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CELLULAR MEMBRANES: MEMBRANE MARKER ENZYME ACTIVITIES IN SYNCHRONIZED MOUSE LEUKEMIC CELLS L5178Y

H. BRUCE BOSMANN

Department of Pharmacology and Toxicology, University of Rochester School of Medicine, Rochester, N. Y. 14620 (U.S.A.)

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SUMMARY

The activities of UDPase, a smooth endoplasmic reticulum marker enzyme; esterase, a rough endoplasmic reticulum marker enzyme, and 5'-nucleotidase, a plasma membrane marker enzyme were measured as a function of cell cycle time in L5178Y cells synchronized with excess thymidine and colcemid. UDPase was active mainly in the S period of the cell cycle with a peak 6.5 h post mitosis. 5'-Nucleotidase was active mainly in the S period of the cell cycle with a peak 5.0 h post mitosis. Esterase was active throughout the cell cycle but mainly in early S period. Each enzyme followed a peak pattern.

INTRODUCTION

The use of marker enzymes for the identification and analysis of membranes or subcellular fractions is of increasing importance. Examples of enzymes used in this manner and the particle or membrane they identify are: succinic dehydrogenase (EC 1.3.99.1), inner mitochondrial membrane[1]; monoamine oxidase, outer mitochondrial membrane[2]; collagen:glucosyl transferase, plasma membrane[3]; and acid phosphatase (EC 3.1.3.2), lysosome[4]. In each instance at least two criteria must be fulfilled for the enzyme to be useful as a marker: (a) the enzyme must be present rather exclusively in the particle or membrane and (b) the enzyme must be rather tightly bound to the membrane or particle or tightly held within the particle, it identifies so as not to be lost or solubilized during isolation. Three enzymes quite often used to identify the membranes of the cell are UDPase (EC 3.6.1.6), described as a smooth endoplasmic reticulum marker enzyme[5], esterase (EC 3.1.1) a rough endoplasmic reticulum marker enzyme[6] and 5'-nucleotidase (EC 3.1.3.5) a plasma or surface membrane marker enzyme[7,8]. The present report documents the activity of these three membrane marker enzymes in synchronized L1578Y cells.

The activity of a variety of enzymes in synchronous cultures has been studied[9] primarily in cells of non-mammalian origin because of the difficulty of synchronizing mammalian cells. In measuring activity of an enzyme in the cell cycle it should be noted that one is measuring the net result of synthesis, degradation, activation, inhibition, availability and cofactor availability and not merely synthesis of the enzyme. Furthermore, even though excess substrate and necessary cofactors are supplied in the assay for the enzyme, the limitation *in vivo* or availability of these may, in fact, be what controls enzyme activity. Measurement of enzyme activity in synchronized cells has produced widely different patterns of activity[9]; *e.g.* in synchronized HeLa cells alkaline phosphatase (EC 3.1.3.1) is detected at a more or less constant rate over the cell cycle[10] while glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and lactate dehydrogenase (EC 1.1.1.27) exhibit three peaks of activity, 3, 7 and 10 h post mitosis[11].

MATERIALS AND METHODS

Cell culture

L5178Y cells (mouse lymphoma cell line) were grown in suspension culture in sealed containers in FISCHER's medium[12] with 10 % horse serum and were utilized in the exponential growth phase. FISCHER's medium in liquid form and horse serum were supplied by Grand Island Biological Co., Buffalo, N. Y; to the supplied medium, penicillin to 500 units/ml and streptomycin to 0.05 mg/ml were added. Cell numbers were determined in a Coulter counter or by counting in a hemocytometer counting chamber. Cells were synchronized by the method of DOIDA AND OKADA[13], by applying one treatment with excess thymidine followed by one treatment with colcemid and deoxycytidine. At each half-hour or hour after release from the colcemid block 30 ml of cell suspension were centrifuged out of solution at 2500 × *g* for 5 min and 1 ml of 0.1 % Triton X-100 was added. The cells were homogenized by 30 strokes with a Ten Broeck homogenizer and assayed immediately or frozen until ready for use. The extracts were always assayed for enzyme activity on either the same day as extraction or on the following day; however, experiments indicated that the enzymes described herein were completely stable for at least 1 week in the frozen extract. Simultaneously with the sampling for extraction, 3 ml of cells were centrifuged out of solution, resuspended in 0.2 ml of Fischer's medium containing 10 μC of [^{3}H]thymidine (15C/mmole) (New England Nuclear Corp.) and incubated at 37° for 5 min. The incubation was terminated with 10 % trichloroacetic acid, centrifuged, the insoluble pellet washed two times with 10 % trichloroacetic acid and once with ethanol–diethyl ether (2:1, v/v), dissolved in 1 M NaOH, plated on a glass filter disc and counted in a liquid scintillation counter. Counts/min from zero time incubations which were precipitated immediately were subtracted from these results. This radioactive incorporation of [^{3}H]thymidine is an indication of DNA synthesis in the cell and is a useful measure of synchrony and the S period. Protein was determined by the method of LOWRY *et al.*[14]. Crystalline bovine serum albumin was used as a standard. Protein per cell increased linearly from about 0.9 mg/10^7 cells to about 1.7 mg/10^7 cells after about 9.5 h and then dropped back to 0.9 mg/10^7 cells. In general there was a doubling of the cells at 9.5–10 h after release from the M block.

Enzyme assay

Esterase activity in the 0.1 % Triton X-100 extracts was determined with *p*-nitrophenyl acetate as substrate by the method of BIER[15]. The 5′-nucleotidase activity was assayed with 5′-AMP as substrate by the method of HEPPEL AND HILMOE[16], and the UDPase activity was determined by the method of PLAUT[17].

RESULTS AND DISCUSSION

Initially, it was deemed necessary to determine levels of activity of each of the enzymes in 0.1 % Triton X-100 extracts of logarithmic phase L5178Y cells. The results, presented in Table I indicate that UDPase was present at the highest activity in the L5178Y cells followed by 5′-nucleotidase and finally esterase. Each of the enzymes was linear with respect to volume of extract and boiling of the extract before assay reduced the activity to zero for 5′-nucleotidase and esterase, and to only 10 mμmoles/h for UDPase (Table I). Furthermore, no activity was present in zero time controls for any of the enzyme activities.

TABLE I

ACTIVITIES OF THREE MEMBRANE MARKER ENZYMES IN LOGARITHMIC L5178Y CELLS

Cells were harvested in logarithmic phase of growth and extracted with 0.1 % Triton X-100 as given in the text. 100 μl of extract represent 0.425 mg of protein and approx. $3.3 \cdot 10^6$ cells. (Boil) represents samples in which the extract was boiled 2 min before assay. (0 time) represents samples which were not incubated but in which the assay was terminated immediately after addition of the extract. Conditions of assays were as given in the text. Volumes were balanced with glass-distilled water. Data are given as mμmoles/h and as means ± S. D.

Conditions	*UDPase*	*5′-Nucleotidase*	*Esterase*
100 μl of extract	450 ± 11	96 ± 4	41 ± 3
80 μl of extract	360 ± 14	78 ± 1	33 ± 1
60 μl of extract	300 ± 21	56 ± 2	25 ± 2
40 μl of extract	186 ± 4	40 ± 3	17 ± 1
20 μl of extract	89 ± 4	19 ± 2	8 ± 1
0 μl of extract	0	0	0
100 μl of extract (Boil)	10 ± 4	0	0
100 μl of extract (0 time)	0	0	0

Fig. 1 presents the data on the activity of the three enzymes in the cell cycle of L5178Y cells and the results of the incorporation of [^{3}H]thymidine. The incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was elevated from 2 to 8 h post mitosis. There was virtually no UDPase activity in periods exclusive of S; activity started at about 2.5 h, reached a shoulder of about 200 mμmoles/h at 4.5 h and was maximal at about 330 mμmoles/h at 6.5 h post mitosis. After the peak activity of 6.5 h the UDPase activity fell rather rapidly and there was again virtually no activity 9, 9.5 and 10 h post mitosis. 5′-Nucleotidase was active throughout the cell cycle with a very small peak of activity of about 22 mμmoles/h at the G_1–S interphase of 2–2.5 h; the majority of the activity was in the S period with the highest activity of 85 mμmoles/h at 5 and 5.5 h (Fig. 1). Esterase had an even higher relative basal activity of about 8–12 mμmoles/h on either side of peak activity of 24 mμmoles/h which occurred 4.5 h post mitosis. Esterase activity began to rise at 1.5 h, definitely still in the G_1 period.

The data presented in this report indicate that the activities of the three membrane marker enzymes studied are similar in that each is active predominantly in the S period of the L5178Y cell cycle. Since the activity of an enzyme is a net result of many factors it is difficult to determine when in the cell cycle synthesis occurs.

However, it is of interest that in L5178Y the S period is the principal period for synthesis of proteins (enzymes) and glycoproteins while lipids and glycolipids seem to be synthesized exclusively in G_2 and M (ref. 18). If in fact the membrane marker enzymes are active only after they are bound to their respective membranes, these membranes, fragments, or subunits must be synthesized rather early in the cell cycle in the S period perhaps using pre-existing lipids and glycolipids.

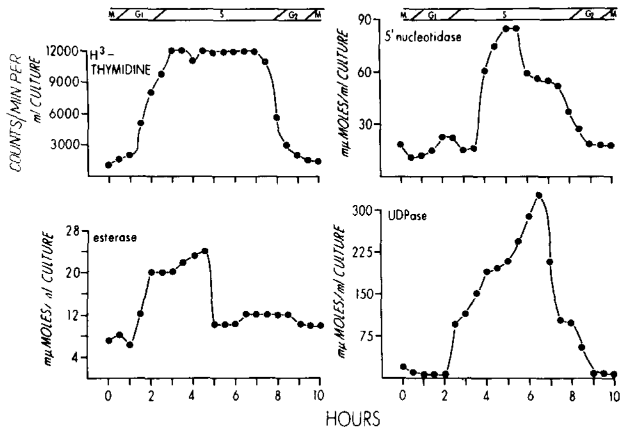

Fig. 1. Activity of three membrane marker enzymes in synchronized L5178Y cells. Cells were released from the M block at zero h. 1 ml of culture contained approx. $1 \cdot 10^6$ cells except at 9.5–10 h when double that amount was present. At each 0.5-h period an aliquot of synchronized cells was extracted with 0.1 % Triton X-100 for enzyme assay or incubated in complete FISCHER's medium with 10 μC of [^{3}H] thymidine for 5 min. Radioactivity was determined in three times trichloroacetic acid-washed, one time ether–ethanol (1:2, v/v)-extracted material. Each point is the mean from seven independent experiments.

Each of the enzyme patterns described for the three membrane marker enzymes seem to fit the peak pattern of MITCHISON[9] as opposed to the step, continuous exponential, or continuous linear patterns[9]. It is of interest that in MITCHISON's[9] report most non-mammalian cells seem to follow non-peak patterns while most mammalian cells have peak patterns for the enzymes studied[9]. The activity patterns for the three membrane marker enzymes reported herein for mammalian cells seem to be peak patterns with the peak in the S period of the cell cycle. The rise portion of the peak is usually equated with synthesis while the decline portion of the peak is usually equated with degradation or instability. It would be of interest to determine in the case of these membrane marker enzymes whether the decline of the activity after the peak results from proteolytic degradation, inherent instability of the enzymes which allows thermal or other perturbations to cause denaturation or conformational changes rendering inactivity, or from non-availability of the enzymes or cofactors at certain times. The second alternative does not seem to pertain since the enzymes are quite stable after extraction and the third alternative does not seem reasonable since the non-ionic detergent extracts each sample equally well and cofactors are supplied in the assay. Thus it seems that not only does the cell possess mechanisms for the synthesis of enzymes at precise times in the cell cycle but that also the cell has mechanisms for the degradation or inactivation of enzymes at equally precise times.

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