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ELECTRON MICROSCOPE AUTORADIOGRAPHY OF LABELLED YEAST PLASMA MEMBRANE

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SUMMARY

The efficacy of certain tagging agents in the selective labelling of the plasma membrane of yeast protoplasts and whole cells has been examined by electron microscope autoradiography. Determination of radioactively labelled tagging agent associated with the cell involved visual counting of developed silver grains in the nuclear emulsion and quantitating the distribution. Based on the measured dimensions of the cells, a method of describing the source of radioactivity with respect to the hypothetical centre of the cell was made. Approx. 96 and 81% of the total label associated with cells tagged by enzyme-catalyzed iodination with ^{131}I and reaction with *N*-[^{3}H]ethylmaleimide, respectively, was localized with that cellular region corresponding to the plasma membrane. ^{3}H dansylation effectively labelled the plasma membrane, but also resulted in significant penetration of the cell. Fluorodinitro-[^{3}H]benzene was found to be completely unselective.

INTRODUCTION

The advantages of chemically labelling the external surface of the cell plasma membrane with specific reagents to aid in its detection and sudy of its topography have been emphasized[1–3]. Application of this technique has been made in investigations on the erythrocyte membrane[1,4–8] and has been extended to studies on the plasma membrane of other cells including liver[9], normal and neoplasmic lymphocytes[10], fibroblasts[11,12] and yeast protoplasts[3]. The general factors required for a satisfactory plasma membrane tagging agent have been detailed[1], but of fundamental importance is complete impermeability of the cell. Determination of this factor has been complicated, although the absence of labelled haemoglobin recovered from tagged intact erythrocytes has been taken as general evidence for the impermeability of various tagging agents[1,4–8]. Extrapolation of these findings to other cells has not been justified and the required pertinent studies have not been made.

An investigation employing the procedure of electron microscope autoradiography has therefore been undertaken to determine the precise location and quantitative distribution of several tagging agents in yeast protoplasts and whole cells.

METHODS

Materials

The source of the radioactive tagging agents and the general conditions for the growth of *Saccharomyces cerevisiae* and the derivation of yeast protoplasts were as described previously[3].

Tagging

The various labelling procedures were carried out with a suspension of $1 \cdot 10^7$–$1 \cdot 10^8$ cells/ml at 30 °C in selected buffered media, as previously reported[3].

Autoradiography

Cell preparation[3]. All solutions used in the fixation of protoplasts contained 15% mannitol as osmotic stabilizer. Washed tagged yeast protoplasts and intact cells were fixed in 2.5% glutaraldehyde followed by post-fixation in 1% OsO_4 and careful washing. Agar rods were prepared, dehydrated, infiltrated with epon and polymerized[3]. Gold sections (90–150 nm) were cut and then mounted on unsupported copper grids which were taped on circular microscope cover slips.

Emulsion preparation and application[13]. Ilford L-4 nuclear emulsion (Ilford Ltd., Ilford, Essex, U.K.) was dissolved in water (0.5 g/ml) at 60 °C, stirred for 15 min at 45 °C and then gently stirred for 2.5–6.0 min in an ice–water bath. After a period of approx. 70 min, monolayer films were formed by dipping the open end of 1 inch × 2 inch specimen tubes in the chilled emulsion and allowing to dry. The film was transferred by inversion of a tube on the surface of grids mounted on a cover slip which was then fastened to an appropriately labelled glass slide with double-faced cellophane tape. The slides were stored over indicating gel, as desiccant, at 4 °C in a light-proof box.

Film development[13]. Following exposure at 4 °C for varying times (2–6 weeks), the emulsions were developed in Microdol-X (Eastman Kodak) for 3 min at 20 °C, stopped 30 s in 1% (v/v) acetic acid and fixed in Kodak rapid fixer (diluted 1:3) for 5 min. The films, after washing with four changes of water over a 30 min period, were stored *in vacuo*.

Analyses[14]. Determination of label distribution was made by visual counting of developed silver grains using a Philips EM300 electron microscope.

Grain density was expressed as the percentage of total silver grains per unit of distance; the unit of distance being equivalent to the determined value for the mean grain diameter. Background count of silver grains not associated with cells was made and a background density calculated using a distance of 22.5 μm (equivalent to one-half width of a grid square). Thus each grid square was treated as a separate counting domaine composed of the area external to cells. The cells occupied less than 10% of the grid square area. Categories of grain location were defined (Fig. 1).

RESULTS AND DISCUSSION

The number and distribution of developed silver grains on the autoradiographs varied with the sample under examination. In general, a low grain count per cell of 0–3 was found, but was of the same magnitude as reported for *Escherichia coli*

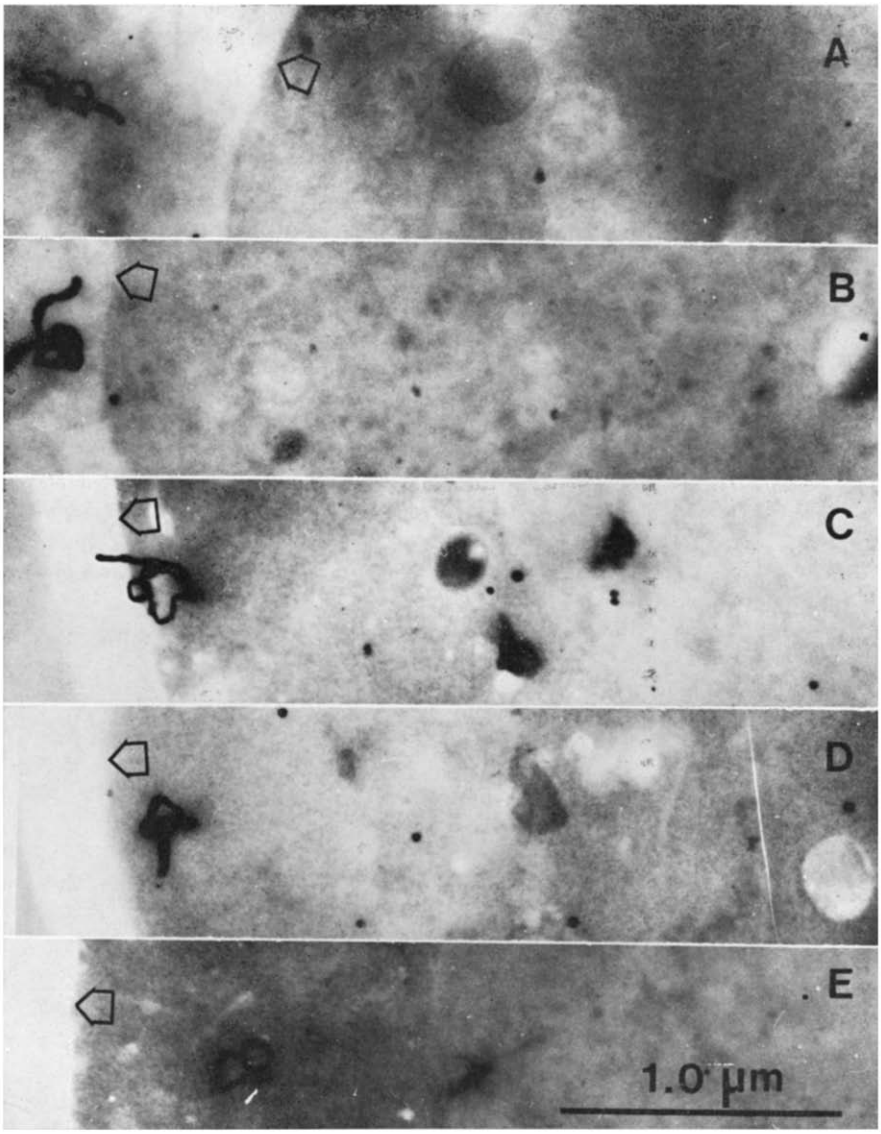

Fig. 1 Electron microscope autoradiographs showing the relative position in yeast cell section of a developed silver grain with respect to the plasma membrane (arrowed):external more than one grain diameter (A), external less than one diameter (B), overlying (C), internal less than one diameter (D) and internal more than one diameter (E).

labelled with [^{3}H]leucine and erythrocytes tagged with ^{125}I (ref. 10). The higher count of 20–50 grains per cell noted with ^{125}I-labelled lymphocytes[10] may have been overestimated due to clusters of apparently individual grains arising from a single hit[15]. It has been pointed out[16] that lack of silver grain development need not necessarily reflect lack of labelling. In fact, the conditions presently employed for the tagging of the yeast cells have been established[17] as providing a compromise

between optimum radioisotope incorporation and minimal discernible disruption of membrane integrity. Thus it appears that the determined low level of incorporation of the recovered label in yeast protoplasts found for enzyme-catalyzed iodination (1–3%) and reaction with *N*-ethylmaleimide (5%) was associated with a high degree of specificity of the tag for the plasma membrane (*vide infra*). On the other hand, the marked incorporation of 53 and 79% for dansyl chloride and fluorodinitrobenzene, respectively, was accompanied by significant labelling of the cellular contents. It may, therefore, be concluded that enhanced grain development cannot be achieved by increasing the level of emitted ionizing radiation[13], but rather awaits the availability of a more sensitive emulsion[15].

Developed silver grains, as calculated from a size frequency curve, had a mean diameter of 0.40 μm (range 0.23–0.83 μm). This value was used as the basic distance unit in calculating the location of silver grains. Protoplasts and whole cells, in section, were observed to be essentially circular, possessing mean diameters of 4.0 and 4.3 μm, respectively. Cell walls were determined to have a mean width of 0.4 μm, whereas the plasma membrane measured a thickness of approx. 0.008 μm. It was therefore possible to calculate the hypothetical centre of the cell (zero point) using the readily apparent plasma membrane as reference point, and to relate the distribution of radioactivity to the cell geometry. Expression of the results in this way is considered to possess certain advantages in that the determined cell centre is independent of radioactivity measurement, can be calculated with a reasonable degree of accuracy due to the geometry of the cell studied, and allows the consideration of radial distribution of silver grains irrespective of the specimen examined. The grain density distribution for whole cells and protoplasts labelled with the various tagging agents is represented in Figs 2 and 3. Grain development under the present conditions was calculated from

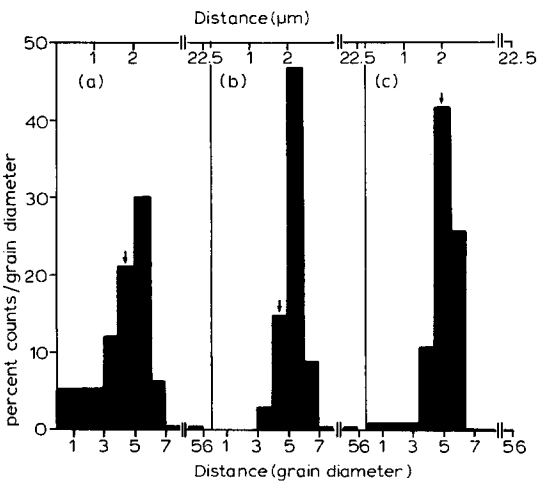

Fig. 2. Developed silver grain density distribution following the uptake of 0.1 μCi $Na^{131}I$/ml over a period of 5–7 min by whole yeast cells (a) in the absence of lactoperoxidase, and by whole yeast cells (b) and yeast protoplasts (c) under conditions of lactoperoxidase-catalyzed iodination[3]. Arrows designate the determined position of the plasma membrane. The data are plotted using the hypothetical centre of the cell as origin; locations from this point are given in terms of distance units which are equivalent to the mean diameter (0.4 μm) of a developed silver grain.

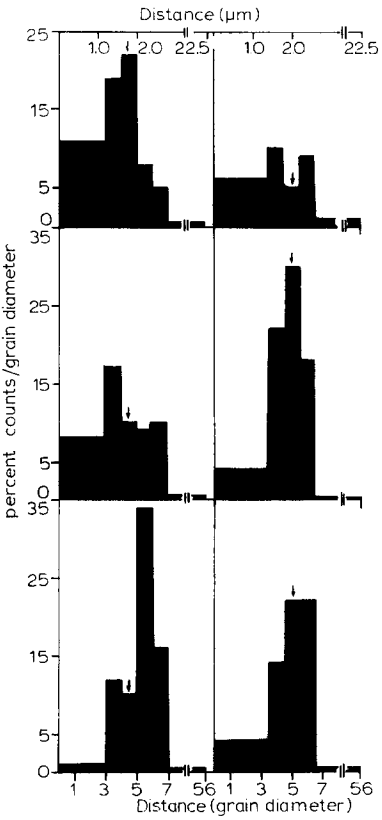

Fig. 3. Developed silver grain density distribution in whole yeast cells (a) and yeast protoplasts (b) tagged with 15 μM 1-fluoro-2,4-dinitro-[3,5-$^3H_2$]benzene (10 μCi/ml) for 5 min (1), 0.5 mM [G-^{3}H]dansyl chloride (8 μCi/ml) for 45 min (2), and 1 mM *N*-[2-^{3}H]ethylmaleimide (8 μCi/ml) for 10 min (3). Autoradiographic measurement and representation of the data are as described in Fig. 2. Arrows designate the determined position of the plasma membrane.

available data[18,19] to occur within an approximate distance of 0.3 μm of the source of radioactivity with a probability of 90%. It is, therefore, considered, with a high degree of certainty, that a developed grain is within one distance unit (average grain diameter of 0.40 μm) of the radiation source. Thus little activity (<1% of the total grain distribution) was detected occurring at distances greater than one distance unit from the outside periphery of the cells (Figs 2 and 3).

Radioactivity in tagged cells appeared to be mainly associated with the plasma membrane although variation in cellular distribution occurred (Fig. 1). Quantitative analyses (Figs 2 and 3) showed the major location of silver grains in the regions of 3.5–6.5 and 3.0–7.0 distance units, measured from the cell centre, for protoplasts and whole cells, respectively, corresponding to the zone of the plasma membrane cell wall.

Variable quantitites of the label were measured in the cytoplasmic contents (delineated by the distance 0–3 from the cell centre in the case of whole cells and 0–3.5 for protoplasts). No silver grains were detected in the cytoplasm of whole

cells labelled enzymatically with ^{131}I, whereas a grain density of $<1\%$ was found in the protoplasts. The cytoplasmic grain density of protoplasts labelled with *N*-ethylmaleimide was found to be slightly higher with a value of 4% per distance unit compared to that of 1% for intact cells (Fig. 3). In the case of the dansylated cells a higher grain distribution occurred in the cytoplasm of whole cells than of protoplasts. An even distribution of tagging agent throughout cells labelled with fluorodinitrobenzene was found.

Although the degree of any labelling of the cell wall was not established, some type of interaction between the cell wall and iodine or *N*-ethylmaleimide might be conjectured since the cytoplasmic occurrence was determined to be less in whole cells than in protoplasts (Figs 2 and 3).

Enzyme-catalyzed iodination would appear to be the labelling method of choice for yeast plasma membrane and the determined high degree of selectivity (96% of the total activity associated with the cell) is in agreement with the findings on erythrocytes[8]. Non-enzyme-catalyzed iodination or passage of ^{131}I into the cytoplasm was relatively low (Fig. 2). The electron microscope autoradiographic visualization of the cellular site of iodination has been previously described for lymphocyte surface membrane[10]. *N*-Ethylmaleimide was also found to have a high specificity (81% of the total activity associated with the cell) for yeast plasma membrane, although suggestive evidence has been presented for the penetration of yeast cells at high reagent concentration[20]. The present results confirm the general lack of specificity for the plasma membrane, high degree of cellular penetration involving presumed membrane disruption and experimental difficulties previously experienced with dansyl chloride[3,21] and fluorodinitrobenzene[3,22,23] and would generally preclude the usefulness of these compounds as specific tagging agents.

The technique of autoradiography possesses the important capability of demonstrating the actual distribution of the tagging agent within intact cells and hence gives a direct measure of the degree of permeability of the plasma membrane. No assumption need therefore be made that the bulk of the recovered tag is associated with the plasma membrane. Thus analyses subsequently made on labelled cellular material, whose location has been definitely established, will permit more precise description of the site of tagging and will validate the application of the tag in ascertaining specific membrane composition and architecture. It is still necessary, however, to substantiate the autoradiographic evidence by the isolation of the tagged membrane. Thus the present results confirm the data obtained on the sub-cellular fractionation of tagged yeast protoplasts[3] and *vice versa*. The importance of corroborating autoradiographic finding by density gradient fractionation of the cell has also been recognized in localizing the sites of membrane synthesis in *Bacillus subtilis*[24]. The value of autoradiography can be extended by judicious choice of different tagging agents to permit the derivation of more definite information as exemplified by the location of enzyme marker systems employing specific radioisotopically labelled inhibitors[25] and the detection of cholesterol in the myelin sheath using [^{3}H]digitonin[26].

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