

Flow Cytofluorometric Analysis of Insulin Binding and Internalization by Swiss 3T3 Cells¹

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The binding of a fluorescein-isothiocyanate derivative of insulin to Swiss 3T3 cells was measured by flow cytometry. The kinetics of the subsequent internalization were also measured; at a concentration of 1 μM labeled insulin approximately 25% of the internalization was insulin-specific. The kinetics of endocytosis were contrasted to those of fluorescent derivatives of histone and dextran. In addition, the fusion of endocytic vesicles containing insulin or dextran with ly-

sosomes was detected by measuring the pH-dependent increase in fluorescein fluorescence caused by the addition of chloroquine. The application of these results to the analysis of growth control by insulin and related hormones is discussed.

Key terms: Insulin, hormone binding, receptor-mediated endocytosis, chloroquine, lysosomes, FITC-dextran, histone, flow cytometry

Insulin shows great diversity in the metabolic processes that it influences in various target tissues. In fibroblasts and other cells of mesenchymal origin, it promotes both protein synthesis and DNA synthesis (8). The role of cell surface receptor binding and internalization in mediating insulin action is at present unclear.

Flow cytometric systems allow the rapid measurement of cellular properties on a cell by cell basis (18). While the majority of applications have been in cell cycle analysis and immunology, flow cytometry has also been used for the quantitation of ligand binding in cell populations. The binding of fluorescent-labeled lectins has been particularly analyzed (4, 5, 7, 30). In this paper we extend this method to the analysis of the binding and internalization of insulin.

Materials and Methods

Cells and reagents: Swiss 3T3 cells (31) were maintained in monolayer culture as previously described (32). For experiments, actively growing monolayers (average cell density of 1×10^4 cells/cm²) were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum (Gibco Grand Island, NY), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate.

Fluorescein-isothiocyanate (FITC), FITC-labeled dextran, insulin (Bovine pancreas), bovine serum albumin (BSA), and chloroquine (diphosphate salt) were obtained from Sigma (Sigma, St. Louis, MO). Chicken erythrocyte histones were labeled with iodoacetamido-fluorescein (IAAF; Molecular Probes, Plano, TX) as described previously (19). Fluorescently labeled bovine insulin was prepared essentially as described by Schechter *et al.* (29), using FITC instead of rhodamineisothiocyanate.

Determination of biological activity of insulin derivatives: Protein synthesis from methionine was assayed essentially as described by Petersen and Blecher (22). Confluent, serum-starved (0.2% fetal calf serum, 1 day) 3T3 monolayers were washed twice with PBS, and then incubated for 30 min at 37°C with or without a wide range of concentrations of insulin (or FITC-insulin) in PBS containing 0.4 mg/ml glucose and 0.15% BSA. [³⁵S]Methionine (Amersham, Arlington Heights, IL) (1 $\mu\text{Ci}/\text{dish}$, 10 nM) was then added for 30 min at 37°C. All subsequent steps were exactly as previously reported (22).

Stimulation of DNA synthesis in confluent 3T3 monolayers was assayed essentially as described by Rozenfurt, *et al.* (27). Confluent 3T3 monolayers (35-mm plates) were washed twice with serum-free medium and incubated in serum-free medium containing 10 ng/ml vasopressin for 12 hr. [³H]Thymidine (2.5 $\mu\text{Ci}/\text{ml}$, 10 mM) was then added and the incubation was continued for an additional 12 hr. Material insoluble in cold 5% trichloroacetic acid was dissolved by incubating in 0.5 ml of 0.1 M NaOH for 5 hr, the solution was neutralized with 0.5 ml of 0.1 M HCl, and then the radioactivity in 200 μl was determined by scintillation counting in 5 ml Liquiscint (National Diagnostics, Somerville, NJ).

Incubation of cells with FITC-insulin: For measurement of hormone binding at 4°C, subconfluent monolayers of 3T3 were prechilled for 30 min at 4°C and then the culture medium was replaced with cold DME containing 42 mM HEPES pH 7.0. FITC-insulin was added and the incubation continued at 4°C. At the desired time, the medium was removed, the plates were washed with PBS, and then 1 ml of 0.5 mM EDTA in PBS, either with or without 0.25% trypsin,

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was added. The plates were incubated at 37°C for 10 min, after which 1 ml of cold PBS containing 10% fetal calf serum and 0.05% sodium azide was added and the cells were removed by pipetting. Samples were kept on ice and analyzed at 4°C normally within 30-60 min.

Internalization was measured by direct addition of FITC-insulin to the culture medium. Cells were harvested as above using trypsin/EDTA and kept on ice until analyzed. To detect fluorescence in lysosomes, cells were removed with trypsin as above except that sodium azide was omitted. The samples were analyzed, 100 μ M chloroquine was added, and the samples were reanalyzed after 30 min.

Flow cytometry: A FACS-IV cell sorter (Becton Dickinson Co., Mountain View, CA) interfaced with a VAX-11/780 computer (Digital Equipment Corp., Maynard, MA) was used for all analyses. Excitation was with the 488 nm line of a Spectra-Physics Model 164-05 argon ion laser, and emission was measured using a 520-nm long pass filter and a 530-nm long pass optical glass filter (Ditric Optics, Marlboro, MA). Fluorescent microspheres (Polysciences, Warrington, PA; no. 719, Lot 4-1591) were used to align and calibrate the instrument. For 1.333 μ diameter microspheres (coefficient of variation (cv) = 1.2%), typical cv were 2% for both scatter and fluorescence. At least 10,000 cells were analyzed for each sample. Mean fluorescences were calculated from the distributions and expressed as bead equivalents (be), as previously described (19). Be were converted to molecules of specific probes using the method described by Bohn and Manske (5). One be is equal to the fluorescence of 1.73×10^{-13} g (1.82×10^7 molecules) of FITC-insulin in PBS at pH 7.4. Fluorescence measurements were made using a Schoeffel RRS 1000 spectrofluorimeter (Schoeffel Instr. Div., Kratos Inc., Westwood, NJ) interfaced to a Tektronix 31 programmable calculator (Tektronix Inc., Beaverton, OR). Kinetics were analyzed by interactive least squares fitting (20).

Results

Properties of the fluorescent derivative of insulin:

The binding of insulin to cell membranes has been divided into high and low affinity classes (10, 15, 23, 28). The biological effects of insulin can also be resolved into independent metabolic and growth promoting functions (16). To determine the effect of FITC-labeling on these two functions, the increase in protein or DNA synthesis by confluent cells upon addition of either insulin or FITC-insulin was measured by radiolabeling. For stimulating protein synthesis, the FITC-insulin had an ED_{50} of 97 nM as compared to 1.5 nM for unlabeled insulin (1.5% relative activity). For stimulating DNA synthesis, the FITC-insulin had an ED_{50} of 280 ± 45 nM, as compared to 160 ± 25 nM for unlabeled insulin. This corresponds to a relative activity of $56 \pm 12\%$. The decreased protein synthesis stimulating activity of the labeled insulin suggests that the labeling interferes with binding to the high affinity receptor (it is likely that protein synthesis stimulation is mediated through this receptor since it occurs at low insulin concentrations). Since the autofluorescence of unlabeled 3T3 cells under our conditions is equivalent to the fluorescence of 3-5 million FITC-insulin molecules, the high affinity class is below the detection limit of the current method. The effects of FITC-labeling on high affinity binding are therefore not of great concern. In contrast, binding to the low affinity sites (which probably consist of receptors for insulin-like hormones responsible for growth control (3, 25)) does not appear to be greatly affected by the labeling procedure thereby justifying the use of the FITC-insulin compound for studies of this class of sites.

Surface binding: In order to detect surface binding, cells were incubated at 4°C in the presence of FITC-insulin for 60 min and then were removed from the culture dishes either

with trypsin, which removes surface bound insulin, or EDTA which does not. Results of a typical experiment are shown in Figure 1. The difference between the two measurements represents the surface binding, approximately 2×10^6 molecules per cell. The same method can be applied to actively growing cells, avoiding any change in receptor number or properties as a result of cooling the cells. The results of these measurements (Fig. 1) are in good agreement with those at 4°C.

Insulin internalization: The temperature dependence of the internalization process is shown in Figure 2. The amount that is trypsin insensitive is more than five times greater at 37°C than at 4°C. That this internalization is at least partly mediated by a receptor which is specific for insulin (or an insulin analog) is shown in Figure 3. The presence of increasing concentrations of unlabeled insulin up to 10 μ M decreases the amount of FITC-insulin internalized during 60 min incubation. At least 25% of the internalization is insulin specific. Insulin binding at 4°C can also be inhibited by unlabeled insulin, but less efficiently. The low degree of specificity observed may be due to insulin dimerization.

Since we estimated the number of FITC-insulin molecules bound to the surface at 1 μ M to be 2×10^6 (Fig. 1), 10-25% specific binding corresponds to between 200,000 and 500,000 receptors per cell. This value is in approximate agreement with our calculation of about 100,000 low affinity receptors

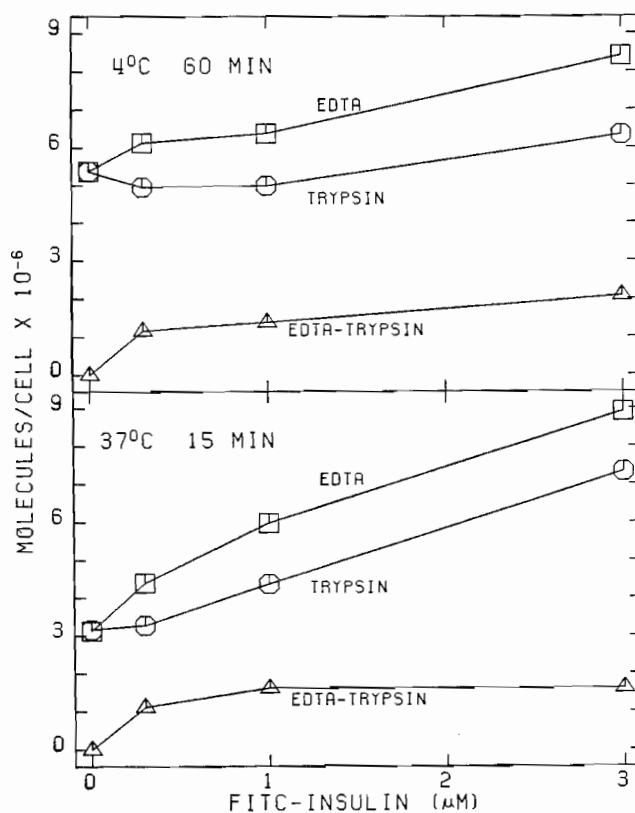


FIG. 1. Quantitation of surface bound FITC-insulin. Monolayer cultures were incubated with various concentrations of FITC-insulin for either 60 min at 4°C or 15 min at 37°C after which the cells were removed with EDTA (\square) or trypsin/EDTA (\circ) and analyzed at 4°C as described in "Materials and Methods." The difference between the two measurements (\triangle) represents the surface bound material.

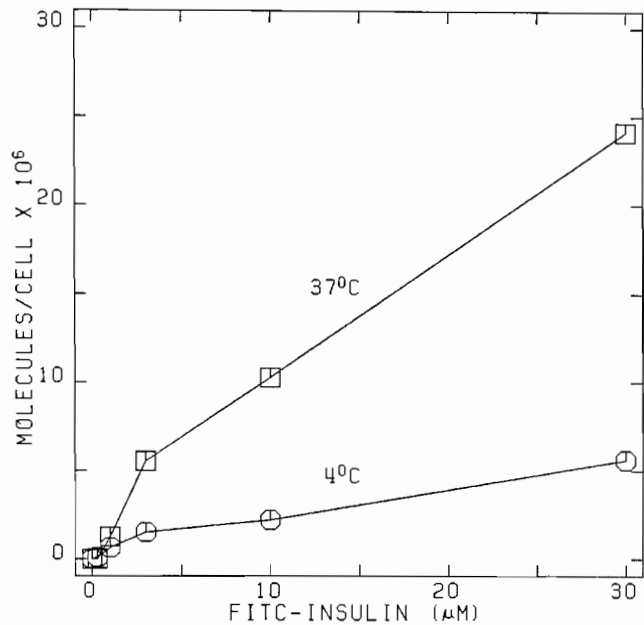


FIG. 2. Temperature dependence of FITC-insulin internalization. Cells were incubated for 60 min either at 4°C (○) or 37°C (□) and then removed with trypsin/EDTA.

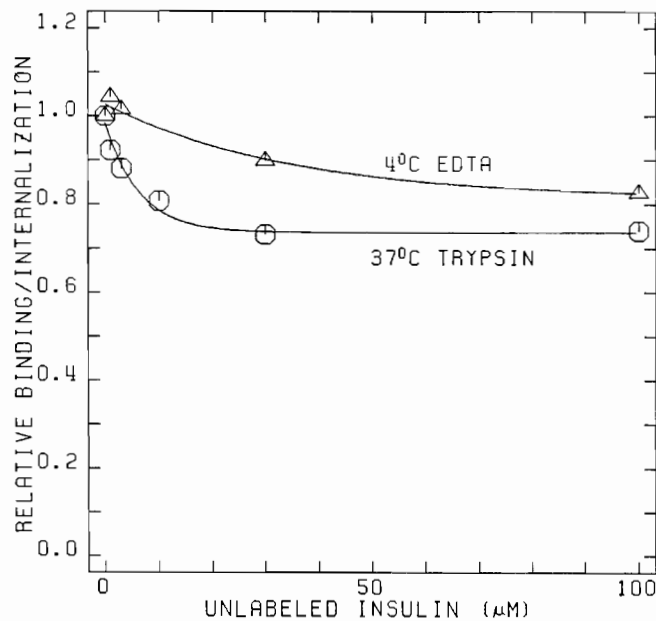


FIG. 3. Inhibition of FITC-insulin binding and internalization by unlabeled insulin. Cells were incubated for 60 min with 1 μ M FITC-insulin at 4°C (Δ) or 37°C (○) in the presence of different concentrations of unlabeled insulin and then removed with EDTA (Δ) or trypsin/EDTA (○). The average standard deviation SD is 0.1. The lines represent first-order fits to the data.

per cell using the data of Rubin *et al.* for uninduced 3T3-L1 cells (28).

The kinetics of insulin endocytosis at 1 μ M are shown in Figure 4. The amount internalized per cell reaches a maximum after 1 hr. This maximum probably results from a combination of internalization of the majority of specific receptors and

attainment of an equilibrium between nonspecific endocytosis and exocytosis.

Comparison of internalization and lysosomal fusion kinetics for different endocytic processes: FITC-dextran has been used previously to quantitate fluid-phase pinocytosis

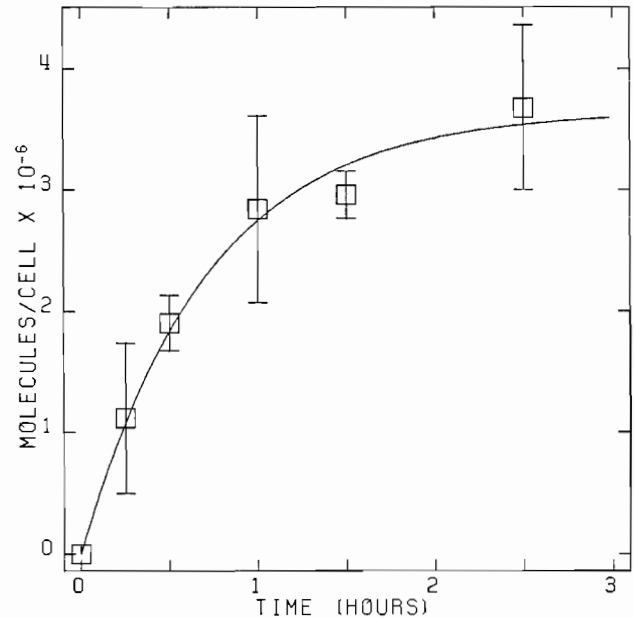


FIG. 4. Kinetics of FITC-insulin internalization. Cells were incubated at 37°C with 1 μ M FITC-insulin for various times and then removed with trypsin/EDTA and analyzed. The line is a first-order fit to the data.

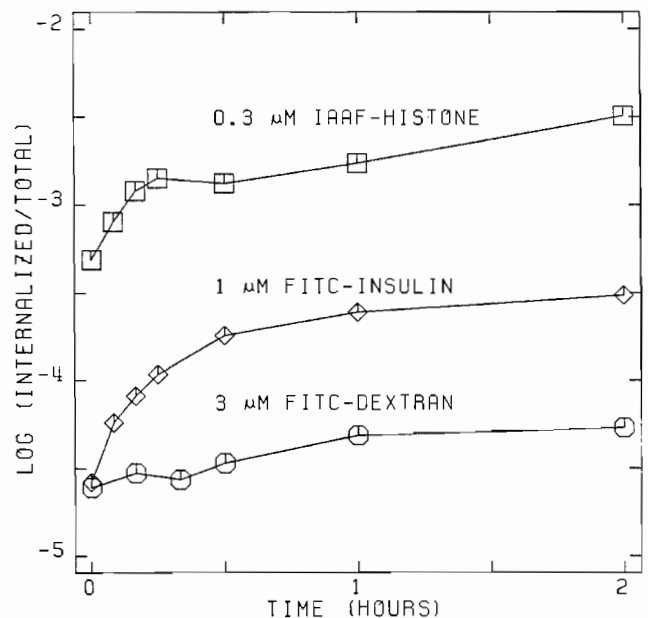


FIG. 5. Comparison of internalization of different probes. Cells were incubated at 37°C with 20 μ g/ml (0.3 μ M) IAAF-histone (□), 1 μ M FITC-insulin (\diamond) or 200 μ g/ml (3 μ M) FITC-dextran (○) and then removed with trypsin/EDTA. The values of the mean fluorescence per cell were converted to molecules per cell and then multiplied by the cell concentration to yield the internalized concentration.

in single cells by microspectrofluorimetry (2). IAAF-histone is internalized by nonspecific adsorptive endocytosis after rapid binding to the cell membrane (19). We have compared the internalization kinetics of these probes with those of FITC-insulin (Fig. 5). The internalization of FITC-insulin is more than 10-fold lower than that of IAAF-histone and it reaches a maximum value while the IAAF-histone fluorescence continues to increase with time. The insulin internalization is approximately 10-fold greater than that of FITC-dextran, and the rapid initial kinetics for FITC-insulin indicate that the internalization follows surface binding.

Since the excitation spectrum of fluorescein is sensitive to pH, the ratio of the emission intensity with excitation at 490 nm to that at 450 nm has been used to measure the lysosomal pH of living cells (21). This method has also been used to measure the effect of phagosome-lysosome fusion on lysosomal pH, and to measure the kinetics of pH change within phagosomes (12). In addition to the change in excitation properties, the emission intensity at a fixed excitation wavelength decreases with pH (21). Since the anti-malarial drug chloroquine increases the pH of lysosomes (21), it can be used to measure the fraction of fluorescein labeled probes in lysosomes by comparing the fluorescence of samples before and

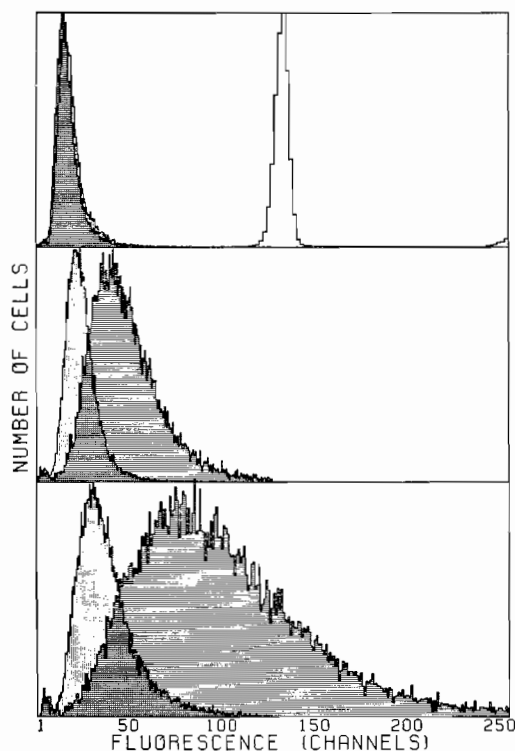


Fig. 6. Detection of FITC-insulin in lysosomes using chloroquine. Typical fluorescence histograms obtained by flow cytometry are shown. Cells were incubated without FITC-insulin (top), or with $1 \mu\text{M}$ FITC-insulin for 30 min (middle) or 120 min (bottom), and then analyzed as described in "Materials and Methods" before (vertical shading) or after (horizontal shading) the addition of chloroquine. A histogram for fluorescent beads run at the same time in also shown (unshaded). All histograms were obtained with the same settings, except for the 120-min plus chloroquine sample and the beads standard which were obtained at 0.5 and 0.25 times the normal gain, respectively (the histograms have been normalized accordingly).

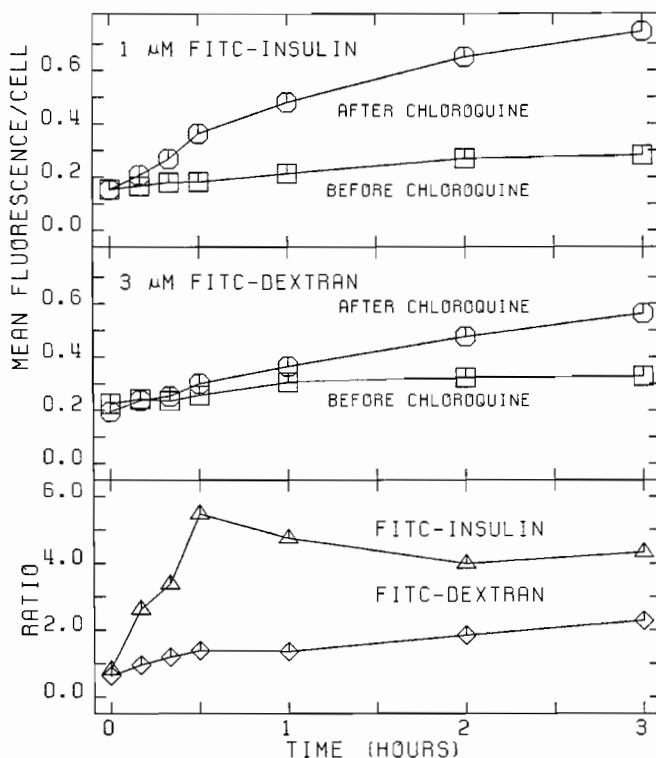


Fig. 7. Comparison of the lysosomal fusion kinetics of FITC-insulin and FITC-dextran. Cells were incubated with $1 \mu\text{M}$ FITC-insulin or $3 \mu\text{M}$ FITC-dextran, removed with trypsin/EDTA, and then analyzed before (\square) and after (\circ) the addition of $100 \mu\text{M}$ chloroquine. The ratio of these measurements for FITC-insulin (Δ) and FITC-dextran (\diamond) is also shown.

after the addition of chloroquine (19). Using this method, fluorescence histograms such as those in Figure 6 are obtained. The results of this analysis for FITC-insulin and FITC-dextran are shown in Figure 7. After a 30 min incubation, the FITC-insulin fluorescence with chloroquine is approximately five times that without chloroquine (after correcting for autofluorescence). Since this is close to the maximum change expected (19), the majority of internalized fluorescence must be present in lysosomes at 30 min. This is in marked contrast to the results for FITC-dextran. The significance of the kinetics of lysosomal fusion of FITC-insulin containing vesicles is discussed below.

Discussion

The results we have presented show that hormone binding, internalization and lysosomal fusion can be measured by flow cytofluorometry. This method can be used to detect variation within cell populations, and flow sorting can then be used to isolate cells of interest for cloning and/or further biochemical analysis. In addition, hormone binding can be correlated with other cellular properties (e.g., cell cycle stage, binding of other hormones, cell volume, surface area) on a cell-by-cell basis.

Results from a number of experimental systems (reviewed by Goldstein *et al.* (13) and Geisow (11) indicate that many molecules which are internalized by receptor mediated endocytosis enter lysosomes within 60 min. For example, ferritin-labeled epidermal growth factor (EGF) (14), [^{125}I]human

growth hormone (1) and immunolabeled α_2 -macroglobulin (33) are found in lysosomes within 30–60 min. Since Maxfield *et al.* (17) have presented evidence that insulin, EGF, and α_2 -macroglobulin are internalized *via* a common pathway, our finding of a lysosomal fusion time of approximately 30 min for FITC-insulin is consistent with previous results. Since at 1 μ M FITC-insulin internalization occurs through both specific and nonspecific sites (Fig. 3), the large fraction of FITC-insulin which we found in lysosomes at 30 min agrees well with previous findings that the majority of insulin degradation is mediated through the low-affinity and “non-specific” sites (6). Internalization and lysosomal fusion have been suggested as a mechanism by which receptor down-regulation and hormone action is accomplished, especially for molecules involved in growth control (9).

3T3 cells require high levels of serum when grown in normal medium (26) and require the addition of 1 μ M insulin when grown in serum-free medium (24). In contrast, SV101 cells, which are derived from 3T3 cells by transformation with SV40 virus, have low serum requirement and do not require insulin for growth in serum-free medium (24). We have used the methods described above to correlate the growth requirement for insulin with amount of FITC-insulin binding and internalization (Murphy RF, Powers S, Cantor CR, Pollack R, manuscript in preparation). Experiments are in progress which are designed to correlate, at the single cell level, the loss of growth control (and FITC-insulin internalization) with the expression of T-antigen during transformation.

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