Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3598–3601, April 1996
Cell Biology

Protein-free cell culture on an artificial substrate with covalently immobilized insulin

(growth factor/cell growth/biomaterial)

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Communicated by Robert Langer, Massachusetts Institute of Technology, Cambridge, MA, December 5, 1995 (received for review April 13, 1995)

ABSTRACT **Insulin was immobilized on a surface-hydrolyzed poly(methyl methacrylate) film. Chinese hamster ovary cells overexpressing human insulin receptors were cultured on the film in the absence of serum or soluble proteins. Small amounts of immobilized insulin (1–10% of the required amount of free insulin) were sufficient to stimulate cell proliferation. In addition, the maximal mitogenic effect of immobilized insulin was greater than that of free insulin. Immobilized insulin activated the insulin receptor and downstream signaling proteins, and this activation persisted for longer periods than that obtained with free insulin, probably explaining the greater mitogenic effect of the immobilized insulin. Finally the immobilized-insulin film was usable repeatedly without marked loss of activity.**

Mammalian cell culture is a key technique for both basic biological research and its industrial applications. The importance of substrata or biomaterials, including both biological and synthetic extracellular matrices, for cell culture as well as for the production of artificial tissues and organs has become increasingly evident (1–4). However, it is difficult to regulate cellular functions, such as proliferation, secretion, movement, and differentiation with the use of biomaterials alone. The immobilization of biosignaling molecules such as growth factors and cytokines in such a manner that they retain their activity would therefore contribute significantly to the design of functional biomaterials.

Although the binding of biosignaling molecules to their cell surface receptors results in the formation of complexes that are internalized and sorted, with some receptors recycled to the cell surface, it is not known which steps in this process are required for signal transduction. Horwitz *et al.* (5) showed that covalently immobilized interleukin 2 preserved the viability of an interleukin 2-dependent cell line, and they concluded that the immobilized cytokine retains at least some of the activity of the soluble protein and that internalization of the receptor may not be essential for signal transduction.

We have previously shown that insulin immobilized on various artificial and biological substrata increases the growth of anchorage-dependent cells (mouse STO fibroblasts, mouse sarcoma cells, and bovine endothelial cells) to a greater extent than the free protein (6–9). These observations demonstrate that it is possible to culture cells on artificial substrata without the addition of growth factors to the culture medium. To clarify the increased growth-promoting effect of immobilized insulin, we have now investigated the mechanism of signal transduction by the immobilized ligand with Chinese hamster ovary (CHO) cells that overexpress human insulin receptors, in order to detect changes in receptor activity more readily. We also demonstrated the repeated use of the immobilized-insulin substratum without substantial loss of activity.

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MATERIALS AND METHODS

Substrate Preparation. Insulin was immobilized on a partially hydrolyzed poly(methyl methacrylate) (PMMA) film as described (6, 7). The PMMA film (diameter, 15 or 100 mm) was cast from a toluene solution on a glass plate. The film was partially hydrolyzed by dipping in 4 M NaOH for 90 min at 50°C and was then immersed in 10% (wt/vol) citric acid overnight at room temperature. The surface-hydrolyzed PMMA film was washed with double-distilled water until the pH of the washes reached pH 7.0 and was then incubated at 4°C for 2 h in 0.5 M Mes buffer (pH 4.5) containing water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1 mg/ml) as a coupling agent. The film was subsequently incubated in Mes buffer (10 ml for four films with a diameter of 15 mm and 100 ml for a film with a diameter of 100 mm) containing ^{125}I-labeled bovine insulin at various concentrations at 4°C for 48 h and then washed >30 times with 10 ml of phosphate-buffered saline (PBS) until the release of ^{125}I-labeled insulin became undetectable. The amount of immobilized insulin was determined on the basis of the radioactivity associated with the film. Immobilization of nonlabeled insulin was performed according to the same procedure. The surface-hydrolyzed PMMA film without immobilized ligand was used as a control. The films were disinfected with 70% (vol/vol) ethanol and washed with sterilized PBS.

Cell Culture. CHO cells overexpressing human insulin receptors (CHO-T cells; 10^6 receptors per cell) were subcultured in Ham's F-12 medium containing 10% (vol/vol) fetal bovine serum under 5% CO_2 in air at 37°C (10). After culture in the absence of serum for 2 days, cells were harvested by incubation at 37°C for 5 min with PBS containing 0.02% (wt/vol) EDTA and 0.15% (wt/vol) trypsin, suspended in Ham's F-12 medium (2×10^5 cells per ml), and cultured under 5% CO_2 in air at 37°C for 48 h in the 24-well plates containing various PMMA films (diameter, 15 mm).

Determination of DNA Synthesis. Cultured cells were solubilized by heating in 1 M NaOH at 70°C for 30 min or until complete lysis was confirmed with a light microscope. The lysate was then neutralized with 1 M HCl and homogenized. The homogenate (100 μl) was added to 3 ml of a solution containing 100 mM NaCl, 10 mM EDTA, 10 mM Tris·HCl (pH 7.0), and 4′,6-diamidino-2-phenylindole (100 ng/ml). The amount of DNA synthesized in the cells was determined by measuring the amount of complex formed between DNA and the dye (11). The fluorescence intensity of the complex at 450 nm was measured at an excitation wavelength of 360 nm. The amount of DNA in cells grown on control PMMA films in the absence of insulin decreased to 52% ± 7% (mean ± SD; n = 8) of the initial amount after culture for 48 h.

Abbreviations: PMMA, poly(methyl methacrylate); CHO, Chinese hamster ovary; IR, insulin receptor; IRS-1, insulin receptor substrate 1; PI, phosphatidylinositol.

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Association of Insulin with Cells. Cells (2×10^5/ml) were incubated with free or immobilized ^{125}I-labeled insulin in Ham's F-12 medium under 5% CO_2 in air at 37°C for the prescribed time and then harvested by incubation with PBS containing 0.02% EDTA at room temperature for 1 h. The complete removal of cells from the film by this treatment was confirmed with a light microscope. The radioactivity associated with the harvested cells was measured.

Insulin Receptor Autophosphorylation and Activation of Cellular Proteins. Time course assays were performed as described (10, 12). CHO-T cells that had been cultured in the absence of serum for 2 days were harvested by incubation with PBS containing 0.02% EDTA, suspended in Ham's F-12 medium (10^6 cells per 10 ml), and added either to an immobilized-insulin PMMA film (0.39 μg/cm^2; diameter, 100 mm) or to a control PMMA film (diameter, 100 mm) in the presence of free insulin (20 μg/ml).

After incubation at 37°C for the indicated time with immobilized or free insulin, the cells were washed with PBS, frozen in liquid nitrogen, scraped into 1 ml of ice-cold lysis buffer [137 mM NaCl, 20 mM Tris·HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 0.4 mM sodium orthovanadate], and solubilized on ice for 10 min. The lysate was centrifuged at 15,000 × *g* in an Eppendorf centrifuge for 15 min, and the entire supernatant was collected and subjected to one of the following biochemical assays.

For assay of the phosphorylation of the insulin receptor (IR) or insulin receptor substrate 1 (IRS-1) on tyrosine residues, protein G-Sepharose beads (15 μl) (Pharmacia) were coated with mouse antibodies to IR or to IRS-1 (20 μg) in 50 μl of WG buffer [50 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% (vol/vol) Triton X-100]. The beads were washed twice with WG buffer (1 ml) and then incubated with the lysate supernatant for 4 h at 4°C on a rotating platform. Immunoprecipitates were washed twice with WG buffer (1 ml) and then heated at 100°C for 3 min with 30 μl of SDS sample buffer [62.5 mM Tris·HCl (pH 6.8), 10% glycerol, 2% SDS, 5% (vol/vol) 2-mercaptoethanol, 0.001% (wt/vol) bromophenol blue] (13). After centrifugation at 1000 × *g* for 30 sec, a portion (10 μl) of the resulting supernatant was subjected to SDS/PAGE on an 8.5% gel, and the separated proteins were transferred to an Immobilon-P (Millipore) nitrocellulose filter. The filter was incubated with rabbit polyclonal or mouse monoclonal antibodies to phosphotyrosine for detection of IR and IRS-1, respectively. The bound antibodies were detected with horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin and alkaline phosphatase-conjugated antibodies to mouse immunoglobulin, respectively, followed by ECL detection system (Amersham).

Phosphatidylinositol (PI)-3-kinase activity was measured directly in IRS-1 immunoprecipitates in a reaction mixture (50 μl) containing PI (0.2 mg/ml), 20 mM Hepes (pH 7.1), 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$, and 40 μM [γ-^{32}P]ATP (0.1 μCi/ml; 1 Ci = 37 GBq). PI was dried with nitrogen gas, sonicated in a bath sonicator (three times for 5 min each time with cooling in between), and incubated with the immunoprecipitate for 5 min at room temperature before addition of MgATP to start the reaction. The reaction was stopped after 5 min by the addition of 4 M HCl (15 μl) and chloroform/methanol (1:1, vol/vol) (130 μl). Tubes were shaken for 30 sec, and the lower layer (30 μl) was spotted onto a silica gel 60 plate (Merck) that had been coated with 1% (wt/vol) potassium oxalate and activated at 100°C for 1 h. Plates were developed, and the radioactive reaction product was determined with a radioanalytic imaging system.

Repeated Utilization of Substrata. Cells cultured on 24-well plates containing immobilized-insulin PMMA films (0.39 μg/cm^2; diameter, 15 mm) were detached from the films by incubation in 0.02% EDTA at room temperature for 1 h or 0.15% trypsin at 37°C for 10 min, after which no cells were detected on the films by phase-contrast microscopy. The films were sterilized and placed on a new 24-well plate for the next cell culture.

RESULTS

PMMA films on which various amounts of insulin were immobilized were obtained by changing the feed concentration of insulin (Fig. 1). The amounts of immobilized insulin (0.1–0.3 μg/cm^2) indicate formation of the protein monolayer (14).

It is important to confirm that the immobilized ligand does not subsequently dissociate from the film, as pointed out by Horwitz *et al.* (5). We therefore incubated immobilized ^{125}I-labeled insulin with 10% (vol/vol) serum-containing or serum-free culture medium at 37°C for 48 h. In neither instance was an increase in radioactivity in the medium or a decrease in immobilized ^{125}I-labeled insulin on the film observed (data not shown). In addition, incubation with 4 M urea and 8 M guanidine hydrochloride for 48 h did not induce leakage of immobilized ^{125}I-labeled insulin, as described previously (14).

Association of insulin with the CHO-T cells was also analyzed (Fig. 2). Although cells incubated with free insulin associated with this growth factor, cells incubated with an even larger amount of immobilized insulin were not associated with this growth factor after removal of cells from the film.

The amount of total DNA in CHO-T cells cultured for 48 h was markedly increased in the presence of small amounts of immobilized insulin; comparable effects required 10–100 times as much free insulin (Fig. 3). In addition, the maximal stimulation of DNA synthesis in response to immobilized insulin was greater than that in response to free insulin.

Phosphorylation of the IR (Fig. 4*A*) and IRS-1 (Fig. 4*B*) on tyrosine residues, as well as activation of PI-3-kinase (Fig. 4*C*), was induced by both free and immobilized insulin. The time courses of IR phosphorylation and PI-3-kinase activation differed markedly between the two conditions. Both IR phosphorylation and PI-3-kinase activity continued to increase for up to 12 h (in Fig. 4*B*, data for 12-h incubation not shown) in response to immobilized insulin, whereas in response to free insulin both phenomena achieved a transient maximum within 30 min. These results indicate that immobilized insulin interacts specifically with IRs and that the increase in cell growth induced by immobilized insulin is attributable to the persistent phosphorylation and activation of IR substrates.

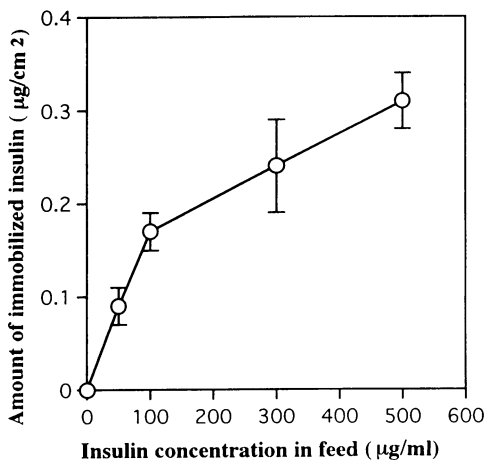

FIG. 1. Effect of insulin concentration in the feed on the amount of insulin immobilized on surface-hydrolyzed PMMA films. The amount of immobilized insulin was calculated from the radioactivity of ^{125}I-labeled insulin associated with the film. Data are means ± SD (n = 5).

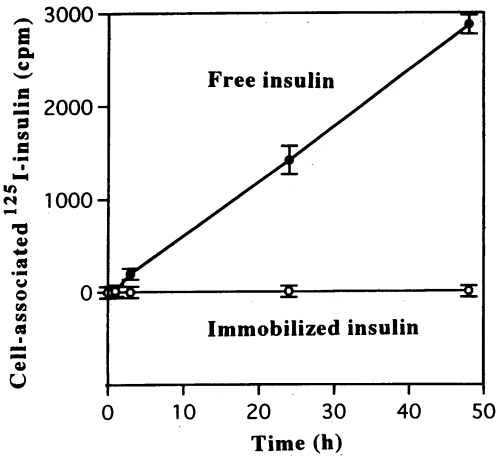

FIG. 2. Time course of insulin association with CHO-T cells. Cells were cultured in the presence of free ^{125}I-labeled insulin (0.1 μg per well, 10^6 cpm) or immobilized ^{125}I-labeled insulin (0.55 μg per well, 10^6 cpm) for the indicated time. Cells were then detached by incubation with 0.02% EDTA, and the radioactivity associated with the harvested cells was measured. Incubation with free insulin was performed on control PMMA films. Data are means ± SD (n = 12).

The effect of repeated use of the PMMA film-immobilized insulin on its activity was assessed by culturing CHO-T cells for 2 days, removing the cells by treatment with EDTA or trypsin, and then repeating the procedure. Whereas trypsin treatment markedly reduced the growth-promoting activity of immobilized insulin (Fig. 5*A*), as a result of hydrolysis of the growth factor (Fig. 5*B*), EDTA treatment had no marked effect on insulin activity or hydrolysis. The slight decrease in relative growth rate after repeated use of immobilized insulin with EDTA may result from coverage of the film with proteins secreted from the growing cells.

DISCUSSION

We have shown that immobilized insulin induced a pronounced stimulation of CHO-T cell growth. The growth of mouse STO fibroblasts (7), mouse sarcoma cells (8), and bovine endothelial cells (9) was previously shown to be increased to a greater extent by immobilized insulin than by free insulin, although the effect on these cells was less marked than that on CHO-T cells. Hofmann *et al.* (15) showed that the

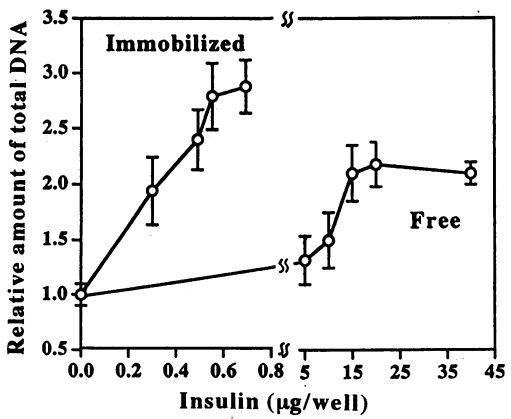

FIG. 3. Relative amount of total DNA in CHO-T cells during incubation in the presence of various concentrations of free or immobilized insulin for 48 h. Incubation of cells (2×10^5/well) with free insulin was performed on control PMMA films. The amount of total DNA in cells cultured on control PMMA films in the absence of free or immobilized insulin was taken as 1.0. Data are means ± SD (n = 8).

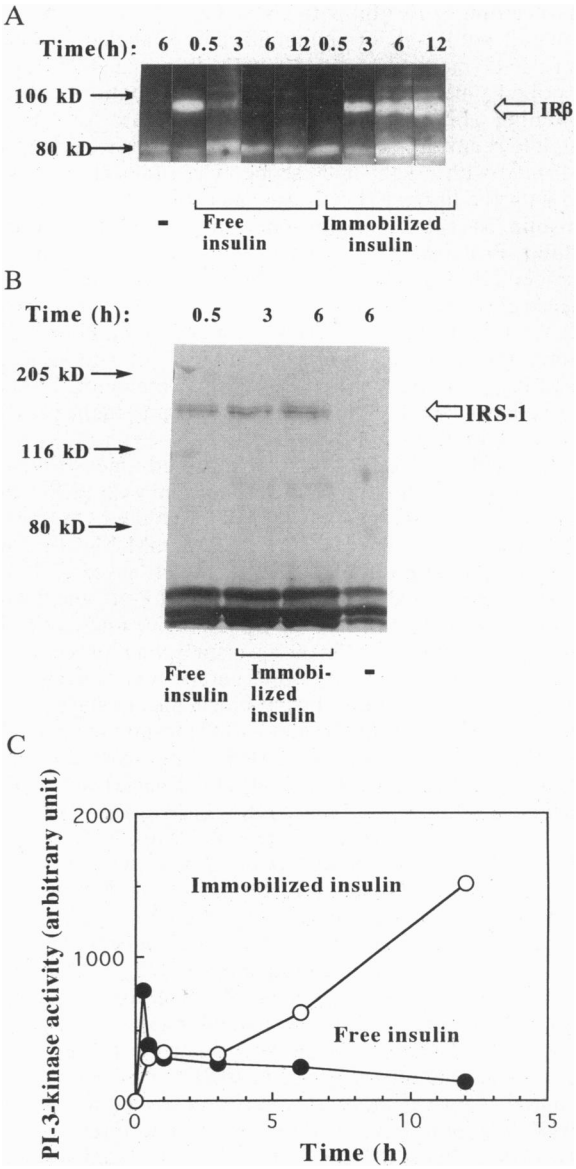

FIG. 4. Time courses of tyrosine phosphorylation of the IR β subunit (*A*) and of IRS-1 (*B*) and time course of activation of PI-3-kinase (*C*) in CHO-T cells in response to free or immobilized insulin. Cells were cultured on control PMMA films in the absence (−) or presence of free insulin or on immobilized-insulin PMMA films for the indicated times and then assayed as described in *Materials and Methods*. Data in *C* are representative of three separate experiments.

stimulatory effects of insulin were enhanced by transfection of insulin receptors into mouse fibroblasts.

The mitogenic effect of immobilized insulin appears to be specific. Thus, immobilized albumin and γ-globulin have no effect on either cell adhesion or cell growth, whereas immobilized collagen and fibronectin enhance cell adhesion but not cell growth (16). Furthermore, antibodies to insulin inhibit cell proliferation induced by immobilized insulin (14). In addition, whereas immobilized insulin increased the growth of anchorage-dependent cells, it had no effect on anchorage-independent cells such as hybridoma cells (8). Adhesion or direct interaction of cells with the substratum is therefore necessary for growth stimulation. We have now shown that direct interaction of immobilized insulin with CHO-T cells triggers key steps in insulin signal transduction and that the time course of these events differs from that associated with free insulin.

Recent studies have demonstrated phosphorylation of integrin or neighboring proteins without internalization of extra-

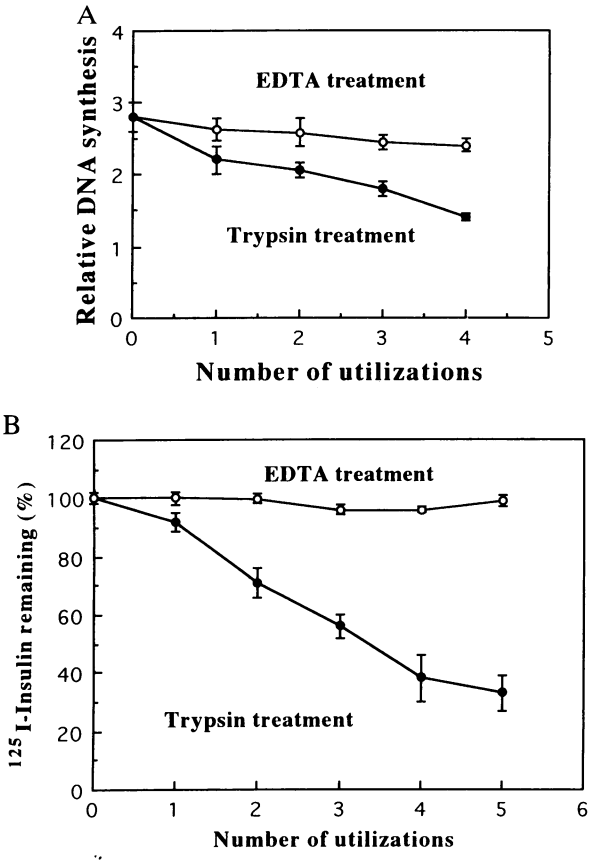

FIG. 5. Effect of repeated use of PMMA film-immobilized insulin on CHO-T cell proliferation (*A*) and the amount of growth factor remaining associated with the film (*B*). After each 2-day culture, the film was washed with 0.02% EDTA or 0.15% trypsin and either the amount of DNA synthesis was determined (*A*; DNA synthesis by cells cultured for the first time on control PMMA films in the absence of free insulin was taken as 1.0) or the amount of immobilized ^{125}I-labeled insulin associated with the film was measured (*B*; the radioactivity associated with virgin immobilized film was taken as 100%). Data are means ± SD (n = 5 in *A* and n = 12 in *B*).

cellular matrix (17), as well as ligand-induced cell transformation and growth stimulation by a noninternalizing epidermal growth factor receptor (18, 19). These results suggest that immobilized insulin may induce autophosphorylation of the IR without internalization.

The observation that immobilized insulin stimulated CHO-T cell growth in smaller amounts than free insulin may be attributable to immobilization providing a high local concentration of insulin that results in efficient interaction with IRs and multivalent cross-linking of them. The greater maximal mitogenic effect of immobilized insulin may be due to inhibition of receptor down-regulation by internalization. In addition it is possible that immobilized insulin prevents the free lateral diffusion of the activated receptor in the plane of the membrane, thereby inhibiting its potential interaction with regulators of receptor activity such as tyrosine phosphatases or serine/threonine-dependent protein kinases. The persistent activation of the receptor and neighboring proteins indicates that the enhanced stimulatory effect on cell growth by immobilized insulin primarily results from the long-term activation associated with these explanations.

Insoluble insulin conjugates have been synthesized to investigate the mechanism of insulin action (20–23). Although these conjugates facilitated extensive purification of solubilized IRs by affinity chromatography (24), definitive conclusions regarding the effectiveness of immobilized insulin could not be drawn because of the possibility that immobilization was incomplete (25). Furthermore, given our observation of the marked time delay between exposure of CHO-T cells to immobilized insulin and the detection of signal transduction events, previous studies examining acute effects of insulin conjugates may not have allowed sufficient time for evaluation of activity (24–26). In addition, because previous studies used porous beads that were not accessible to the cells as the immobilizing support, it was difficult to evaluate quantitatively the relation between the amount of immobilized insulin and biological effects (5, 26).

Although it is difficult to certify absolutely that no release of immobilized insulin from the substratum occurs, the present study sheds light on the mechanism of action of immobilized insulin by demonstrating the time course of key signal transduction events and opens the way to the biomaterial application of immobilized insulin by repeated utilization. By taking the cross-talk of receptors of different types into consideration (27), coimmobilization of cell adhesion factors with insulin may further enhance cell growth (9, 28). Thus, biomaterial design with the use of biological signaling molecules should yield further important advances in cell culture systems.

We thank R. Langer for critical review of the manuscript and K. Saito for help with the radioisotope experiments.

1. Peppas, N. A. & Langer, R. (1994) *Science* **263,** 1715–1718.
2. Singhvi, R., Stephanopoulos, G. N., Wang, D. I. C., Kumar, A., Lopez, G. P., Whitesides, G. M. & Ingber, D. E. (1994) *Science* **264,** 696–698.
3. Hubbell, J. A., Palsson, B. O. & Papoutsakis, E. T., eds. (1994) *Biotech. Bioeng.* **43,** nos. 7 and 8.
4. Roskelly, C. D., Desprez, P. Y. & Bissell, M. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12378–12382.
5. Horwitz, J. I., Toner, M., Tompkins, R. G. & Yarmush, M. L. (1993) *Mol. Immunol.* **30,** 1041–1048.
6. Ito, Y., Liu, S. Q. & Imanishi, Y. (1991) *Biomaterials* **12,** 449–453.
7. Liu, S. Q., Ito, Y. & Imanishi, Y. (1992) *Biomaterials* **13,** 50–58.
8. Ito, Y., Uno, T., Liu, S. Q. & Imanishi, Y. (1992) *Biotech. Bioeng.* **40,** 1271–1276.
9. Liu, S. Q., Ito, Y. & Imanishi, Y. (1993) *J. Biomed. Mater. Res.* **27,** 909–915.
10. Endemann, G., Yonezawa, K. & Roth, R. A. (1990) *J. Biol. Chem.* **265,** 396–400.
11. Zheng, J., Ito, Y. & Imanishi, Y. (1994) *Biomaterials* **15,** 963–968.
12. Yonezawa, K., Yokono, K., Shii, K., Ogawa, W., Ando, A., Hara, K., Baba, S., Kaburagi, Y., Yamamoto-Honda, R., Momomura, K., Kadowaki, T. & Kasuga, M. (1992) *J. Biol. Chem.* **267,** 440–446.
13. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
14. Liu, S. Q., Ito, Y. & Imanishi, Y. (1992) *J. Biophys. Biochem. Methods* **25,** 139–148.
15. Hofmann, C., Goldfine, I. D. & Whittaker, J. (1989) *J. Biol. Chem.* **264,** 8606–8611.
16. Ito, Y., Liu, S. Q., Nakabayashi, M. & Imanishi, Y. (1992) *Biomaterials* **13,** 789–794.
17. Hynes, O. (1992) *Cell* **69,** 11–25.
18. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N. & Rosenfeld, M. G. (1990) *Science* **247,** 962–964.
19. Reddy, C. C., Wells, A. & Lauffenburger, D. A. (1994) *Biotech. Prog.* **10,** 377–384.
20. Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 450–457.
21. Butcher, R. W., Croffird, O. B., Gammeltoff, S., Gliemann, J., Gavin, J. R., Goldfine, I. D., Kahn, C. R., Rodbell, M., Roth, J., Jarett, L., Lefkowitz, R. J., Levine, R. & Marinetti, G. V. (1973) *Science* **182,** 396–397.
22. Oka, T. & Topper, Y. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1630–1633.
23. Kolb, H. J., Renner, R., Hepp, K. D., Weiss, L. & Wieland, O. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 248–252.
24. Cuatrecasas, P. (1973) *Science* **182,** 397–398.
25. Venter, J. C. (1982) *Pharmacol. Rev.* **34,** 153–257.
26. Garwin, J. L. & Gelehter, T. D. (1974) *Arch. Biochem. Biophys.* **164,** 52–59.
27. Vuori, K. & Ruoslahti, E. (1994) *Science* **266,** 1576–1578.
28. Ito, Y., Zheng, J. & Imanishi, Y. (1995) *Biotech. Prog.* **11,** 677–681.