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Cytometry 5:275-280 (1984)

Reduced Insulin Endocytosis in Serum-Transformed Fibroblasts Demonstrated by Flow Cytometry[1]

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Neoplastic transformation often results in the loss of growth control and concomitant changes in cell surface properties. The changes in endocytosis of a variety of probes after serum or anchorage transformation were measured for mouse fibroblasts by flow cytofluorometry. No major differences in dextran (fluid phase) or histone (nonspecific-adsorptive) endocytosis were observed among four cell lines with different growth properties. However, decreased receptor-mediated internalization of α_2-macroglobulin was observed for cell lines transformed to either serum or anchorage independence. Furthermore, increased wheat germ agglutinin and decreased insulin endocytosis were observed, but only in serum transformants. The changes specific to serum transformants were not accounted for by changes in binding of wheat germ agglutinin or insulin. The possible implications of these observations regarding serum transformation and the insulin requirement for growth in serum-free medium are discussed.

Key terms: Insulin, endocytosis, FITC-dextran, α_2-macroglobulin, serum-free medium, transformation, flow cytometry

Many cellular changes have been reported to accompany the conversion of a normal cell to a neoplastic cell (16). Transformation of fibroblasts by agents such as simian virus 40 (SV40) can result in the elimination of many growth requirements, such as those for anchorage (34), serum (32), epidermal growth factor (EGF) (8), and insulin (29). Changes in the cell surface and the cytoskeleton have been of particular interest because of their proposed roles in controlling cell growth (11,28,30). Unfortunately, it has not been possible to develop these observations into an explanation for the mechanism that underlies the ability of a transformed cell to grow under restrictive conditions. For example, the alterations in nutrient transport which have been observed may be secondary to the growth state of the cell and not intrinsically different in normal and transformed cells (6,11,24). However, it is reasonable to expect that certain molecular constituents of the cell surface or cytoskeleton will be of primary importance in the loss of growth control.

Endocytosis is a cellular function that involves both the cell surface and the cytoskeleton (cytochalasins have been shown to inhibit endocytosis in a variety of systems (1,4,33,38). It has been suggested to play a role in mitogenic stimulation by growth factors (13), but quantitative comparisons of fluid phase and receptor-mediated endocytosis by normal and transformed cells have not previously been reported. We have therefore begun an analysis of the changes in endocytosis that accompany transformation to either serum or anchorage independence. We have used flow cytometry to analyze the binding and endocytosis of various fluorescent probes by mouse fibroblast cell lines. Flow cytometry allows the rapid and accurate quantitation of fluorescent and scattered light resulting from the passage of individual cells through a focused laser beam. Because dead or broken cells can be eliminated from analysis and because no separation of free and bound ligand is necessary, flow cytometry has been used to study both ligand binding (5,18,20,23,35) and endocytosis (12,19,22,23).

MATERIALS AND METHODS

Cell Culture

Cell lines were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% (v/v) fetal calf serum (FCS) with 100 μg/ml streptomycin and 100 u/ml penicillin. The conditions for growing cells in partially defined serum-free medium have been described previously (29). The totally defined serum-free conditions will

[1]This work was supported in part by postdoctoral fellowship DRG-352-F from the Damon Runyon Walter Winchell Cancer Foundation (R.F.M.), National Institutes of Health grants GM-14825 and GM-27576 (C.R.C.), and National Cancer Institute Public Health Service grant CA-25066 (R.P.).

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be published elsewhere (Powers and Pollack, in preparation). The serum requirement was determined by plating approximately 1,000 cells/cm^2 in DME containing either 1% or 10% calf serum and counting duplicate dishes on day 1 and day 4. The number of doublings in 1% serum was divided by the number of doublings in 10% serum to yield the relative growth rate (RGR). The anchorage requirement was determined by plating 10^5, 10^4, and 10^3 cells in 3 ml 1.3% methocel medium over a 2-ml 0.5% agarose underlay in 60-mm dishes. Plates were fed weekly with 5 ml methocel medium and scored for colonies greater than 0.2 mm in diameter after 3 weeks' incubation. The number of such colonies was divided by the number of cells plated to yield colony forming efficiency (CFE). The insulin requirement in serum-free medium was determined in a manner analogous to the determination of the serum requirement, except that the number of doublings without insulin was divided by the number of doublings with insulin to yield the relative growth rate. The concentration dependence of the insulin requirement was determined by plating 200 cells onto 160-mm dishes in MCDB402 containing various concentrations of insulin, 5 μg/ml transferrin, 10 ng/ml fibroblast growth factor (FGF), and 5 μg/ml gimmel factor (2). The medium was replaced with fresh medium every 4 days and cell numbers were measured with a Coulter counter 12 days after plating.

Incubation of Cells with Fluorescent Probes

The preparation and properties of the fluorescein isothiocyanate (FITC)-conjugated insulin have been previously described (23). The FITC-insulin had 56% of the activity of unlabeled insulin for stimulating DNA synthesis. Gel electrophoresis revealed that more than 95% of the insulin was labeled. 5-Iodoacetamido fluorescein (IAAF)-conjugated histone was prepared as described (22). FITC-dextran and FITC-wheat germ agglutinin (FITC-WGA) were obtained from Sigma. FITC-α_2M was the generous gift of Dr. F. Maxfield.

For binding measurements, subconfluent monolayers were washed once with cold phosphate-buffered saline (PBS), and then incubated for 60 min at 4°C with 0.1 μM FITC-WGA or 1 μM FITC-insulin in PBS with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The cells were harvested by washing once with PBS and detaching the cells with 0.5 mM ethylenediaminetetraacetic acid (EDTA)/PBS. An equal volume of 10%FCS/PBS was added and the suspended cells were kept on ice until analysis to prevent internalization of bound probe.

For measurements of the endocytosis of FITC-insulin, FITC-WGA, FITC-dextran, and IAAF-histone, subconfluent cultures (60-mm dishes) were incubated with probes (added directly to the growth medium) for 150 min at 37°C. This is the time necessary for FITC-insulin endocytosis to reach steady state (23). Similar internalization kinetics were observed for FITC-WGA internalization by 3T3 and for FITC-insulin internalization by SV101. The final concentration of the probes were: FITC-insulin, 1 μM; FITC-WGA, 0.1 μM; FITC-dextran, 1 μM; IAAF-histone, 0.78 μM. Nonspecific insulin binding or internalization was determined for samples coincubated with 30 μM unlabeled insulin. Subconfluent cultures that had been washed twice with DME were incubated for 30 min at 37°C with 80 μg/ml FITC-α_2M in serum-free DME (nonspecific internalization was measured using 3 mg/ml unlabeled α_2M). For all probes, plates were washed once with 0.025% trypsin/0.5 mM EDTA/PBS, and cells were detached by incubating for 10 min in 1 ml of the same solution. These conditions eliminate all FITC-insulin surface binding, reducing the fluorescence to background levels (23). 1 ml of 10%FCS/0.05%NaN_3/PBS was added and the suspended cells were kept on ice until analysis.

Fluorescence Measurements

Mean fluorescence per cell was determined using a FACS IV (Becton Dickinson, Mountain View, CA). Excitation was with the 488-nm line of an argon ion laser, and emission was measured using a 520-nm long-pass dielectric filter and a 530-nm long-pass optical glass filter. Sample temperature was maintained at 4°C by a circulating water bath containing ethylene glycol/water. Fluorescence measurements were converted to number of molecules per cell using fluorescent bead standards (22,23) and were corrected for autofluorescence of unlabeled cells. Cell volumes were measured using a model ZF Coulter counter equipped with a model P64 channel analyzer (Coulter Electronics, Hialeah, FL).

RESULTS

Growth Properties of the Four Cell Lines

The starting point for our examination of the changes in endocytic properties following neoplastic transformation was a set of four mouse fibroblast cell lines with all possible combinations of phenotypes with respect to two growth properties which often accompany transformation: a reduced serum requirement and a reduced requirement for anchorage to a solid substrate. As seen in Table 1, Swiss 3T3 displayed a high dependence upon 10% serum for optimal growth, and did not form colonies at all when suspended in methocel. Thus, 3T3 is normal with respect to both growth requirements. SV101, an SV40-transformed derivative of 3T3 (27,36), grew well in 1% serum and also efficiently formed colonies in methocel. It is therefore transformed with respect to both requirements. Swiss 3T6 is derived from mouse embryo fibroblasts, as was 3T3, but unlike 3T3 it was passaged at high densities. It grew relatively well in 1% serum, but did not form colonies in methocel. Aγ4, derived from SV101 by negative selection for serum requirement (37), showed a strong growth dependence upon 10% serum, but retained the parental cell line's ability to form colonies in methocel (Table 1). 3T6 and Aγ4 complete the set: 3T6 is anchorage-normal but serum-transformed, and Aγ4 is anchorage-transformed but serum-normal.

In order to correct our endocytosis measurements for differences in cell size, we measured the average cell

Table 1
Properties of the Four Cell Lines

Cell line	Growth requirements[a] Serum (RGR)	Growth requirements[a] Anchorage (CFE)	Cell volume (μ^3)	Surface area[b] (μ^2)	Light scattering[c]
3T3	NN 0.26	<0.001	2,660	928	69 ± 14
Aγ4	NT 0.02	1	3,070	1,020	73 ± 8
SV101	TT 0.67	7	2,140	803	56 ± 6
3T6	TN 0.65	<0.001	2,370	858	63 ± 10

The anchorage and serum requirements of a particular cell line are indicated by a two letter abbreviation, e.g., TN for 3T6 which is serum transformed and anchorage normal.
[a]The relative growth rate (RGR) and colony forming efficiency (CFE) were measured as described in Materials and Methods.
[b]Calculated from cell volume assuming spherical geometry.
[c]Mean forward angle light scattering (channels) ± 1 SD.

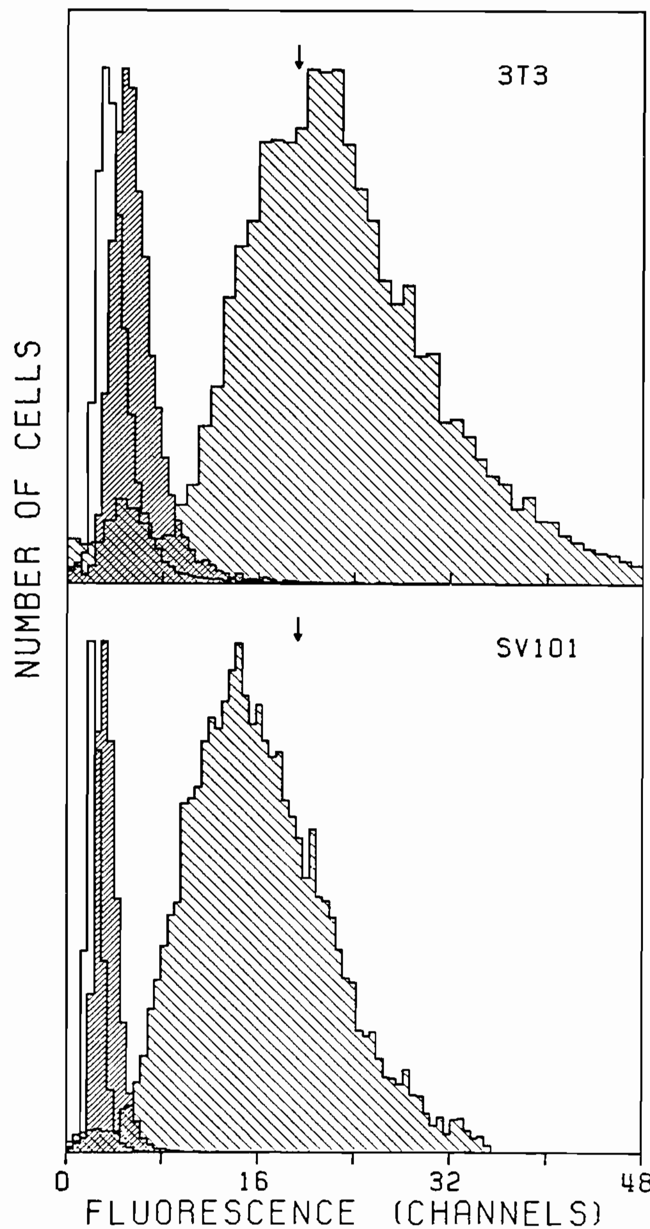

FIG. 1. Receptor-mediated endocytosis of α_2-macroglobulin by normal and SV40-transformed fibroblasts. Monolayers were incubated with 80 μg/ml FITC-α_2-macroglobulin for 30 min at 37°C in the absence (\\\) or presence (///) of 3 mg/ml unlabeled α_2-macroglobulin, trypsinized, and then analyzed by flow cytometry in the presence of 100 μM chloroquine (to prevent quenching of the FITC fluorescence by low pH). Histograms for unlabeled cells (unshaded) and the position of fluorescent bead standards (arrow) are also shown.

volume for each cell line using a Coulter counter, and calculated the average surface area (Table 1). The average amounts of laser light scattered in the forward direction by each cell line are also shown in Table 1. In this size range, the light scattering values are roughly proportional to cell volume (R = 0.974), and similar values were obtained if measurements were corrected using light scattering (data not shown).

Endocytosis Measurements for Different Probes

To determine whether loss of growth control affects endocytosis in general, we used three fluorescent probes which are internalized by different mechanisms. FITC-dextran is internalized by fluid-phase pinocytosis [without binding to the cell surface, (3)]. IAAF-histone is internalized by nonspecific adsorptive endocytosis (22). FITC-α_2-macroglobulin is a protease inhibitor found in serum that undergoes receptor-mediated endocytosis (21,26). We have measured the internalization of these probes by the four cell lines using flow cytometry. An example of the results of this analysis for α_2M internalization by 3T3 and SV101 is shown in Figure 1. The high degree of specificity of the internalization is shown by the effect of the addition of an excess of unlabeled α_2M. For both cell lines, the internalization is 92% specific. SV101 specifically endocytosed 38±5% less α_2M than 3T3.

The results of measurements of steady-state endocytosis for the other cell lines and probes are shown in Table 2. No major differences in the amount of FITC-dextran or IAAF-histone endocytosis were observed, especially when these values are normalized for surface area (using the data of Table 1). We conclude that neoplastic

Table 2
Comparison of Different Types of Endocytosis in Normal and Transformed Fibroblasts

Cell line	Fluid phase FITC-dextran total: Molecules per cell $\times 10^{-6}$	Fluid phase FITC-dextran total: Molecules per μm^2 $\times 10^{-3}$	Nonspecific IAAF-histone total: Molecules per cell $\times 10^{-6}$	Nonspecific IAAF-histone total: Molecules per μm^2 $\times 10^{-3}$	Receptor-mediated α_2-macroglobulin, Total: Molecules per cell $\times 10^{-6}$	Receptor-mediated α_2-macroglobulin specific: Molecules per cell $\times 10^{-6}$	Receptor-mediated α_2-macroglobulin specific: Molecules per μm^2 $\times 10^{-3}$
3T3	NN 0.385 ± 0.068	0.42	163	176	21.5 ± 1.1	17.5	18.9
Aγ4	NT 0.361 ± 0.027	0.35	180	176	11.6 ± 3.5	6.2	6.1
SV101	TT 0.280 ± 0.037	0.35	189	235	14.1 ± 5.4	10.4	12.9
3T6	TN 0.232 ± 0.022	0.27	147	171	9.7 ± 0.7	5.3	6.2

See Materials and Methods for concentrations and conditions. The indicated errors are 1 SD.

Table 3
Endocytosis and Binding of Wheat Germ Agglutinin and Insulin

Cell line	FITC-WGA Binding, Total: Molecules per cell $\times 10^{-6}$	FITC-WGA Endocytosis, Total: Molecules per cell $\times 10^{-6}$	FITC-WGA Endocytosis, Total: Molecules per μm^2 $\times 10^{-3}$	FITC-Insulin Binding, Total: Molecules per cell $\times 10^{-6}$	FITC-Insulin Binding, Specific: Molecules per cell $\times 10^{-6}$	FITC-Insulin Endocytosis, Total: Molecules per cell $\times 10^{-6}$	FITC-Insulin Endocytosis, Total: Molecules per μm^2 $\times 10^{-3}$	FITC-Insulin Endocytosis, Specific: Molecules per cell $\times 10^{-6}$
3T3	0.47	3.3 ± 0.5	3.6	2.0 ± 0.5	0.74	4.0 ± 1.0	4.3	0.25 ± 0.03
Aγ4	0.72	4.1 ± 1.0	4.0	2.0 ± 0.2	0.45	3.8 ± 0.7	3.7	0.56 ± 0.29
SV101	0.58	5.3 ± 0.4	6.7	1.5 ± 0.3	0.39	2.1 ± 0.2	2.6	0.19 ± 0.12
3T6	0.35	5.4 ± 0.3	6.3	2.0 ± 1.0	0.48	2.3 ± 0.3	2.7	0.17 ± 0.05

See Materials and Methods for concentrations and conditions. The indicated errors are 1 SD.

transformation need not impair the mechanisms involved in these bulk endocytic processes but may affect receptor-mediated endocytosis.

In contrast to the results for α_2M, SV101 and 3T6 endocytosed more FITC-WGA than 3T3 or Aγ4 (Table 3). This difference is not explained by differences in the amount of lectin bound per cell. Previous results which demonstrated that increased agglutinability after transformation resulted not from changes in the number of lectin binding sites but from changes in the mobility of these sites (7,25,30); thus we interpret our results to mean that increased lectin site mobility contributes to increased lectin endocytosis.

Table 3 also shows the results of measurements of insulin binding and endocytosis. For these measurements, we used the supraphysiologic concentration of insulin needed to stimulate growth of these cell lines, as discussed below. The behavior of the cell lines was reversed (compared to WGA) with SV101 and 3T6, each of which internalizes approximately half as much FITC-insulin as the serum-sensitive 3T3 and Aγ4. The cell lines all had similar amounts of total FITC-insulin binding. A partial explanation for the internalization differences could be made based on the specific binding measurements, since SV101 and 3T6 had approximately two-thirds as many specific sites as 3T3. However, Aγ4 also had this low number of sites, but exhibited increased total and specific insulin internalization. Thus, during selection from SV101, Aγ4 did not increase its number of insulin binding sites, but increased the amount of insulin endocytosis.

It should be pointed out that at 1 μM, most of the insulin internalization is nonspecific, i.e., not inhibited by a 30-fold excess of unlabeled insulin. Since our fluorescent probe is biologically active, this is not likely to be due to the nature of the fluorescent insulin, and it reflects the amount of insulin internalization occurring at growth-stimulatory insulin concentrations (see below). Nonspecific binding of radioactive insulin increases significantly when the concentration is increased from 0.1 to 1 nM (15). Thus, at the 1,000-fold higher concentration we have used, it is not surprising that we observe this high degree of nonspecific internalization.

Two lines of argument support the concept that the reduced insulin endocytosis we have observed in serum transformants is specific for insulin. First, the specific insulin internalization measurements show a reduction similar to that observed for total insulin internalization. Second, none of the nonspecific probes (dextran, histone, or WGA) yielded a comparable result. However, interpretation of our results in terms of specific receptors must be reserved until analysis of both insulin and insulin-like growth factor receptors is carried out with radioactive ligands.

High Growth Requirement for Insulin in Serum-free Medium

Growth in serum-free medium provides a rigorous method for determining the specific hormonal and growth factor requirements of a given cell line (2). When Swiss 3T3 fibroblasts are grown at subconfluent densities in serum-free medium, the strongest requirement

we have observed is for insulin (29). Figure 2 shows a dose-response curve for 3T3 growth in serum-free medium containing various concentrations of insulin. The maximum response occurs above 1 μM. At this high insulin concentration, the majority of specific insulin binding is to low-affinity sites, presumably receptors for insulin-like growth factors (31). Thus, although insulin endocytosis at 1 μM cannot be used as a measure of specific receptor internalization, this concentration is more relevant to insulin stimulation of growth than concentrations that are nonmitogenic for fibroblasts.

Correlation of Insulin Requirement with Insulin Endocytosis

We have determined the specific growth requirements for insulin in serum-free medium for 3T3, SV101, and Aγ4 (Powers and Pollack, in preparation). The results of this analysis and similar measurements for 3T6 are shown in Table 4. All cell lines grow well in the presence of insulin, but only the serum transformants do so in its absence.

The quantitative differences in insulin requirement paralleled those we have observed for insulin endocytosis (R = −0.95). This is also true for insulin endocytosis per μm^2 (R = −0.87). No correlation with total insulin binding per μm^2 was observed (R = 0.045).

DISCUSSION

Our measurements of endocytosis for four cell lines indicate that transformation to serum or anchorage independence is not accompanied by significant changes in the amount of bulk endocytosis, either fluid phase or adsorptive. This is in agreement with the finding by Davies (10) that the rate of endocytosis of sucrose was similar for growing Balb/c-3T3 and SV40-transformed 3T3. Both serum and anchorage transformants show reduced receptor-mediated endocytosis of α_2M. Pastan et al. (26) have reported that, when observed by indirect immunofluorescence, α_2M internalization was visibly decreased in cell lines transformed by the Moloney strain of murine sarcoma virus, relative to the parental cell lines; this was not the case for SV40-transformed Balb 3T3 or a variety of other transformants. The discrepancy between their observations and our results for SV40-transformed Swiss 3T3 is probably due to the insensitivity of qualitative interpretation of microscopic images. It will be of interest to see whether other receptor-mediated processes are affected by transformation.

Serum transformation was accompanied by an increase in internalization of WGA, which was not paralleled by changes in WGA-binding sites. Our WGA results are in agreement with the suggestion that increased WGA-agglutinability of transformed cells results from increased mobility of WGA-binding sites and not from an increase in number of these sites (7,25,30). The contrast between the increased WGA endocytosis and decreased α_2M and insulin endocytosis is probably due to the multivalency of WGA, although it may be due to the fact that WGA binds to a number of different glycosylated surface proteins. Our WGA results may be explained by the hypothesis that serum transformation leads to increased membrane fluidity [possibly limited to certain regions (14)] and that increased fluidity facilitates aggregation (and internalization) of sites by the multivalent lectin. Increases in membrane fluidity of growing cells (relative to stationary cells) have been reported (9), and alterations in receptor-cytoskeleton interactions resulting from changes in membrane fluidity have been suggested (17).

The results we have obtained for insulin endocytosis are in contrast to those obtained for WGA endocytosis.

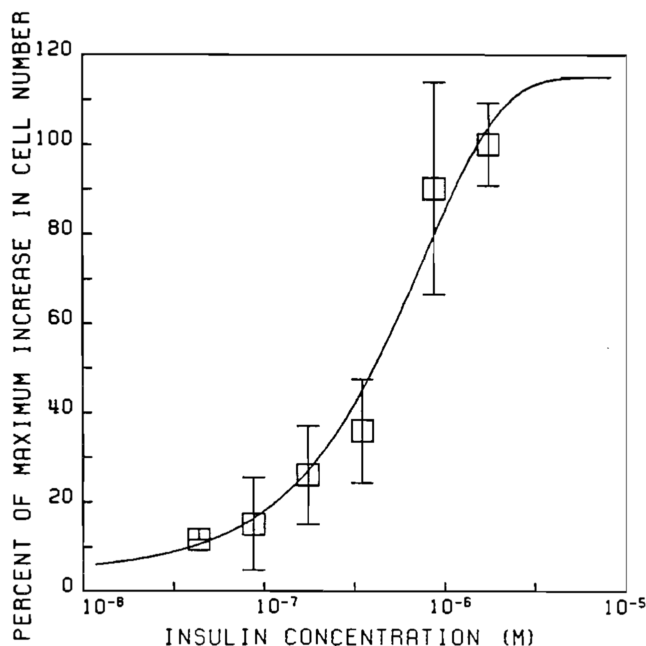

FIG. 2. Concentration dependence of insulin requirement for 3T3 growth in serum-free medium. Cells were incubated for 12 days in serum-free medium containing various concentrations of insulin as described in Materials and Methods and then cell numbers were measured with a Coulter counter. The line is an unconstrained first-order fit to the data.

Table 4
Insulin Requirements of the Four Cell Lines in Serum-Free Medium

Cell Line	Experiment 1	Experiment 2
3T3	0.34 ± 0.006	0.33 ± 0.063
Aγ4	0.39 ± 0.016	0.11 ± 0.152
SV101	0.87 ± 0.021	0.72 ± 0.152
3T6	0.67 ± 0.071	0.52 ± 0.102

Mean values and SD for quadruplicate measurements of the relative growth rate are shown. Serum-free medium for experiment 1 contained 5 μg/ml transferrin, 10 ng/ml FGF, and 5 μg/ml gimmel factor, with or without 10 μg/ml (1.6 μM) insulin (29). The medium for experiment 2 contained 5 μg/ml transferrin, 10 ng/ml FGF, 40 ng/ml EGF, and 10^{-7} M dexamethasone, with or without 10 μg/ml (1.6 μM) insulin (data for 3T3, Aγ4, and SV101 from Powers and Pollack, in preparation).

Serum-transformed fibroblasts endocytosed less insulin than serum-normal fibroblasts. The quantitative correlation between insulin endocytosis and the insulin requirement for growth in serum-free medium suggests that these two phenomena are related, although further experimentation will be necessary to substantiate this idea. The fact that optimal growth stimulation by insulin in serum-free medium occurs above 1 μM (Fig. 2), a concentration at which both high- and low-affinity receptors are saturated and at which only 25% of insulin internalization is specific, is consistent with this suggestion.

ACKNOWLEDGMENTS

We thank Mr. Kazunori Yamamoto for excellent technical assistance and Ms. Rose Marie Cubbon and Dr. Arline Deitch for their generous help with the Coulter volume measurements.

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