodecylsulphate/polyacrylamide slab gel electrophoresis was as described (9). Analysis of the autoradiograms was done by scanning in a spectrofotometer (Gilford 230, Oberlin, Ohio, USA).

RESULTS

Induction of 48kDa mRNA by phorbol ester. Bowes melanoma cells were treated for 6 h with 100 ng/ml of 12-O-tetradecanoyl-phorbol 13-acetate or left untreated. Messenger RNA was extracted, purified and translated in the rabbit reticulocyte lysate system in the presence of L-(^{35}S)methionine as a label. As a control a rabbit globin mRNA was added. The samples were separated by sodium dodecyl sulphate/polyacrylamide slab gel electrophoresis. The gels were dried and autoradiographed as described. From figure 1 it is clear that three major bands appear (in the 50,000 dalton zone) from phorbol ester-induced mRNA. First, an endogenous 50,000 dalton protein, also seen in the blank mix of the reticulocyte lysate (closed triangle). Second, a 52,000 dalton protein, seen both in mRNA translations from untreated cells and, more prominently, from phorbol ester-treated cells. This 52,000 protein was identified as the unprocessed reticulocyte lysate produced, tissue-type plasminogen activator (open quadran-

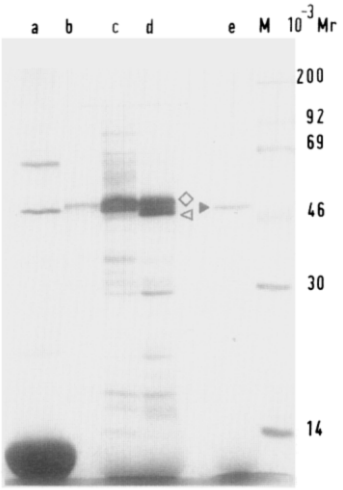

Fig. 1. Autoradiogram of the translation products of melanoma cell RNA in reticulocyate lysates. Confluent cultures of melanoma cells were treated for 6 h with 100 ng/ml of 12-O-tetradecanoyl-phorbol 13-acetate or left untreated. RNA was extracted, purified and equal amounts were translated in the nuclease-treated rabbit reticulocyte lysate in the presence of L-(^{35}S)methionine as a label. Control translations were done with globin mRNA and bidistilled water (blank). After translation the samples were separated on a polyacrylamide slab gel. A (^{14}C)-labelled protein mixture was used as molecular mass standardization. Lanes are as follows : lane a, control translation of rabbit globin mRNA; lane b, immunoprecipitation with antiserum against tissue-type plasminogen activator of translation from lane d; lane c, translation of mRNA from untreated melanoma cells; lane d, translation from phorbol ester-induced mRNA; lane e, blank mixture of reticulocyte lysate; lane M, molecular mass standarization. Open and filled triangles and open diamond indicate 48,000, 50,000 and 52,000 dalton proteins.

gles). Third, a 48,000 protein clearly visible in the translation of the phorbol ester-induced mRNA preparation. The induction of 48kDa-mRNA was determined semi-quantitatively by scanning the gels and integration of the scanning surfaces. Figure 2 shows a scanning plot and autoradiography strip of untreated (1) and treated (2) melanoma cells. From the figure 2 it is clear that 12-O-tetradecanoyl-phorbol 13-acetate treatment increases or decreases the amount of certain cellular mRNAs. The increase is mostly pronounced for the 48kDa protein indicated by the arrow. From the scanning we could indirectly estimate that the mRNA for 48kDa was induced more than 5-fold (determined on 3 independent experiments).

Messenger RNA for 48kDa is co-induced with that of tissue-type plasminogen activator. Melanoma cells were treated for 6 h with 0, 1, 10 and 100 ng/ml of tetradecanoyl-phorbol-acetate. Parallel cultures were cultivated in serum-free medium for production of tissue-type plasminogen activator protein, parallel cultures were used for mRNA extraction. The RNA was purified as described and translated both in reticulocyte lysates for 48kDa mRNA determination and in Xenopus laevis oocytes for tissue-type plasminogen activator mRNA determination. The plasminogen activator protein from oocytes and melanoma cultures was quan-

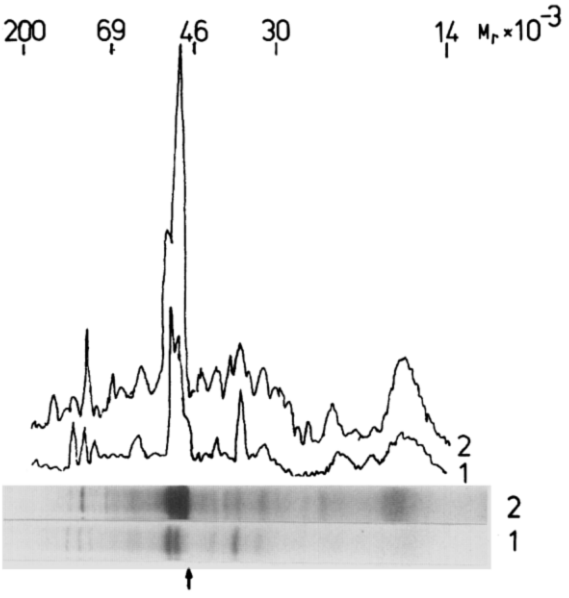

Fig. 2. Autoradiogram and scanning plots of melanoma mRNA translation products. Melanoma cells were treated and mRNA extracted and translated as in Fig. 1. Separate lanes from translations of uninduced mRNA (1) and phorbol ester-induced mRNA (2) were scanned in a Gilford spectrophotometer and plotted. Top of the figure shows the molecular mass standardization (10^{-3} M_r) from a parallel lane of the same gel slab.

titated by fibrin plates. Table 1 summarizes the results of the scannings and the enzymatic assays for tissue-type plasminogen activator. It is obvious that

Table 1. Simultaneous induction in melanoma cells of mRNA for 48kDa and tissue type plasminogen activator

Phorbol ester dose (ng/ml)	48K mRNA (scanner units)	Tissue type plasminogen activator production		
		In oocyte culture fluid (IU/ml)		In melanoma culture fluid (IU/ml)
		Exp. 1	Exp. 2	
0	93	0.038	0.145	15
1	100	0.015	0.280	17
10	144	0.128	0.750	21
100	490	0.540	0.880	30

Melanoma cells were treated for 6 h with different amounts of 12-O-tetradecanoyl-phorbol 13-acetate. mRNA was extracted, purified and equal amounts were translated in reticulocyte lysates for 90 min and in Xenopus oocytes for 48 h. The reticulocyte lysate translation products were subjected to electrophoresis, autoradiography and scanning. The oocyte supernatants and the parallel 24 h production fluids from the melanoma cells were tested for tissue-type plasmingen activator production.

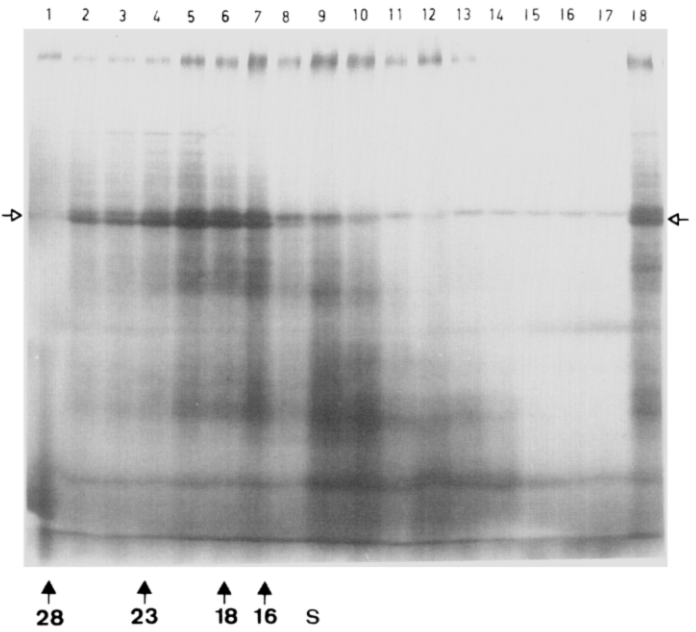

Fig. 3. Autoradiogram showing the mRNA translation of sucrose gradient ultracentrifugation fractions from melanoma cells. Melanoma cells were treated with phorbol ester for 6 h. Approximately 10 A_{260} units of poly(A)-rich RNA was separated by linear sucrose gradient ultracentrifugation. Individual fractions (1-17) as well as unfractionated mRNA (lane 18) were translated in reticulocyte lysates as described. Closed arrows indicate 28, 23, 18, 16S ribosomal markers, respectively. The 48kDa protein is indicated by an open arrow.

the mRNA of 48kDa and tissue-type plasminogen activator are coinduced, dependent on phorbol ester dose; 1 ng/ml yielding no detectable increase and 100 ng/ml yielding an approximately 5-fold increase. Also, for both mRNAs the highest yields were obtained by phorbol ester treatment for 6 h. Treatment for longer time intervals (16 h) caused less production of 48kDa and tissue-type plasminogen activator mRNA than 6 h. With the oocyte-system plasminogen activator mRNA present only at the extraction time is measured. Within the melanoma cells, however, mRNA (at 6 h) can be translated into protein for longer time intervals, giving increased cumulative plasminogen activator production for periods longer than 6 h.

Characterization of the mRNA for 48kDa. Melanoma cells were treated with 100 ng/ml of 12-O-tetradecanoylphorbol 13-acetate for 6 h and RNA was extracted as described. Approximately 10 A_{260} units of poly(A)rich RNA were then separated by ultracentrifugation on a 5-30 % w/v linear sucrose gradient. Ribosomal RNA preparations from E.coli and from melanoma cells were included as RNA size markers. After tapping the gradients each fraction of mRNA was precipitated and prepared for translation. After translation the proteins were separated on a slab gel and the dried gel was autoradiographed. Figure 3 shows a typical gradient profile. 48kDa could be translated from mRNA in several fractions, frac-

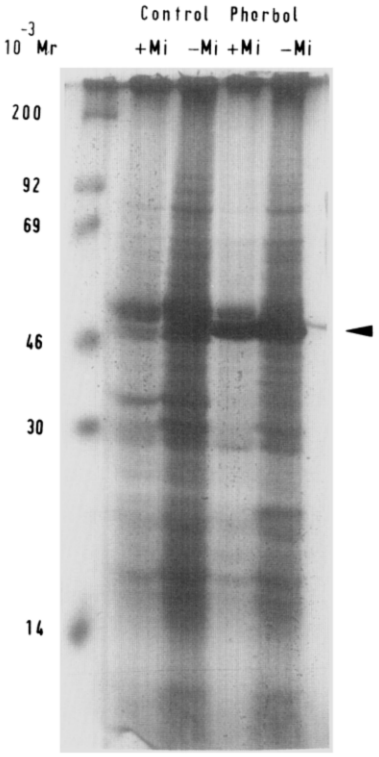

Fig. 4. Autoradiogram showing the processing of the 48kDa protein. Melanoma cells were treated and mRNA extracted as described. Equal amounts of phorbol ester-induced and uninduced mRNA were translated in rabbit reticulocyte lysates in the presence (+ Mi) or absence (- Mi) of canine microsomal membrane fractions. The radiolabelled proteins were separated on a slab gel and visualized by autoradiography. The arrow indicates the 48kDa phorbol ester-induced protein. The left lane is the molecular mass standardization. The right lane is a blank mixture of reticulocyte lysates.

tion 5 containing the peak. As could be calculated from the ribosomal RNA sedimentation profiles the 48kDa mRNA has a sedimentation coefficient of 20 $\pm$ 0.2 S (N = 4), a value compatible with the molecular mass of the protein. The translation product of 48kDa-mRNA was further characterized by translation in reticulocyte lysate aided with canine pancreas microsomal membranes (Fig. 4). It is clear that the addition of glycosylating and signal peptide-cleaving membranes could not change the molecular mass of the phorbol ester-induced 48kDa.

DISCUSSION

Messenger RNA for the phorbol ester-induced 48kDa protein from human melanoma cells was isolated and characterized. It was isolated from the total cellular RNA pool by phenol extraction, affinity chromatography on oligo(dT)-cellulose and sucrose gradient ultracentrifugation. It was characterized by *in vitro* translation in rabbit reticulocyte lysates.

The induction of 48kDa by 12-O-tetradecanoyl-phorbol 13-acetate in melanoma cells occurs within 6 h, a time interval sufficient to obtain maximal production of tissue-type plasminogen activator mRNA by the same cell type. The induction of 48kDa mRNA is clearly visible on autoradiograms. The band intensity could be explained by a pronounced induction of the mRNA and/or high methionine content of the protein. Labelling experiments with L-(^{35}S)methionine on melanoma cells are in favor of the former (10). From independent experiments the induction was at least 5-fold for 48kDa and tissue-type plasminogen activator (2,9). Moreover, both were induced in a dose-dependent fashion. Phorbol ester treatment drastically changed the cellular protein and mRNA content; some mRNAs are increased, others decreased. However, some mRNAs seem to be specifically affected, e.g. the mRNAs for tissue-type plasminogen activator and 48kDa.

Classical procedures for the isolation of mRNA were used to obtain sufficient material for characterization and cloning experiments. Although sucrose gradient ultracentrifugation gave mRNA for 48kDa in several fractions, the sedimentation coefficient could be determined in several independent experiments to be 20S. The mRNA contains thus approximately 2600 nucleotide residues. This value is compatible with the molecular mass of a 48kDa protein. The distribution on the gradient was also in favour of the abundant induction of the mRNA.

Translation of mRNA from untreated and phorbol ester-treated cells in reticulocyte lysates aided wit canine microsomal membranes showed no alterations in the molecular mass of the 48kDa protein. This suggests that 48kDa is an unglycosylated unsecreted protein. The melanoma cell RNA was also translated in the nuclease-treated wheat germ system : 48kDa was only visible after translation from phorbol ester-induced RNA (data not shown). Injection of mRNA in Xenopus oocytes and translation in the presence of L-(^{35}S)methionine as a label could not reveal interpretable data due to labelling of endogenous proteins. A specific antibody will be needed to visualize 48kDa from oocytes. The translation experiments could partially identify the 48kDa protein with the described melanoma proteins (10). Both are monomeric, one-chain polypeptides. Also, the fact that the mRNA product seems unprocessed is in favour of its intracellular or membrane-bound localization (9).

The obtained RNA, the induction method and the characterization as an intensively labelled protein band after translation of the RNA in reticulocyte lysates should be sufficient as tools in the molecular cloning of the 48kDa gene. This should make feasable the study of its possible role in secretion processes of glycoproteins in eucaryotic cells.

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