Diffusion of Low Density Lipoprotein-Receptor Complex on Human Fibroblasts

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ABSTRACT Diffusion of the complex consisting of low density lipoprotein (LDL) bound to its receptor on the surface of human fibroblasts has been measured with the help of an intensely fluorescent, biologically active LDL derivative, dioctadecylindocarbocyanine LDL (diI(3)-LDL). Fluorescence photobleaching recovery and direct video observations of the Brownian motion of individual LDL-receptor complexes yielded diffusion coefficients for the slow diffusion on cell surfaces and fast diffusion on membrane blebs, respectively. At 10°C, <20% of the LDL-receptor complex was measurably diffusible either on normal human fibroblasts GM-3348 or on LDL-receptor-internalization-defective J. D. cells GM-2408A. At 21°C and 28°C, the diffusion coefficients of the LDL-receptor complex were 1.4 and 4.5 × 10^{-11} cm²/s with diffusible fractions of ~75 and 60%, respectively, on both cell lines. The lipid analog nitrobenzoxadiazolephosphatidylcholine (NBD-PC) diffused in the GM-2408A cell membrane at 1.5 × 10^{-8} cm²/sec at 22°C. On blebs induced in GM-2408A cell membranes, the diI(3)-LDL receptor complex diffusion coefficient increased to ~10^{-9} cm²/s, thus approaching the maximum theoretical predictions for a large protein in the viscous lipid bilayer. Cytoskeletal staining of blebs with NBD-phallacidin, a fluorescent probe specific for F-actin, indicated that loss of the bulk of the F-actin cytoskeleton accompanied the release of the natural constraints on lateral diffusion observed on blebs. This work shows that the internalization defect of J. D. is not due to immobilization of the LDL-receptor complex since its diffusibility is sufficient to sustain even the internalization rates observed in the native fibroblasts. Nevertheless, as with many other cell membrane receptors, the diffusion coefficient of the LDL-receptor complex is at least two orders of magnitude slower on native membrane than the viscous limit approached on cell membrane blebs where it is released from lateral constraints. However, LDL-receptor diffusion may not limit LDL internalization in normal human fibroblasts.

Low density lipoprotein (LDL), like insulin and epidermal growth factor (EGF), is one of a class of proteins that binds to a specific high-affinity cell surface receptor (2, 3) and is then internalized by the cell at a clathrin-coated area of membrane, a coated pit (4, 5). In normal human fibroblasts, >70% of the LDL receptor (LDL-R) population appears localized in coated pits even before LDL binding occurs (6). Thus, it is unclear whether diffusion to a coated pit on the membrane by either the bare LDL receptor or LDL bound to the LDL receptor, the LDL-receptor complex (LDL-RC), is a necessary step in the regulation of cholesterol synthesis and degradation. In contrast, the insulin and EGF-receptor complexes are reported to diffuse and form clusters before internalization at coated pits (8, 9). The mobility of a ligand-receptor complex can be studied by fluorescence photobleaching recovery (FPR) by monitoring the characteristics of the relaxation of concentration gradients induced in a distribution of fluorescence-labeled receptor complex on the cell surface. However, in the case of LDL-RC on normal fibroblasts some LDL-RC may internalize during the course of the FPR measurement. In this case, surface fluorescence may be difficult to distinguish from internalized fluorescence, so that interpretation of the data becomes ambiguous.

Brown and Goldstein have characterized a line of human fibroblasts, J. D. (also designated as GM-2408A), which appears unable to internalize the LDL-RC even after LDL has
mounted for experiments as described below. An alternative lesion consists of some constraint of the diffusibility of the LDL receptors that prevents their reaching the coated pits from their (random) membrane insertion sites. We can test these hypotheses by measuring the diffusion coefficient of the LDL-RC on J. D. cells, calculating the limiting rate of diffusion to coated pits, and comparing the result with the residual internalization rate. Goldstein et al. (14) have analyzed the kinetics of internalization of LDL on normal human fibroblasts and have observed that our preliminary value of the diffusion coefficient determined on J. D. cells (15) is just sufficient to account for the LDL internalization rate on normal cells which may thus be diffusion-limited. This paper reports additional results, including more details of our experiments, and explores some factors that may limit the LDL receptor diffusibility.

Only a few LDL receptors per square micrometer exist on fibroblasts (7). Thus, if each LDL receptor were labeled with a single fluorophore the fluorescence signal would be too small for an FPR experiment. However, in a previous report, we described the synthesis and characterization of a highly fluorescent LDL derivative (16), dioctadecyldiacarbocyanine-LDL (diI-LDL), that possesses the binding and enzyme inhibitory properties of native LDL and makes FPR experiments with LDL possible. This probe which has negligible nonspecific binding (0.5-3%) is used in the experiments described below. Many of the essential controls for this paper were reported in our previous paper (16).

Our experiments address two questions. First: On what time scale is the LDL-RC diffusible on the cell surface? Results of diI-LDL receptor complex diffusion on J. D. cells at 10°C, 22°C, and 28°C and on GM-3348 normal fibroblasts at 10°C are reported. As a standard with which to compare these LDL diffusion results, we also measured the diffusion on the lipid probe nitrabenzoazidazol-phosphatidyl choline (NBD-PC) on J. D. cells at 22°C. Second: Does the LDL-RC receptor complex interact with some component of the cell cytoskeleton that regulates its mobility? We investigate this question by measurement of the random diffusive motion of individual diI-LDL complexes on membrane blebs where they are released from cytoskeletal constraints (17, 18). The relationship between the bleb membrane and the bulk of the F-actin cytoskeleton is determined by fluorescence staining with the actin-specific probe NBD-phallacidin (19-21).

**MATERIALS AND METHODS**

**Cell Labeling**

GM-2408A and GM-3348 fibroblasts were grown as previously described (16). LDL and diI-LDL were prepared as in reference 16. All buffers were purchased from Gibco, Grand Island Biological Company (Grand Island, NY) and were maintained at 0-4°C for diI-LDL labeling of cells. Cells grown on 22 × 23 mm glass coverslips were washed twice in phosphate buffered saline (PBS), once in Medium 199 plus HEPES at pH 7.3 (buffer A), incubated with a 9-12 μg/ml solution of diI-LDL in buffer A for 1-2 h at 4°C, washed three times in PBS, and incubated for 10 min in Hank’s Balanced Salt Solution supplemented with 2 mg/ml albumin (Sigma Chemical Co., St. Louis, MO), 2 mM calcium, and 10 mM Tris at pH 7.3. Cells were washed one more time in buffer A and then mounted for experiments as described below.

**Cell Labeling with NBD-PC**

The diffusion coefficient of the lipid probe NBD-PC (Molecular Probes, Eugene, OR) was measured to serve as a comparison for the LDL diffusion measurements, and to demonstrate that the data for free diffusion in the minoceptor beam (as described below) confirmed to theory. NBD-PC at 100 μg/ml in a solution of ethanol was added to buffer A at a ratio of 1/100.

Cells washed as described above were incubated with the solution of NBD-PC for 15 min at 4°C, washed twice in PBS, once in Medium 199, and mounted for FPR experiments.

**Fluorescence Photobleaching Recovery Measurements**

Diffusion of diI-LDL and NBD-PC on cells was measured by FPR (10, 22) using a pattern of alternating light and dark stripes as described by Smith and McConnell (23). Experiments were done with a Zeiss Universal Microscope with either a ×40 or ×100 neofluar oil immersion objective. The pattern of alternating light and dark stripes was imaged on the sample by placing a 100 or 200 lines-per-inch Ronchi ruling at the image plane behind the nespiece and objective lens optics and illuminating the ruling normal to its surface with the beam from a Spectra Physics Argon-Ion laser. Monitor and bleach beams were separated by a fixed 0.5-μm-thick quartz optical flat (Virgo Optics, Stirling, NJ) placed at 45° to the laser beam (24). The intense bleach beam was normally blocked by a shutter but could be recombined with the monitor beam by a second matched quartz flat placed parallel to the first. The recovery curves were analyzed by an algorithm similar to that described by Powell (26). A typical recovery curve for this technique is shown in Fig. 2. The smaller dots represent the fluorescence intensity at equal discrete intervals of time, whereas the large dots are the average of (2j + 1) consecutive intervals centered about the point where m = (j x j + j) and j = 0, 1, 2, . . . . The curve is fitted to the average points for computational economy, and to weight the early portion of the curve more heavily than the tail.

In contrast to FPR recovery curves for gaussian point bleach patterns which can recover to 100% of their initial prebleach value (10), the upper bound for “striped” bleach” recovery is 0.5 of the value between the prebleach amplitude and the amplitude of the first postbleach curve (23). The limiting amplitude at large times for a curve with low nonspecific background which recovers nearly 100% is given by:

\[ I_{\text{max}} = \frac{(4n^2 + 4n + 1) - (4n^2 + 4n + 1)}{2} \]

and the percent recovery is:

\[ R = \frac{(4n^2 + 4n + 1)}{I_{\text{max}} - I_{\text{min}}} \]

where \( n \) is the intensity at time t. \( I_{\text{max}} \) is the average prebleach intensity and \( I_{\text{min}} \) is the postbleach intensity. Bleaching pattern contrast was confirmed to a few percent by NBD-PC lipid probe recovery in multilayers.

**Cell Bleb Formation**

Cell blebs were produced by two methods on J. D. and GM-3348 cells that had been grown in the usual manner. (a) In the first method, cells were incubated at 37°C for 40 min with 25 mM formaldehyde and 1 mM dithiothreitol in PBS, labeled with 8-12 μg/ml diI-LDL alone in buffer A, or in buffer A with an 800 μg/ml excess of either albumin monomer (Miles Biochemical, Elkhart, IN) or LDL, washed twice in PBS, and mounted for viewing (24). (b) In the second method, blebs were produced on cells by incubations for 1 h at 22°C with a solution of dimethyl-3-dithiobispropionimidate (DBP). (Dr. W. Carley, personal communication). The cells were washed three times in PBS, incubated in a 150-nM solution of NBD-Ph in PBS, washed three times, and mounted for viewing in phase and fluorescence microscopy. NBD-Ph fluorescence was excited between 450 and 480 nm on a Nikon Optiphot microscope, and blebs were scored as to whether they showed NBD-Ph fluorescence. LDL is not prevented from binding to its receptor by such formaldehyde treatments (16, 25).

**Measurement of Diffusion on Blebs**

Blebs formed by method a, above, were labeled with diI-LDL and viewed through a neofluar × 100 oil objective using a 546-nm fluorescence excitation. LDL fluorescence was observed through a 590-nm barrier filter (Schott Optical, Duryea, PA). A series of real time fluorescence images from a SIT camera.
attached to the microscope were recorded at 1/30-s intervals on video tape. The displacements of the fluorescent images of the diI(3)-LDL molecules were measured as functions of time and plotted on clear acetate sheets placed over the TV monitor images of the video recordings. Particle positions at each end of the interval \( \delta t \) were determined and the displacement \( x(\delta t) \) was computed for a given particle. For fixed intervals \( \delta t \), the average squared displacement \( \langle x(\delta t)^2 \rangle \) for multiple particle displacements was computed. The diffusion coefficient \( D \) was determined by linear regression from a plot of \( x^2 / \delta t \) at each value of \( \delta t \), using the relationship of (27, 28):

\[
\langle x(\delta t)^2 \rangle = 4D \delta t.
\]  

(3)

**Bleaching Pattern Calibration**

The microscope ocular ruling that was used to measure bleaching grid periodicity was calibrated for each lens by measuring a 2-mm-long grid divided into 10-\( \mu \)m segments. The periodicity of the focused Ronchi pattern was then determined.

**Microscope Stage, Cell Culture Chamber**

These FPR experiments entailed extended observations of cells at well-defined temperatures using \( \times \) 100 oil immersion optics. This requires a cell coverslip mount with the following properties: closed construction to avoid pH changes and evaporation of buffer, good thermal ballast, compatibility with standard and temperature-regulated microscope stages, and ease of use. A coverslip of cells is mounted cell-side-down on a neoprene 0-ring recessed into a metal frame which presses against the coverslip face to form a watertight seal around the glass. Another coverslip mounted in a similar manner forms the bottom surface window of the cell. Two small holes drilled into the side provide access to the inner chamber for medium transfer or probe insertion. The holes can be covered with tape to close the chamber. Since the mount is nearly the size of a glass slide, it can be moved with standard microscope translators. The large heat capacity of the chamber maintains temperatures with 0.1°C on temperature-controlled stages without added insulation. Cell coverslips once mounted can be left in place on the chamber, returned to the incubator for further cell growth, and later reused for further observations.

**RESULTS**

**Diffusion of the Fluorescent Lipid NBD-PC**

A representative striped bleach, FPR recovery curve for NBD-PC diffusion on GM-2408A cells at 22°C is shown in Fig. 1. The initial fluorescence intensity decrease, as in all FPR experiments, is due to the irreversible photobleaching of probe by a brief intense pulse of laser light that is spatially identical to the monitor beam (10). The fluorescence intensity recovers to 93% of the theoretical maximum as determined by the computerized fitting. The recovery percentage is slightly larger since the curve is not corrected for a typical background of 5%.

The exponential relaxation characteristics of striped FPR bleaching (23) are evident in contrast to the nonexponential relaxation of gaussian spot bleach experiments (10). For eight measurements, the average diffusion coefficient of NBD-PC is \((1.5 \pm 0.3) \times 10^{-8} \text{ cm}^2 / \text{s} \) and the average recovery of fluorescence is 90 ± 9%. These results confirm that the cells have typical lipid fluidity (26) as determined by the diffusivity of the lipid probe.

**Diffusion of diI(3)-LDL on GM-2408A**

In Fig. 2 a-c there are FPR recovery curves representative of diI(3)-LDL diffusion at temperatures of 10°, 21°, and 28°C.
on the LDL-receptor internalization defective human cell line GM-2408A. Fig. 2 a shows a FPR recovery curve for 10°C. A distinguishing feature is the minimal recovery of fluorescence over 200 s. In these experiments we attribute the recovery of the FPR curve to a small subpopulation of molecules yielding the calculated experimental diffusion coefficient. In experiments with low percent recovery, a large subpopulation of fluorescent molecules exists with a smaller diffusibility that is no longer accurately determinable. An extreme upper bound for the diffusion coefficient of this nondiffusable fraction is estimated using the total experimental time and the bleaching pattern periodicity. At 10°C, the clearly diffusible fraction of LDL receptors is small, averaging 20 ± 20% (nine measurements). Its diffusion coefficient is 0.5–3.0 × 10⁻¹¹ cm²/s. Experiments ranged over 200–800 s; thus the 80% immobile fraction is characterized only by a diffusion coefficient substantially <0.25–1 × 10⁻¹¹ cm²/s. At 21°–22°C (11 measurements), the diffusible fraction increases to 75 ± 15%, Fig. 2 b. In this case a diffusion coefficient of (1.4 ± 1.1) × 10⁻¹¹ cm²/s describes the majority of the LDL-receptor complexes. The significant difference between these results for the two temperatures is the higher percentage recovery at 21°–22°C as compared to 10°C.

At 27°–28°C (eight measurements) the diffusible fraction of 60 ± 20% has a diffusion coefficient of (4.5 ± 1.5) × 10⁻¹¹ cm²/s (Fig. 2 c) and the extreme upper limit for the diffusion coefficient of the immobile fraction is ~2.0 × 10⁻¹¹ cm²/s.

**Diffusion of diI(3)-LDL on Normal Fibroblasts**

At 10°C diI(3)-LDL diffusion on GM-3348 fibroblasts was similar to that at 10°C on J. D. cells. At this temperature the LDL-receptor complex is not internalized by the normal cell (11). A representative curve for the 10°C diffusion on GM-3348 is shown in Fig. 3. The diffusible fraction of 20 ± 15% diffused at 0.5–2.0 × 10⁻¹¹ cm²/s (seven measurements) whereas the more immobile fraction of 80% diffused more slowly than 0.3 × 10⁻¹¹ cm²/s.

**diI(3)-LDL Diffusive Motion on Blebs**

The diI(3)-LDL receptor complex can be observed with video image intensifiers as distinct, individual fluorescent spots undergoing Brownian motion in the plane of the bleb surface. This motion can be localized in the vicinity of the membrane by differential focusing and readily distinguished from the more rapid and nonplanar three-dimensional tumbling of diI(3)-LDL in solution and the imperceptible motion of diI(3)-LDL on unblebbed cells. Because the motion of LDL receptors on blebs is rapid, the fluorescence recorded on photographic film is a time average over all positions of molecules on the bleb surface (Fig. 4 a). To record individual molecular positions (not shown), we used a SIT video camera; photos are not shown. To test the specificity of bleb labeling, blebs treated with diI(3)-LDL were labeled in the presence of either a 100-fold excess of LDL or albumin. Cells labeled in the presence of albumin showed bleb labeling, whereas the LDL-completed cells did not. We determined that the motion of the diI(3)-LDL receptor complex is diffusive and random, i.e. Brownian, by the following experiment. Numerous measurements were made of the displacement of the diI(3)-LDL receptor complex for fixed intervals of time using the image intensified video camera, the squares of the displacements were computed and averaged, and the averaged values were plotted as in Fig. 5 against the time intervals δt. For pure diffusion the diffusion coefficient D is determined from the slope of the plot of <(x(δt)²)> versus δt, which is equal to 4D. For the two graphs in Fig. 5, D is 1.8 × 10⁻⁹ cm²/s and 0.9 × 10⁻⁹ cm²/s. We attribute the factor of 2 range in D to biological variability since it far exceeds the uncertainties of individual experiments. The linearity of the dependence of x² on δt is characteristic of diffusive motion.

**Reduced Association of F-actin Cytoskeleton with the Bleb Membrane**

We investigated submembranous actin distribution with the fluorescent actin probe NBD-phallacidin (19–21). GM-2408A and GM-3348 fibroblasts were treated with NBD-Ph after blebs had been formed with either DBP or dilute formaldehyde. In a randomly selected group of 27 blebs developed by DBP to >3 μm in diameter, none stained with NBD-Ph even though the adjacent nonblebbed membrane did (Fig. 4 c). Those formaldehyde-permeabilized cells that did stain with NBD-Ph had no fluorescence in blebed regions (some did not stain because of insufficient permeability). Thus the F-actin cytoskeleton that is normally resolvable by fluorescence microscopy with NBD-Ph in cells is not detected at blebed membranes.

**DISCUSSION**

**LDL-Receptor Mobility on J. D. Cells**

This work has aimed to study the diffusibility of the LDL-receptor complex on human fibroblasts. However, the time necessary for a diffusion measurement by FPR on normal fibroblasts overlaps the time for LDL-receptor complex internalization at coated pits. Thus we turned to the mutant fibroblast line that Brown and Goldstein have characterized, J. D. (GM-2408A) (7), which is defective in LDL internalization but not in LDL binding (30). They had hypothesized that the internalization defect results from absence of a coated pit binding site (7) in the defective receptor rather than prevention of diffusion of receptor to coated pits. The properties of the LDL receptor on J. D. enabled unambiguous measurements of LDL-receptor complex mobility at elevated temperatures, so we could determine whether the lesion in J. D. involves inhibition of diffusion of the complex or absence of binding to coated pits.

The results of this work show that the LDL-receptor complex does indeed diffuse sufficiently rapidly and in sufficient quantity to reach coated pits at temperatures and rates compatible.

![Figure 3](https://example.com/figure3.png)
with coated pit internalization. Byron Goldstein and co-workers (14) have analyzed the results of our preliminary report to establish this consistency. Our data show that at temperatures above 21°C the LDL-receptor complex diffuses at least as rapidly on J. D. cells as 1.4-4.5 x 10^-11 cm^2/s with 60-80% of the bound receptor diffusible. To estimate simply the time required for a receptor complex to reach a coated pit by diffusion on the surface, we assume that the mean coated pit spacing is 2b = 1 μm and the diameter of one is 2s = 0.1 μm (30). These values represent, for example, a cell of 5,000 μm^2 surface area containing 10,000-20,000 receptors (7) with coated pits occupying 1-2% of the total surface area. The time, t_c, required for capture by a receptor has been given by (31):

\[ t_c = \frac{b^2}{2D} \left[ \ln(b^2/s^2) - \frac{3}{4} \right] \]  

and measured values of D. For our assumptions the capture time t_c is 125-300 s. This is within the lifetime of a coated pit (3, 7) so that, if coated pit binding did occur, J. D. should be responsive to increasing LDL concentrations. Experiments have shown that J. D. does not respond to increasing amounts of LDL (11).

**Does the LDL-Receptor Complex Interact with Some Component of the Cell Cytoskeleton**

The above result which is consistent with loss of binding of the LDL-receptor complex on J. D. to coated pits does not exclude its interaction with other membrane-associated structures. The theoretical calculations of Saffman and Delbruck (32, 33) and measurements of lipid diffusion enable us to estimate the coefficient of unhindered LDL complex diffusion in the lipid membrane. LDL-receptor complex diffusion, if unhindered, should be only slightly slower than lipid analog diffusion. Using the measured diffusion coefficient of the membrane lipid probe NBD-PC at 22°C of D = 10^-8 cm^2/s on J. D. cells, the calculated diffusion coefficient of the LDL-receptor complex should exceed 10^-8 cm^2/s. However, our data show that the diffusion coefficients for Dil(3)-LDL are 2-3 orders of
magnitude smaller on J. D. cells. Thus, even though the LDL receptor appears to have a coated pit binding defect, its mobility appears to be constrained by interaction with or binding to some other cell components. These constraints are evidently released by bleb formation. Our data show that the diffusion coefficient of individual LDL-receptor complex units or clusters increases on blebs to \(2 \times 10^{-9} \text{ cm}^2/\text{s}\), a value in agreement with the Saffman and Delbruck conclusion that \(D\) depends only logarithmically on molecular weight (32, 33). Incidentally, the motion of the complex on blebs is specifically seen in these experiments to be Brownian motion, i.e., diffusion. Many other cell membrane receptors diffuse as slowly as \(10^{-11} \text{ cm}^2/\text{s}\) on the cell surface, and similar release of other membrane receptors from constraints on diffusion by bleb formation have been found in a series of experiments in our laboratory (31).

**Could F-actin be Involved in an LDL-Receptor Complex Transmembrane Linkage?**

Transmembrane linkages of membrane proteins involving actin either directly or indirectly have been proposed (35-38). We determined, using NBD-phallacidin, that the F-actin cytoskeleton is substantially depleted from membrane blebs. This suggests a causal relationship between the F-actin cytoskeleton and inhibition of protein diffusibility. However, more subtle mechanisms of diffusibility release may accompany disruption of local cytoskeletal binding. Further studies on the LDL receptor are in preparation (D. Tank, W. Fredericks, L. Barak and W. Webb, private communication).

**Conjecture on LDL-Receptor Complex Diffusion Results in Normal Fibroblasts**

At \(10^\circ\text{C}\) the LDL-receptor complex behaves similarly on normal and J. D. fibroblasts. Diffusion is slow at best, is limited to <20\% of the receptor population, and the diffusion coefficients are \(10^{-11} \text{ cm}^2/\text{s}\) for the other 80\%. Internalization of the LDL-receptor complex interferes with measurements of diffusion above \(15^\circ\text{C}\) on normal cells. However, if the lesion on J. D.'s LDL receptor is only expressed at a coated pit and inhibition of protein diffusibility. However, more subtle mechanisms of diffusibility release may accompany disruption of local cytoskeletal binding. Further studies on the LDL receptor are in preparation (D. Tank, W. Fredericks, L. Barak and W. Webb, private communication).

**Is it Necessary for the LDL-Receptor Complex to Diffuse to Coated Pits on Normal Cells?**

The Brown and Goldstein model assumes movement of the LDL-RC or receptor to coated pits within the coated pit lifetime, and our experimental results show that the diffusion rate of the complex on J. D. cells is quite consistent with their description. Our results and their model predict that the LDL-receptor complex should be able to move to coated pits on normal fibroblasts within the coated pit lifetime (14). Nevertheless diffusion of the LDL-receptor complex on the normal fibroblast membrane may be irrelevant.

Alternative models where the LDL receptor or LDL-receptor complex is not required to diffuse to coated pits are also consistent with the known properties of LDL-receptor complexes on normal fibroblasts. For instance, the LDL receptor may be essentially always associated with coated pits on normal fibroblasts. Indeed, aggregated receptors can capture ligand from solution about the cell almost as well as homogeneously spaced surface receptors (31, 39). If receptors are preferentially inserted in coated membrane, their diffusion on the membrane does not seem essential. Evidence for preaggregation of LDL [6], insulin [2], and EGF [3] receptor-ligand complexes may induce the formation of subjacent coated membrane to which the receptor binds. (It has been reported that IgM aggregation on the surface of lymphocytes induces the formation of clathrin-coated areas directly under the IgM receptors [40].)

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