Aberrant regulation of the metabolism of the insulin receptor in Swarm rat chondrosarcoma chondrocytes

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Treatment of Swarm rat chondrosarcoma chondrocytes for 3 days in media containing either non-recombinant pig or recombinant human insulin (1 μ g/ml) increased the rate of proteoglycan synthesis $\sim$ 6-fold compared with cells cultured in the absence of insulin. The concentrations of human and pig insulin that stimulated the cells to double their rate of proteoglycan synthesis were $\sim$ 1 ng/ml and $\sim$ 2 ng/ml respectively. Because physiological concentrations of insulin do not influence proteoglycan synthesis in non-transformed chondrocytes, the findings indicated a possible abnormality in the insulin-dependent regulation of the insulin receptor in these tumour cells. Like most cells, chondrosarcoma chondrocytes down-regulated their insulin receptors when incubated with insulin for 30 min. However, the number of plasma-membrane and intracellular insulin receptors did not decrease when the chondrocytes were exposed to insulin chronically for 4 days. Chondrocytes were cultured in media containing ^2H -, ^{13}C - and ^{15}N -labelled amino acids, and the heavy-isotope density-shift method was used to investigate both the rate of degradation and the rate of synthesis of the insulin receptor. Although the rate of synthesis of the receptor was slightly faster in the insulin-treated cultures, as assessed by a slightly faster rate of appearance of the 'heavy' receptor, the rate of degradation of the receptor was slower in the insulin-treated cultures. The half-lives for the 'light' receptors were approx. 18 h and 10 h for chondrocytes cultured in insulin-containing and insulin-free media respectively. These studies *in vitro* indicate that the apparent up-regulation of insulin receptors that occurs in this transformed cell upon long-term exposure to insulin is primarily the result of a decreased rate of receptor degradation.

INTRODUCTION

The Swarm rat chondrosarcoma chondrocyte synthesizes and secretes molecules that make up the extracellular matrix of cartilage. Growth of this tumour *in vivo* depends on insulin (Salomon *et al.*, 1979) as well as other hormones (Salomon *et al.*, 1979; McCumbee *et al.*, 1981). We have reported that the rate of proteoglycan synthesis by this transformed cell is considerably higher than that obtained by any other mesenchymal cell upon exposure *in vitro* for > 24 h to physiological concentrations of non-recombinant insulin, insulin-like growth factor (IGF)-I or IGF-II (Stevens *et al.*, 1981; Stevens & Hascall, 1981; Foley *et al.*, 1982). It has been reported that physiological concentrations of insulin also increase the rate of synthesis of collagen (Bembenek *et al.*, 1982) and hyaluronic acid (Mason *et al.*, 1982).

Competitive binding experiments with radiolabelled and unlabelled 3,3',5-tri-iodo-L-thyronine (T_3), insulin, IGF-I and IGF-II have revealed distinct plasma-membrane binding proteins and/or receptors on this cell for each of these hormones (Foley *et al.*, 1982; Stevens *et al.*, 1986). Using anti-insulin-receptor antibodies, we found that insulin stimulates proteoglycan synthesis via the insulin receptor rather than the IGF-I receptor or the IGF-II receptor (Foley *et al.*, 1982). Furthermore, in

contrast with most other cell types (Gavin *et al.*, 1974), long-term exposure of chondrosarcoma chondrocytes to pig insulin resulted in an increase in the number of insulin receptors on the plasma membrane (Stevens *et al.*, 1983). This unusual insulin effect appeared to be relatively specific, because insulin did not significantly increase the plasma-membrane binding of ^{125}I - T_3 (Stevens *et al.*, 1986) or ^{125}I -IGF-II (Stevens *et al.*, 1983). Likewise, culturing the chondrocytes in medium that contains sufficient concentrations of rat IGF-II to induce a 2-fold increase in the basal rate of proteoglycan synthesis did not influence the extent of plasma-membrane binding of either ^{125}I -insulin or ^{125}I -IGF-II (Stevens *et al.*, 1983). Because of these findings, we proposed that the insulin-induced increase in the number of plasma-membrane insulin receptors might be an abnormality in metabolic control that facilitates growth of the tumour *in vivo*.

As described herein, we used recombinant human insulin to demonstrate that it is unlikely that a contaminant in the commercial preparations of insulin induces proteoglycan synthesis and expression of the insulin receptor. Both the total and plasma-membrane-localized insulin receptors increased in number after 4 days of culture in insulin-containing media. This insulin-stimulated increase in the number of insulin receptors is primarily the result of a decreased rate of receptor

Abbreviations used: Binding buffer, buffer containing EDTA, Hepes, NaCl, MgSO_4 , KCl, glucose and BSA; BSA, bovine serum albumin; DMEM, Dulbecco's high-glucose modified Eagle's medium containing BSA, penicillin, streptomycin, Hepes, Bes and Tes; IGF, insulin-like growth factor; T_3 , 3,3',5-tri-iodo-L-thyronine; WGA, wheat-germ agglutinin.

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degradation, rather than a translocation of the receptor from an intracellular compartment to the cell surface, or increased synthesis of the receptor *de novo*.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: Dulbecco's high-glucose modified Eagle's medium, penicillin and streptomycin (Grand Island Biological Co., Grand Island, NY, U.S.A.); Hanks' balanced salt solution (M.A. Bio-products, Walkersville, MD, U.S.A.); bovine immunoglobulin (Mann Research Laboratories, New York, NY, U.S.A.); recombinant human insulin (26.5 units/mg) (Eli Lilly and Co., Indianapolis, IN, U.S.A.); pig insulin (25.9 units/mg), bovine lung aprotinin (12000 units/mg), crystallized globulin-free bovine serum albumin (BSA), insulin-free BSA, trypsin, soya-bean trypsin inhibitor, Triton X-100, Hepes, Tes, Bes and poly(ethylene glycol) (M, 8000) (Sigma, St. Louis, MO, U.S.A.); Zwittergent 3-12 (Calbiochem-Behring, La Jolla, CA, U.S.A.); [³⁵S]sulphate (~4000 Ci/mmol) and receptor-grade ¹²⁵I-insulin (400 Ci/mg or 2200 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.); a ²H-, ¹³C-, ¹⁵N-labelled algal amino acid mixture in which 1 g contains 31 mg of labelled lysine, 11 mg of histidine, 57 mg of arginine, 87 mg of aspartic acid, 53 mg of threonine, 62 mg of serine, 110 mg of glutamic acid, 15 mg of proline, 53 mg of glycine, 120 mg of alanine, 68 mg of valine, 22 mg of methionine, 50 mg of isoleucine, 92 mg of leucine, 40 mg of tyrosine, 48 mg of phenylalanine, and 1.2 mg of cysteine (Merck, Sharp and Dohme Isotopes, Quebec, Canada); Hydrofluor (National Diagnostics, Somerville, NJ, U.S.A.); wheat-germ agglutinin (WGA)-agarose (E.-Y. Laboratories, San Mateo, CA, U.S.A.); hydrophilic type GV Durapore membranes (Millipore Corp., Bedford, MA, U.S.A.); Sephadex G-25 columns (Pharmacia, Piscataway, NJ, U.S.A.); ultrapure guanidine hydrochloride (Schwarz/Mann, Cambridge, MA, U.S.A.).

Culture and [³⁵S]sulphate labelling of chondrosarcoma chondrocytes

Swarm rat chondrosarcoma chondrocytes were isolated by a trypsin/collagenase treatment of the minced tumour as previously described (Kimura *et al.*, 1979). Approx. 2.5×10^6 or 7×10^6 chondrocytes were added to 35 mm- or 60 mm-diam. plastic culture dishes respectively, containing 2–7 ml of Dulbecco's high-glucose modified Eagle's medium supplemented with 0.25% (w/v) globulin-free BSA, 100 units of penicillin/ml, 100 µg of streptomycin/ml, 15 mM-Hepes, 10 mM-Bes and 10 mM-Tes, pH 7.2 (DMEM). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air. Insulin was dissolved in 0.01 M-HCl at a concentration of 1 mg/ml, diluted in DMEM to the appropriate concentration, and added to the culture dishes. The standard culture conditions for most experiments consisted of maintaining the chondrocytes in either DMEM alone or DMEM containing 1 µg of insulin/ml (0.2 µM). The culture media were replaced at 24 h intervals; 24 h after the initial plating of the cells was designated as day 1 for each experiment. Some cultures were incubated for 3 h with fresh media containing 25 µCi of [³⁵S]sulphate/ml in order to determine the rate of proteoglycan synthesis. The amount of ³⁵S-labelled proteoglycans secreted into

the culture media and the amount associated with the monolayers were determined by Sephadex G-25 chromatography of samples of the media and cell extracts (Stevens *et al.*, 1981). Student's two-tailed *t* test was used to determine statistical differences in the rate of incorporation of [³⁵S]sulphate and statistical differences in the specific binding of ¹²⁵I-insulin (see below).

Measurement of total, cell-surface and intracellular insulin receptors

Binding of ¹²⁵I-insulin to the plasma-membrane-localized insulin receptor was carried out as follows. Each culture was incubated in room air at 4 °C for 6 h with 1 ml of ice-cold Binding buffer (1 mM-EDTA, 0.1 M-Hepes, 0.12 M-NaCl, 1.2 mM-MgSO₄, 2.5 mM-KCl, 10 mM-glucose and 0.5% insulin-free BSA, pH 8.0) containing 10⁵ d.p.m. of ¹²⁵I-insulin (~0.02 pmol) in the presence or absence of 10 µg of pig insulin/ml (2 µM). The radioactive solution was aspirated from each plate, the monolayer was quickly washed with 3 × 2 ml of ice-cold Binding buffer, and the cells were solubilized with 1.5 ml of 4 M-guanidine hydrochloride containing 0.1% (w/v) Zwittergent 3-12. The radioactivity present in each extract was quantified with a Searle Analytic γ-radiation counter.

Chondrosarcoma cells were incubated with trypsin in order to assess the relative distribution of insulin receptors that were in the plasma membrane or in an intracellular compartment (Marshall, 1985). Ice-cold Hanks' balanced salt solution (4 ml) containing 0.1% insulin-free BSA and trypsin (1 mg/ml) was added to each 60 mm culture dish of chondrocytes. The cultures were incubated at 4 °C for 20 min. Before solubilization and quantification of the number of receptors, the trypsin-treated cultures were washed with 3 × 6 ml of ice-cold Hanks' balanced salt solution containing 1% insulin-free BSA, 0.1 mg of soya-bean trypsin inhibitor/ml, 10 mM-glucose, 15 mM-Hepes, 10 mM-Bes and 10 mM-Tes, pH 7.2. The chondrocytes remained adherent to the plastic culture dishes under these conditions, but > 90% of the cell-surface insulin receptors were degraded, as assessed by their subsequent failure to bind ¹²⁵I-insulin.

Usually insulin receptors were solubilized from 60 mm dishes containing 7×10^6 chondrocytes. The culture media were aspirated, and the monolayers were washed three times at 22 °C with 6 ml of Hanks' balanced salt solution containing 1% insulin-free BSA, 0.1 mg of soya-bean trypsin inhibitor/ml, 10 mM-glucose, 15 mM-Hepes, 10 mM-Bes and 10 mM-Tes, pH 7.2. Between the second and the third washes, the chondrocytes were incubated at 37 °C for 30 min, to promote degradation of plasma-membrane receptor-bound insulin. The chondrocytes were removed from the plastic culture dishes by gently scraping. Cells were centrifuged (150 g), resuspended at a density of 3×10^6 cells/ml in 50 mM-Tris/HCl containing 1% (w/v) Triton X-100 and 300 units of aprotinin/ml, pH 7.4, and sonicated at 4 °C with a Branson Sonifier (30 pulses, 50% power cycle). After centrifugation at ~8000 g for 10 min at 4 °C, the Triton X-100 extracts were diluted 5-fold with phosphate-buffered saline (0.14 M-NaCl/3 mM-KCl/10 mM-sodium phosphate), pH 7.2, and applied at 22 °C to 1 ml columns of WGA-agarose to remove non-specific ¹²⁵I-insulin-binding activities (Harrison & Itin, 1980; Lotan *et al.*, 1977). Each lectin column was washed with 5 ml

Fig. 1. Dose/response effect of recombinant human insulin (●) and non-recombinant pig insulin (○) on the stimulation of proteoglycan biosynthesis

Chondrocytes were cultured for 3 days in various concentrations of insulin before being radiolabelled with [^{35}S]sulphate for 3 h. Values for incorporation of [^{35}S]sulphate into proteoglycan per culture are means $\pm$ S.D. ($n = 3$).

of phosphate-buffered saline, pH 7.2, containing 0.2% Triton X-100. Over 90% of the solubilized insulin receptors were recovered in the eluates when the columns were washed with 3 ml of phosphate-buffered saline containing 0.2% Triton X-100 and 0.5 M-*N*-acetylglucosamine. A 50 μl portion of each extract was added to 20 μl of Binding buffer containing 50000 d.p.m. of ^{125}I -insulin (~ 0.01 pmol) in the absence or presence of 10 μg of pig insulin/ml. After a 2 h incubation at 22 $^{\circ}\text{C}$, 0.5 ml of Hanks' balanced salt solution containing 0.1% (w/v) bovine immunoglobulin was added, followed by 0.5 ml of 25% (w/v) poly(ethylene glycol) in water. The samples were mixed well, and incubated in an ice bath for at least 30 min. The mixtures were filtered under reduced pressure on hydrophilic Durapore membranes (Cuatrecasas, 1972) to remove free ^{125}I -insulin from that bound to the receptor. After washing the membranes with 3 $\times$ 1 ml of ice-cold 8% poly(ethylene glycol) in 0.1 M-phosphate buffer, pH 7.5, the membrane-bound radioactivities were quantified.

Alternatively, a 5 min centrifugation at $\sim 8000 g$ at 4 $^{\circ}\text{C}$ was used to separate the ^{125}I -insulin/insulin-receptor complex from free ^{125}I -insulin. The supernatants were aspirated; the pellets were washed with 1 ml of ice-cold 8% poly(ethylene glycol) and analysed for radioactivity.

Heavy-isotope density-shift experiments

The heavy-isotope density-shift method of Reed & Lane (1980) was used to monitor the synthesis and degradation of the chondrosarcoma chondrocyte's insulin receptor. After culturing for 3½ days under standard culture conditions, the cell monolayers were washed twice with Hanks' balanced salt solution, and 5 ml of fresh DMEM containing heavy (^2H , ^{13}C , ^{15}N) amino acids were added. For the preparation of heavy DMEM, 100 ml of heavy-isotope-labelled amino acids was added to 100 ml of amino acid-free DMEM along with 3 mg of non-labelled cysteine, 6 mg of glutamine, 0.8 mg of histidine, 2 mg of lysine and 0.8 mg of tryptophan. After various times at 37 $^{\circ}\text{C}$ in 5% CO_2 , the cells were washed with 3 $\times$ 6 ml of Hanks' balanced salt solution containing

Fig. 2. Specific binding of ^{125}I -insulin to total (a) and to cell-surface (b) insulin receptors for chondrocytes cultured for 1–5 days in DMEM (—), or DMEM containing 1 μg of pig insulin/ml (---)

The data for the first 4 days of culture are means $\pm$ S.D., for three separate experiments. In only one of the three experiments were the chondrocytes cultured for 5 days. Each experiment was done in triplicate.

1% insulin-free BSA and 10 mM-glucose. Between the second and the third washes, the cells were incubated at 37 $^{\circ}\text{C}$ for 30 min in 5% CO_2 to dissociate receptor-bound insulin. The 'light' and 'heavy' insulin receptors were solubilized and purified by the WGA-agarose chromatographic technique described above. Samples (3 ml) of the eluates from the lectin columns were mixed with 2 ml of CsCl (1.098 g of CsCl/ml) in 50 mM-Tris/HCl, pH 7.4, containing 400 units of aprotinin/ml. The samples were centrifuged for 38 h at 4 $^{\circ}\text{C}$ in a Beckman SW55Ti rotor at 50000 rev./min (304000 g_{max}); 40 fractions were collected, starting from the top of the tube. Each fraction was assayed for ^{125}I -insulin-binding activity by the hydrophilic-Durapore-membrane technique. There was a 75–85% recovery of ^{125}I -insulin-binding activity after density-gradient centrifugation. The area under the curve for the 'light receptor' (generally fractions 10–20) and the area under the curve for the 'heavy receptor' (generally fractions 21–31) were determined and compared with one another.

Table 1. Short-term effect of insulin on the distribution of insulin receptors

Swarm rat chondrocytes that were cultured in insulin-free DMEM for 1–4 days were washed and preincubated for 30 min with DMEM containing or lacking 1 μ g of insulin/ml. The percentages of the insulin receptors that were located on the surfaces of the cells were determined by quantifying the total, cell-surface and intracellular binding of 125 I-insulin. The data are means of an experiment done in triplicate.

Day of culture	No insulin preincubation		Insulin preincubation	
	Total bound (c.p.m.)	(% cell surface)	Total bound (c.p.m.)	(% cell surface)
1	1.7×10^3	67	1.7×10^3	50
2	3.1×10^3	70	2.9×10^3	54
3	2.6×10^3	76	2.6×10^3	65
4	2.3×10^3	73	2.1×10^3	56

Table 2. Short-term effect of exposure of chondrocytes to insulin-free medium on the distribution of insulin receptors

Swarm rat chondrocytes that were cultured in DMEM containing 1 μ g of insulin/ml for 1–4 days were washed, and preincubated for 30 min in DMEM containing or lacking 1 μ g of insulin/ml. The percentages of insulin receptors that were located on the cell surfaces were determined by quantifying the total, cell-surface and intracellular binding of 125 I-insulin. The data are means of an experiment done in triplicate.

Day of culture	Insulin preincubation		No insulin preincubation	
	Total bound (c.p.m.)	(% cell surface)	Total bound (c.p.m.)	(% cell surface)
1	2.2×10^3	32	2.2×10^3	52
2	3.7×10^3	37	3.4×10^3	60
3	4.1×10^3	47	4.3×10^3	68
4	4.3×10^3	38	4.4×10^3	64

RESULTS

Proteoglycan synthesis by chondrocytes exposed to recombinant and non-recombinant insulin

Chondrocytes were cultured for 3 days in the absence or presence of either recombinant human or non-recombinant pig insulin, and the rates of proteoglycan synthesis were assessed. Although both insulin preparations stimulated the rate of synthesis of 35 S-labelled proteoglycans relative to non-insulin treated cells ($P < 0.001$ at insulin concentrations > 1 ng/ml), the rat tumour chondrocytes were slightly more sensitive to the recombinant insulin than to non-recombinant insulin (Fig. 1). At the physiological concentrations of 1 ng of human insulin/ml and 2 ng of pig insulin/ml, the rates of proteoglycan synthesis were approximately double that of cells maintained in DMEM alone. Although the rate of proteoglycan synthesis was ~ 6 -fold higher for cells exposed to pharmacological concentrations of either species of insulin, an approx. 10-fold higher concentration of pig insulin was required to achieve the maximal rate of incorporation of radioactivity. Subsequent studies on the metabolism of the insulin receptor were carried out on cells exposed to 1 μ g of either pig or human insulin/ml (0.2 μ M) to ensure that the general anabolic rate of the cells (as assessed by the rate of proteoglycan synthesis) was maximal.

Effect of long-term exposure of chondrosarcoma chondrocytes to high doses of insulin

By determining the relative 125 I-insulin-binding activities of detergent-extracted and WGA-agarose-purified

insulin receptors, the total number of insulin receptors present in cells cultured in DMEM alone was compared with that of cells cultured in the presence of insulin. As depicted in Fig. 2(a), the extracts of cells maintained for 1 day in DMEM and pig insulin specifically bound ~ 20 % more 125 I-insulin than did extracts derived from cells cultured in DMEM alone ($P < 0.1$). By day 4 of culture, the specific binding of 125 I-insulin to the receptors in the detergent extracts was ~ 2 -fold higher in the insulin-treated cultures ($P < 0.01$). This finding appeared to be mainly due to a decrease in the binding of the radiolabelled hormone to the receptors present in those cells that were cultured in DMEM alone. Scatchard-plot analyses revealed similar association constants for the solubilized receptors whether or not the cells were exposed to insulin (results not shown), indicating that the difference in 125 I-insulin binding reflected differences in the number of high-affinity receptors. Similarly, when cells were cultured for 3 and 4 days in the presence of human recombinant insulin rather than pig insulin, the specific binding of 125 I-insulin to the extracted receptors was 2.4-fold (mean, $n = 2$) higher than that of cells maintained in DMEM alone. The ratio of the trypsin-susceptible 125 I-insulin-binding activity to the total 125 I-insulin-binding activity in cells cultured in insulin-containing media was consistently lower than that in cells cultured in just DMEM. During the first 3 days of the experiments depicted in Fig. 2(b), the trypsin-susceptible 125 I-insulin-binding activity for cell-surface receptors of chondrocytes cultured in insulin-containing media was actually lower than that of chondrocytes cultured in DMEM alone. After 4 days, however,

Fig. 3. Heavy-isotope analysis of ^{125}I -insulin binding to receptors

Chondrocytes were cultured in heavy-isotope-labelled amino acids for 4–16 h in the presence (●) or the absence (○) of insulin. After disruption of the cells and WGA-agarose chromatography to remove non-specific binding, the extracts were centrifuged and the fractions were incubated with ^{125}I -insulin to determine the distribution of 'light' (L) and 'heavy' (H) insulin receptors.

chondrocytes cultured in insulin-containing DMEM had more trypsin-susceptible ^{125}I -insulin-binding activity than did those cultured in insulin-free DMEM.

Acute effect of insulin on internalization of plasma-membrane insulin receptors

In order to assess the effects of insulin on internalization of insulin receptors, the culture conditions were altered for only a short period of time, and the distributions of plasma-membrane and intracellular-localized receptors were determined. After 1, 2, 3 or 4 days of culture, 25% of the cultures were solubilized immediately, 25% were trypsin-treated before being solubilized, 25% were exposed at 37 °C for 30 min to DMEM containing 1 μg of insulin/ml before being solubilized, and 25% were incubated first with insulin and then with trypsin before the cells were disrupted. As assessed by their trypsin susceptibility, the chondrosarcoma cells which were maintained in DMEM alone had 67–76% of their total receptors on the cell surface for each of the 4 days of culture (Table 1). After the short period of time of exposure to insulin, 50–65% of the

total receptors were trypsin-sensitive, representing internalization of 14–25% of the plasma-membrane-localized receptors into a trypsin-inaccessible compartment.

A similar experiment was then performed on cells that were cultured in insulin-containing DMEM for 1–4 days, to determine if removal of insulin from the culture media for a short period of time resulted in an increase of plasma-membrane receptors. Only 32–47% of the total insulin receptors were expressed on the cell surface of chondrocytes cultured for 1–4 days in the presence of high concentrations of insulin (Table 2). However, if these insulin-treated cells were washed and incubated for 30 min in insulin-free DMEM, the proportion of the total insulin receptors that were present on the surface increased to 52–68%, representing an externalization of 45–68% of the intracellular-localized receptors. The up-regulation of the number of insulin receptors that occurred on long-term (> 4 days) (Fig. 2), but not short-term (Table 1), exposure of these cells to insulin therefore indicates that either increased receptor synthesis or decreased receptor degradation overrides the cells' attempt to down-regulate these receptors via the normal internalization-translocation mechanism.

Fig. 4. ^{125}I -insulin-binding activity of cells cultured in heavy isotope-labelled amino acid media in the presence (a) or the absence (b) of insulin

The data are means $\pm$ S.D. for three separate experiments for the ^{125}I -insulin-binding activity to total receptors (■, □), old or 'light' receptors (●, ○) and new or 'heavy' receptors (▲, △), relative to that of starting cells.

Effect of insulin on the rate of synthesis and the rate of degradation of insulin receptors

The heavy-isotope density-shift method was used to assess the effect of insulin on the metabolism of its receptor after $3\frac{1}{2}$ days of culture. Before the chondrocytes were exposed to culture media containing heavy amino acids, one prominent peak of ^{125}I -insulin binding activity was obtained in the density-gradient fractions of the cell extracts (Fig. 3). After 4–8 h of culture in the media containing heavy amino acids, a second peak of ^{125}I -insulin-binding activity appeared at a higher density, and the ratio of ^{125}I -insulin-binding to receptors in the 'heavy' and 'light' fractions increased as the incubation time increased. In the experiment shown, the amount of ^{125}I -insulin bound to 'light' receptors decreased by $\sim 60\%$ and $\sim 25\%$ for chondrocytes cultured for 16 h in the absence and presence of insulin, respectively, relative to that of the starting cells. Fig. 4 represents the average of the data from three separate experiments in which the ^{125}I -insulin-binding activity of 'light' (old) and 'heavy' (new) insulin receptors is compared with that of the starting cells cultured in DMEM containing pig insulin (Fig. 4a) or in DMEM alone (Fig. 4b). When cells were cultured in heavy media for 16 h, total ('light plus heavy') ^{125}I -insulin binding activity increased slightly in the insulin-treated cultures (Fig. 4a) but decreased slightly in non-insulin-treated cultures (Fig. 4b). These latter findings are similar to those obtained with cells cultured in normal DMEM (Fig. 2). The heavy-isotope density-shift method revealed that the major consequence of insulin treatment was an alteration in the regulation of the rate of receptor degradation. The ratio of ^{125}I -insulin binding for the old (or the 'light') receptor at the 16 h time point to the starting cells was 0.49, 0.34 and 0.76 for the three heavy-amino-acid isotope experiments in which cultures were exposed to insulin, and 0.16, 0.13 and 0.43 for the corresponding non-insulin-treated cultures ($P < 0.02$). As assessed by the rate of disappearance of the 'light' receptor, the half-lives of the insulin receptors

were estimated to be ~ 18 h for cells cultured in DMEM containing insulin and ~ 10 h for cells cultured in DMEM alone.

DISCUSSION

Chondrocytes synthesize and secrete macromolecules that comprise the extracellular matrix of cartilage. We, as well as others, have demonstrated that the Swarm rat chondrosarcoma chondrocyte is uniquely responsive *in vitro* to physiological concentrations of pig insulin, producing large amounts of cartilage-like proteoglycans (Stevens *et al.*, 1981; McCumbee & Lebovitz, 1980), type II collagen (Bembenek *et al.*, 1982), hyaluronic acid (Mason *et al.*, 1982) and other secretory proteins (Stevens *et al.*, 1981). Using human recombinant insulin (Fig. 1), we demonstrate in the present study that it is insulin itself, rather than a possible contaminant, that is the factor responsible for the increased rate of synthesis of proteoglycans.

We have previously reported that exposure of chondrosarcoma chondrocytes to pig insulin *in vitro* for 4 days induces an increase in the number of high-affinity insulin receptors on the plasma membrane (Stevens *et al.*, 1983). Since exposure of these cells to insulin does not result in increased plasma-membrane binding of ^{125}I -IGF-II (Stevens *et al.*, 1983) or ^{125}I - T_3 (Stevens *et al.*, 1986), this stimulative effect is relatively specific for the insulin receptor. The apparent up-regulation of plasma-membrane receptors could be due to inhibition of receptor degradation, stimulation of translocation of the receptor from an intracellular compartment to the cell surface, or an increased rate of synthesis of the receptor. In the present investigation we have used a trypsin-treatment procedure and low-temperature binding conditions to prevent insulin-receptor recycling (Marshall, 1985; Reed *et al.*, 1984). This was done to assess the relative distribution of cell-surface and intracellular receptors for cells exposed to insulin for either short periods of time

(30 min; Table 1) or for extended periods of time (> 16 h; Fig. 2). On exposure of the Swarm rat chondrosarcoma chondrocytes to $1 \mu\text{g}$ of insulin/ml ($0.2 \mu\text{M}$) for 30 min, there was a modest 14–25% decrease in binding of ^{125}I -insulin to cell-surface-localized receptors compared with cells incubated in DMEM alone (Table 1). This finding indicated that these tumour cells behave like non-transformed cells in that, at least for a limited period of time, they can down-regulate their insulin receptors. Although a 50% decrease in the number of cell-surface insulin receptors was achieved at physiological concentrations of insulin ($< 10 \text{ nM}$) in cultured mouse fibroblasts (Kadle *et al.*, 1984), human hepatocytes and rat hepatocytes (Kalant *et al.*, 1984), higher concentrations of insulin ($> 0.1 \mu\text{M}$) were required to give the same effect in human colon carcinoma, human melanoma (Mountjoy *et al.*, 1983), human U-937 monocytes (Carpentier *et al.*, 1984) and rat mammary tumour cells (Sorge & Hilf, 1982). Furthermore, a lack of down-regulation was observed in certain human breast-carcinoma cell lines (Mountjoy *et al.*, 1983). Thus this modest decrease in the number of plasma-membrane insulin receptors on the rat chondrosarcoma chondrocyte after a short-term exposure to insulin may be a feature common to some transformed cells.

On exposure to insulin for 5 days, the chondrosarcoma chondrocytes possessed more total receptors and more plasma-membrane receptors than did cells maintained in DMEM alone (Fig. 2). Since these cells are not dividing when cultured in the absence of fetal-calf serum (Stevens *et al.*, 1981), insulin is either inhibiting receptor degradation or stimulating receptor synthesis. The heavy-isotope experiments revealed that the half-life of the receptor was ~ 18 h for chondrocytes cultured in the presence of insulin and ~ 10 h for chondrocytes cultured in the absence of insulin (Figs. 3 and 4). Thus the primary effect of insulin *in vitro* is due to a decrease in the rate of degradation of the insulin receptor. This finding is contrary to that obtained for all non-transformed cells that are exposed to insulin for extended periods of time. Although insulin does not have any effect on its own receptor's metabolism in myocytes (Standaert & Pollet, 1984) and chick liver cells (Krupp & Lane, 1981), insulin tends to stimulate, rather than inhibit, the degradation of its receptor in other cells. For instance, the half-lives of the insulin receptors in 3T3 mouse fibroblasts are 10.2 h and 4.2 h for cells cultured in the absence and presence of insulin respectively (Knutson *et al.*, 1982). One reason why the Swarm rat chondrosarcoma chondrocyte is so insulin-responsive *in vivo* and *in vitro* may be because it cannot properly regulate the number of functional insulin receptors on its cell surface. The net result of this abnormality in insulin-receptor regulation is that, as the cell is exposed to increasing amounts of insulin, rather than shutting down its overall anabolic pathway, this tumour cell becomes programmed to produce more cartilage matrix components.

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