Ligand–receptor interactions involved in the stimulation of Swiss 3T3 fibroblasts by insulin-like growth factors and insulin

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1. The binding of ^{125}I-labelled insulin-like growth factor 1 (^{125}I-IGF-1) to Swiss mouse 3T3 fibroblasts was time- and concentration-dependent. Unlabelled IGF-1 had a slightly higher potency than multiplication-stimulating activity (MSA) in inhibiting the binding of ^{125}I-IGF-1, and insulin gave a parallel inhibition curve at 300–1000-fold lower potency. Chemical cross-linking of bound ^{125}I-IGF-1 to its receptors, followed by polyacrylamide-gel electrophoresis under reducing conditions, revealed a major band of M_r 130000, the labelling of which was inhibited by IGF-1 or high concentrations of insulin. 2. The binding of ^{125}I-IGF-1 was not affected by either co-incubation or preincubation of the cells with a range of heterologous growth factors and mitogens. However, IGF-1 and MSA each induced down-regulation of ^{125}I-IGF-1 binding sites. 3. The maximal stimulations of DNA synthesis induced by IGF-1, MSA and insulin, in the presence of a synergizing mitogen, were similar. The dose–response curve for insulin was not parallel to those for IGF-1 and MSA; in particular, low concentrations of insulin induced a greater stimulation than expected on the basis of its potency in the inhibition or down-regulation of ^{125}I-IGF-1 binding. 4. The preincubation of ^{125}I-IGF-1 with Swiss 3T3 cells at 37 °C decreased its ability to bind to a second batch of cells. This inactivation did not occur when the preincubation was performed at 4 °C or in the presence of cycloheximide. Chemical cross-linking revealed that the cells released an IGF-binding protein, giving a complex of M_r about 48000. 5. It is concluded that type I IGF receptors mediate the stimulation of Swiss 3T3 cells by insulin-like mitogens, but that insulin probably stimulates the cells through insulin receptors. The cells can modulate the amount of ligand binding, both by down-regulation of the receptors and by the secretion of an IGF-binding protein.

INTRODUCTION

The stimulation of quiescent cells into the cell cycle can be achieved by using a variety of growth factors or mitogens. In most cell types in culture, insulin or insulin-like growth factors (IGFs) are potent mitogens, either alone or in combination with other factors. The insulin-like growth factors 1 and 2 are peptides closely related to insulin in amino acid sequence, three-dimensional structure and range of biological activities (Humbel, 1984; Froesch *et al.*, 1985). Three distinguishable receptor types for these peptides (insulin, type I IGF and type II IGF receptors) display highest affinities for insulin, IGF-1 and IGF-2 respectively, but there is extensive cross-reaction of each peptide with each receptor, except for insulin with the type II IGF receptor (Massague & Czech, 1982; Rechler & Nissley, 1985). The insulin and type I IGF receptors are known to mediate various cellular responses, but the function of the type II IGF receptor is not known (Rechler & Nissley, 1985).

The Swiss mouse 3T3 fibroblast cell line has been widely used to study mechanisms of growth-factor action, and shows a characteristic pattern of synergistic stimulation by combinations of growth factors of different functional classes (Rozengurt & Mendoza, 1985). Much work has focused on those factors which stimulate phosphatidylinositol breakdown, increase cyclic AMP or act through the epidermal growth factor (EGF) receptor. Insulin synergizes with each of these classes in the stimulation of the quiescent cells through G_1 phase to DNA synthesis (Rozengurt & Mendoza, 1985), but its intracellular messenger system is not known. Insulin is generally used at pharmacological concentrations, which may, as in other cell systems, imply an action at the type I IGF receptor (Van Wyk *et al.*, 1985). In the present study we investigate the binding of ^{125}I-IGF-1 to Swiss 3T3 cells and its inhibition by insulin, IGF-1 and multiplication-stimulating activity (MSA; rat IGF-2) and compare these effects with the mitogenic stimulation of the cells by these peptides.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), newborn-calf serum, antibiotics and trypsin were obtained from Flow Laboratories. Genetically engineered [Thr^{59}]-IGF-1 manufactured by Amgen, [*methyl*-^{3}H]thymidine and ^{125}I were obtained from Amersham International. MSA was obtained from Collaborative Research. IGF-1 and MSA were solubilized in acid as recommended, and stored frozen as batches until required. IGF-1 was iodinated by the soluble lactoperoxidase method (Thorell & Johansson, 1971), and the ^{125}I-IGF-1 (specific radioactivity between 1120 and 1350 Ci/mmol in four

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; EGF, epidermal growth factor; IGF, insulin-like growth factor; MSA, multiplication-stimulating activity; TGFβ, transforming growth factor β; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

separate preparations) was separated from free ^{125}I and aggregated material by gel filtration on Sephadex G-50 (Pharmacia) using phosphate-buffered saline (Brown & Blakely, 1983) containing bovine serum albumin (BSA) at 1 mg/ml as carrier. The binding potency of IGF-1 was not substantially affected by iodination, since the total amount of IGF-1 specifically bound to the cells at a given total concentration of IGF-1 was similar for different proportional mixtures of labelled and unlabelled IGF-1. BSA, insulin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and prostaglandins were from Sigma Chemical Co. Bombesin and vasopressin were from Bachem and Cambridge Research Biochemicals respectively, and transforming growth factor β (TGFβ) was from Peninsula Laboratories. Epidermal growth factor (EGF) was purified from mouse submaxillary glands as described previously (Savage & Cohen, 1972). Dilutions of all reagents in the appropriate medium were prepared from concentrated stock solutions immediately before use.

Methods

Stock cultures of Swiss mouse 3T3 cells were maintained and passaged as described previously (Brown & Blakeley, 1983). For experimental use, cells were seeded into 24-well cluster trays or 3.5 cm dishes (Nunc) and incubated for 6–8 days, by which time they were confluent and quiescent, at about 6×10^4 cells/well or 3×10^5 cells/dish.

The incorporation of [^{3}H]thymidine (final concn. 1 μCi/ml and 1 μM) into DNA was assayed as described previously (Brown & Blakeley, 1983; Corps *et al.*, 1985). The binding of ^{125}I-IGF-1 was assayed by a procedure developed from that of previous authors (e.g. Ballard *et al.*, 1986) to be directly comparable with the conditions which we use routinely for mitogenic stimulation. Thus, binding assays at 37 °C were performed on cells incubated in the same medium as that for [^{3}H]thymidine incorporation assays, i.e. serum-free DMEM containing BSA (1 mg/ml), in a humidified atmosphere of 10% (v/v) CO_2 in air. Binding assays at room temperature (20–22 °C) and 4 °C were performed in a binding medium consisting of serum-free DMEM without bicarbonate containing BSA (1 mg/ml) and buffered with 20 mM-Hepes, pH 7.4. Cells were rinsed twice with the medium appropriate to the experiment, and then incubated in the presence of ^{125}I-IGF-1 (1 ng/ml) and other additions as described in the text and legends. Non-specific binding was determined by including an excess of unlabelled IGF-1; alternatively, insulin (500 μg/ml) was used to inhibit binding to type I IGF and/or insulin receptors (see the Results section). At the end of the incubation the cells were placed on ice and washed four times at 4 °C with 1 ml of phosphate-buffered saline containing BSA (1 mg/ml) and KI (0.1 μM). The bound radioactivity was solubilized by adding 0.5 M-NaOH/0.2% Triton X-100, and was counted in an LKB gamma counter. Detailed variations of the incubation protocol are described in the text and legends.

The chemical cross-linking of ^{125}I-IGF-1 to its receptors on Swiss 3T3 cells was performed with disuccinimidyl suberate (DSS), essentially as described previously for the insulin and EGF receptors (Pilch & Czech, 1980; Blay & Brown, 1985). All incubations were performed at 4 °C. Cells (three 3.5 cm dishes per experimental treatment) were incubated for 18 h with ^{125}I-IGF-1 (3 ng/ml) and unlabelled peptides as described in the legend to Fig. 4. They were then washed twice with 1 ml of phosphate-buffered saline containing BSA and KI (as above), rinsed and given 1 ml of phosphate-buffered saline. DSS in dimethyl sulphoxide, or dimethyl sulphoxide alone, was added to give a final concentration of 0.5 mM-DSS in 1% (v/v) dimethyl sulphoxide, and the cells were incubated for 15 min before quenching the reaction by replacement of the solution with 150 mM-Tris/HCl, pH 7.4. The cells from each treatment were then combined by scraping them into 2 ml of phosphate-buffered saline, and centrifuged at 300 ***g*** for 10 min. The cell pellet was lysed in 40 μl of lysis buffer [10 mM-NaCl/1.5 mM-$MgCl_2$/1% (v/v) Nonidet P40/10 mM-Tris, pH 7.4], supplemented with 10 μl of concentrated (5×) electrophoresis sample buffer (Laemmli, 1970) with or without reducing agent, and heated to 100 °C for 4 min. Portions were subjected to SDS/polyacrylamide-gel electrophoresis in a 4% (w/v)-acrylamide stacking gel and a 6% (w/v) separating gel (Laemmli, 1970). The gel was fixed in 50% (v/v) methanol in 10% (v/v) acetic acid, dried on to filter paper, and exposed for 14 days to Fuji RX film at −70 °C with intensifying screens.

The chemical cross-linking of ^{125}I-IGF-1 to binding proteins secreted into the supernatant medium by Swiss 3T3 cells was also performed with DSS. Cells were rinsed twice with serum-free DMEM, incubated for 60 min at 37 °C, rinsed again and then incubated overnight at 37 °C. The supernatant medium was removed, centrifuged to remove any cellular debris, and then incubated for 15 min at 37 °C with ^{125}I-IGF-1 (1 ng/ml) to allow binding to occur. DSS was then added, and the mixture was incubated for 15 min at room temperature before adding concentrated (5×) electrophoresis sample buffer and heating to 100 °C for 4 min. Portions were subjected to SDS/polyacrylamide-gel electrophoresis in a 4% (w/v)-acrylamide stacking gel and a 12.5% (w/v) separating gel, which was then fixed for autoradiography as described above.

RESULTS

Stimulation of Swiss 3T3 cells by insulin-like growth factors

In common with most other growth factors for Swiss 3T3 cells, IGF-1, insulin and MSA induced only a small stimulation of [^{3}H]thymidine incorporation (2–4-fold over basal) in the absence of a synergizing co-mitogen (Fig. 1*a*). Insulin, IGF-1 and MSA did not synergize with each other when added in any combination; indeed, any increased responses above the maximum obtained with the individual peptides were considerably less than additive (Fig. 1*a*). In the presence of a co-mitogen (EGF or bombesin) the stimulations obtained were 20–50-fold over basal and equivalent to greater than 50% of the stimulation induced by 10% serum (Fig. 1*a*). All three peptides gave similar maximal stimulations. IGF-1 was the most potent, inducing half-maximal stimulation at 3–10 ng/ml, whereas about 200 ng of MSA/ml was required for the same effect (Fig. 1*b*). Insulin induced half-maximal stimulation at 30–100 ng/ml, but the dose–response curve for insulin was not parallel to those obtained for IGF-1 or MSA (Fig. 1*b*). Possible reasons for this non-parallel behaviour are considered below. The relative stimulatory potencies of the three peptides were independent of the co-mitogen used.

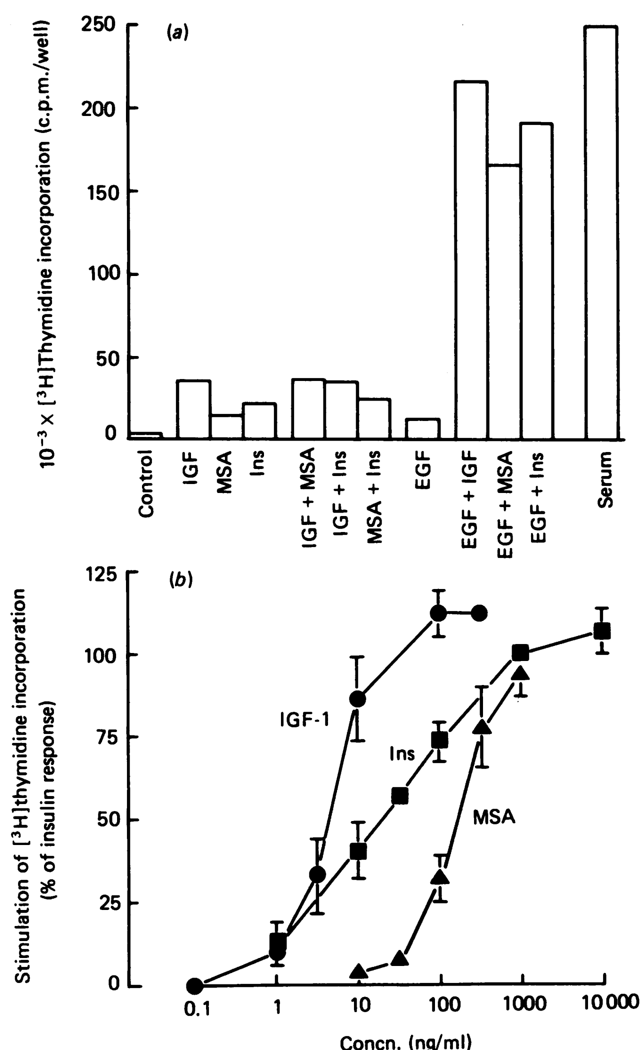

Fig. 1. Stimulation of Swiss 3T3 cells by insulin-like mitogens

(*a*) Cells were incubated for 42 h at 37 °C with IGF-1, MSA or insulin (Ins) at 0.1, 0.1 or 1.0 μg/ml respectively, in the presence or absence of EGF (10 ng/ml), and the incorporation of [³H]thymidine into acid-precipitable material was determined; the columns labelled 'Control' and 'Serum' show [³H]thymidine incorporation by cells incubated without mitogen and with 10% (v/v) newborn-calf serum respectively. Results are means of duplicate samples which differed by less than 6% from the mean. (*b*) In a separate series of similar experiments, IGF-1 (●), MSA (▲) or insulin (■) was added at the concentrations indicated, in the presence of EGF (10 ng/ml) or bombesin (100 pM). The response to this concentration of bombesin alone was similar to that to EGF in panel (*a*). Results are means ± S.E.M. for three to five experiments at each concentration of each mitogen. To account for variation between experiments in the absolute values obtained, the results within each experiment were scaled to a percentage of the stimulation induced by insulin (1 μg/ml) in the same experiment. Thus 0% and 100% represent the incorporation of [³H]thymidine in the presence of EGF (or bombesin) alone, and EGF (or bombesin) plus insulin, respectively.

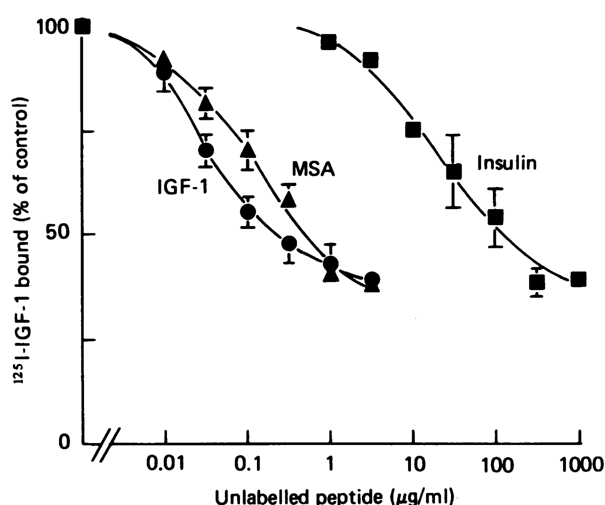

Fig. 2. Inhibition of ¹²⁵I-IGF-1 binding to Swiss 3T3 cells by unlabelled IGF-1, MSA and insulin

Cells were incubated for 90 min at room temperature with ¹²⁵I-IGF-1 (1 ng/ml) and the indicated concentrations of unlabelled IGF-1 (●), MSA (▲) or insulin (■). Results are means ± S.E.M. for three to six experiments at each concentration of each unlabelled peptide, the results in each experiment being expressed as a percentage of the total binding of ¹²⁵I-IGF-1 in the absence of unlabelled peptides, which was 6–10 pg/well (100–160 pg/10^6 cells) in the different experiments.

Binding of ¹²⁵I-IGF-1 to Swiss 3T3 cells

The binding of ¹²⁵I-IGF-1 at mitogenic concentrations to Swiss 3T3 cells was studied at 37 °C, room temperature and 4 °C. The optimum pH for specific binding was 7.4–7.6, whereas non-specific binding showed little variation with pH in the range 6–8. The highest specific binding was obtained at 4 °C, where it increased over 8 h and remained constant until at least 24 h. At 37 °C the maximal specific binding achieved (2.5 pg/well at 1 ng/ml, up to 90 pg/well at 100 ng/ml) was somewhat lower than at 4 °C, presumably because of factors such as down-regulation and binding-protein production (see below). The maximal binding at 37 °C was achieved between 1 and 2 h of incubation, and was generally maintained for at least 4 h, but in some experiments using 10–100 ng of ¹²⁵I-IGF-1/ml there was a small decrease (up to 30%) in binding over this period.

The dose–response curves for the inhibition of ¹²⁵I-IGF-1 binding by IGF-1, MSA and insulin were parallel (Fig. 2): IGF-1 was the most potent, and the potency of MSA was 30–60% of that of IGF-1, whereas the curve for insulin was displaced by 300–1000-fold from that for IGF-1. The maximum inhibition obtained in response to each unlabelled peptide was similar, and the addition of maximally inhibitory concentrations of MSA and insulin together did not result in a greater inhibition than that achieved by either alone (results not shown). These binding characteristics indicate that at low mitogenic concentrations of ¹²⁵I-IGF-1 the only significant binding is to type I IGF receptors, and that the binding remaining after inhibition by insulin represents non-specific binding rather than binding to type II IGF receptors (Rechler & Nissley, 1985). This interpretation of the binding data was supported by the chemical cross-linking of ¹²⁵I-IGF-1 to its receptors on Swiss 3T3 cells (Fig. 3). Under reducing conditions, the principal band observed had an approximate M_r of 130000, which is characteristic of the type I IGF receptor (Massague & Czech, 1982), and the labelling was blocked by 0.1–1 μg of IGF-1/ml or 500 μg of insulin/ml. A higher-M_r band

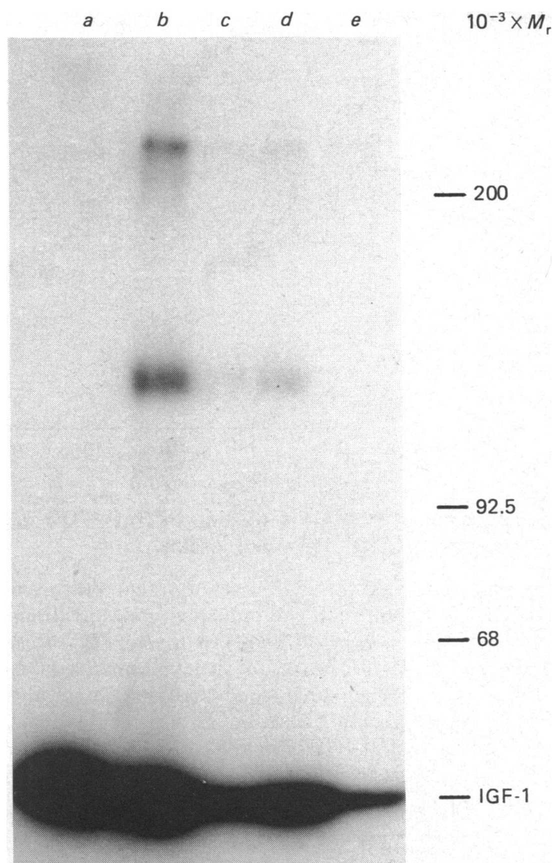

Fig. 3. Cross-linking of ^{125}I-IGF-1 to its receptors on Swiss 3T3 cells

Cells were incubated for 18 h at 4 °C with ^{125}I-IGF-1 (3 ng/ml) and unlabelled peptides as described below. The bound ^{125}I-IGF-1 was then cross-linked to its receptors with DSS, and analysed by polyacrylamide-gel electrophoresis under reducing conditions, as described in the text. Each lane is equivalent to the same number of cells: *a*, no cross-linker included; *b*, ^{125}I-IGF-1 alone; *c*, insulin (500 μg/ml); *d*, IGF-1 (100 ng/ml); *e*, IGF-1 (1 μg/ml). The M_r markers were as follows: myosin heavy chain, 200000; phosphorylase *b*, 92500; bovine serum albumin, 68000.

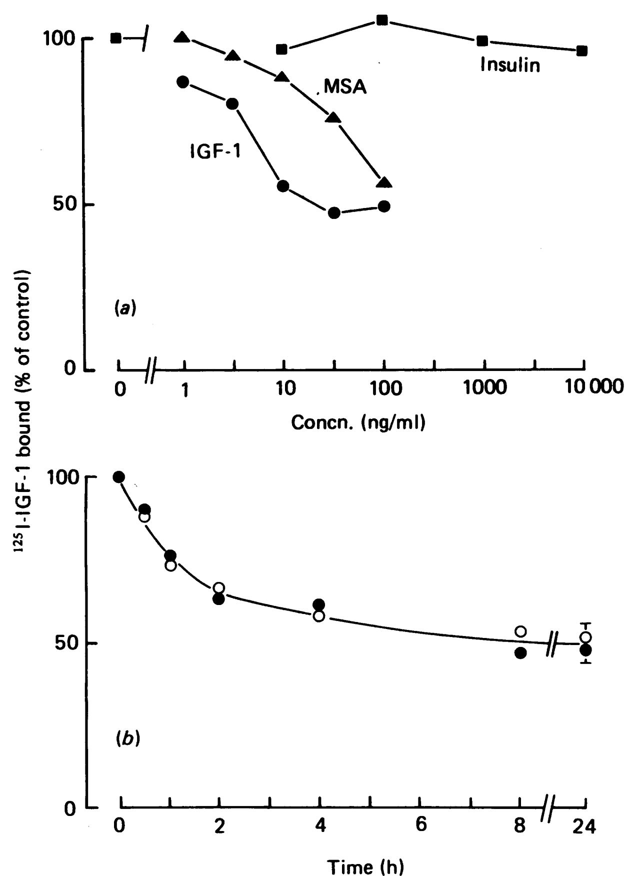

Fig. 4. Down-regulation of specific ^{125}I-IGF-1 binding sites on Swiss 3T3 cells

(*a*) Cells were incubated for 18 h at 37 °C with the indicated concentrations of IGF-1 (●), MSA (▲) or insulin (■). They were then rinsed twice with DMEM containing BSA (1 mg/ml), and incubated for 1 h at 37 °C to allow removal of surface-bound peptides before being transferred on to ice and rinsed with ice-cold binding medium. They were then incubated for 18 h at 4 °C with ^{125}I-IGF-1 (1 ng/ml) in the presence or the absence of excess unlabelled insulin. The results shown are the insulin-inhibitable (specific) binding expressed as a percentage of that in control cells (130 pg/10^6 cells), and are the means of duplicate or triplicate samples which differed by less than 5% from the mean. Results consistent with these were obtained in at least two other experiments with each peptide, except that the highest concentration of insulin (10 μg/ml) gave a decrease in binding of up to 20% in some experiments. (*b*) Cells were incubated for the times indicated at 37 °C with IGF-1 at 10 ng/ml (○) or 100 ng/ml (●), and the specific binding of ^{125}I-IGF-1 was then determined after washing as described above. The results, expressed as a percentage of the specific binding in control cells, are means of four to six samples from two experiments, except for the 24 h time points, which are means ± S.E.M. from five experiments.

(greater than 200000) was also observed (Fig. 3), but the formation of this band was also blocked by insulin; it is thus likely to be a chemically cross-linked dimer of the 130000-M_r subunit, rather than a type II IGF receptor, which has an M_r of 260000 (Massague & Czech, 1982) but does not bind insulin (Rechler & Nissley, 1985).

Effects of heterologous mitogens on ^{125}I-IGF-1 binding

The binding of ^{125}I-IGF-1 to Swiss 3T3 cells was not affected by the following heterologous mitogens used at mitogenic concentrations: TPA, bombesin, vasopressin, prostaglandins $F_{2\alpha}$ and E_1, EGF and TGFβ. This result was obtained both when the cells were incubated for 2 h at 37 °C with the mitogens and ^{125}I-IGF-1 together, and also when the cells were preincubated with the mitogens for 18 h at 37 °C before washing the cells and performing the binding assay at 4 °C (with or without insulin to distinguish type I IGF-receptor binding from type II receptor or non-specific binding). Thus none of these mitogens appeared to affect type I IGF receptor affinity, in contrast with the affinity-modulation of the EGF receptor on these cells by various mitogens which activate protein kinase C, such as phorbol esters and bombesin (Shoyab *et al.*, 1979; Brown *et al.*, 1984). Also, there appeared to be no enhancement of ^{125}I-IGF-1 binding such as that induced by platelet-derived growth factor in

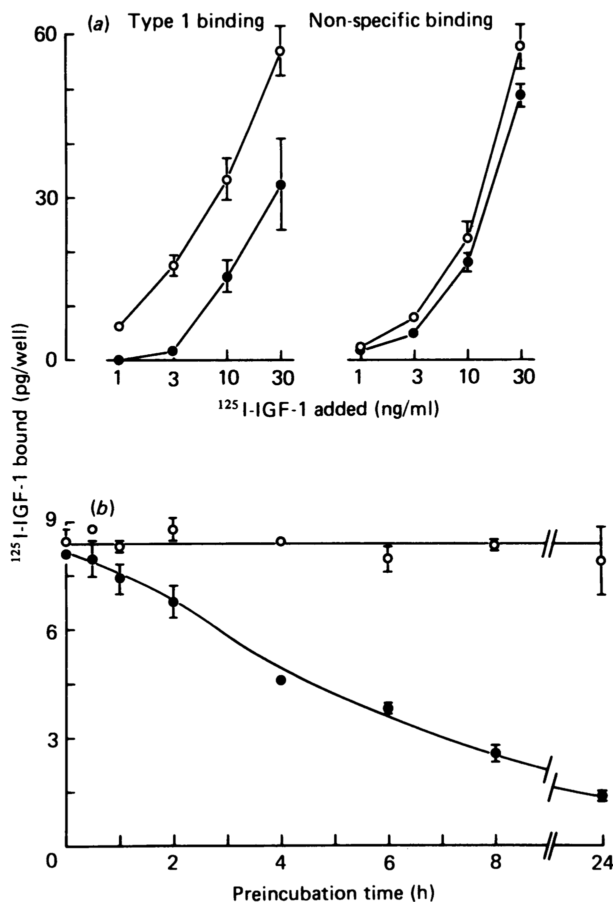

Fig. 5. Inactivation of ^{125}I-IGF-1 by preincubation with Swiss 3T3 cells

^{125}I-IGF-1 was preincubated in binding medium at 37 °C either with Swiss 3T3 cells (●) or in parallel incubations in the absence of cells (○). After the time indicated, the supernatants containing ^{125}I-IGF-1 were transferred on to ice, and were then incubated with a separate batch of cells for 18 h at 4 °C in the presence or absence of excess unlabelled insulin. (*a*) Effect of 18 h preincubation on the specific (left panel) and non-specific (right panel) binding of various concentrations of ^{125}I-IGF-1. (*b*) Time course of the effect on the binding of 1 ng of ^{125}I-IGF-1/ml. Results are means ± S.E.M. for triplicate samples.

BALB/c 3T3 cells (Clemmons *et al.*, 1980), or by follicle-stimulating hormone in cultured rat granulosa cells in longer incubations (Adashi *et al.*, 1986).

Down-regulation of type I IGF receptors

The incubation of Swiss 3T3 cells with mitogenic concentrations of IGF-1 at 37 °C decreased the amount of binding of ^{125}I-IGF-1 in a subsequent assay at 4 °C (Fig. 4). The reduction still occurred after the cells had been washed extensively and incubated for 1 h at 37 °C (conditions which removed over 80% of surface-bound IGF-1; results not shown) before the binding assay was performed. This down-regulation effect was due entirely to a decrease in binding to type I IGF receptors, since there was no significant effect on the binding remaining in the presence of excess insulin. The maximal decrease in type I IGF receptor binding was about 50%, and was achieved with 10–100 ng of IGF-1/ml, whereas 1–3 ng/ml gave smaller but significant effects (Fig. 4*a*). The concentrations inducing maximal decreases in binding gave similar time-courses of down-regulation (Fig. 4*b*), a 25% decrease in binding occurring after 1 h and 50% between 4 and 8 h. This extent of down-regulation was maintained for at least 18 h, and was not affected when either of the co-mitogens EGF or prostaglandin E_1 was included together with the IGF-1 (results not shown).

The ability of MSA and insulin to induce down-regulation of type I IGF receptors was also tested (Fig. 4*a*). MSA induced down-regulation with a potency about 10-fold lower than that of IGF-1. Insulin generally had no significant effect (Fig. 4*a*), although the highest concentration used (10 μg/ml) induced a small decrease in binding (up to about 20%) in some experiments (results not shown).

Secretion of an IGF-binding protein that modulates the cellular binding of IGF-1

During the course of these studies, we observed that the preincubation of ^{125}I-IGF-1 with Swiss 3T3 cells for 18 h at 37 °C decreased its ability to bind to a second population of cells, compared with ^{125}I-IGF-1 that had been preincubated for the same time without cells (Fig. 5*a*). The degree of inactivation, which applied only to type I receptor-specific binding and not to non-specific binding, was greatest at low concentrations of IGF-1 (Fig. 5*a*). Thus the type I receptor-specific binding of 1 ng of ^{125}I-IGF-1/ml was decreased by 90 ± 6% (mean ± S.E.M. from six experiments), whereas that of 30 ng of ^{125}I-IGF-1/ml was decreased by 43 ± 10% (3), to a value equivalent to that of 10 ng of ^{125}I-IGF-1/ml preincubated without cells. The decrease in binding activity did not occur when the preincubation was performed at 4 °C, but was independent of the temperature at which the binding assay was performed (37 °C or 4 °C; results not shown). The degree of inactivation increased with the time of preincubation, such that a 50% decrease in the specific binding of 1 ng of ^{125}I-IGF-1/ml had occurred by 4 h (Fig. 5*b*).

There are several possible mechanisms by which such a decrease in binding activity could occur. The slow release of IGF-binding components adsorbed from serum is unlikely, since the degree of inactivation was not affected by the length or number of rinses of the cells before the preincubation (results not shown). Two results suggest that the decrease in binding activity is due to the secretion of a transferable inhibitory agent, rather than the metabolism of ^{125}I-IGF-1, by the Swiss 3T3 cells. First, the decrease in binding was only 9 ± 5% when the preincubation was performed in the presence of cycloheximide (100 μM), compared with 90 ± 14% in its absence (mean ± S.E.M., $n = 3$). Secondly, binding medium that had been incubated for 18 h at 37 °C with cells but without ^{125}I-IGF-1 inhibited the binding of ^{125}I-IGF-1 (1 ng/ml) in subsequent binding assays at 4 °C (57 ± 7% inhibition; mean ± S.E.M., $n = 4$). Direct evidence that this inhibitory effect was due to the production by the cells of an IGF-binding protein was obtained by chemical cross-linking (Fig. 6). When ^{125}I-IGF-1 was incubated with supernatants from Swiss 3T3 cells and then treated with DSS and subjected to gel electrophoresis, a major band was detected at an M_r of about 48000. This band was not observed when the cross-linking agent was not added or when the supernatants were taken from parallel wells that did not contain cells.

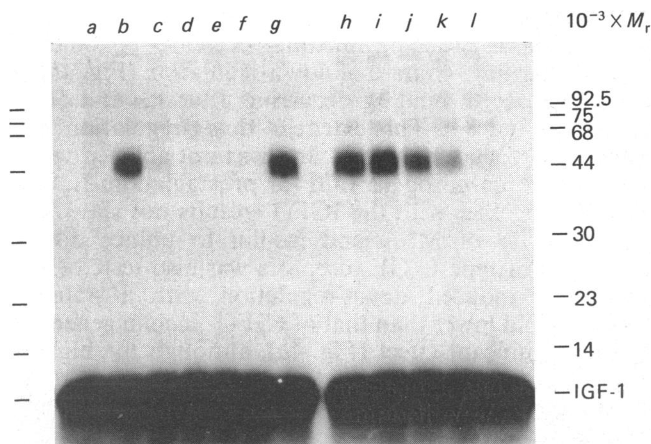

Fig. 6. Cross-linking of ^{125}I-IGF-1 to supernatants from Swiss 3T3 cells

^{125}I-IGF-1 (1 ng/ml) was incubated for 15 min with supernatant and unlabelled peptides as described below. The bound ^{125}I-IGF-1 was then cross-linked with DSS, and analysed by polyacrylamide-gel electrophoresis under reducing conditions, as described in the text. Each lane contains the same volume of incubation mixture: *a*, no cross-linker included; *b*, ^{125}I-IGF-1 alone; *c–f*, unlabelled IGF-1 (3, 10, 30, 100 ng/ml respectively); *g*, insulin, 10 μg/ml; *h*, ^{125}I-IGF-1 alone; *i–l*, MSA (3, 10, 30, 100 ng/ml respectively). The M_r markers were as follows: phosphorylase *b*, 92500; transferrin, 75000; bovine serum albumin, 68000; ovalbumin, 44000; carbonic anhydrase, 30000; soya-bean trypsin inhibitor, 23000; lysozyme, 14000.

The labelling of this band was inhibited by the inclusion of unlabelled IGF-1 or MSA but not insulin (Fig. 6), which is characteristic of IGF-binding proteins from other sources (Nissley & Rechler, 1984). IGF-1 was more potent than MSA in inhibiting the binding of ^{125}I-IGF-1 to the binding protein.

DISCUSSION

Various studies on the mitogenic actions of insulin and related peptides have suggested that their effects on cell growth are generally, but not exclusively, mediated through the type I IGF receptor (see Nissley & Rechler, 1984). Work using human cells and an inhibitory antibody (αIR-3) specific for the type I IGF receptor has confirmed that the mitogenic action of IGF-2 is mediated by the type I receptor (Conover *et al.*, 1986), as is also the stimulation induced by high concentrations of insulin (Van Wyk *et al.*, 1985; Flier *et al.*, 1986). However, in some studies, at lower concentrations of insulin sufficient to saturate the insulin receptor, but not to interact significantly with the type I IGF receptor, the stimulation observed was not sensitive to inhibition by αIR-3 and was attributed to an insulin-receptor-mediated effect (Flier *et al.*, 1986). In the present study using Swiss mouse 3T3 fibroblasts, the basic properties of ^{125}I-IGF-1 binding indicate the presence of a type I IGF receptor. The amount of this receptor on the cell surface is not affected by mitogens other than the occupying ligands, which cause down-regulation as in other cell types (Rosenfeld & Dollar, 1982; De Vroede *et al.*, 1984).

The potency of insulin in inhibiting the binding of ^{125}I-IGF-1 to Swiss 3T3 cells was about 300–1000-fold lower than that of IGF-1, and the inhibition curves were parallel (Fig. 2). This is consistent with the relative affinities of these peptides for type I IGF receptors. However, the mitogenic dose–response curves for IGF-1 and insulin were not parallel (Fig. 1*b*), such that a given concentration of insulin induced a greater stimulation of DNA synthesis than would be expected if insulin was acting through the type I IGF receptor. The probable explanation is that insulin, at least at lower mitogenic doses, is acting through the insulin receptor and not interacting with the type I IGF receptor, as found for some human fibroblasts (Flier *et al.*, 1986). This suggestion is supported by the lack of down-regulation of the type I IGF receptor by 1 μg of insulin/ml (which gave near-maximal stimulation), whereas IGF-1 concentrations inducing a similar stimulation produced a 50% decrease in type I-specific binding in the down-regulation assay (Fig. 4). Other, less likely, explanations include the possibility that insulin interacts with the type I receptor in a manner which results in greater mitogenic signal generation for a given degree of occupancy. Conclusive evidence on this point awaits the development of inhibitory antibodies (analogous to αIR-3; see above) against mouse insulin and type I IGF receptors.

The potency of MSA in inhibiting the binding of ^{125}I-IGF-1 to Swiss 3T3 cells was 30–60% of that of IGF-1 (Fig. 2). However, the relative potency of MSA in both the down-regulation and the mitogenic stimulation assays (about 10% and 3% respectively) was lower than that expected on the basis of this apparent interaction with the type I receptor. This discrepancy cannot be due to difficulties in defining the concentrations used in the different assays, since portions of the same stock solutions, and the same dilution protocols, were used in setting up the different assays. We therefore have no simple explanation for the different relative potencies observed, unless MSA has a lower 'intrinsic activity' than IGF-1. Intriguingly, however, it has recently been reported that there may be two different high-affinity binding sites on the type I IGF receptor from human placental membranes: one of these sites is sensitive to the blocking antibody αIR-3 and the other site has the higher affinity for IGF-2 (Casella *et al.*, 1986). If there are two analogous sites on the type I IGF receptors on Swiss 3T3 cells and the coupling of the MSA-preferring site to intracellular signal generation is weaker, then the discrepancy which we observe between the binding and functional activities of MSA might be resolved.

The incubation of ^{125}I-IGF-1 with Swiss 3T3 cells led to a decrease in its binding activity, apparently owing to the production of an IGF-binding protein. Binding proteins giving a cross-linked complex of M_r 40000–50000 have previously been shown to be produced by other cells in culture [see Nissley & Rechler (1984) for review, and Clemmons *et al.* (1986) and Romanus *et al.* (1987) for more recent examples from fibroblasts]. The effect of the binding protein, combined with the down-regulation of receptors occurring over the mitogenic dose–response range (Fig. 4), presumably explains why the maximal extent of type I receptor-specific binding obtained at 4 °C was not achieved at 37 °C. Given that the stimulation of quiescent cells requires prolonged incubation with insulin or IGFs (Leof *et al.*, 1982), then a decrease in binding ability within the time-scale of a

mitogenic incubation may affect considerations of the relationship between binding and stimulation. Analysis of Fig. 5(*b*), for example, shows that very little receptor-active ^{125}I-IGF-1 remained detectable after an 8 h incubation of low concentrations of ^{125}I-IGF-1 with cells. In these experiments there was little mitogenic stimulation, despite an initial amount of detectable binding. At doses of ^{125}I-IGF-1 sufficient to give full mitogenic stimulation, the binding ability of IGF-1 was decreased, but not abolished, by the binding protein.

Finally, although both insulin and IGF-1 are apparently able to mediate the stimulation of Swiss 3T3 cells by acting through their separate receptor populations, the combination of IGF-1 and insulin resulted in less than additive stimulation. The intracellular signal pathways activated through the different receptors thus appear to be insufficiently different from each other to combine synergistically. Both the insulin receptor (Gammeltoft & Van Obberghen, 1986) and the type I IGF receptor (Jacobs *et al.*, 1983) possess intrinsic tyrosine kinase activity. The relevant physiological substrates for these two kinases have not been identified, but we can predict either that the range of substrates for the one will be a subset of the range for the other, or that they will influence different points of the same biochemical pathways. In contrast, the substrates for the EGF receptor tyrosine kinase must be involved in different pathways [see Klein *et al.* (1985) for example of different, but overlapping, specificities], allowing synergy between EGF and either IGF or insulin.

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REFERENCES

Adashi, E. Y., Resnick, C. L., Svoboda, M. E. & Van Wyk, J. J. (1986) J. Biol. Chem. **261**, 3923–3926

Ballard, F. J., Read, L. C., Francis, G. L., Bagley, C. J. & Wallace, J. C. (1986) Biochem. J. **233**, 223–230

Blay, J. & Brown, K. D. (1985) Biochem. J. **225**, 85–94

Brown, K. D. & Blakeley, D. M. (1983) Biochem. J. **212**, 465–472

Brown, K. D., Blay, J., Irvine, R. F., Heslop, J. P. & Berridge, M. J. (1984) Biochem. Biophys. Res. Commun. **123**, 377–384

Casella, S. J., Han, V. K., D'Ercole, A. J., Svoboda, M. E. & Van Wyk, J. J. (1986) J. Biol. Chem. **261**, 9268–9273

Clemmons, D. R., Van Wyk, J. J. & Pledger, W. J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 6644–6648

Clemmons, D. R., Elgin, R. G., Han, V. K. M., Casella, S. J., D'Ercole, A. J. & Van Wyk, J. J. (1986) J. Clin. Invest. **77**, 1548–1556

Conover, C. A., Misra, P., Hintz, R. L. & Rosenfeld, R. G. (1986) Biochem. Biophys. Res. Commun. **139**, 501–508

Corps, A. N., Rees, L. H. & Brown, K. D. (1985) Biochem. J. **231**, 781–784

De Vroede, M. A., Romanus, J. A., Standaert, M. L., Pollet, R. J., Nissley, S. P. & Rechler, M. M. (1984) Endocrinology (Baltimore) **114**, 1917–1929

Flier, J. S., Usher, P. & Moses, A. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 664–668

Froesch, E. R., Schmid, Chr., Schwander, J. & Zapf, J. (1985) Annu. Rev. Physiol. **47**, 443–467

Gammeltoft, S. & Van Obberghen, E. (1986) Biochem. J. **235**, 1–11

Humbel, R. E. (1984) Horm. Proteins Pept. **12**, 57–79

Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) J. Biol. Chem. **258**, 9581–9584

Klein, H. H., Freidenberg, G. R., Cordera, R. & Olefsky, J. M. (1985) Biochem. Biophys. Res. Commun. **127**, 254–263

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Leof, E. B., Wharton, W., Van Wyk, J. J. & Pledger, W. J. (1982) Exp. Cell Res. **141**, 107–115

Massague, J. & Czech, M. P. (1982) J. Biol. Chem. **257**, 5038–5041

Nissley, S. P. & Rechler, M. M. (1984) Horm. Proteins Pept. **12**, 127–203

Pilch, P. F. & Czech, M. P. (1980) J. Biol. Chem. **255**, 1722–1731

Rechler, M. M. & Nissley, S. P. (1985) Annu. Rev. Physiol. **47**, 425–442

Romanus, J. A., Yang, Y. W.-H., Nissley, S. P. & Rechler, M. M. (1987) Endocrinology (Baltimore) **121**, 1041–1050

Rosenfeld, R. G. & Dollar, L. A. (1982) J. Clin. Endocrinol. Metab. **55**, 434–440

Rozengurt, E. & Mendoza, S. A. (1985) J. Cell Sci. Suppl. **3**, 229–242

Savage, C. R., Jr. & Cohen, S. (1972) J. Biol. Chem. **247**, 7609–7611

Shoyab, M., De Larco, J. E. & Todaro, G. J. (1979) Nature (London) **279**, 387–391

Thorell, J. I. & Johansson, B. G. (1971) Biochim. Biophys. Acta **251**, 363–369

Van Wyk, J. J., Graves, D. C., Casella, S. J. & Jacobs, S. (1985) J. Clin. Endocrinol. Metab. **61**, 639–643

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