Dibutyryladenine 3':5'-Cyclic Monophosphate-Mediated Changes in Rat Cells Involve Macromolecular Alterations in Vinblastine-Precipitable Proteins

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ts-NT₃-KR rat cell cultures show the loss of three components in the molecular-weight region 200 000–250 000 when exposed to dibutyryl cyclic AMP, under conditions of both restriction and expression of the transformed phenotype. Vinblastine is able to precipitate preferentially from control cultures the species that are decreased by exposure to the cyclic nucleotide. Serum-starved cultures exposed to dibutyryl cyclic AMP reveal differences in their vinblastine precipitates, depending on whether the expression of the transformation phenotype is restricted or not.

Several studies have shown that treatment of transformed cultures with cyclic AMP derivatives restores the 'normal' morphology of cells, leading to an increased flattening and elongation of the monolayers (Hsie & Puck, 1971; Johnson *et al.*, 1971). However, some paradoxical effects have been observed in cultured adrenal tumour cells (Kwan & Wishnow, 1974) and in NRK cells transformed by temperature-sensitive mouse sarcoma virus (Somers *et al.*, 1977) and Rous sarcoma virus (Rieber & Bacalao, 1976), cases in which dibutyryl cyclic AMP leads to cell-rounding and decreased flattening of the cultures.

As an involvement of microtubular proteins has been invoked to explain the morphological alterations mediated by dibutyryl cyclic AMP in cells (Hsie & Puck, 1971; Rebhun, 1976), we have now investigated a possible cyclic nucleotide-mediated qualitative alteration in microtubule-related proteins precipitable with vinblastine (Marantz *et al.*, 1969; Olmstead *et al.*, 1970).

The results described below show that preparation of extracts from cells grown in the presence of dibutyryl cyclic AMP, in a milieu suitable for the isolation of vinblastine-precipitable proteins (Marantz *et al.*, 1969; Olmstead *et al.*, 1970), permits the detection of a selective alteration in Coomassie Blue-stainable components of about 200 000–250 000 daltons, precipitable with vinblastine, which can be detected in proliferating control cells, but not in the corresponding rounded cultures exposed to dibutyryl cyclic AMP. Vinblastine precipitation of extracts from serum-starved cells permits the detection of a 35 000-dalton species only in cells that manifest a

normal phenotype, but not in cells that express transformed properties.

Materials and Methods

Cell cultures

The experiments described were carried out with ts-NT₃-KR cells. This cell line is a cloned derivative of normal rat kidney cells transformed by the temperature-sensitive *ts* 339 derivative of the B77 strain of Rous sarcoma virus. The ts-NT₃-KR cells exhibited the characteristics of transformed cells at 33°C and of the normal phenotype at 37°C (Rieber & Bacalao, 1976). Cell cultures were grown in Eagle's minimal essential medium (Flow Laboratories, Rockville, MD, U.S.A.; cat. no. 1A-0206) supplemented with foetal calf serum to a final concentration of 10%. To improve the temperature-mediated differential expression of the transformation phenotype in ts-NT₃-KR cells, such cultures were supplemented with 1% (w/v) dimethyl sulphoxide and 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI, U.S.A.; cat. no. 0660-01). Whenever indicated, dibutyryl cyclic AMP was added at the time of seeding the cultures to a concentration of 0.1 mM, unless otherwise indicated.

Preparation of cell-free extracts

Cell cultures were washed three times with 0.15 M-NaCl to remove traces of serum-containing medium, and subsequently monolayers were detached with a 'rubber policeman' in 0.15 M-NaCl containing 1 mM-phenylmethane sulphonyl fluoride to prevent proteo-

lytic activity. After harvesting at 500g for 10min, cell pellets were resuspended in 0.25M-sucrose in 0.01M-sodium phosphate buffer, pH 6.5, containing 1mM-phenylmethanesulphonyl fluoride. Pellets were ruptured by freezing in a mixture of solid CO₂/propan-2-ol and thawing at 30°C, by repeating the process three times. Centrifugation at 10000g for 10min yielded a supernatant which was used for protein quantification by measuring absorbance at 280nm for subsequent vinblastine precipitation or electrophoretic fractionation.

Vinblastine precipitation of microtubule-associated proteins

Cells harvested as indicated above were resuspended in medium A containing 0.25M-sucrose, 1mM-magnesium acetate, 0.1mM-GTP and 0.01M-sodium phosphate buffer, pH 6.5, in an adaptation of the procedures of Marantz *et al.* (1969) and Olmstead *et al.* (1970). Cells were frozen and thawed three times in medium A (20 × 10⁶ cells/ml) and centrifuged at 10000g for 10min. The supernatants were then mixed with an equal volume of vinblastine sulphate (2mg/ml in medium A) and kept at 37°C for 1h. Vinblastine precipitates were collected by centrifugation at 20000g for 45min and used for electrophoresis, for comparison with identical samples to which no vinblastine had been added, whenever indicated.

Polyacrylamide-gel electrophoresis

This was carried out by the high-resolution procedure of Laemmli (1970), by using 9cm-long 7.5% polyacrylamide running gels and 1cm-long stacking 3.3% polyacrylamide gels prepared in Tris/glycine buffer containing 0.1% sodium dodecyl sulphate. Before electrophoresis, samples containing approx. 50 µg of protein were dissociated by boiling for 3min in a mixture containing 2% sodium dodecyl sulphate, 0.1M-2-mercaptoethanol, 2mM-phenylmethanesulphonyl fluoride and 0.1M-Tris/HCl, pH 6.8.

Gels were run at 2.5mA per tube until the Bromophenol Blue tracking dye migrated to within 5mm of the end of the gels. Gels discs were then fixed for 36h in acetic acid/propan-2-ol/water (7:30:63, by vol.) and subsequently stained for 2h in 0.05% Coomassie Blue in fixing fluid.

Approximate molecular weights were estimated by comparison with protein standards of known molecular weight (aldolase, albumin, ovalbumin, soya-bean trypsin inhibitor, cytochrome c) run under conditions identical with those used for the actual samples.

Results and Discussion

In early studies on the morphological changes mediated by dibutyryl cyclic AMP, Hsie & Puck

(1971) postulated an involvement of microtubular proteins. Hence we investigated now a possible cyclic nucleotide-mediated qualitative alteration in microtubule-related proteins precipitable with vinblastine. For this purpose, cells were ruptured in a sucrose/phosphate buffer reported to be useful for the subsequent isolation of vinblastine-reactive proteins (see the Materials and Methods section). A preliminary examination of the protein patterns from extracts of cells before vinblastine precipitation is presented in Fig. 1.

Extracts from rapidly proliferating cells grown in the absence of the cyclic nucleotide resemble each

Fig. 1. Effect of dibutyryl cyclic AMP on the electrophoretic patterns of *ts*-NT₃-KR cells grown at 37°C and 33°C. *ts*-NT₃-KR cultures were grown at the temperatures indicated in each case in medium supplemented with 10% serum to test the effect of 24h exposure to 1mM-dibutyryl cyclic AMP. Subsequently, subconfluent monolayers were washed and processed in sucrose/phosphate buffer and run in 7.5% polyacrylamide gels, as detailed in the Materials and Methods section for Coomassie Blue staining of proteins. (a) Extract from untreated cells grown at 37°C. (b) Extract from untreated cells grown at 33°C. (c) Extract from cells grown at 37°C in the presence of dibutyryl cyclic AMP. (d) Extract from cells grown at 33°C in the presence of dibutyryl cyclic AMP. The black line indicates the region of more obvious alteration as a result of exposure to the cyclic nucleotide.

Fig. 2. Effect of vinblastine treatment on the proteins recognized in dibutyryl cyclic AMP-treated and control cells

Cultures grown at 37°C as indicated in Fig. 1 were subsequently collected and used for comparison of soluble proteins and vinblastine-precipitable proteins, in extracts prepared in sucrose/phosphate/GTP (see the Materials and Methods section). (a) Extracts from untreated cells grown at 37°C. (b) Vinblastine precipitates from extracts from untreated cells grown at 37°C. (c) Extracts from cells grown at 37°C in the presence of 1 mM-dibutyryl cyclic AMP. (d) Vinblastine precipitates from extracts of cells grown at 37°C in the presence of 1 mM-dibutyryl cyclic AMP. The arrows and the black line indicate the regions altered on vinblastine precipitation.

other regardless of whether cells are grown at 37°C or 33°C (Figs. 1a and 1b). However, addition of the cyclic nucleotide to the cultures caused a marked alteration at the top of the gel (Figs. 1c and 1d) in the 200 000–250 000-dalton region, in cells exposed to dibutyryl cyclic AMP at both 37°C and 33°C. Analysis of the same extracts from proliferating cells grown at 37°C presented in Fig. 1, after treatment with vinblastine sulphate (see the Materials and Methods section), revealed that the vinblastine-precipitable proteins from untreated cells appeared to be enriched in the two slower-migrating proteins (Figs. 2a and 2b) that appeared decreased not only in extracts from cells exposed to the cyclic nucleotide (Figs. 1c and 1d) but also in the same extracts subjected to vinblastine sulphate precipitation.

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Fig. 3. Effect of low serum concentration and dibutyryl cyclic AMP on the vinblastine-reactive proteins

ts-NT₃-KR cells were grown for 72 h in medium supplemented with 0.5% serum and 1 mM-dibutyryl cyclic AMP where indicated, and examined for vinblastine-reactive proteins as described in the Materials and Methods section. (a) Vinblastine precipitates from untreated cells grown at 37°C. (b) Vinblastine precipitates obtained from extracts of cells grown at 37°C in the presence of dibutyryl cyclic AMP. (c) Vinblastine precipitates from untreated cells grown at 33°C. (d) Vinblastine precipitates from cells grown at 33°C in the presence of dibutyryl cyclic AMP. The arrow indicates the presence of a vinblastine-reactive protein decreased preferentially in transformed cells grown with dibutyryl cyclic AMP.

However, in both control cells and cells treated with dibutyryl cyclic AMP, vinblastine precipitates (Figs. 2b and 2d) appeared to be devoid of the third fastest migrating species detected before vinblastine precipitation (Fig. 1).

Another interesting feature of the vinblastine precipitates of cells treated with the cyclic AMP derivative was the presence of a 35 000-mol.wt. protein species precipitated by vinblastine only in cells with restricted expression of transformation at 37°C, but not in 'transformed' cells also exposed to the cyclic nucleotide (0.1 mM) after 72 h in medium supplemented with 0.5% serum (Fig. 3d), and the relative decrease in two slowly migrating components in vinblastine precipitates from cells at 33°C, (Fig. 3c), as compared with comparable fractions of cells grown at 37°C (Fig. 3a).

Separate experiments (M. Rieber & A. T. Meza, unpublished work), reveal that the 200000–250000-dalton molecules, shown to be modified by dibutyryl cyclic AMP in the above results, differ from fibronectin-like molecules of similar molecular weight (Vuento *et al.*, 1977) in that fibronectin can be shown by iodination to be surface-associated with ts-NT₃-KR cells that express a normal phenotype, even when the cells are grown with dibutyryl cyclic AMP.

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