Detection of Surface-bound Ligands by Freeze-Fracture Autoradiography

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ABSTRACT   This article describes a new freeze-fracture autoradiographic technique for the detection of radioactive ligands associated with the surface of cells in monolayer or suspension culture. Since freeze-fracture replicas are produced in the conventional way, all membrane features normally seen in freeze-fracture are retained, and autoradiographic grains produced by the labeled ligands are seen superimposed on unaltered exoplasmic membrane fracture faces.

To assess the feasibility and resolution of this technique, we compared the surface distribution of α_2-macroglobulin and cholera toxin, labeled either with ^{125}I or with colloidal gold, on 3T3-L1 fibroblasts. Both by autoradiography and cytochemical gold labeling, α_2-macroglobulin was associated specifically with coated pits, whereas cholera toxin was preferentially found over smaller, apparently non-coated membrane invaginations.

Together with data on the surface localization of ^{125}I-transferrin on HL-60 myelomonocytic cells, these results demonstrate the application of this technique for the accurate determination of ligand distribution over large areas of plasma membrane. The simplicity and reproducibility of the method should now allow freeze-fracture autoradiography to become a standard technique for investigating the distribution of both endogenous and exogenous cell surface-associated molecules, as well as the redistribution of such molecules under different experimental conditions.

The introduction of the freeze-fracture technique, which splits membranes and exposes their hydrophobic interior, has made it possible to examine extensive *en face* views of natural and synthetic membranes by electron microscopy (1–3). Recently Pinto da Silva and Kan described a modification of the classical freeze-fracture method that enables cytochemical markers bound to the outer surface of the external leaflet of the membrane to be visualized at high resolution (4). According to this technique, the markers coupled to colloidal gold are seen superimposed on the unaltered images of freeze-fractured exoplasmic membrane fracture faces.

In the present study we have extended this methodology to the autoradiographic localization of polypeptide ligands coupled to ^{125}I. This simple, feasible, and reproducible freeze-fracture autoradiographic technique has the potential to reveal the distribution of radioactive ligands over large areas of cell surface.

MATERIALS AND METHODS

Cell Culture: HL-60 myelomonocytic leukemia cells were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cell doubling time was ~36 h and cultures were divided every 2–3 d to maintain a density of from 2.5×10^5 to 1.0×10^6 cells/ml.

3T3 L1 fibroblasts, provided by Dr. Ora M. Rosen (Albert Einstein College of Medicine, New York), were grown to confluence on plastic coverslips (15-mm diam) in 35-mm petri dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cultures were fed every 2 d with Dulbecco-Vogt's minimum essential medium containing 10% fetal calf serum.

Reagents: Human apotransferrin (Sigma Chemical Co., St. Louis, MO) was converted to ferrotransferrin by incubation for 30 min at 37°C with 2 mol $FeCl_2$ per mol transferrin, and labeled with ^{125}I as previously described (5). Cholera toxin (Calbiochem-Behring Corp., La Jolla, CA) and α_2-macroglobulin (Boehringer Mannheim, Mannheim, Federal Republic of Germany) were iodinated by the chloramine T method according to Bennett and Cuatrecasas (6) and Mosher et al. (7), respectively.

Colloidal gold (15-nm particles) was prepared according to Frens (8); its pH was adjusted to 6.9 for coupling to cholera toxin (9) and 6.0 for coupling to α_2-macroglobulin (10). The method used for coupling colloidal gold to proteins, and the concentration and purification of the resulting protein–gold complexes were previously described (11).

Incubation Conditions: After a washing with balanced salt solution binding buffer (pH 7.4) supplemented with 1% bovine serum albumin (fraction V), HL-60 cells (5–10 × 10^6 cells/ml) were incubated with 30 μg/ml ^{125}I-transferrin for 30 min at 4°C in the same buffer. Unbound transferrin was then

removed by washing three times with ice-cold buffer, and cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. 3T3 L1 fibroblasts adhering to plastic coverslips were first preincubated for 2 h at 37°C in culture medium (see above) without fetal calf serum. Cells were then equilibrated for 10 min at 4°C, washed twice with Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin (fraction V), pH 7.4, and further incubated in 1 ml of Krebs-Ringer bicarbonate buffer containing the labeled ligand for 2 h at 4°C (~100 µg/ml α_2-macroglobulin-gold, ~20 ng/ml ^{125}I-α_2-macroglobulin, ~30 µg/ml cholera toxin-gold, ~10 ng/ml ^{125}I-cholera toxin). At the end of the incubation, cells were washed three times with Krebs-Ringer bicarbonate buffer at 4°C and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min; for incubations using radioactive ligands, a duplicate coverslip with unfixed cells was cut into small pieces, which were counted in a gamma-counter (Beckman Instruments Inc., Palo Alto, CA). For each incubation with ^{125}I-labeled ligands identical incubations were carried out in parallel in the presence of an excess of unlabeled cholera toxin or unlabeled α_2-macroglobulin, added to the incubation medium 15 min before the iodinated ligand to determine nonspecific binding (cell-associated radioactivity in the presence of an excess of unlabeled hormone). In all cases the nonspecific binding represented <15% of total binding.

Freeze-Fracture: Fixed, labeled HL-60 cells were impregnated for 2 h in 30% (vol/vol) glycerol in 0.1 M cacodylate buffer, pH 7.4, sandwiched between double-replica copper supports, frozen in Freon 22 cooled with liquid nitrogen, and freeze-fractured at −110°C. Fixed, labeled 3T3 L1 fibroblasts adherent to plastic coverslips were left for 2 h in 30% (vol/vol) glycerol in 0.1 M cacodylate buffer, pH 7.4, cut into 1.5 × 0.5 mm squares, inverted on specimen carriers (Balzers, Liechtenstein), and freeze-fractured at −110°C in a Balzers BAF 301 apparatus according to Pauli et al. (12). Fractured surfaces were replicated by platinum/carbon evaporation. As described by Pinto da Silva and Kan (4), the replicas were washed by successive floatings on distilled water only and then mounted on Parlodion-coated grids (Mallinckrodt Chemical Works, St. Louis, MO) in such a way that the remaining attached cell fragments were sandwiched between the replica and the copper grid.

Autoradiography: Grids with replicas were processed for autoradiography by the method of Caro et al. (13). They were mounted on clean glass slides and covered with a layer of Ilford L4 emulsion (Ilford Ltd., Basildon, Essex, England). The grids were dried overnight at room temperature and then stored at 4°C for 1–2 wk. Grids were developed in freshly prepared Microdol X (Eastman Kodak Co., Rochester, NY) at 20°C for 4 min, rinsed in water, fixed in Kodak F 24 for 5 min, and washed four times in distilled water baths. The grids were then removed from the glass slide, dried for 24 h, and examined in a Philips EM 300 electron microscope (Philips Electronic Instruments, Eindhoven, The Netherlands).

In addition, some autoradiographic exposures were carried out as previously described (14) on thin sections of cells that had been exposed to the radioactive ligands as described above, and then fixed, dehydrated, and embedded in Epon 812 for conventional electron microscopy.

RESULTS AND DISCUSSION

After surface labeling of HL-60 myelomonocytic leukemia cells with ^{125}I-transferrin, the labeled ligand can be localized on conventional thin sections by autoradiography at the electron microscopic level (Fig. 1A). The plasma membrane surface visualized by this technique is, however, limited to the thickness of the thin section (~700 Å). When surface labeled HL-60 cells were freeze-fractured, replicated, cleaned by the method proposed by Pinto da Silva and Kan (4), and layered with a photographic emulsion, the ^{125}I-transferrin bound to the outer surface of the outer, or exoplasmic, half of the membrane can similarly be detected by autoradiography (Fig. 1B). This technique has major advantages over thin section autoradiography, since the radioactively labeled ligand is directly visualized on large membrane surfaces; therefore, the surface distribution of the ligand can be more accurately established.

To ascertain the feasibility of the gold label–fracture technique and of the freeze-fracture autoradiography method, we next compared these two methods by investigating the surface distribution of ligands known to associate with specific domains of the cell surface. We first analyzed the surface distribution of α_2-macroglobulin, previously described for thin sections to associate preferentially with coated pits (10). When 3T3 L1 fibroblasts were incubated for 2 h at 4°C in the presence of α_2-macroglobulin-gold, gold particles attached to the outer surface of the exoplasmic membrane fracture face of the plasma membrane were associated with large polymorphous bulges corresponding to coated pits (Fig. 2A). Similarly, when ^{125}I-α_2-macroglobulin was used, autoradiographic grains were selectively over or near coated pits (Fig. 2C). Whereas
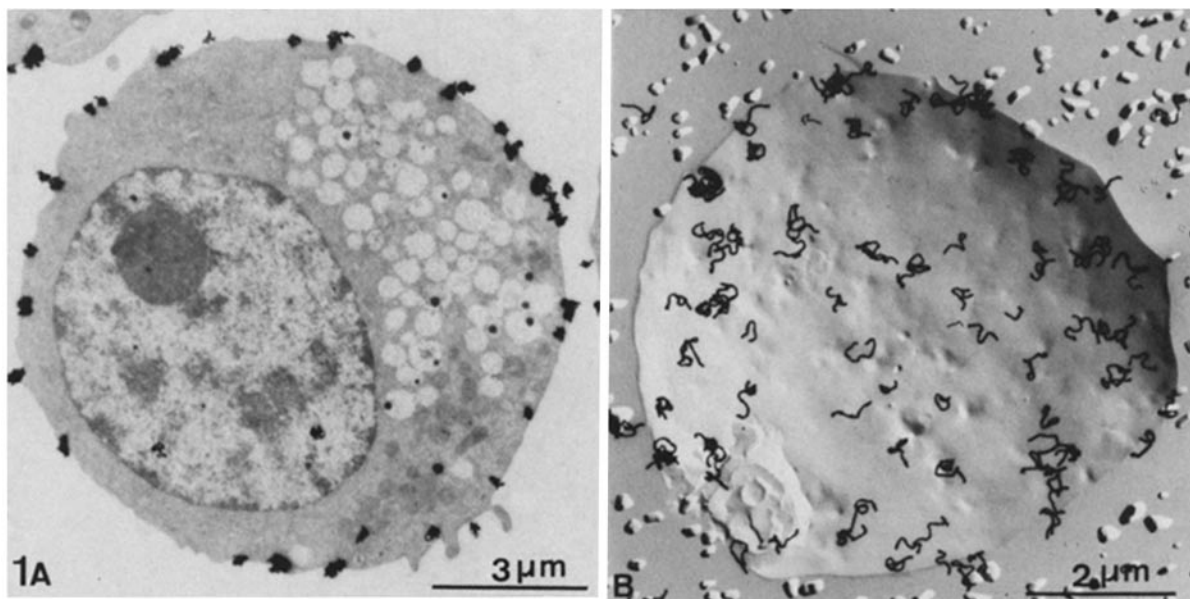

FIGURE 1   Thin section (A) and freeze-fracture (B) autoradiograph of HL-60 myelomonocytic cells incubated for 30 min at 4°C in the presence of ^{125}I-transferrin. As seen on thin sections, autoradiographic grains are mostly associated with the plasma membrane. On freeze-fracture replicas, the distribution of autoradiographic grains produced by ^{125}I-transferrin associated with the outer surface of the external leaflet of the plasma membrane is viewed *en face*, providing information on the distribution of labeled ligand over large areas of the cell surface. (A) × 7,000. (B) × 9,500.

α_2-macroglobulin, like most polypeptide hormones, growth factors, and viruses, preferentially associates with coated pits, other ligands (e.g., cholera and tetanus toxins) have previously been described to concentrate in smaller, apparently non-coated, invaginations (9). When cholera toxin was coupled to colloidal gold and 3T3 L1 fibroblasts were incubated with this conjugate, gold particles were frequently found in such small non-coated invaginations. This observation was confirmed on freeze-fracture replicas prepared by the label-fracture technique (Fig. 2B). When ^{125}I-cholera toxin was used and an Ilford L4 emulsion was layered over the freeze-fracture replica, autoradiographic grains produced by the source of radioactivity (^{125}I-cholera toxin) were associated with the outer surface of the external leaflet of the plasma membrane and were similarly found over surface regions rich in non-coated invaginations (Fig. 2D). When a double labeling experiment using ^{125}I-cholera toxin and α_2-macroglobulin-gold was performed, each ligand was associated with the appropriate surface domain: autoradiographic grains were near non-coated pits whereas gold particles were associated with large bulging regions that represented coated pits (Fig. 2D).

The data presented here extend the application of the "label fracture" method proposed by Pinto da Silva and Kan to the surface localization of bound polypeptide ligands, and we describe a modification that allows the detection of radioactively labeled molecules bound to the cell surface. Despite the
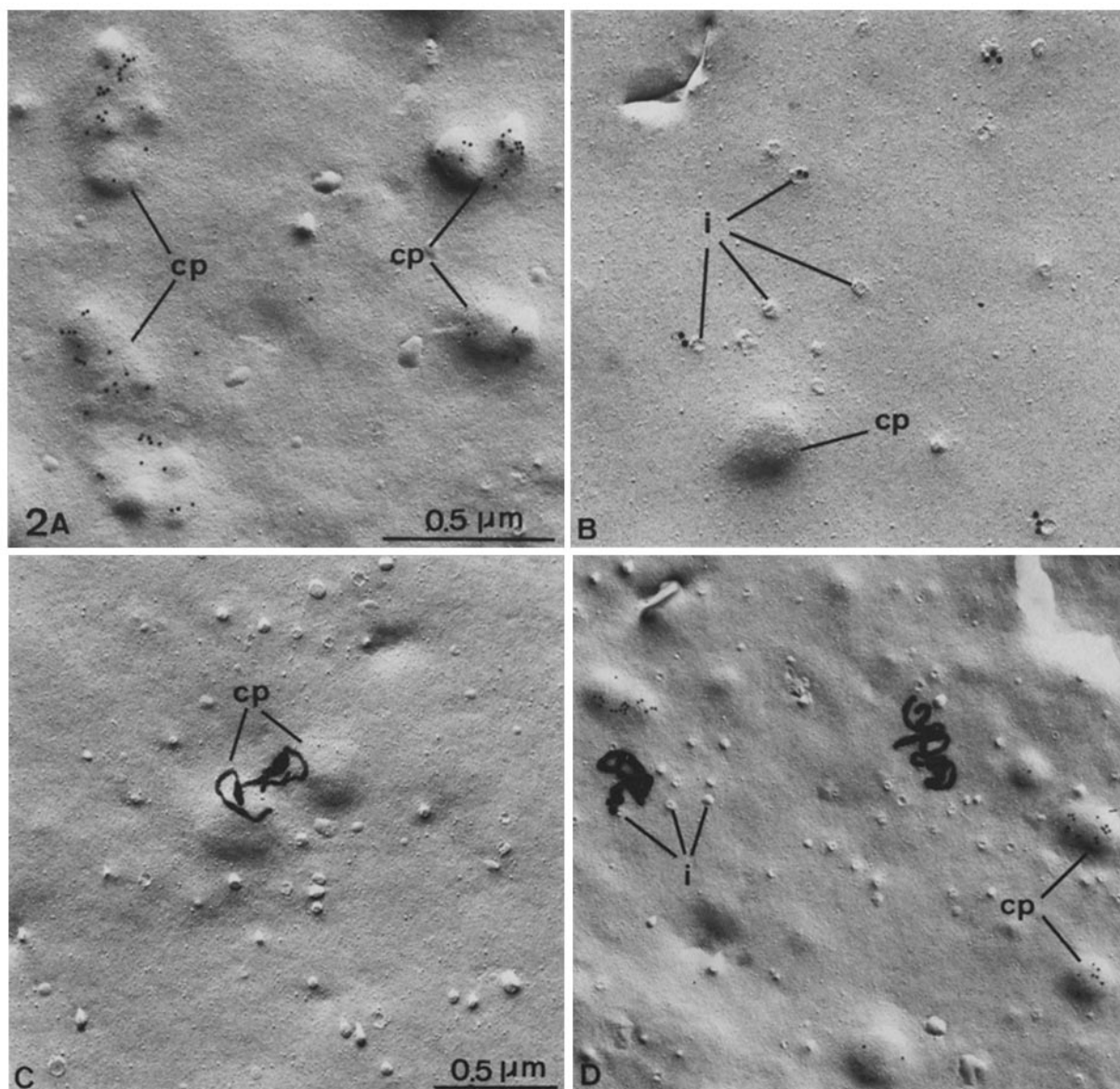

FIGURE 2 (A and B) Label-fracture of 3T3-L1 fibroblasts incubated in the presence of α_2-macroglobulin-gold (A) or cholera toxin-gold (B) for 2 h at 4°C. α_2-macroglobulin-gold is found within large polymorphous bulges corresponding to coated pits, whereas microinvaginations (i) remain unlabeled. In contrast, cholera toxin-gold associates with these small invaginations whereas coated pits (cp) are devoid of labeling. (C and D) Freeze-fracture autoradiographs of 3T3-L1 fibroblasts incubated in the presence of ^{125}I-α_2-macroglobulin (C) or doubled labeled with ^{125}I-cholera toxin and α_2-macroglobulin-gold (D). In C, autoradiographic grains produced by the subjacent ^{125}I-α_2-macroglobulin are superimposed on coated pits (cp). In D, the grains produced by ^{125}I-cholera toxin are, in contrast, found over regions rich in non-coated microinvaginations (i) of the membrane, whereas coated pits are heavily-labeled with α_2-macroglobulin-gold. (A) × 48,000. (B) × 52,000. (C) × 35,000. (D) × 30,000.

relatively poor resolution of the autoradiographic technique at the electron microscope level, the adjunction of radioactively labeled molecules to ligands for their localization by autoradiography has important advantages over the coupling of electron dense probes such as colloidal gold to the same ligands. First, the addition of a radioactive isotope to a ligand usually has a minimal effect (if any) on the biological activity of the molecule into which it is incorporated. Second, most biochemical characterization of the interaction between ligands and their target cells is carried out with radioactively labeled compounds identical to those used for morphological analysis. Third, the added radioactive isotope has less chance of interfering with ligand handling by the target cell than is the case with relatively large electron dense probes coupled to the same ligands.

The freeze-fracture autoradiographic technique presented in this paper, which is based on the retention of the radioactive label at the surface of exoplasmic membrane halves after freeze-fracturing and washings in distilled water, is simple and easily reproducible. This is in contrast with initial attempts at freeze-fracture autoradiograpy, which were based on the application of a monolayer of Parlodion-stabilized, dry autoradiographic emulsion ex vacuo or in vacuo to fractured replicated frozen specimens and where, after 1 or 2 wk at −80°C, the attached cellular material was digested away (15, 16). Based on the same principle, other related techniques have also been proposed (17–19). Besides their tediousness, all these methods have the disadvantage of being limited to heavily labeled specimens, since low temperature reduces the number of developable silver grains (15). In addition, when applied to tissues and cells, the specimen is still present during exposure, so isotopes at some distance from the fractured tissue surface could result in grain formation in the emulsion. Subsequently, the monolayer freeze-fracture autoradiography method of Fisher enabled isotopes associated with the outer surface of cells in monolayers to be detected with no interference from underlying tissue (20). However, the replicas resulting from this method differ in appearance from conventional freeze-fracture replicas. The surface is rougher and there is little definition between smooth areas and particles, probably because of the involvement of a freeze-drying step before replication (21). Such limitations, which precluded the widespread use of the previously published methods, have now been overcome since (a) All membrane features normally detectable on conventional freeze-fracture replicas (e.g., intramembrane particles), which are obscured with the monolayer freeze-fracture autoradiography method, remain visible by the described method. (b) Nonadherent tissue is washed away from fractured exoplasmic membrane fracture faces before application of photographic emulsion. Therefore, only label associated with the outer leaflet of the plasma membrane produces autoradiographic grains. (c) The autoradiographic emulsion is layered at room temperature directly over the replicas on the copper electron microscope grids. (d) The present technique is not restricted to monolayers of cells adhering to solid substrates (e.g., glass) but can be extended to cells in suspension and possibly even to intact tissue.

In the present study, we have demonstrated the feasibility of a freeze-fracture autoradiography technique to localize plasma membrane–bound radioactive ligands. In addition to providing an easily reproducible method for the visualization of surface labeling of close-to-native ligands on large membrane areas, examination of such freeze-fracture autoradiography specimens should also allow the direct relation of the radioactivity with the distribution of specific groups or classes of intramembrane particles not only on the exoplasmic membrane fracture face but also on the protoplasmic face visualized on complementary replicas (4). Within the limit of electron microscope autoradiography resolution, this methodology has also the potential to detect the capacity of the exoplasmic leaflet of the membrane to incorporate radioactive components, and to establish their distribution within the plane of the membrane.

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