COVALENT ATTACHMENT OF ENZYME AS A MEMBRANE-LABEL FOR VIABLE EUCARYOTIC CELLS

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ABSTRACT

An enzyme, β-D-galactosidase, was covalently coupled to mammalian cells by means of a bifunctional reagent. The coupling procedure did not cause appreciable loss of cell viability (<6%) as measured by plating efficiency and membrane integrity. After 24 h in culture, the cells exhibited an average of 2.6×10^4 molecules of β-D-galactosidase per cell. Histological evidence indicated that the enzyme was localized on the cell surface and distributed uniformly among the cell population. Considerations for choosing enzyme-label include sensitivity of assay by enzymatic, immunologic and histochemical methods, and the possibility of isolating labeled membrane components by enzyme-specific affinity chromatography.

KEY WORDS enzyme-label · cell membrane · β-D-galactosidase · cell surface modification · bifunctional reagent

Reagents capable of specifically modifying outer elements of the cell are useful for investigating functional and structural properties of membranes (1). An ideal reagent would (*a*) be incapable of penetrating the membrane due to some physical property such as large molecular size or electrostatic charge; (*b*) perturb the membrane structure minimally; (*c*) be detectable with great sensitivity; (*d*) remain firmly attached to membrane components during fractionation. This rationale is the basis of a popular method using an enzyme, lactoperoxidase, to catalyze the labeling of cell membranes with radioactive iodine (2, 3). We report here a nonradioactive method for membrane labeling in which an enzyme, β-D-galactosidase, is itself coupled to the cell by means of a cross-linking reagent, bisdiazobenzidine (BDB).[1] This offers the following advantages: (*a*) a more sensitive assay than that using radioisotopes[2] without deleterious effects of radiation; (*b*) less risk of labeling internal elements than in the lactoperoxidase method (1, 4) thus providing an unambiguous indicator of cell surface components for membrane fractionation studies; (*c*) practically no risk of transferring the label due to metabolic turnover, especially during studies of the fate of membrane components; (*d*) identifiability of membrane structures by isolating the enzyme-membrane complexes by specific methods such as affinity- and antibody-columns; (*e*) correlation of biochemical and morphological information by histochemical localization of the enzyme using either direct or immunological procedures; (*f*) choice of coupling specificity through the use of different cross-linking chemicals, including cleavable bifunctional reagents; (*g*) cell surfaces may be modified

[1] *Abbreviations used in this paper are*: BDB, bisdiazobenzidine; E4, Dulbecco's modification of Eagle's Basal medium; FCS, fetal bovine serum; HSA, human serum albumin; ONPG, o-nitro-phenyl-β-D-galactopyranoside; PBS, Dulbecco's phosphate-buffered saline.

[2] Assuming a limit of detectability of 10 cpm, a radioisotopic assay could measure 1.7×10^5 molecules of β-D-galactosidase if the specific radioactivity is one molecule of ^{131}I per molecule of protein (465,000 mol wt). In contrast, the sensitivity of fluorogenic assays of β-D-galactosidase can be extended to measurements of individual enzyme molecules (7).

by addition of well-characterized protein antigens (e.g., fragments of β-D-galactosidase) for use in immunologic experiments.

MATERIALS AND METHODS

Cells

Primary cultures of mouse embryo fibroblasts from Taylors Own mice (IRCF) were used for most of the experiments. The cells were grown in round, 2.5-liter Winchester bottles with Dulbecco's modification of Eagle's medium (E4) supplemented with 10% fetal calf serum (FCS). Cell suspensions were obtained by decanting the tissue culture medium from the bottles and rinsing the walls of the bottles twice with 20 ml of a solution containing 0.05% trypsin–0.15% EDTA, leaving a small volume (~5 ml) of the solution after the last rinse. The cells were detached after gentle agitation at 37°C, and 40 ml of E4-FCS was added to the cell-suspension. The cells were then washed five times with 40 ml of Dulbecco's phosphate buffered saline (PBS), pH 7.2 (5), and finally resuspended in 10 ml of PBS. The final washings were done at 0°–4°C.

Enzyme

Purified preparations of β-D-galactosidase (EC 3.2.1.23) were obtained either commercially (Worthington Biochemical Corp., Freehold, N. J.) or by the method of Craven et al. (6), omitting the column chromatography; specific activities were 4.3×10^5 (nominal value) and 5.9×10^5 EU/mg, respectively. The assay of the enzyme and the definition of an enzyme unit (EU) have been previously described (7). Enzyme solutions were dialyzed against PBS for 5–12 h at 2°C before their use in experiments. Tritium-labeled enzyme was purified by the same procedure used for the unlabeled enzyme. The source of enzyme was bacteria which had been grown in the presence of [4,5-^{3}H]DL-leucine (New England Nuclear, Boston, Mass., 5.4 Ci/mmol).

Cross-linking Reagent

Bisdiazobenzidine (BDB) was used for coupling β-D-galactosidase to the cells. The diazonium salt was prepared at 0°C by mixing 45 ml of a 27.7 mM solution of benzidine (Hopkin and Williams Ltd., Chadwell Heath, Essex, England.) made in 0.2 N HCl with 5 ml of 0.51 M $NaNO_2$ made in glass-distilled water. The mixture was allowed to stand at 0°C for 30 min with occasional stirring. It was rapidly distributed into 2-ml aliquots, frozen in acetone-dry ice, and stored at −20°C. This stock solution was almost colorless and did not show signs of deterioration (appearance of a yellowish color) after 3 mo.

Coupling of Enzyme to Cells

The coupling procedure was done at room temperature (24°C). Just a few seconds before coupling, 0.5 ml of the BDB stock solution (thawed ~5 min before) was diluted with 7.5 ml of a 0.15 M Na-K phosphate buffer pH 7.3 (215 ml of 0.15 M Na_2HPO_4 mixed with ~40 ml 0.15 M KH_2PO_4 to give pH 7.3 at 24°C) which had been equilibrated at room temperature. One ml of the BDB dilution was added to 9 ml of a cell suspension with $\sim 10^7$ cells/ml. After exactly 2 min, a given volume of reaction mixture was added to a tube containing enough β-D-galactosidase solution to obtain a final enzyme concentration of 5.6×10^4 U/ml (~0.1 mg/ml) and the reaction was allowed to proceed for an additional 8 min. At the end of this time period, 5 ml of E4-10% FCS was added to the tube to quench the coupling reaction. A control in which horse serum albumin (HSA) was substituted for β-D-galactosidase was made with the same cell suspension treated with BDB.

After the coupling procedure, the cells were spun down and resuspended in cold E4-10% FCS.

Assay of Cell-bound Enzyme in Monolayers or Suspensions

In most experiments the enzyme was measured at 37°C while the cells were attached to petri plates. The plates (Falcon, 5-cm Diam) were emptied, rinsed twice with 2 ml PBS, and then filled with 2 ml of a PBS solution containing 3×10^{-3} M *o*-nitrophenyl-β-D-galactopyranoside (ONPG), the substrate for the enzyme. The enzymatic reaction was stopped after a given time (determined by the yellow color of the solution) by mixing 0.5 ml of 0.6 M sodium carbonate with 1 ml of the plate fluid. The amount of enzyme was calculated from the concentration of hydrolyzed substrate measured by optical density at 420 nm (7) after removal of nonadherent cells by millipore filtration. One β-D-galactosidase unit represents the activity of 2.6×10^9 molecules of enzyme (7). Blank values were obtained from HSA-coupled cells. The optical density of the blanks did not differ significantly from that of controls without enzyme or cells.

In cell suspensions, the enzyme was measured at 37°C after washing the cells three times with PBS and resuspending in PBS. For the assay, 0.1 ml of the suspension was added to 1.9 ml of ONPG (in PBS) to make the final concentration of substrate 3×10^{-3} M. The reaction was stopped by separating the cells either by filtration through a millipore membrane or by centrifugation. One ml of the supernate was mixed with 0.5 ml of 0.6 M sodium carbonate and its optical density was measured at 420 nm.

Counting and Viability of Cells

A Coulter counter with a 100-μm aperture was used to enumerate cells. The instrument was checked with cell suspensions which have been counted in a hemocytometer. To count the attached cells, the plates were rinsed twice with PBS (the rinses were added to the growth medium for counting) and then 0.5 ml of the trypsin-EDTA solution was added: After the cells were detached as judged by microscopic observation, 9.5 ml of the saline was added and the number of cells measured in the Coulter counter.

Cell viability was determined by ability of the cells to attach and divide on tissue culture plates and by fluorochromasia (8). The cultures were incubated at 37°C in a 10% CO_2 atmosphere using E4-10% FCS as the growth medium.

Radioautography of Cell Sections Labeled with Radioactive Enzyme

Cells (lymphocytic leukemia EL4) treated with BDB and ^{3}H-labeled β-D-galactosidase as described above were fixed in 2% glutaraldehyde. After subsequent washing, cells were postfixed in a 1% solution of osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Epon. Sections 1.0 μm thick were cut with glass knives on an ultratome and then transferred to microscope slides. The slides were coated with Ilford L4 nuclear emulsion, and allowed to stand at room temperature for 60 d. After developing the film, sections were stained with Giemsa and observed using a ×100 objective under dark field.

RESULTS

A summary of the procedure for coupling β-D-galactosidase to cell membranes is shown in Table I. Representative data are also included. No significant differences in cell suspensions were observed with 1.5×10^{-4} M BDB. The time sequence for coupling was taken from erythrocyte experiments (9). Variations in these times were not investigated. A cell was considered viable by two criteria, intactness of the plasma membrane as measured by fluorochromasia (8) and ability of the cells to attach to plates and divide. In the experiment chosen for Table I, the difference between normal and BDB-treated cells in terms of plating efficiency was 4%, 87% of the treated cells being capable of attaching to plates. The difference in viability measured by fluorochromasia was 6%. The viability of control cells treated with HSA was similar to that of normal or β-D-galactosidase-coupled cells. This procedure was successfully used for labeling other murine cell lines including lymphomas and myelomas.

The enzyme bound to the cells was measured *in situ* at various intervals after the cells had been growing on plates for several hours. Under these conditions, it was easy to eliminate both dead cells and unbound enzyme by washing the cell monolayers with PBS. The possibility that cytophilic β-D-galactosidase (present in the supernate from the β-D-galactosidase-treated cell suspension) might adhere nonspecifically to cell membranes was examined by adding enzyme to the HSA-treated cells (Table I) and comparing these cells to enzyme-free controls. No indication of cytophilic enzyme was observed in any of our experiments. The significance of these measurements is enhanced by the observation that the cells were growing normally (the generation time was 21 h) and had enough time for elimination of any non-integral membrane component.

After ~20 h in culture, the cells had an average enzymatic activity corresponding to 2.6×10^4 molecules of β-D-galactosidase per cell. The enzymatic activity measured during the next 24 h decreased as shown in Fig. 1. The reason for this was not investigated.

Evidence that most of the enzyme label is located on the cell surface was obtained by examining autoradiographs of serial sections of cells previously coupled to ^{3}H-labeled β-D-galactosidase. A representative photomicrograph showing silver grains is presented in Fig. 2. We examined

TABLE I

Summary of Procedure for Coupling β-D-Galactosidase to Viable Cells

1. 9 ml of a suspension of washed cells in PBS (10^7 cells/ml, 95% viable).*
2. Add 1 ml BDB 1:15 to the cells and allow reaction to proceed for 2 min at room temperature (24°C).
3. Remove a 2-ml portion of the reaction mixture and add it to 0.1 ml of β-D-galactosidase (2 mg/ml), and as a control, 2 ml to 0.1 ml of HSA (2 mg/ml). Allow coupling to proceed for 8 min at 24°C.
4. Add 5 ml of E4-10% FCS to each tube, wash the cells twice and resuspend them in 5 ml cold E4-10% FCS.
5. Plate 4×10^5 cells on 50-mm petri dishes. Viability (after 2.5 h) 87%.

* Cell viability was measured by ability to attach to plates and divide.

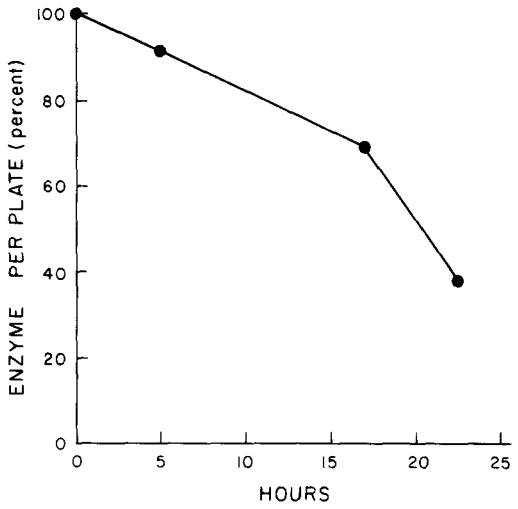

FIGURE 1 Cell-bound enzymatic activity as a function of time in tissue culture. Cells were labeled with β-D-galactosidase and assayed as described in the text.

cell sections and scored the frequency of silver grains near the cell surface versus those inside the cell. As an average diameter of the cell sections was 6.8 μm and as grains derived from tritium tend to be within ~1 μm of the isotope location, we defined an "outside" grain as one falling within an imaginary annulus with an outside diameter equal to 1.1 of the cell diameter and an inside of 0.9. We found 55 outside grains versus seven inside in a total of 370 cell sections examined. Since the sections were 1 μm thick, two out of seven sections represent either the "top" or the "bottom" of a

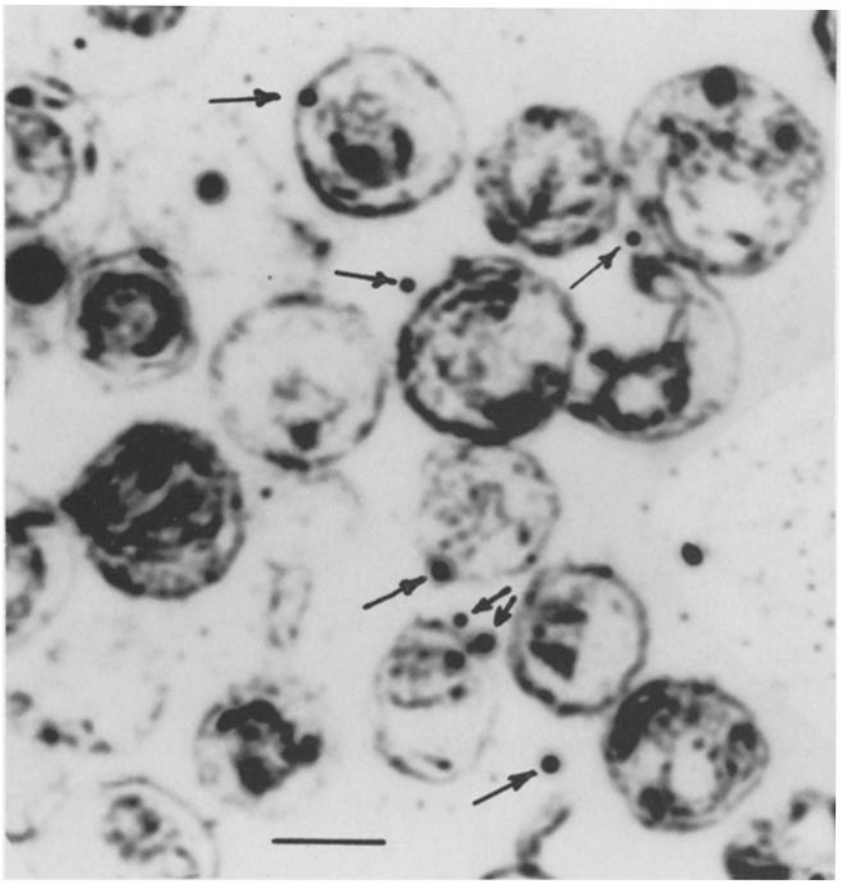

FIGURE 2 Autoradiograph of cell sections labeled wih tritiated β-D-galactosidase as described in the text. Serial sections stained with Giemsa were examined under dark field with a ×100 objective. Under these conditions, silver grains appeared as highly refractile perfectly round particles which were clearly differentiated from cell structures. The approach used to establish the location of the grains is described in the text. The arrows indicate silver grains. Bar, 5 μm.

cell, accordingly, one would expect that 12.8% of the outside grains would look as inside grains. Therefore, the observed distribution of grains (12.7%) indicates that practically all the enzyme was located on the cell surface. Also, we measured a random frequency of outside versus inside grains. This was obtained by repeatedly (200 times) scoring the position of silver grains on a circle of 5 μm diameter randomly placed on the area of the cell sections. The "random" sampling showed 36 outside grains versus 36 inside. These values agree with the expected one since the area of the imaginary annulus is equal to that of the imaginary inside circle. Furthermore, these experiments served to demonstrate that the enzyme-label was uniformly distributed throughout the cell population, since no sections with more than three silver grains were observed.

DISCUSSION

The data shown above demonstrate the feasibility of covalently binding a macromolecular label to mammalian cells without affecting their viability. An enzyme, β-D-galactosidase, was chosen as the label under the assumption that its molecular weight (465,000) would restrict the location of the enzyme to the exterior of the plasma membrane. Evidence in support of this assumption is presented here and also has been independently ob-

tained by cell fractionation.[3] A second consideration for choosing β-D-galactosidase was that it can be assayed with equal or greater sensitivity than a radioactive label[2] and that histochemical and immunological tests for this enzyme are available.

The amount of cell-bound enzyme measured at intervals after the coupling reaction showed a decrease in activity as the culture grew. This could represent shedding of membrane components (10, 11), although the possibility of enzyme inactivation has to be considered.

These two possibilities could be distinguished if the fate of the enzyme label is followed by means independent of enzymatic activity. (Quantitative recovery of enzymatic activity in the growth media cannot be taken as definitive evidence.) In general, studies of the physiological fate of a molecule are more conclusive if both functional and structural tags are present in the molecule. In enzymes, the functional tag is inherent; therefore, an additional tag, such as a fluorescent or radioactive group, provides double means of identification.

The method for covalently attaching an enzyme to the cell membrane has potential uses for the specific isolation of membrane components. For instance, the following scheme could be used for that purpose. (*a*) Enzyme is attached to cell surfaces using a cleavable bifunctional coupling reagent; (*b*) soluble membrane preparations are made by any of the available techniques; (*c*) complexes containing enzyme are separated and purified by the known specific procedures used for purification of the given enzyme; (*d*) membrane components are uncoupled from the enzyme by cleaving the cross-linking reagent; (*e*) the enzyme is removed from the preparation by immunoabsorption chromatography; (*f*) membrane components remaining in the preparation are purified by conventional methods or, alternatively, used for antibody production which, in turn, could serve for subsequent isolation and characterization of membrane components in the absence of a membrane label.

[3] Dr. Lionel A. Manson (Wistar Institute, personal communication) used our procedure to label membranes of different mouse cells including spleen and three tissue culture lines, L-5178Y (lymphoma), P815Y (mastocytoma), and A-10 (adenocarcinoma). After homogenization and fractionation (12) he found most of the β-D-galactosidase (91–99%) stably associated with membranes and little (1.4%) or no enzyme in the cytosol. He also observed that either 10% glycerol or antibody against β-D-galactosidase served to stabilize the enzyme when homogenization was done after washing the cells with protein-free medium.

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