# Surface Redistribution of $^{125}$I-Insulin in Cultured Human Lymphocytes

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ABSTRACT    The cultured human lymphocyte (IM-9) binds $^{125}$I-insulin by a receptor-mediated process; the receptor, in turn, is regulated by the ligand. In the present study we have examined quantitatively the morphologic events involved in $^{125}$I-insulin interaction with the surface of the lymphocyte. At 2 min of incubation at 15° or 37°C, the ligand localizes preferentially at the villous surface of the cell, whereas with longer periods of incubation, the ligand distributes indistinguishably between the villous and nonvillous surface. When rebinding is blocked, $^{125}$I-insulin localizes preferentially at the nonvillous surface of the cell. When the total cell surface is considered, there is little preferential association with coated pits; when only the nonvillous surface is considered, a preferential association with coated pits is found and is quantitatively increased in the absence of rebinding of the ligand. This cell has an abundant villous surface (~55% of the total surface); and, as seen on freeze-fracture replicas, the plasma membrane of the villous surface contains a 60% greater density of intramembrane particles than the nonvillous surface.

The data suggest an ordered pattern of insulin interaction with the cell surface (i.e., binding to villi followed by redistribution to the nonvillous portion of the cell containing coated pits). These events probably reflect the mechanism by which the cell segregates specific receptors and related proteins in the plane of the membrane so that they can be selectively removed.

Polypeptide hormones and related ligands bind to a finite number of specific sites on the cell surface. These receptors have been characterized by a variety of biochemical techniques and, in certain instances, have been shown to exist in particular domains of the cell surface. Anderson et al. (1) have demonstrated that ferritin-labeled, low-density lipoprotein (ferritin-LDL) localizes initially and preferentially in coated pits of human fibroblasts, and their data have been confirmed for $^{125}$I-LDL by quantitative electron microscope (EM) autoradiography (2). In the normal fibroblast, localization of LDL receptors in coated pits is unrelated to occupancy by the ligand (1). Similarly, we have demonstrated that $^{125}$I-epidermal growth factor ($^{125}$I-EGF) initially, and preferentially, localizes to such coated pits in human fibroblasts (3), and Willingham et al. (4) have shown that $\alpha_2$-macroglobulin initially localizes diffusely on the membrane of cultured rodent fibroblasts, but binding of the ligand induces clustering of the ligand-receptor complex

into coated pits. They also suggest that EGF and insulin behave in a similar fashion (5). More recent studies carried out in cultured human lymphocytes, and using insulin and anti-insulin receptor antibody labeled with $^{125}$I (6, 7) or fluorescein (8, 9), have demonstrated that the insulin-receptor complex is mobile in the plane of the plasma membrane. Until now, however, we have been unable to show a preferential localization of labeled insulin to coated invaginations of either cultured human lymphocytes or freshly isolated rodent hepatocytes (6, 10). Thus, until now, we have been unable to identify specific domains of the cell surface with which insulin interacts.

In the present investigation we have quantitatively studied the interaction of $^{125}$I-insulin with the surface of cultured human lymphocytes. We find an ordered pattern of interaction involving initial localization of the insulin-receptor complex at the villous surface of the cell followed by redistribution to the nonvillous surface and concentration in coated pits.

## MATERIALS AND METHODS

### Cells and Reagents

Human cultured lymphocytes of the IM-9 cell line were used for all experiments. Cells were grown at 37°C in RPMI 1640 medium containing 10% fetal calf serum and 25 mM HEPES (International Biological Laboratories, Inc., Rockville, Md.). Glutamine (0.29 mg/ml) was added just before feeding. Cells were "fed" three times a week by dividing the culture 1:3 and adding fresh medium. Cells in late log phase or early stationary phase of growth were split 1:2 in fresh medium 24 h before use.

$^{125}$I-Insulin was prepared at a specific activity of 200 $\mu$Ci/$\mu$g by a modification (11) of the chloramine-T method (12). The labeled insulin was purified by filtration on Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) at 4°C before each experiment.

### Incubation Conditions

Cells were removed from the growth medium by centrifugation at 600 g for 10 min and washed once by resuspension in the buffer used for the incubation. Approximately 4–5 × 10⁶ cells/ml were incubated in assay buffer (50 mM HEPES, 120 mM NaCl, 1.2 mM magnesium sulfate, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 1 mg/ml bovine serum albumin, pH 7.6) with 5–6 ng/ml $^{125}$I-insulin at 15° and 37°C for various periods of time in 17 100 mm plastic Falcon tubes (Falcon Plastics, Div., Becton, Dickinson & Co., Oxnard, Calif.). Identical incubations were carried out in the presence of 10 $\mu$g/ml unlabeled insulin to determine nonspecific binding. At appropriate time intervals, 1.5 ml of cells were gently aspirated into conical plastic tubes, centrifuged at 200 g at 4°C for 5 min, and the supernate was aspirated. 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was then added to the cell pellet and fixation was carried out for a minimum of 4 h at room temperature. The glutaraldehyde was then aspirated and replaced with 0.1 M phosphate buffer (pH 7.4) until further processing. Radioactivity in the cell pellet was determined in a gamma counter.

### Analysis of Dissociation of Cell-associated Radioactivity

At the end of 2 h of association at 37°C, in a separate set of incubations carried out exactly as described above, aliquots of cells (100 $\mu$l or 1.5 ml) were taken and transferred to a series of tubes that contained 10 ml of assay buffer and 1 $\mu$g/ml unlabeled insulin at 15°C. At intervals (15, 30, 60, and 90 min) the tubes were centrifuged, and the cells were fixed for EM analysis (1.5-ml samples) or the radioactivity in the cell pellet was counted (100-$\mu$l samples).

### Preparation for Conventional Electron Microscopy and Autoradiography

After three successive washes in phosphate buffer, the cell pellet was postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, dehydrated in graded ethanol, and embedded in Epon (Shell Chemicals Co., Houston, Tex). Grids that contained the sections were coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England). After 3–5 wk, the grids were developed in Microdol X (Eastman Kodak Co., Rochester, N. Y.). Samples were then examined in a Philips EM 300 electron microscope (Philips Instruments, Eindhoven, The Netherlands) and grains photographed only on cells that were judged to be well preserved. Photographs were taken at a magnification (× 9,000) calibrated with a reference grid (2.160 lines/mm) (Ernest F. Fulham Inc., Schenectady, N. Y.).

### Preparation for Freeze-Fracture

The glutaraldehyde-fixed pellet was immersed in 30% glycerol buffered with 0.1 M phosphate buffer (pH 7.4) for at least 30 min, rapidly frozen in Freon 22 cooled in liquid nitrogen, then fractured and shadowed in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to the technique of Moor and Mühlethaler (13). Replicas were cleaned by sequential treatment with sodium hypochlorite and dimethyl formamide, rinsed in distilled water, recovered on copper grids, and examined in a Philips EM 300 electron microscope. Photographs were taken at magnifications (× 9,000 and × 45,000) calibrated with a reference grid (see above).

### Quantitative Analysis

THIN SECTION: Evaluation of the percentage of the total cell surface occupied by villous and nonvillous surfaces was carried out by morphometry.

Thin sections of three different Epon blocks for each incubation time studied (one block per experiment) were examined. Eight pictures of lymphocytes from each block (a total of 24 pictures for each experimental condition) were made at an initial magnification of × 3,000. For each section, random sampling was ensured by photographing each corner of two consecutive squares of the supporting grid. Each picture contained ~3–5 lymphocyte sections, resulting in a total of ~100 lymphocyte sections analyzed for each time-point.

Morphometric determinations were made on negatives with a test screen (250 × 215 mm) in the form of double-period square lattice (with a 1:9 ratio) used on a table projection unit (14). The rectangular frame enclosed 891 test points, of which 99 were set off as "heavy points." The coarse grid was useful for estimating the cell volume. The fine grid was used to estimate the surface density of the villous and nonvillous surfaces. The surface densities of villous[1] (Sv$_v$) and nonvillous (Sv$_n$) surfaces were determined by $Sv = 2I/(9 \cdot P_T \cdot d)$, where $I$ is the number of intersections between villous cell surface (I$_v$) or nonvillous cell surface (I$_n$) and the horizontal lines of the fine grid, $P_T$ is the number of the test points of the coarse grid enclosed by the lymphocytes, and $d$ is the length of the test line (14). Because the sum of the surface densities of villous and nonvillous surfaces results in the surface density of the whole cell, it was possible to calculate the proportion of the total cell surface occupied by villous and nonvillous surfaces.

Evaluation of the percentage of coated and noncoated invaginations was carried out by analyzing ~200 invaginations in each experimental condition.

AUTORADIOGRAPHY: For each association time studied, three different experiments were analyzed and a minimum of 600 grains associated with the plasma membrane were photographed (~200 in each experiment). Quantifications were carried out on positive prints enlarged three times (final magnification, × 27,000). A grain was considered associated with the plasma membrane when its center was ±250 nm from the plasma membrane. Assuming a half-distance (HD) of 80–100 nm (15), this distance of 250 nm should include >85% of the grains originating from a source associated with the plasma membrane. Grains were divided into five classes based on their association with specialized segments of the plasma membrane: (a) coated pits, (b) villi, (c) nonvillous and noncoated segments, (d) shared association villous/nonvillous surface, and (e) uninterpretable. Grains were considered associated with villi or coated pits if their center was <250 nm from these specialized segments; they were categorized in d when both nonvillous and villous segments were present within a distance of 250 nm from the grain center; and they were considered uninterpretable when the plane of section was inappropriate for a clear identification of the plasma membrane or when the structures underlying the grain could not be unequivocally identified. At each time-point ~8% of the grains were classified as uninterpretable.

FREEZE-FRACTURE: In each experimental condition tested, a minimum of 24 membrane faces (protoplasmic [P] and extracellular [E] faces) present in two different replicas (2 × 12) were photographed sequentially at an initial magnification of × 9,000 for the determination of the frequency of membrane invagination. Membrane faces were carefully selected for flatness. Quantitative evaluations were carried out on positive prints (enlarged three times) on a graphic tablet (type 4953; Tektronix, Inc., Beaverton, Oreg.) connected with a microprocessor (IMSAI, type 8080) allowing the different parameters to be recorded and statistically analyzed.

The number and size of intramembrane particles were evaluated on pictures made at a higher magnification (× 45,000). A minimum of 24 P faces were photographed for each experimental condition. Numbering of intramembrane particles was performed on positive prints enlarged three times (final magnification, × 105,000) by counting these features inside a square grid calibrated so as to represent 0.25 $\mu$m². The number found was multiplied by 4 to obtain the result per 1 $\mu$m². In the two extreme conditions studied (2 min, 15°C and 180 min, 37°C), 25 P faces of microvilli were photographed and the numbering of intramembrane particles was performed on positive prints enlarged three times (final magnification, × 135,000) by counting the features on the flatter zone of the microvilli. Counting was carried out inside small squares (0.004 $\mu$m²), and for each villi the counting was done on the largest number of squares possible.

All size measurements were performed with the aid of an × 8 magnifier containing a reticle calibrated in tenths of a millimeter.

## RESULTS

### Surface Characteristics of the IM-9 Cultured Human Lymphocytes

As seen in the scanning microscope, the surface of IM-9 lymphocytes was covered by numerous cytoplasmic projections: the microvilli (Fig. 1). In the experimental conditions

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[1] We considered as microvilli those cytoplasmic projections rich in microfilaments and devoid of intracytoplasmic organelles.
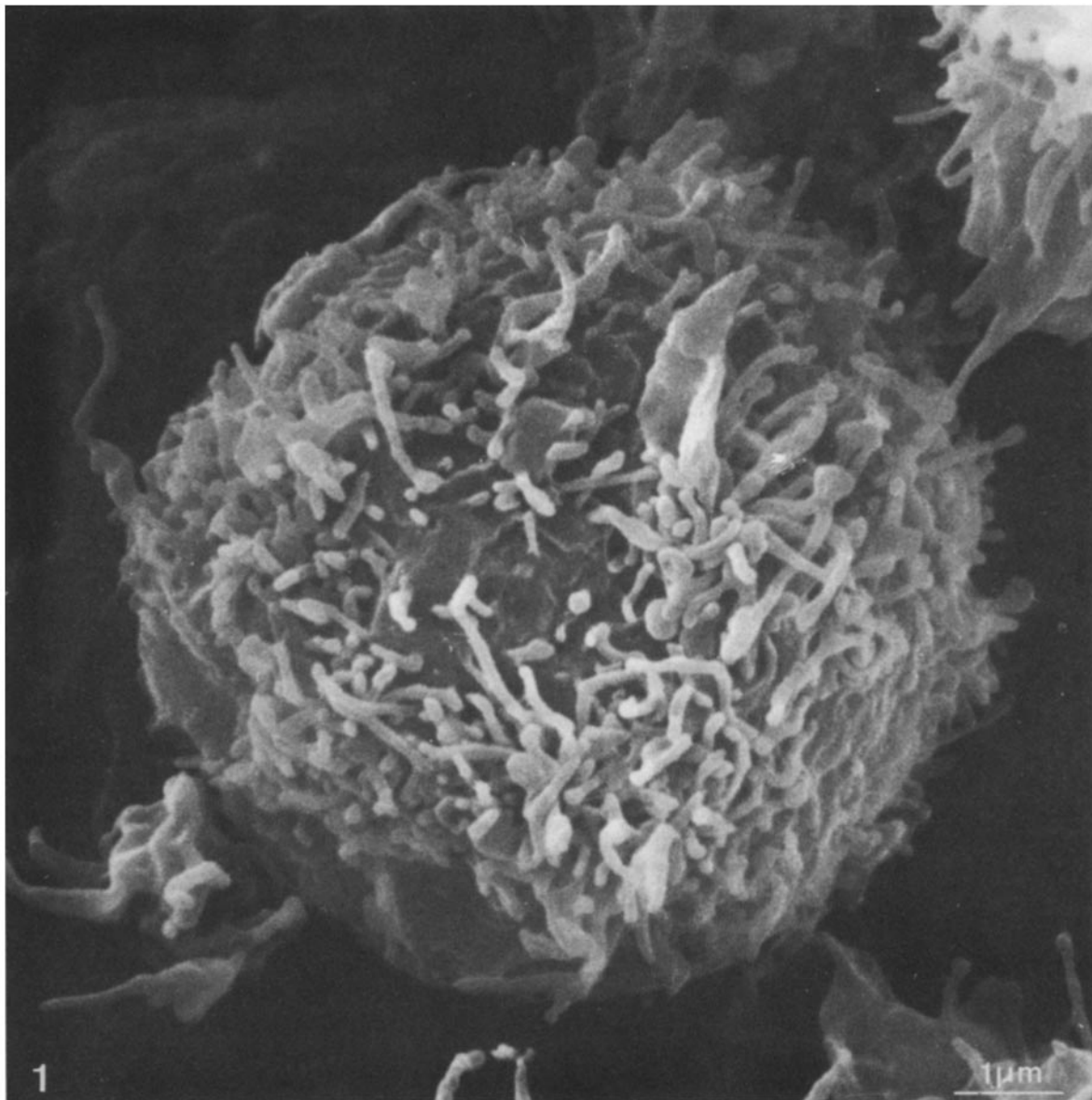
FIGURE 1   Low power view of an IM-9 cultured human lymphocyte seen in the scanning electron microscope. The cell is covered by numerous microvilli. × 15,000.

analyzed, the cells were well preserved and numerous biochemical studies have demonstrated that they contained insulin receptors with characteristics similar to those found in other cell types (for review, see reference 11).

## General Characteristics of $^{125}$I-Insulin Binding to IM-9 Lymphocytes

When IM-9 lymphocytes were incubated with $^{125}$I-insulin for short periods of time (2 min) at 15° or 37°C, developed grains were frequently seen associated with the cell periphery (Fig. 2a). This observation was previously confirmed by a quantitative analysis showing that the labeled material (most of which was intact insulin) was initially restricted to the plasma membrane (16). At higher magnification a large proportion of grains was seen to overlie microvilli (Fig. 2b). After longer incubation periods at 37°C (120–180 min), or when further binding was prevented by dilution in the presence of an excess unlabeled insulin (dissociation experiments), 15–25% of the cell-associ-

ated labeled material was internalized (16, 17). Grains that remained localized on the plasma membrane were, for the most part, associated with nonvillous segments (Fig. 2c)[2]. Under these conditions, grains were frequently found over or in the vicinity of coated pits (Fig. 3).

## Relationship of Autoradiographic Grains to Microvilli

To determine whether the grains associated with the plasma membrane (±250 nm from the plasma membrane) had a

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[2] As determined by gel filtration, after 180 min of incubation at 37°C, 80% of the cell-associated radioactivity corresponded to intact insulin (Van Obberghen, Carpentier, and Gorden, unpublished observations). Furthermore, the typical degradation products of insulin (small iodotyrosyl peptides or iodine) were removed from the preparation during the dehydration procedure required for preparing the tissues for electron microscopy and, therefore, did not contribute significantly to developed grains (see reference 10).
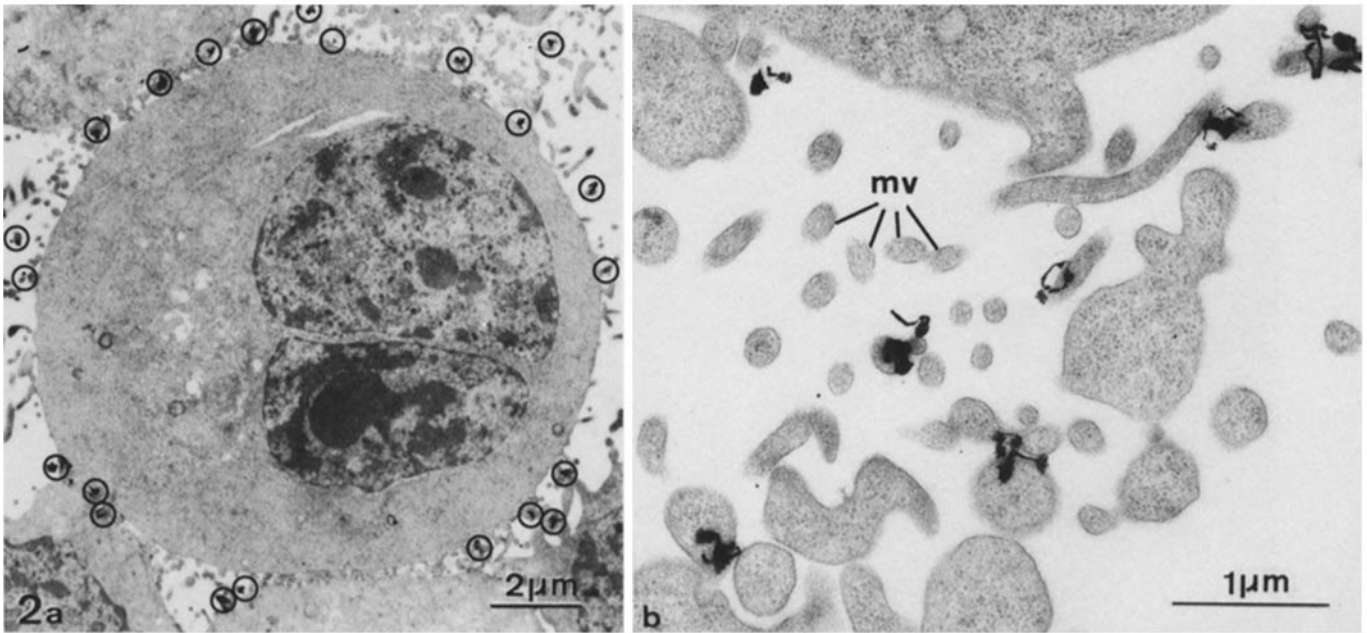
FIGURE 2  (a) General view, at low magnification, of IM-9 cultured human lymphocytes incubated with $^{125}$I-insulin for 2 min at 37°C. Grains (within circles) are localized at the cell periphery where they are frequently seen associated with microvilli. (b and c) Comparison at higher magnification of the grain distribution on the cell surface at the beginning (2 min) (b) and the end of the incubation (180 min) (c) at 37°C. After 2 min of incubation grains are predominantly found over, or close to, microvilli (mv), whereas after 180 min of incubation grains are more frequently seen on the nonvillous surface of the cell. a, × 6,000; b, × 21,000; c, × 21,000.
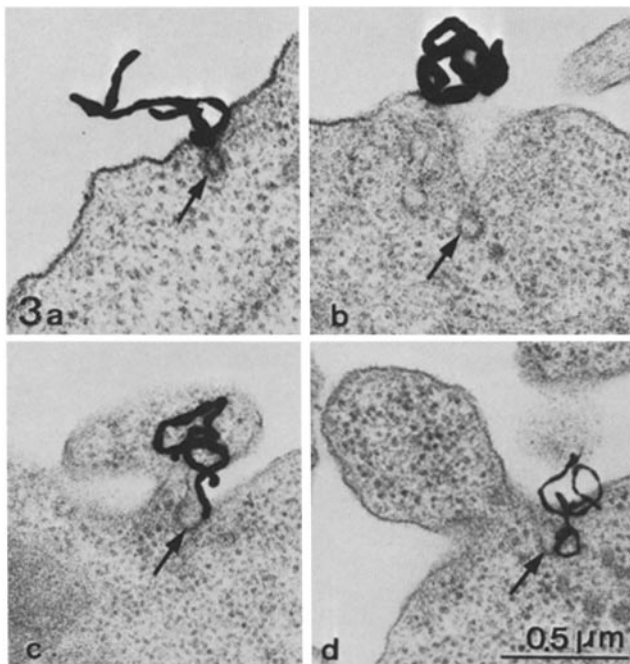
preferential relationship to microvilli, we determined, first, the percentage of the total cell surface occupied by microvilli and, second, the relationship of grains to these structures.

As measured by morphometry, microvilli of IM-9 lymphocytes constituted 53–55% of the total cell surface; this percentage remained practically constant with incubation time at both 15° and 37°C (Table I). After 2 min of incubation at 15° and 37°C, 68% of the grains associated with microvilli, but at both temperatures this value decreased progressively with time and reached 55% after 180 min at 37°C (Table I). Thus, as determined by the $\chi^2$ test (Table I), and by the ratio of percent grains related to microvilli over percent microvilli (Fig. 4), $^{125}$I-insulin showed an initial preferential association with microvilli, but with incubation time, this preferential association was lost. During dissociation (see above) the ratio of percent grains related to microvilli over percent microvilli reached values

FIGURE 3  Selected images of developed autoradiographic grains localized in the vicinity of coated pits on the plasma membrane of IM-9 cultured human lymphocytes. Coated pits (arrows) are found on flat regions of the plasma membrane (a), at the base of cytoplasmic projections (c and d), or at the bottom of fingerlike processes (b). A, × 38,000; B, × 33,000; C, × 33,000; D, × 33,000.

TABLE I

Relationship of Grains to Microvilli

| Conditions | Number of grains ana-lyzed | Grains associ-ated with villi | | Villous surface |
|---|---|---|---|---|
| | | % | | % |
| 2 min, 15°C | 668 | 68 | P < 0.001* | 53 |
| 120 min, 15°C | 673 | 60 | NS | 53 |
| 2 min, 37°C | 887 | 68 | P < 0.001 | 53 |
| 60 min, 37°C | 642 | 59 | NS | 55 |
| 180 min, 37°C | 618 | 55 | NS | 55 |
| 30-min dissociation‡ | 123§ | 46 | P < 0.01 | 56 |
| 90-min dissociation‡ | 80§ | 41 | P < 0.001 | 57 |

* Because of the low number of grains that remained associated with the plasma membrane during the dissociation experiment, grains found at 10 and 30 min of dissociation were pooled under *30 min dissociation* and those found at 60 and 90 min were pooled under *90 min dissociation*.
‡ Statistical determinations were carried out by the $\chi^2$ test.
§ Cells were first incubated for 180 min at 37°C in the presence of 5 ng/ml $^{125}$I-insulin; the medium was then removed and replaced by fresh buffer containing an excess unlabeled insulin (1 μg/ml) (see Materials and Methods).

below 1, which indicated a preferential association of the ligand with nonvillous segments of the plasma membrane.

## Relationship of Autoradiographic Grains to Coated Pits

To determine whether grains associated with the plasma membrane had a preferential relationship to coated pits, we determined, first, the percentage of the cell surface occupied by coated pits and, second, the relationship of grains to these membrane specializations.

To evaluate the percentage of the cell surface occupied by coated pits, we first measured the frequency of membrane invaginations (coated or noncoated) per 100 μm² of plasma membrane. This quantification was carried out on freeze-fracture replicas where membrane invaginations appear as small pits on the P face of the fractured membrane (Fig. 5) and as small mounds on the E face of the membrane. Because the fracturing procedure cut most microvilli (Fig. 5), this quantification gave us an estimate of the frequency of invaginations per 100 μm² of nonvillous surface of the cell. Knowing the percentage of the total cell surface occupied by the nonvillous segments (see above), it was then possible to calculate the frequency of invaginations per 100 μm² of total cell surface. Values obtained remained constant with incubation time and temperature and were not affected by insulin treatment (data not shown). On the basis of the evaluation (carried out in thin sections) of the percentage of total invaginations that were coated³, one could know the percentage of coated invaginations per unit surface area. Multiplying the percentage of coated invaginations per 100 μm² by the mean surface of coated invagination ($\pi D^2$ were D (the mean diameter) is 44 ± 1 nm, $n = 260$), one could then evaluate the percentage of the cell surface occupied by coated pits. Values obtained could be expressed per unit area of total cell surface or per unit area of nonvillous cell surface (Table II). When expressed per unit area of total cell surface, the results were comparable to pre-

³ In the various experimental conditions tested, a mean of 47% of total cell surface invaginations were coated (range was 46.1–48.4%).

viously published data obtained by other methods in other cell types (1, 3).

After 2 min of incubation at 15° and 37°C, respectively, 1.7 and 2.1% of the total number of grains analyzed and 5.3 and 6.7% of the grains localized on the nonvillous surface were associated (±250 nm) with coated pits (Table II). The ratio of the percentage of grains related to coated pits on the nonvillous surface over the percentage of nonvillous surface occupied by coated pits indicated, therefore, that after 2 min of incubation at 15° or 37°C, grains preferentially associated with coated pits (Fig. 6). This preferential association with coated pits increased progressively with time and temperature, and reached a value of 14.0 during dissociation(see above) (Fig. 6).

## Freeze-Fracture Analysis of Microvilli

Freeze-fracture replicas of IM-9 lymphocyte plasma membrane reveal globular subunits, the so-called intramembrane particles (Fig. 5). These structures represent, at least in part, the morphologic equivalents of membrane proteins (18–20) and presumably membrane receptors (21–23).

The frequency of intramembrane particles per μm² of nonvillous P face remained unchanged during incubation with physiological concentrations of insulin, and these values were similar to those observed in control conditions (absence of insulin) (Table III). When measured after 2 min of incubation at 15°C, and after 180 min at 37°C in the absence or presence of 5 ng/ml $^{125}$I-insulin, the frequency of intramembrane particles on P faces of microvilli was in both cases 60% higher than on the nonvillous surface of the cell (Fig. 7 and Table III). After 2 min of incubation at 15°C, the intramembrane particles located on villi exhibited a larger diameter than did the intramembrane particles in nonvillous regions of the plasma membrane (Fig. 8). A similar finding was observed after 180 min of incubation at 37°C.
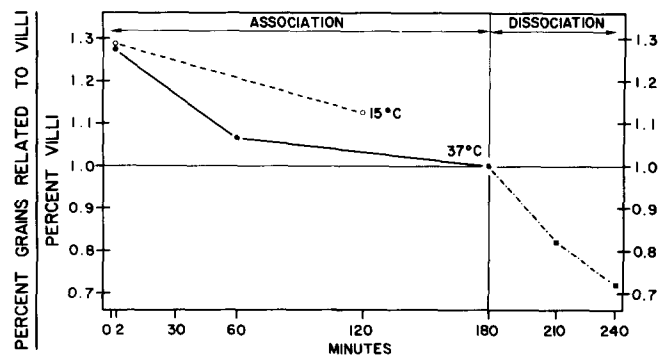


FIGURE 4   Relationship of autoradiographic grains to microvilli. Results are expressed as the ratio of the percentage of grains related to microvilli over the percentage of the total cell surface occupied by microvilli. During the association period, cells were incubated with 5 ng/ml $^{125}$I-insulin at 15° (O- - -O) or 37°C (●———●). During dissociation (■-·-■), after extensive washings, cells were incubated in the absence of $^{125}$I-insulin and presence of 1 μg/ml unlabeled insulin. Ratios above 1 indicate a preferential association with microvilli, and ratios below 1 indicate a preferential association to nonvillous segments of the plasma membrane. At both 15° and 37°C, the initial preferential localization of grains is to microvilli. As a function of time, this preferential association disappears progressively. During dissociation, grains preferentially associate with the nonvillous surface of the cell.
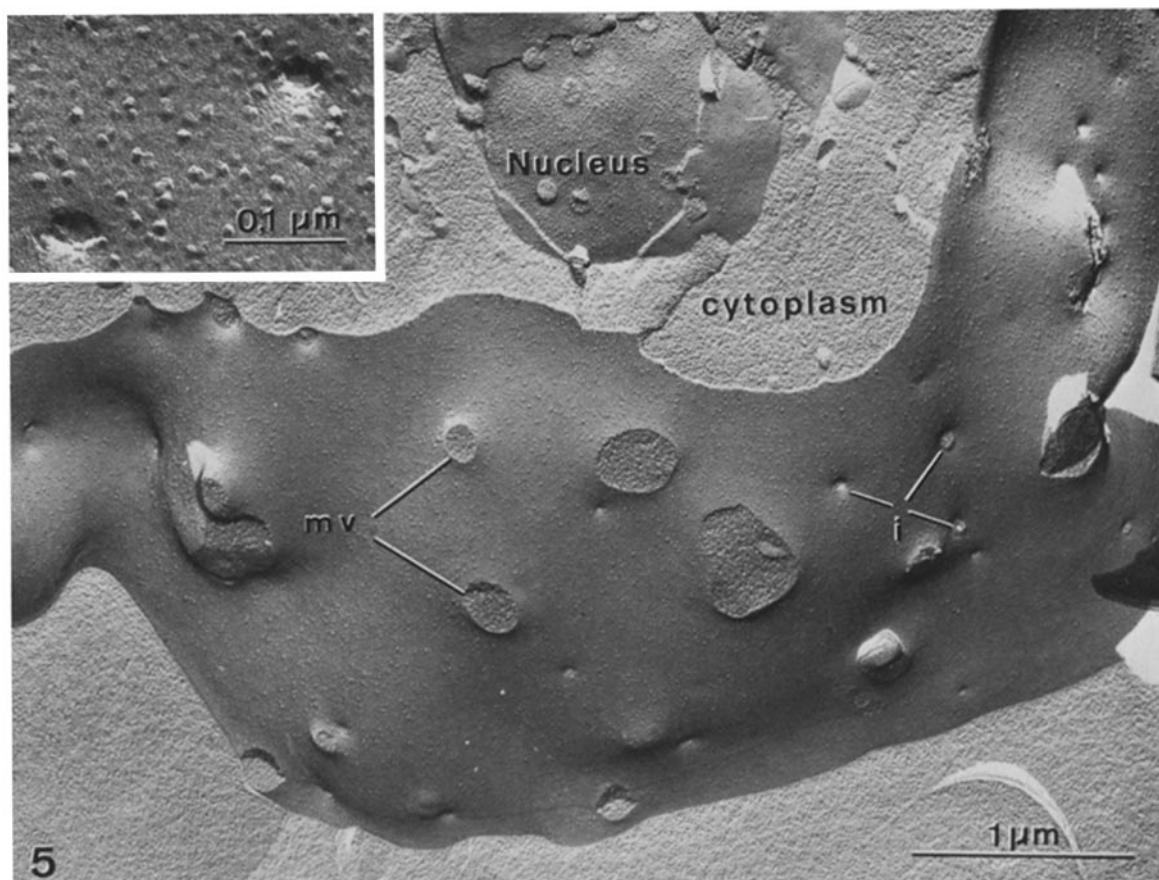
FIGURE 5 Freeze-fracture replica showing the P face (inner leaflet) of the membrane, the cross-fractured cytoplasm, and the nucleus of an IM-9 lymphocyte incubated in the presence of 5 ng/ml $^{125}$I-insulin for 2 min at 37°C. On the P face, the necks of membrane invaginations appear as small depressions (i), and microvilli are frequently fractured at their base (mv). Intramembrane particles are numerous and randomly distributed (inset). × 29,000; inset, × 160,000.

TABLE II

Relationship of Grains to Coated Pits

| Conditions | Total cell surface | | Nonvillous cell surface | |
|---|---|---|---|---|
| | Grains related to coated pits | Coated pits | Grains related to coated pits | Coated pits |
| | % | % | % | % |
| 2 min, 15°C | 1.7 | 1.0 | 5.3 | 1.8 |
| 120 min, 15°C | 2.3 | — | 6.1 | — |
| 2 min, 37°C | 2.1 | 1.0 | 6.7 | 1.9 |
| 60 min, 37°C | 2.8 | 1.0 | 7.0 | 1.8 |
| 180 min, 37°C | 4.0 | 0.9 | 9.0 | 1.7 |
| Dissociation* | 10.2 | 0.9 | 23.4 | 1.6 |

* Because of the low number of pictures available at each time of dissociation, the different time points were pooled.

## DISCUSSION

We and others have previously demonstrated by morphological techniques that the initial binding of insulin is restricted to the plasma membrane of the target cell (8, 16, 24–28). These observations were in total agreement with previous biochemical data. In the present study, we have further characterized this initial interaction and have analyzed by quantitative EM au-

toradiography the distribution of $^{125}$I-insulin on the surface of IM-9 cultured human lymphocytes in various experimental conditions. Our findings indicate that, after 2 min of incubation at 15°C, $^{125}$I-insulin is not randomly distributed on the cell surface but, rather, preferentially associates with microvilli and coated segments. With an increase in incubation time and temperature, the preferential association with microvilli progressively decreases, whereas the preferential association with coated segments increases. When further binding is prevented by dilution in the presence of an excess of unlabeled insulin (dissociation experiment), labeled material that remains associated with the cell surface preferentially localizes to nonvillous segments and, more precisely, to coated pits on the nonvillous surface of the lymphocytes.

These data are consistent with the concept of an ordered pattern of ligand binding to the cell surface: (a) the microvilli represent a large surface for initial contact with the ligand, (b) the hormone-receptor complex then moves from the villous to the smooth surface of the cell, (c) the complex then redistributes toward the Golgi pole of the cell (6, 9), and concomitantly concentrates in coated regions of the cell, presumably for internalization. The frequent finding of autoradiographic grains associated with uropods (29), typically located at the Golgi pole of the cell and characterized by numerous cytoplasmic projections, coated pits, and coated vesicles, supports this concept.

Present data, which are the first to demonstrate the initial

binding of a polypeptide hormone to microvilli, are in agreement with previous studies showing localization of surface immunoglobulins on microvilli of B lymphocytes (30), of anionic sites on cytoplasmic projections of baby hamster kidney (BHK) cells (31), and of Semliki Forest virus on microvilli of BHK-21 cells (32).

The present study clarifies our previous failure to demonstrate initial preferential localization of insulin to coated pits (6). The high percentage of grains initially associated with microvilli (68%) masks a concomitant preferential association of the ligand with coated segments that are restricted to the nonvillous surface of the cell. A similar "masking effect" should also be taken into account in the interpretation of other studies in which a preferential association of the ligand with coated pits was not detected (10, 27, 33-36). The proportion of grains that initially associate with coated pits remains low, however. These results contrast with the high percentages of LDL and

EGF found initially concentrated in coated pits on the plasma membrane of cultured fibroblasts (1, 3) but are close to the ~5% of virus particles directly bound to coated pits of BHK-21 cells (32).

Data concerning the association of autoradiographic grains with microvilli and coated pits in the different incubation conditions studied were all expressed in terms of percentage of the total number of grains analyzed. The analysis of the evolution of grain distribution is therefore subject to several interpretations. When $^{125}$I-insulin is incubated at low temperatures with cultured human lymphocytes, the ligand progressively associates with the cell. Binding is a function of the number of receptor sites and the affinity of the receptor for the ligand. When the temperature is raised to 37°C, the total number of binding sites does not change but the affinity decreases and the ligand dissociates from the cell. A small amount (~15%) of the labeled material is internalized by the cell. We have interpreted our data to mean that the ligand
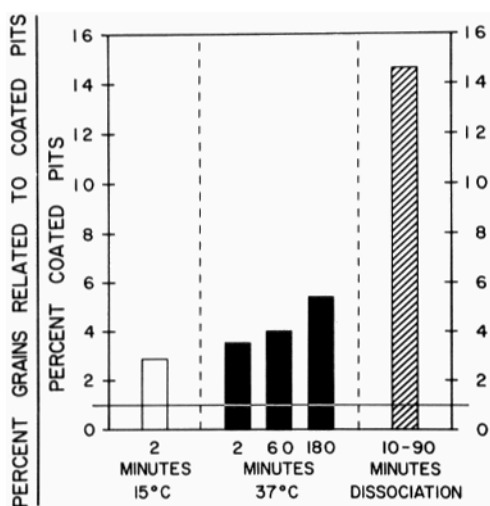


FIGURE 6   Relationship of autoradiographic grains to coated pits on the nonvillous surface of the cell. Results are expressed as the ratio of the percentage of grains related to coated pits over the percentage of the nonvillous surface of the cell occupied by coated pits. Ratios above 1 indicate a preferential association with coated pits. By 2 min at both 15° and 37°C, grains preferentially associate with coated pits. As a function of incubation time at 37°C, this preferential association increases progressively. During dissociation, the preferential association ratio reaches a value above 14.
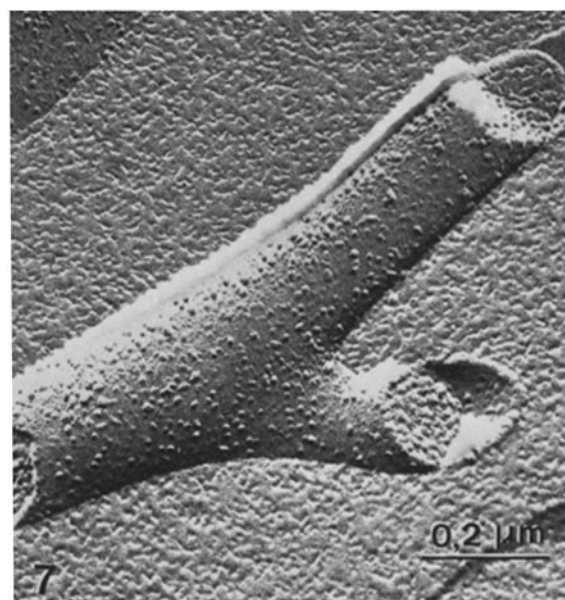


FIGURE 7   Freeze-fracture replica of microvilli of IM-9 cultured human lymphocytes. Determinations of concentration and size of intramembrane particles were restricted to regions with adequate shadowing. × 86,000.

TABLE III

Frequency of Intramembrane Particles/$\mu$m$^2$— P Face

| Conditions | Control | | $^{125}$I-Insulin (5 ng/ml)* | |
|---|---|---|---|---|
| | Nonvillous | Villous | Nonvillous | Villous |
| | P < 0.001 | | P < 0.001 | |
| 2 min, 15°C | 1,080 ± 35 | 1,736 ± 60 | 1,086 ± 25 | 1,736 ± 56 |
| | n = 24 | n = 21 | n = 27 | n = 25 |
| 2 min, 37°C | — | — | 1,090 ± 34 | — |
| | | | n = 26 | |
| 60 min, 37°C | — | — | 1,064 ± 28 | — |
| | | | n = 24 | |
| | P < 0.001 | | P < 0.001 | |
| 180 min, 37°C | 1,084 ± 32 | 1,808 ± 56 | 1,066 ± 32 | 1,720 ± 72 |
| | n = 28 | n = 22 | n = 30 | n = 25 |

n, Number of membrane faces analyzed. Statistical determinations were carried out by the Student's t test.
* No statistically significant differences were noted between the frequencies of intramembrane particles on P faces from control or 5 ng/ml $^{125}$I-insulin-treated IM-9 lymphocytes.
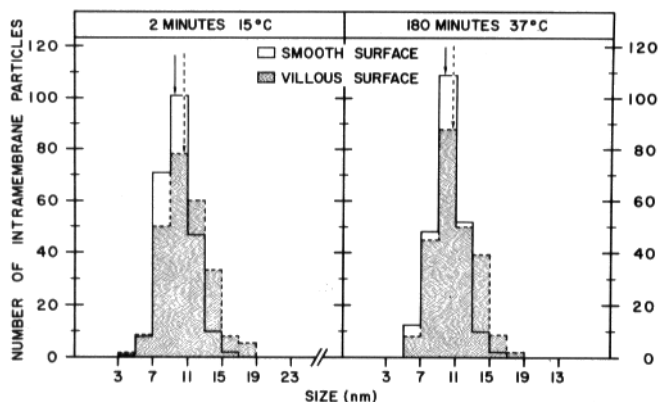
FIGURE 8  Size distribution of intramembrane particles on P faces of IM-9 cultured human lymphocyte plasma membrane after 2 min of incubation at 15°C and 180 min of incubation at 37°C in the presence of 5 ng/ml $^{125}$I-insulin. In each condition, the size distribution of intramembrane particles located on the nonvillous surface of the cell (open columns) is compared to the size distribution of intramembrane particles located on the villous surface of the cell (stippled column). The mean sizes are represented by the vertical arrows. Numbers of particles analyzed ($n$) and mean sizes ($\bar{x}$). 2 min, 15°C nonvillous surface: $n = 240$, $\bar{x} = 98.7 \pm 1.3$, villous surface: $n = 241$, $\bar{x} = 109.0 \pm 1.7$; 180 min, 37°C nonvillous surface: $n = 248$, $\bar{x} = 101.7 \pm 0.8$, villous surface: $n = 239$, $\bar{x} = 109.2 \pm 1.6$. Similar mean sizes and size distributions of intramembrane particles are observed in the absence of $^{125}$I-insulin (data not shown).

initially binds to the villous surface of the cell and redistributes by movement in the plane of the plasma membrane. An alternative interpretation could involve differential affinities between receptors on the villous and nonvillous surface of the cell. Thus, a rapid dissociation from the villous surface and a slower dissociation from the nonvillous surface would lead to an apparent redistribution of the ligand. It is also true that these alternative explanations for redistribution of the ligand are not mutually exclusive and that both events could occur simultaneously. For example, one could speculate that compartmentalization exists in coated pits that concentrate the ligand, giving the ligand an apparently greater avidity for its receptor. At the present time there is, however, no information to suggest that receptors at different loci in the membrane have different affinities. An additional feature favoring the translocation hypothesis relates to the binding of a unique immunoglobulin (anti-insulin receptor antibody) isolated from a patient with an extreme form of insulin resistance (37). This immunoglobulin competes for insulin binding and is an insulin agonist (37, 38). This material binds diffusely to the cultured lymphocyte at low temperatures but redistributes to the Golgi pole of the cell at 37°C (7, 9). This antibody, in contrast to insulin, predominantly binds irreversibly to the cell (39). Thus, differential affinities are not relevant to the antibody redistribution.

The studies in the present paper have yielded two additional findings that support the idea that the microvilli represent a discrete entity with a structure distinct from that of the body of the plasma membrane: (a) quantitative evaluation of the freeze-fracture replicas showed that microvilli contained 60% more intramembrane particles per square micrometer of surface than did the nonvillous surface of the cell, and (b) the mean diameter of the intramembrane particles of the microvilli was significantly larger than that of the particles of the nonvillous membrane regions. Similar characteristics were previously noted for

intramembrane particles found within large pits (coated pits) on the plasma membrane of cultured human fibroblasts (40).[4] It is of interest to note that the present data, showing a higher concentration of intramembrane particles in specialized regions of the cell where a preferential binding of insulin is observed, are in agreement with previous data showing an increased concentration of intramembrane particles in the plasma membrane of adipocytes with increased insulin-binding capacities (diabetes, fasting) (41) and a decreased concentration of particles in an opposite condition (ob/ob mice) (42).

Finally, these studies demonstrate quantitatively specialized forms of compartmentalization all at the level of the plasma membrane. Functionally, these events could provide a mechanism for "trapping" the specific ligand and segregating the specific receptors for purposes of removal from the cell surface. This type of specificity is a prerequisite to receptor-mediated receptor regulation.

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[4] In IM-9 lymphocytes, the small size of coated invaginations does not allow an appropriate visualization of the invaginated membrane and excludes such a quantitative analysis.

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