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Characterization of endoplasmic reticulum by co-localization of BiP and dicarbocyanine dyes

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Summary

The original concept of *endoplasmic reticulum* derived from the observation of a reticular network in cultured fibroblasts by electron microscopy of whole cells. It was previously reported that the fluorescent dye, $DiOC_6(3)$, stains a similar network as well as mitochondria and other organelles in living cells. Here, we investigate the significance of the structures labeled by $DiOC_6(3)$ in CV-1 cells, a monkey epithelial cell line. First, we show that the network stained in living CV-1 cells is preserved by glutaraldehyde fixation and then we co-label it with an antibody against BiP (immunoglobulin binding protein), a protein commonly accepted to be present in the endoplasmic reticulum. Anti-BiP labeled the same network as that labeled by $DiOC_6(3)$, so this network now is identified as being part of the endoplasmic reticulum. $DiOC_6(3)$ labels many other membrane compartments in addition to the endoplasmic reticulum. This, along with its lipophilic properties, suggests that $DiOC_6(3)$ stains all intracellular membranes. However, the extensive reticular network in the thin peripheral regions of cultured cells is easily distinguished from these other membranes. Thus, staining by $DiOC_6(3)$ is a useful method for localizing the endoplasmic reticulum, particularly in thin peripheral regions of cultured cells.

Key words: endoplasmic reticulum, $DiOC_6(3)$, BiP.

Introduction

The name *endoplasmic reticulum* (ER) was given to a "lace-like" network observed by transmission electron microscopy in the thin periphery of osmium-fixed, whole-mounted cells (Porter et al., 1945; Porter and Kallman, 1952; Porter, 1953). With the development of sectioning methods for electron microscopy, profiles of membrane-bounded compartments were recognized, and the extension of many of these compartments as lamellar sacs (cisternae) and tubes was demonstrated in reconstructions of serial thin sections (Porter and Blum, 1953). A correspondence between the morphology of the networks seen in whole-mount, cultured cells and the membrane-bounded compartments seen in sectioned cells was noted (Palade and Porter, 1954), and extended membrane compartments were established to be universal components of cells (Palade, 1956; Porter, 1961). It is now thought that the endoplasmic reticulum is the site of several crucial cell functions, including protein synthesis, lipid synthesis, and calcium sequestration and release (Satoh et al., 1990; Terasaki and Sardet, 1991).

Certain fluorescent dyes, including $DiOC_6(3)$, stain networks very similar in appearance to those seen in the original electron micrographs of whole-mount, cultured cells (Terasaki et al., 1984). The staining technique is simple, produces clear, distinct images, and is well suited for use with cells in culture (Terasaki et al., 1986; Lee and Chen, 1988; Dailey and Bridgman, 1989; Sanger et al., 1989; Lee et al., 1989) as well as plant cells (Quader et al., 1987; Allen and Brown, 1988; Lichtsheidl and Weiss, 1988). However, there remains a lack of certainty about what $DiOC_6(3)$ stains - it remains to be demonstrated that the network marked by the dye in cultured cells is actually the endoplasmic reticulum. Most of what is now known about the ER is based on membrane preparations from differential centrifugation or on thin-section microscopy, and it has not been formally demonstrated by currently accepted criteria that the network stained by $DiOC_6(3)$ is the endoplasmic reticulum. For instance, it has been found that certain other organelles such as lysosomes (Robinson et al., 1986; Swanson et al., 1987; Heuser, 1989) and the *trans*-Golgi network (Griffiths and Simons, 1986; Cooper et al., 1990) can also take on tubular forms.

This paper addresses the issue of the correspondence of the reticular staining seen with $DiOC_6(3)$ in the periphery of cultured cells to the distribution of a protein in the ER, BiP (immunoglobulin binding protein). BiP was originally identified in studies on the synthesis and secretion of immunoglobulin G (IgG), the main secretory product of B lymphocytes. Heavy chain IgG but not light chain IgG is synthesized in mouse pre-

B lymphocytes, as well as hybridomas derived from them, but the uncombined heavy chain IgG is not secreted from these cells (Haas and Wabl, 1983). Haas and Wabl (1983) found that the heavy chain IgG is bound to a 78 kDa protein, and they named this binding protein BiP. A rat monoclonal antibody to BiP was later developed, and this antibody was found to immunoprecipitate both BiP and BiP-heavy chain IgG complexes (Bole et al., 1986). Also, by immunoelectron microscopy, the antibody localized BiP to the membrane compartment commonly designated as endoplasmic reticulum (Bole et al., 1989). When the antibody was used to study mature B lymphocytes secreting complete immunoglobulin, BiP was found to bind transiently to newly synthesized heavy chain IgG before the heavy chain combined with light chain IgG to form complete immunoglobulin. It was therefore proposed that the binding of heavy chain with BiP prevents secretion of incomplete immunoglobulin (Bole et al., 1986).

BiP was subsequently found to be present in other types of cells. Work from many laboratories has all been consistent with BiP being present in the ER and absent in post-Golgi compartments. In particular, Kozutsumi et al. (1988) used the antibody of Bole et al. (1986) in studies with CV-1 cells and found that the antibody immunoprecipitates BiP from permeabilized rough microsomes from these cells. The known characteristics of BiP therefore indicate that it is present in the ER of many cell types, including CV-1 cells, and that its distribution can be determined with the antibody of Bole et al. (1986). It is now possible to compare the staining pattern of $DiOC_6(3)$ with the distribution of immuno-labeled BiP, though this requires devising a new procedure for co-labeling with $DiOC_6(3)$ and BiP.

Materials and methods

Cell culture

CV-1 cells (derived from African green monkey kidney) were obtained from the American Type Culture Collection (Rockville, MD). They were grown in Dulbecco's modified Eagle's medium (DME) with 10% calf serum in a 37°C incubator with 5% CO_2 and 95% air.

Reagents

$DiOC_6(3)$ (3,3'-dihexyloxacarbocyanine iodide), obtained from Eastman Kodak Co. (Rochester, NY), was stored as a stock solution of 0.5 mg/ml in ethanol. $DiIC_7(3)$, $DiIC_{12}(3)$, rhodamine B and ovalbumin conjugated with Texas Red were obtained from Molecular Probes (Eugene, OR). Rhodamine B hexyl ester was synthesized by Molecular Probes (Eugene, OR).

Fluorescent dye staining

Cells were fixed in 0.25% glutaraldehyde in a cacodylate buffer (0.1 M sucrose, 0.1 M sodium cacodylate, pH 7.4) for 3-5 min at room temperature, stained with 2.5 μg/ml $DiOC_6(3)$ in cacodylate buffer for about 10 s, and then washed and mounted in the cacodylate buffer for observation. Rhodamine B and rhodamine B hexyl ester were made as stock solutions of 0.5 mg/ml in DMSO, while $DiOC_7(3)$ was made at the same concentration in ethanol. Observations were limited to approximately the first 20 min after the end of fixation for several reasons: (1) the dye later starts to accumulate in large vesicles, probably lysosomes, obscuring the network staining; (2) stained blebs gradually appear on the plasma membrane; and (3) somewhat later, autofluorescence due to the glutaraldehyde fixation appears.

Double-labeling method for $DiOC_6(3)$ and anti-BiP staining

Coverslips with cells were first fixed lightly in 0.025% glutaraldehyde in cacodylate buffer for 5 min at room temperature, stained for 10 s with 2.5 μg $DiOC_6(3)$/ml in the cacodylate buffer, and then washed and mounted in a silicon rubber chamber. After taking photographs, the immersion oil on the back of the coverslip was removed, and the cells were fixed in methanol for 5-10 min at −20°C. The coverslips were then placed back into PBS and processed for immunofluorescence.

Rat monoclonal antibody to BiP was generously provided by Dr. David Bole. The hybridoma supernatant was used at full strength for labeling. After an incubation for 45 min, the coverslip was washed briefly in PBS and then rhodamine-labeled goat anti-rat IgG (Pelfreeze, Rogers, AR) was applied in a 1:50 dilution in PBS. After 45 min of incubation, the coverslip was washed several times over a period of 10 min and then mounted for fluorescence microscopy.

Immunofluorescence of rapidly frozen cells

Procedures were similar to those used by Dailey and Bridgman (1989) for immunofluorescence localization of myosin. Coverslips on which cells were grown were dried of excess fluid and then plunged rapidly into propane:ethane (2:1 mixture) at liquid nitrogen temperature. After 10 s, the coverslip was transferred to liquid nitrogen, and after 10 s more, the coverslip was transferred to a scintillation vial containing 10 ml of freeze substitution mixture; this mixture was kept at −80°C in a freezer or in a solid CO_2/acetone slurry. When fixations in methanol, 1% formaldehyde (as formalin) in methanol, acetone, and 1% formaldehyde in acetone, were compared; the acetone/formaldehyde mixture preserved the continuity of the endoplasmic reticulum slightly better. For the freeze substitution step, the scintillation vial was transferred from −80°C to either a −20°C freezer or a 4°C refrigerator. After one hour, the coverslip was transferred to PBS for further processing with BiP antibody, as described above. While some cells on the coverslip cracked due to shrinkage, there were always cells that remained intact.

Double-labeling with Texas Red ovalbumin and $DiOC_6(3)$

Cells were incubated for 24 h in the culture medium in the presence of 100 μg Texas Red ovalbumin/ml (Swanson, 1989), fixed in 0.25% glutaraldehyde and stained with $DiOC_6(3)$ as above.

Fluorescence microscopy

A Zeiss ICM microscope with a 100 watt mercury arc lamp in the standard epifluorescence mode was used. A Zeiss plan 100× (1.25 NA) objective lens was used for all observations and for photography. Photographs were taken with either Kodak Tri-X or TMAX 400 film.

Results

Staining by dicarbocyanine and rhodamine dyes

Several dicarbocyanine and rhodamine dyes stain a reticular network in glutaraldehyde-fixed cells (Terasaki et al., 1984). The dicarbocyanine DiOC$_6$(3) has been the most useful because it produces a very bright signal and bleaches relatively slowly, but all short chain (6 carbon or shorter) dicarbocyanine dyes of the DiOC$_n$(m+1), DiIC$_n$(m+1) and DiSC$_n$(m+1) series that were tested, as well as several rhodamine-based dyes (rhodamine 6G and tetramethyl-rhodamine ester) have similar staining properties (Terasaki et al., 1984). The molecular structures of the dicarbocyanine and rhodamine dyes are compared in Fig. 1.

Several lines of evidence indicate that these dyes associate primarily with membrane bilayers. All of the dyes are lipophilic (Sims et al., 1974), and are extracted from stained cells by detergents and non-polar solvents (Terasaki et al., 1984). In order to test the involvement of lipophilicity in staining, rhodamine B hexyl ester was synthesized (Molecular Probes, Inc., Eugene, OR); this dye stained the reticular network while the less lipophilic parent compound, rhodamine B, did not. Further evidence that the dyes stain membranes is that all of the dyes stain mitochondria, lysosomes and other

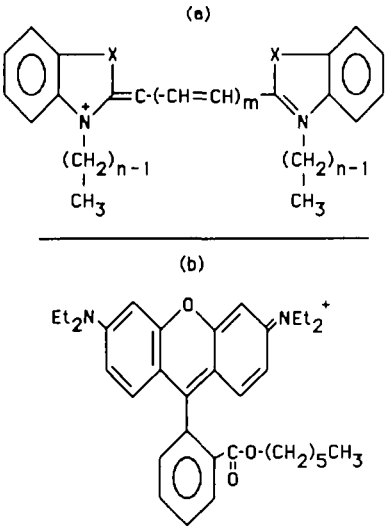

Fig. 1. Molecular structure of dicarbocyanine dyes and rhodamine B hexyl ester. (a) General structure of commercially available dicarbocyanine dyes DiXC$_n$(m+1), where X=oxygen, isopropyl group, or sulfur, n=1 to 18, and m=0, 2, 4 or 6. Short chain dicarbocyanine dyes (n<7) stain intracellular membranes in glutaraldehyde-fixed cells while long chain dyes (n>12) stain only the plasma membrane. DiOC$_6$(3) has X=O, n=6 and m=2. (b) Rhodamine B hexyl ester has similar staining properties as the short chain dicarbocyanines. It has a markedly different molecular structure but shares lipophilicity and possession of a positive charge. The parent compound, rhodamine B, has a carboxyl group in place of the hexyl ester group; it is less lipophilic and does not stain intracellular membranes. This is evidence that a sufficient degree of lipophilicity is required for the staining to occur.

punctate compartments of various sizes, as well as the network in glutaraldehyde-fixed cells. This staining pattern is consistent with the idea that all intracellular membranes are stained - if small vesicles also stain, they might not be visible because of the small amount of dye associated with them.

Further evidence for membrane staining by these dyes is the behavior of longer carbon chain-length dicarbocyanine dyes. DiOC$_{18}$(3), DiIC$_{18}$(3) and DiIC$_{16}$(3) stain the plasma membrane of many different cell types (see Haugland, 1989, for references). Both long carbon chains of these dyes intercalate into the membrane bilayer (Axelrod, 1979). Presumably, the shorter carbon chains of dyes such as DiOC$_6$(3) do not anchor the dyes in the membrane bilayer as strongly, so that these dyes can permeate across the plasma membrane and associate with the bilayer portion of the internal membranes as well. Dyes with intermediate chain lengths were tested; DiIC$_{12}$(3) behaved like the long length dyes, while DiIC$_7$(3) behaved like the short length dyes. Dyes with chain lengths between 7 and 12 are not commercially available, so we were unable to determine at which chain length the staining properties changed.

The properties of DiOC$_6$(3) and related dyes, as well as their staining characteristics, indicate that they do not associate with components specific to particular membranes, but instead associate with the bilayer portion that is common to biological membranes. It seems likely then, that DiOC$_6$(3) and related dyes stain all intracellular membranes in glutaraldehyde-fixed cells through an association with membrane bilayers, and that the network pattern stained by these dyes is a membrane network.

Comparison of DiOC6(3) labeling of living and fixed cells

In living cells, the short chain dicarbocyanine dyes, as well as the rhodamine dyes, stain only the mitochondria at low concentrations (e.g. 0.1 μg/ml DiOC$_6$(3)). The staining is due to accumulation of the positively charged dyes by the large negative mitochondrial membrane potential (Johnson et al., 1981). In fixed cells, the network and other compartments are stained by the same low concentrations that stain only mitochondria in living cells. When living cells are stained by higher dye concentrations, the network, large vesicles and the outline of the cell become visible, and mitochondria usually become swollen. This is consistent with a saturation of mitochondria with the dye, followed by partitioning of the dye into the other membranes (Terasaki, 1989). When living cells stained by high dye concentration are photographed, fixed in glutaraldehyde, and then photographed again, the network staining patterns are identical, indicating that the network is not an artifact of glutaraldehyde fixation (Fig. 2). This also indicates that glutaraldehyde preserves the network pattern well.

Comparison of DiOC$_6$(3) and anti-BiP labeling

BiP is a protein that has been demonstrated to be

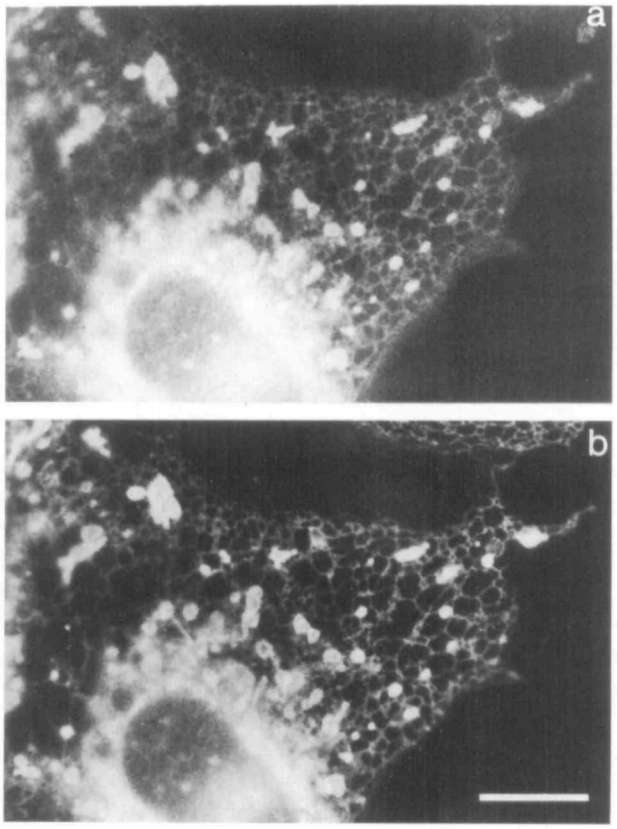

Fig. 2. The same cell, stained while living and then after fixation with $DiOC_6(3)$. (a) A living CV-1 cell was stained with $DiOC_6(3)$ and photographed. (b) The coverslip was quickly un-mounted, fixed with glutaraldehyde and re-stained with $DiOC_6(3)$. The patterns are almost identical, with somewhat more contrast in the fixed cells. This similarity shows that the network stained in glutaraldehyde-fixed cells is not an artifact of fixation. Bar, 10 μm.

present in the ER in many cells (see Introduction). We used anti-BiP (Bole et al., 1986) for immunofluorescence localization of BiP in CV-1 cells. Cells fixed in methanol showed a reticular pattern of anti-BiP staining similar to that seen after staining with $DiOC_6(3)$. The double-labeling procedure was used to compare staining by the two methods in more detail. $DiOC_6(3)$ stained a continuous network pattern in the periphery of CV-1 cells (Fig. 3a) as well as mitochondria and vesicles. The anti-BiP immunofluorescence staining was beaded and discontinuous, but the fluorescent spots defined a network pattern (Fig. 3B) and mitochondria were not labeled by the antibody.

Images of the network pattern from several double-labeled cells were carefully inspected; in regions where the network was not too dense, the patterns produced by $DiOC_6(3)$ and by anti-BiP were identical. In denser regions, where the spacing between branch points of the dye-stained network was small relative to the spacing between immunofluorescent spots, the immunofluorescence staining did not form a discernible network pattern. However, the over-all density of immunofluorescence was similar to that of the fluorescent dye in corresponding regions. Near the cell center, the overlapping, three-dimensional distribution of staining made it impossible to compare the two staining patterns in sufficient detail to determine whether they were identical.

The cold methanol post-fixation used for the anti-BiP staining very probably disrupts the membrane network, leading to the discontinuous beaded staining pattern; disruption of continuous ER membranes in various, generally adverse conditions has been observed by thin-section electron microscopy (e.g. see Ito, 1962). When CV-1 cells are subjected to hypotonic shock, a pattern similar to that of the anti-BiP staining is observed (Terasaki et al., 1986). A common feature in these patterns is the presence of spots at the intersections of the network.

Similarly disrupted networks were usually observed in formaldehyde-fixed cells stained with $DiOC_6(3)$ (not shown) or with anti-BiP (Triton X-100 permeabilized cells; not shown); sometimes, however, the network appeared more continuous in the formaldehyde-fixed cells. A rapid-freezing protocol used previously for localization of myosin (Bridgman and Dailey, 1989) also preserved the network pattern (Fig. 4).

The $DiOC_6(3)$ network is not part of the endocytic membrane system

Macrophages can contain elongated, tubular lysosomes that branch to form membrane networks (Robinson et al., 1986; Swanson et al., 1987; Heuser, 1989). To examine the possibility that the network labeled by $DiOC_6(3)$ in CV-1 cells is an endocytic compartment, CV-1 cells were labeled with Texas Red-labeled ovalbumin, a fluorescent marker for pinocytosis (Swanson, 1989). Labeled cells were fixed with glutaraldehyde and stained with $DiOC_6(3)$. The fluorescence of the two labels was observed separately using a fluorescein set for $DiOC_6(3)$ (Fig. 5A) and a rhodamine filter for Texas Red-labeled ovalbumin (Fig. 5B). The Texas Red-labeled ovalbumin accumulated in spots, corresponding to vesicles, and did not accumulate in networks or extended linear patterns. However, many of the spots labeled by $DiOC_6(3)$ are vesicles of the endocytic pathway. Indeed, most of the Texas Red-labeled ovalbumin spots were also stained by $DiOC_6(3)$, suggesting that, contrary to a previous claim (Terasaki et al., 1984), $DiOC_6(3)$ labels all endocytic compartments.

Characterization of the distribution of ER

The network stained by both $DiOC_6(3)$ and immunofluorescence is remarkably two-dimensional in the peripheral regions of the cells. In contrast, other components of the peripheral regions are not present in a single plane. For instance, microtubules or intermediate filaments overlap each other, forming several layers.

The distinct boundary of the nuclear envelope was observed with the plane of focus in the middle of the cell. Connections between the tubular ER and the nuclear envelope could be seen where mitochondria were absent (Fig. 6).

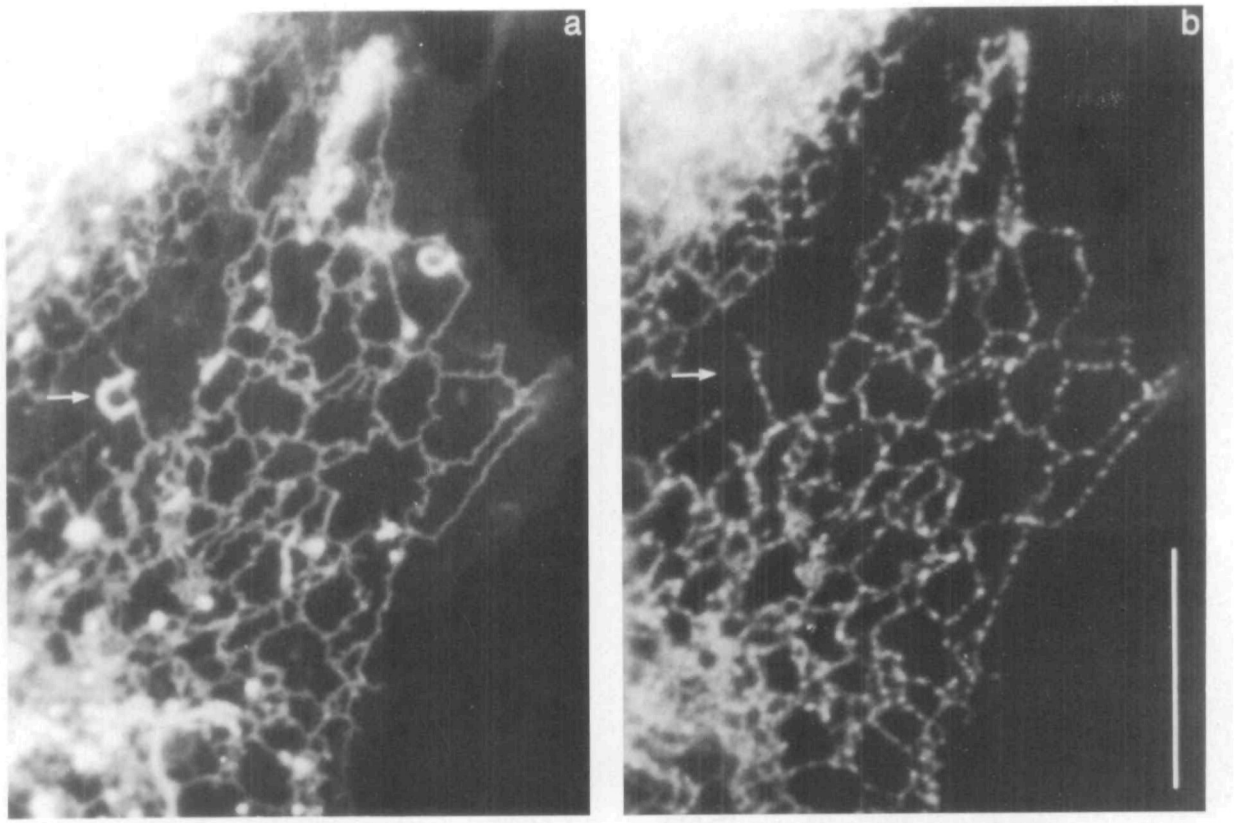

Fig. 3. Double labeling with DiOC$_6$(3) and anti-BiP. (a) CV-1 cell fixed with glutaraldehyde, stained with DiOC$_6$(3), and photographed. (b) The same cell permeabilized and processed for immunofluorescence using the antibody to BiP. The network patterns stained by the two procedures are identical. DiOC$_6$(3) additionally stains other entities: arrow indicates a mitochondrion stained by DiOC$_6$(3) but not by anti-BiP. Bar, 10 μm.

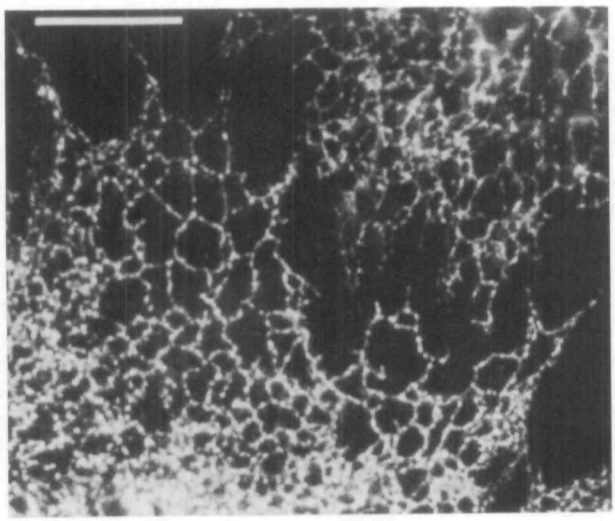

Fig. 4. Rapid frozen, freeze substituted cell stained with anti-BiP. The network pattern is similar to that in methanol-fixed cells (Fig. 3b). Bar, 10 μm.

Discussion

When the fluorescent dye DiOC$_6$(3) was first introduced as a stain for the endoplasmic reticulum (Terasaki et al., 1984), it was concluded that the dye "probably stains the ER". Here we have undertaken further investigation of the staining patterns of DiOC$_6$(3). In our analysis of what the dye stains, we consider first the evidence for the presence of an interconnected membrane network in the periphery of CV-1 cells. Then, using immunofluorescence localization data, we demonstrate that this network is part of the ER.

Evidence for a membrane network in the periphery of CV-1 cells

A straightforward approach to establish the existence of a membrane network in cultured cells is to examine thin sections cut parallel to the substrate. However, the diameter of the membrane tubules is approximately the same as the thickness of the section (approximately 70 nm), so it should be difficult to include large areas of even a two-dimensionally disposed network within a single section. Thin sections generally contain only small segments of tubular membranes, but occasionally contain networks of these membranes (e.g. see Terasaki et al., 1984).

There are several whole-mount methods that do show the membrane network in the periphery of cultured cells. In the original work with whole-mount electron microscopy, a reticular network was stained by osmium tetroxide (Porter et al., 1945; Porter, 1953).

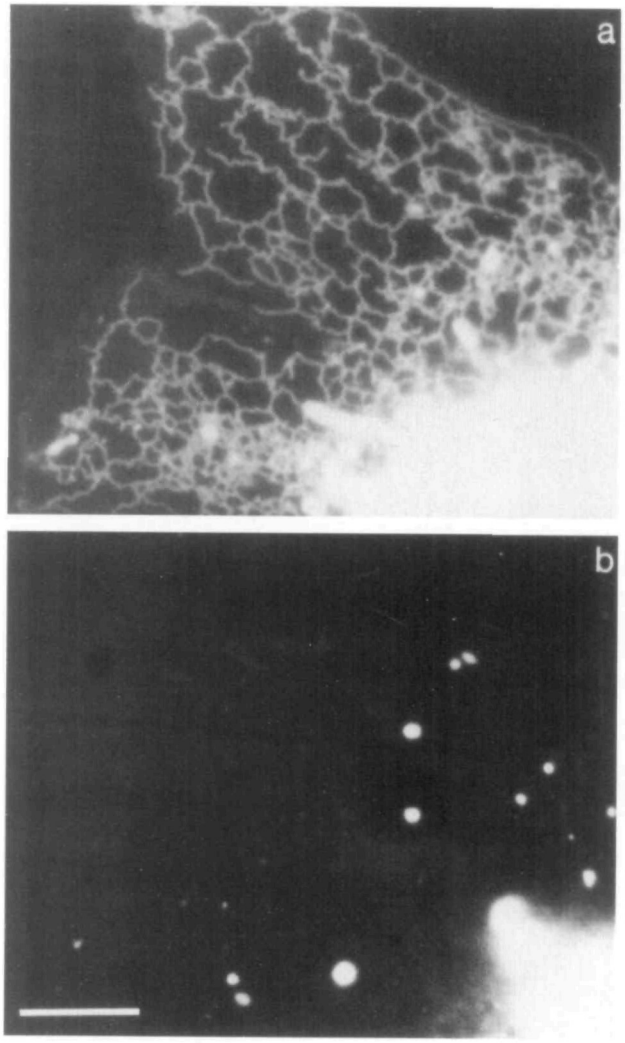

Fig. 5. Double labeling with $DiOC_6(3)$ and Texas Red ovalbumin. This CV-1 cell was exposed to 100 μg/ml Texas Red ovalbumin in growth medium for 24 h. The fluorescently labeled protein is taken up by an adsorptive fluid-phase process, and is a marker for compartments of the endocytic pathway. After the incubation period, the cell was fixed in glutaraldehyde and stained with $DiOC_6(3)$. The pattern of $DiOC_6(3)$ staining (a) and Texas Red ovalbumin (b) show that the membrane network stained by $DiOC_6(3)$ is not part of the endocytic pathway. Also, $DiOC_6(3)$ labels many of the Texas Red ovalbumin-labeled vesicles. Bar, 10 μm.

More recently, using glutaraldehyde fixation followed by osmium tetroxide and critical-point drying, the network pattern was shown more clearly (Buckley and Porter, 1975). The network is stained by osmium, an electron-dense agent that binds to biological membranes.

A similar network pattern has been seen in living cultured cells by phase-contrast microscopy (Buckley, 1964). The network underwent dynamic changes consistent with a fluid membrane structure. Fluorescent labeled phospholipid analogues were also observed to accumulate in a reticular pattern when delivered to cells via liposomes (Pagano et al., 1981). Evidence was presented that an NBD-labeled phosphatidylethanolamine analogue transfers from liposomes into the outer leaflet of the plasma membrane, flip-flops to the inner leaflet, and then diffuses to internal membranes (Pagano and Sleight, 1985). A network pattern was also observed by electron microscopy and phase-contrast light microscopy in whole-mounted cells fixed by potassium permanganate (Song et al., 1985); a fixation known to contrast membranes very effectively (Luft, 1956).

A reticular distribution of numerous proteins has been observed by immunofluorescence. Munro and Pelham (1987) altered a secretory protein, preventing its secretion, and found by immunofluorescence that the altered protein accumulated in a reticular pattern. Saga et al. (1987) found that a glycoprotein, which binds to newly synthesized collagen, also has a reticular distribution, and Koch et al. (1986) found that a calcium binding protein isolated from rough microsomes has a network-like distribution. More recently, foreign expressed proteins that are thought to remain in the ER have been observed in a reticular distribution (e.g. see Lotteau et al., 1990). Lastly, a network is labeled by the fluorescent dye $DiOC_6(3)$ and a number of other lipophilic dyes (Terasaki et al., 1984). The properties and staining behavior of these dyes are consistent with their staining a membranous network.

All of the above methods localize a network pattern that is similar to and very probably identical with the original network pattern observed by whole-mount electron microscopy. An inspection of the patterns shows that they all have three-way junctions and that the network is interconnected. All of the methods are most likely to stain membranes or proteins within membrane compartments, indicating that the network is composed of membranes.

BiP identifies the membrane network as part of the ER

When the distribution of the network localized by $DiOC_6(3)$ was compared with the distribution of the protein BiP, as localized by immunofluorescence, the two staining methods appeared to be staining the same network. Since a large amount of evidence indicates that the immunoglobulin binding protein BiP is present in the ER (see Introduction), the correspondence of $DiOC_6(3)$ staining and BiP localization definitively identifies the membrane network in the periphery of CV-1 cells as ER by currently accepted criteria. This finding, furthermore, establishes staining by $DiOC_6(3)$ as a means for localizing the ER.

$DiOC_6(3)$ and anti-BiP as markers for the ER

An ideal marker for the ER would localize all of the ER and only the ER. Staining by $DiOC_6(3)$ does not meet this criterion because the staining is not confined to the ER; $DiOC_6(3)$ labels mitochondria (Terasaki et al., 1984), and evidence presented in this paper suggests that $DiOC_6(3)$ labels all intracellular membranes, in contrast to a previous claim that it did not label Golgi apparatus or endosomes (Terasaki et al., 1984). Even

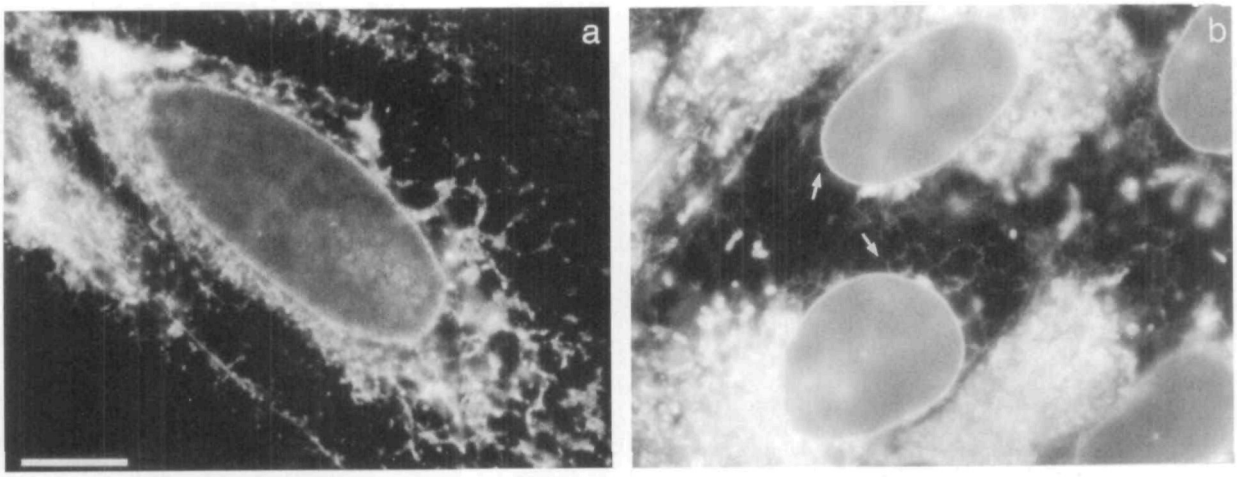

Fig. 6. Staining of the nuclear envelope by anti-BiP and $DiOC_6(3)$. (a) In a methanol-fixed CV-1 cell stained by anti-BiP, a boundary around the nucleus is seen when the microscope is focused above the substratum. (b) In a glutaraldehyde-fixed CV-1 cell stained with $DiOC_6(3)$, the same boundary around the nucleus is seen. In addition, apparent connections with the tubular ER network in the cytoplasm can be seen in regions devoid of mitochondria (arrows). Bar, 10 μm.

though $DiOC_6(3)$ does not specifically stain the ER, it can be used to localize the ER network in the periphery due to the striking differences in the morphology of this network as compared to the other intracellular membranes. Using the morphological criterion of continuity of membranes, staining by $DiOC_6(3)$ should be a useful way to localize the peripheral network of ER in these as well as other types of cells so long as the ER is not confused with tubular lysosomes (Swanson et al., 1987; Heuser, 1989; Robinson et al., 1986) or the *trans*-Golgi network (Griffiths and Simons, 1986; Cooper et al., 1990).

Anti-BiP staining has deficiencies as a marker for the ER that differ from the problems encountered in using $DiOC_6(3)$. Though it is commonly accepted that BiP is in the ER but not in any post-Golgi compartment, BiP may not be present in all of the ER, or may be present at low concentrations in certain parts of it. For instance, BiP was not found by Western blot analysis in a smooth microsome preparation from B lymphocytes (Bole et al., 1986). Also, BiP was not detected in C particle-containing portions of ER, while immunoglobulin was detected in those regions by immunoperoxidase labeling of thin-sectioned B lymphocytes (Bole et al., 1989).

Since it is not certain that anti-BiP labels all of the ER, it cannot be rigorously concluded that the membrane network is the only ER in the periphery of cultured cells. There are also $DiOC_6(3)$-stained vesicles that could be part of the ER, even though they are not continuous with the network and do not contain BiP. We can only be sure that the membrane network in the periphery is part of the endoplasmic reticulum.

Indeed, there remain unresolved issues about the distribution of the endoplasmic reticulum because no morphological feature, biochemical function, or marker enzyme has been demonstrated to be in all of the ER and only the ER. The presence of ribosomes is considered sufficient for a membrane to be identified as being part of the ER, but there are ribosome-free membranes that are also part of the ER (smooth ER). Evidence from intracellular injection of the long-chain dicarbocyanine dye $DiIC_{16}(3)$ into sea urchin eggs suggests that the reticular ER is a single interconnected membrane compartment (Terasaki and Jaffe, 1991), but it has not been proven that ER membranes have such continuity, or whether they are normally continuous but intermittently lose their continuity. With regard to marker enzymes, some proteins, such as glucose 6-phosphatase, are known to be present in the ER, but it is very difficult to show that any such protein is present in all of the ER. Indeed, there are some luminal proteins that have been shown to have a non-uniform distribution in the ER (Vertel et al., 1989). The lack of knowledge about the continuity of ER membranes, or of a characteristic function present in all of the ER, makes it difficult at present to identify and precisely characterize it. However, in conjunction with other methods, fluorescent dyes should be useful for clarifying unresolved issues about the structure and functions of the ER.

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